Behavioural and neural mechanisms involved in fear extinction, inhibition of responding, and the return of fear

Georgina EB Archbold

Department of Psychology
McGill University, Montreal
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For Nanny Joan, who always remembers to ask after my rats.

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Abstract

While learning about cues that predict danger is fundamental to survival, so too is learning about when these same cues no longer present a threat. In Pavlovian fear conditioning, pairing a previously neutral stimulus (tone) with an aversive outcome (shock) results in a conditioned fear response. The ability of a stimulus to elicit fear is gradually reduced if that stimulus is no longer accompanied by an aversive consequence. The diminishing fear response that occurs when, for example, the tone is repeatedly presented without the shock, is known as extinction. The experiments presented in this dissertation characterize temporal factors involved in both extinction learning and retrieval of extinction memory and explore the mechanisms driving the persistence and loss of these memories.

Chapter 2 examines whether extinction conducted shortly after initial fear learning leads to a permanent loss of fear. One disadvantage of using spontaneous recovery as a measure of memory return is that the state of the original memory cannot be definitively verified. Using a paradigm that makes positive predictions for the presence and absence of memory, we find that immediate extinction does not lead to memory erasure. This suggests that even when extinction is conducted within the time window of consolidation, a new extinction memory is formed that inhibits expression of the original fear.

In Chapter 3, it is shown that recall of fear extinction follows a non-monotonic function. Shortly after successful extinction there is significant spontaneous recovery. Conversely, a delay closer to 24 hours produces less recovery and better extinction retention. The results indicate that the recovery from extinction observed shortly after

extinction training is likely due to the aversive experience of extinction itself and reflects a retrieval failure.

Chapter 4 explores the mechanisms involved in the maintenance of extinction memory. This chapter employs the use of an inhibitory peptide that disrupts the stability of GluA2-containing AMPA receptors at the postsynaptic membrane. Infusions of this peptide into the infralimbic cortex after extinction lead to spontaneous recovery. This suggests that GluA2-containing AMPA receptor trafficking in the infralimbic cortex is necessary for the long-term persistence of extinction. Similarly, use of this peptide in the hippocampus suggests that memory in this structure is involved in the contextual control of extinction.

Finally, Chapter 5 builds on the results of Chapter 4 and presents a forgetting mechanism that accounts for the loss of extinction memory with time (spontaneous recovery). Maintaining GluA2-containing AMPA receptors at the synapse in the infralimbic cortex prevents the normally observed recovery of fear.

Overall, the studies in this thesis suggest that extinction is a highly complex learning phenomenon. The relationship between extinction learning and the return of fear is dynamic and dependent on different mechanisms such as the timing of extinction, timing of extinction recall, and the expression of postsynaptic AMPA receptors within the infralimbic cortex and hippocampus. In Chapter 6 these ideas are discussed further.

Résumé

Pour survivre, il est impératif d'apprendre les signes qui prédisent un danger, mais il est également essentiel de pouvoir déterminer quand ces indices ne représentent plus une menace. Dans le conditionnement de peur Pavlovien, l'association d'un stimulus initialement neutre (un son) avec un événement aversif (un choc électrique) provoque une réponse de peur conditionnée. La capacité d'un stimulus à évoquer la peur se réduit progressivement quand ce stimulus n'est plus associé à l'événement aversif. Lorsque la réponse de peur conditionnée diminue, par exemple quand le son est présenté plusieurs fois sans le choc, on parle alors d'extinction. Les études présentées dans cette thèse caractérisent les facteurs temporels impliqués dans l'apprentissage et le rappel de l'extinction, et explorent les mécanismes qui sous-tendent la persistance et la perte de ces souvenirs.

Dans le Chapitre 2, nous nous demandons si l'extinction réalisée immédiatement après un apprentissage de peur conditionnée résulte en une perte permanente de réponse de peur. L'un des désavantages à utiliser la récupération spontanée comme mesure de réapparition du souvenir est qu'il est impossible de vérifier de façon définitive l'état du souvenir d'origine. En utilisant un protocole avec des prédictions positives pour la présence ou l'absence de souvenir, nous observons qu'une extinction immédiate n'efface pas le souvenir. Ceci suggère que même lorsque l'extinction a lieu pendant la fenêtre temporelle de la consolidation, un nouveau souvenir d'extinction est formé et va inhiber l'expression du souvenir de peur initial.

Dans le Chapitre 3, nous démontrons que le rappel du souvenir d'extinction suit une fonction non-monotone. En effet, peu après une extinction réussie, on observe une

récupération spontanée. Avec un délai d'environ 24h, en revanche, on note moins de récupération et au contraire une meilleure rétention de l'extinction. Ces résultats indiquent que la récupération spontanée observée juste après l'extinction est probablement due à l'expérience aversive de la procédure d'extinction en soi, et reflète un échec du rappel du souvenir.

Le Chapitre 4 explore les mécanismes impliqués dans la maintenance du souvenir d'extinction. Pour cela nous avons utilisé un peptide inhibiteur qui dérègle la stabilité des récepteurs GluA2-AMPA au niveau de la membrane post-synaptique. Des infusions de ce peptide dans le cortex infra-limbique après une extinction causent une récupération spontanée. Ceci suggère que le trafic des récepteurs GluA2-AMPA est nécessaire à la persistance au long terme du souvenir d'extinction. De la même façon, l'utilisation de ce peptide dans l'hippocampe suggère que le souvenir présent dans cette structure est impliqué dans le contrôle contextuel de l'extinction.

Enfin, dans le Chapitre 5, nous utilisons les résultats du Chapitre 4 pour proposer un mécanisme d'oubli qui permettrait d'expliquer la perte du souvenir d'extinction avec le temps (récupération spontanée). Le maintien des récepteurs GluA2-AMPA à la synapse dans le cortex infra-limbique empêche la récupération de la réponse de peur observée habituellement.

Globalement, les études de cette thèse suggèrent que l'extinction est un phénomène d'apprentissage extrêmement complexe. Le rapport entre l'apprentissage de l'extinction et le retour de la réponse de peur est dynamique et dépend de différents mécanismes tels que le moment où l'extinction est réalisée, le moment où le souvenir d'extinction est rappelé, et l'expression post-synaptique des récepteurs AMPA dans le

cortex infra-limbique et l'hippocampe. Dans le Chapitre 6, ces idées sont discutées de façon plus approfondie.

Statement of Original Contribution

The research presented in this based dissertation is based mainly on three manuscripts, two of which have been published in peer-reviewed journals. Chapter 2 includes experiments published in the *European Journal of Neuroscience*. These experiments were conducted in light of conflicting evidence to support the ability of immediate extinction to erase fear memories. We employed a novel approach to distinguish between retrieval failure and memory erasure, exploiting the fact the learning to fear a context a second time does not require NMDA receptors in the dorsal hippocampus. The results suggest that immediate extinction of contextual fear does not lead to the permanent loss of fear.

Chapter 3 presents experiments on the recovery of fear shortly after extinction published in *Learning and Memory*. Studies investigating recovery from extinction commonly choose a minimum of 24 hours after extinction training to test for extinction recall. In these experiments we show that shortly after auditory fear extinction, there is significant recovery due to an inability to retrieve the extinction memory. These results suggest that recovery from fear extinction does not follow the previously assumed monotonic function.

Research on the maintenance of extinction memory is limited. The experiments presented in Chapter 4 investigate the role of AMPA receptor trafficking in the long-term persistence of extinction. This is the first set of experiments to study the involvement of GluA2-containing AMPA receptors in extinction memory maintenance. This manuscript presented in Chapter 4 is in preparation for submission. Chapter 5 extends the results presented in Chapter 4 by examining how the loss of GluA2-containing AMPARs from

the synapse leads to spontaneous recovery. This is the first study to suggest that forgetting of extinction memory over time is responsible for the return of fear.

Author Contributions

For the experiments presented in Chapter 2, I designed the experiments through discussions with Dr. Mark Bouton and Dr. Karim Nader. I also performed the surgeries, ran the experiments, analyzed the data, and wrote the manuscript. Editing assistance was provded by Dr. Bouton and Dr. Nader prior to manuscript publication.

The experiments presented in Chapter 3 were designed by myself and Nick Dobbek with the help of Dr. Nader. Nick Dobbek also ran an initial pilot study. I was responsible for collecting and analyzing all the data presented in Chapter 3. I also wrote the manuscript under Dr. Nader's supervision.

The experiments in Chapter 4 and 5 were designed in collaboration with Dr. Oliver Hardt. Kyra McKelvey helped run the pilot studies that led to the experiments presented in this thesis. I ran all the behavioural experiments, performed the animal surgery and infusions, ran the data analyses. I wrote the manuscript presented in Chapter 4 with editorial contributions from Dr. Hardt and Dr. Nader. The work presented in Chapter 5 will be part of a separate larger manuscript by Dr. Hardt in which I will be a contributing author.

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
BA	Basal amygdala
BLA	Basolateral amygdala
CS	Conditioned stimulus
Ce	Central nucleus of the amygdala
DH	Dorsal hippocampus
GABA	Gamma-Aminobutyric acid
GluA2	AMPA receptor subunit
IL	Infralimbic cortex
ITC	Intercalated cells
LA	Lateral amygdala
LTD	Long term depression
LTM	Long term memory
LTP	Long term potentiation
NMDA	N-methyl D-aspartate
NMDAR	NMDA receptor
NSF	N-ethylmaleimide-sensitive factor
PKMζ	Protein kinase M zeta
PL	Prelimbic cortex
STM	Short term memory
US	Unconditioned stimulus
VH	Ventral hippocampus
vmPFC	Ventromedial prefrontal cortex

Chapter 1

General Introduction

1.1 Introduction

Encoding representations of past events and storing them for future reference is paramount for survival. For instance, learning the association between a threatening event and a preceding cue promotes the ability to predict future threats, activating systems necessary to avoid injury and death. Although this mechanism has evolved to keep an organism out of danger, learning when danger is not likely to occur can be equally important. An Arab proverb states that, "a cat bitten once by a snake dreads even rope." While it may be advantageous for the cat to be fearful of future encounters with snakes, avoiding all long skinny objects may interfere with the ability of the cat to carry out his day-to-day activities (i.e., finding food) and it is consequently maladaptive. Both regulation and modification of behaviour in response to changing environmental stimuli is dependent on accurate assessment of accumulated lifetime experience. In humans, exaggerated responding to situations that no longer predict danger can lead to pathologies including social anxiety and post-traumatic stress disorder (PTSD). The etiology of these fear-related anxiety disorders may have their foundation in learned – or conditioned – fear (Mineka and Zinbarg, 2006). Pavlovian fear conditioning offers a model to study the behavioural mechanisms and neurobiology of learned fear. What happens when we then eliminate the cause of fear? One way in which animals learn to modify their previously learned behaviours is through a process known as fear extinction. The work in this thesis focuses on understanding both behavioural and neural mechanisms involved in fear extinction, inhibition of a learned response, and the return of fear.

1.2 Pavlovian Fear Conditioning and Extinction

During learning, an initially neutral conditioned stimulus (CS) is paired with an

unconditioned stimulus (US), which elicits a known unconditioned response (UR). Upon learning, the once neutral CS now comes to evoke a conditioned response (CR). Pavlov (1927) first described this phenomenon in dogs trained to salivate at the sound of a metronome, a process he referred to as conditioned reflexes. Another form of associative learning that is more widely used in the laboratory is that of conditioned fear. In standard fear conditioning models, a rat presented a tone paired with a footshock will learn to engage in defensive responses when the tone is presented in the future.

Notably, Pavlov also observed in his experiments that if the sound of the metronome was repeated but the conditioned reflex was not reinforced with food, saliva secretion decreased with each presentation; whereas the CS had once produced an excitatory response, it was now decidedly inhibited. Pavlov viewed this change in behaviour as a form of internal inhibition and termed it extinction of conditioned reflexes. The term extinction can refer to both the procedure and the behavioural outcome. Typically a fear extinction procedure involves repeatedly presenting a trained CS such as a tone without the previously associated reinforcement such as footshock, following which, behaviour returns to what it was prior to initial learning. Thus, extinction as a behavioural outcome is akin to the absence of a learned response. In the case of fear conditioning extinction leads to the absence of fear.

Although the study of extinction, particularly by learning theorists, has continued since Pavlov's seminal work, it was not until almost 80 years after his first published observations, that there was a renewed interest in further understanding the neurobiological mechanisms involved in this inhibitory learning. This reemergence likely stems in part from our greater understanding of how the brain learns and forms memories

at a structural level and also at the molecular and synaptic level. Further, extinction has become clinically relevant as a therapeutic tool in the treatment of anxiety disorders as well as addictive and compulsive behaviours (Davis et al., 2006; Mineka and Zinbarg, 2006). As such, understanding the mechanisms involved in extinction is important both for applying research findings to clinical settings and for enhancing our current knowledge of basic learning processes.

The overall aim of the studies presented in this thesis was to investigate the processes involved in fear extinction. The first part of this thesis will briefly review the current knowledge on the nature of extinction, particularly as it relates to extinction as new learning. It will also review the fundamental role of context and the neural substrates and mechanisms of plasticity underlying extinction. The first series of experiments (Chapter 2) will readdress the question of whether extinction involves new learning of the CS and absence of the US, or rather unlearning of the initial CS-US association. Recent data suggest that both mechanisms may be involved but the timing of extinction determines which mechanism is recruited. The difficulty with establishing whether a memory has been erased arises from the inability to discern, using a behavioural measure, between a memory that exists but cannot be retrieved and one that is no longer stored. The experiments in this chapter offer a novel approach to circumvent this issue.

The second series of experiments (Chapter 3) examines the temporal dynamics of spontaneous recovery. Spontaneous recovery is the return of responding to the extinguished CS that is typically observed with the passage of time and is frequently observed when there is a long delay between extinction learning and tests of extinction retention. The standard delay is upwards of 24 hours, but whether recovery is observed

within that time remains unclear.

In the final set of experiments (Chapters 4 and 5) the structures and mechanisms necessary for long-term storage and maintenance of extinction memory are explored. To date, inferences about where extinction memory is stored and how it is maintained have been based on studies involving lesions or inactivations of specific brain regions. However, lesions and inactivations have limitations in determining the nature of long-term extinction maintenance. Based on recent work investigating the maintenance of memory for auditory fear and object location (Migues et al., 2010; 2012) as well as the mechanisms involved in preventing memory decay (Hardt et al., 2013), we explore the mechanisms involved in both persistence and loss of extinction.

While fear extinction is observed in both Pavlovian and operant conditioning paradigms, for the purposes of this thesis we focused on a model of Pavlovian (classical) fear conditioning. The general approach was to train rats in one experimental context in which presentation of a tone co-terminated with an electric foot shock. Animals generally learn to strongly associate the tone with the shock and consequently show a fear response when the tone is played alone. The standard measure of fear used in the field is the absence of movement aside from respiration, referred to as freezing (Blanchard et al., 1976). Investigating extinction of the fear response involved repeated presentations of the tone in absence of the shock, often in a new context. Successful extinction is determined by a return in the animal's movement and exploratory behaviour. Conversely, poor extinction retrieval (or lack of extinction memory) is characterized by high levels of responding to the original CS.

1.3 New Learning?

Since Pavlov's first observations of his dogs' extinguished salivary responses, there has been an enduring debate as to whether this form of inhibition reflects 'new learning' or 'unlearning.' Pavlov(1927) noted that with the passage of time, the conditioned response returned, or could spontaneously recover, leading him to conclude that the CS-US association is not lost. Konorski (1967) also favoured the view that extinction involved new learning. While he postulated that the inhibitory process of extinction was weaker than conditioning, he also assumed that both inhibitory connections between the CS and US and the original excitatory connections coexisted "side by side." Spontaneous recovery is now well documented but still remains poorly understood (Rescorla, 2004a). In addition to spontaneous recovery, the strongest support for extinction involving new learning rather than unlearning comes from three other behavioural observations of recovery: renewal, reinstatement, and rapid reacquisition.

In renewal, the extinguished CS is tested in a context different from the extinction context (Bouton and Bolles, 1979a; Bouton and King, 1983). This shift in context causes responding to return. A number of renewal paradigms have been tested such as ABA and ABC. In ABA renewal, training occurs in context A, extinction in B, and testing back in A. A return to the original conditioning context for testing results in greater recovery than a switch to a third neutral context. Reinstatement involves reexposure to the US alone which then prompts a recovery of responding to the CS (Rescorla and Heth, 1975; Bouton and Bolles, 1979b). It is thought that the US "reinstates" responding because the US forms an association with the context that subsequently triggers responding to the CS it is later presented in that context. Exposure to the US alone is not sufficient to promote reinstatement since recovery is only observed when the US is given in the same context

the CS is later tested in (Bouton, 1984). In rapid reacquisition the conditioned response may return at a faster rate than initial learning from subsequent CS-US presentations (Napier et al., 1992; Ricker and Bouton, 1996). Whether rapid reacquisition is indeed rapid can depend on the paradigm, number of initial pairings, or the extent of extinction (Bouton, 2004). Further, Bouton has argued that reacquisition may be a form of the ABA renewal effect. In summary, there is ample evidence from behavioural data to suggest that responding to an extinguished CS recovers, discounting the possibility that extinction is unlearning.

Surprisingly despite Pavlov's initial account of extinction, there have been those who held a more parsimonious explanation, treating extinction as the destruction of what had been learned (Rescorla and Wagner, 1972; McClelland and Rumelhart, 1985). This explanation has not received the same general consensus primarily because it is unable to account for the recovery phenomena outlined above. Nevertheless, in the past decade there has been renewed interest in determining whether extinction may involve unlearning under specific conditions. For example, Myers and colleagues (2006) asked if the timing of extinction in relation to initial learning might establish if a process of erasure or unlearning exists. In rats trained in fear potentiated startle (FPS), extinction was assessed at four time points following initial learning: 10 minutes, 1 hour, 24 hours, and 72 hours. Recovery of fear was evident in both the 24 hour and 72 hour groups; however, very little recovery was observed in the more immediate 10 minute and 1 hour groups. This was true for tests of both renewal and reinstatement. Conversely, spontaneous recovery was detectable in all groups with the exception of the 10 minute group. Animals in this group showed no recovery of fear. These results suggest that

extinction conducted 10 minutes after initial fear learning results in memory erasure.

Changes at the synaptic level lend support to these behavioural data. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype glutamate receptors play an important role in fast excitatory synaptic transmission and long-term potentiation (LTP), a proposed model of long-term memory. In particular, fear conditioning increases the surface expression of GluA1-containing AMPA receptors (AMPAR)(Yeh et al., 2006). The same research group found that immediate extinction of fear potentiated startle was able to reverse this increase (Mao, 2006). Furthermore, extinction conducted immediately after learning was shown not to require L-type voltage-gated calcium channel (LVGCC) (Cain et al., 2005) nor calcium/calmodulin-dependent protein kinase II α (CaMKIIα) (Kimura et al., 2008), although both are required for the formation of new inhibitory memories when extinction training is conducted 24 hours after conditioning. These studies provide molecular evidence for a dual mechanism of extinction, whereby early extinction interferes with initial consolidation to permanently erase the original trace.

Nonetheless, the ability of extinction to erase a fear memory following a short delay after acquisition has not been widely replicable in a number of other paradigms, involving both animals (Rescorla, 2004a; Maren and Chang, 2006; Schiller et al., 2008; Woods and Bouton, 2008; Archbold et al., 2010 see Chapter 2) and humans (LaBar and Phelps, 2005; Milad et al., 2005; Alvarez et al., 2007; but see Norrholm et al., 2008; Schiller et al., 2008). The discrepancies between studies that find recovery and those that find memory erasure after immediate extinction are not clear; however, they may be a result of differences in the behavioural paradigms. Immediate extinction of fear

potentiated startle (FPS) appears more likely to generate unlearning in comparison to Pavlovian fear conditioning or instrumental learning.

Adding to the debate over whether extinction involves new learning or unlearning is the observation that extinction elicited within the reconsolidation window prevents recovery of fear (Monfils et al., 2009). Reactivation of a memory, through re-exposure to associated stimuli, results in its destabilization and subsequent reconsolidation (Misanin et al., 1968; Lewis, 1979; Przybyslawski and Sara, 1997; Nader et al., 2000). Following destabilization the memory becomes labile for a period of time, susceptible to changes and disruption by amnestic agents (Nader and Hardt, 2009; Nader and Einarsson, 2010). While there are a number of demonstrations that extinction and reconsolidation are distinct processes (Duvarci, 2004; Suzuki, 2004), one of the most obvious is that a blockade of reconsolidation does not result in any of the recovery phenomena associated with extinction. It has been argued that reconsolidation might serve to mediate memory updating as the reconsolidation window presents an ideal opportunity for new information or new learning to be incorporated into an existing knowledge base (Hupbach et al., 2007; Lee, 2009; Wang and Morris, 2010). The study by Monfils et al. (2009) exploits this idea by giving extinction training that is preceded by a separate brief reactivation session. This resulted in attenuated fear during spontaneous recovery, renewal, and reinstatement. There is strong evidence to support this finding (Clem and Huganir, 2010; Schiller et al., 2010; Flavell et al., 2011; Rao-Ruiz et al., 2011); however, its universality remains contended (Chan et al., 2010; Costanzi et al., 2011; Soeter and Kindt, 2011).

Whether extinction is new learning or unlearning remains a point of contention. With advances in both neurobiological and behavioural studies the field may be able to

come to some reconciliation. Indeed, it is likely that the two are not mutually exclusive and both new learning and unlearning mechanisms are involved in extinction (Delamater, 2004; Herry et al., 2010; Orsini and Maren, 2012).

1.4 The Importance of Context

If extinction is new learning and not unlearning, a critical question is what determines when this memory is expressed? Processes of recovery following extinction may offer some explanation as they point to a memory that is particularly context-dependent. "Context" is typically defined as the environmental stimuli that form the background for an event, in the case of research, often a specific apparatus in a lab. A physical change in context, whether it be the original conditioning context or even a neutral one, results in recovery of responding. Indeed, extinction is relatively specific to the context in which it is learned and its retrieval appears to be particularly under the control of contextual cues. Bouton (1993a; 2004) has suggested that extinction involves contextual inhibition because the CS is associated with two possible outcomes and consequently resembles an ambiguous word. Thus, ambiguity in the 'meaning' of the CS arises because the occurrence of shock follows presentation of the CS in one context, but not in another. During extinction an animal learns that the CS no longer predicts the US, generating a CS-noUS association that competes with the original CS-US association. Importantly, the original conditioning is less context-specific than extinction. Thus, the context necessarily determines which association will be expressed or "sets the occasion" for the appropriate response (Bouton and Swartzentruber, 1986).

This model predicts that responding to an extinguished CS should return whenever there is a change in context (Bouton and Ricker, 1994). In renewal, presenting the CS in

a context different from that of extinction results in a return of responding (Bouton and Bolles, 1979a). Renewal can occur under several conditions known familiarly in the literature as ABA, ABC, and AAB. In ABA renewal, when conditioning is acquired in Context A and extinction in Context B, there is significant recovery of the conditioned response with a return to the original Context A (Bouton and King, 1983; Harris et al., 2000). Similarly, in ABC renewal when the CS is tested in a third neutral context, Context C, there is robust recovery (Bouton and Bolles, 1979a; Bouton, 1993a). A third form of renewal, although less universally observed, also exists in which both conditioning and extinction occur in the Context A while testing the CS occurs in Context B. In this AAB renewal there is again return of conditioned responding (but see Bouton and King, 1983; Bouton, 1994; Tamai and Nakajima, 2000).

Reinstatement is also dependent on the context. Recovery of responding to the CS only occurs if the US presentation takes place in the same context in which the CS is later tested (Rescorla and Heth, 1975; Bouton and Bolles, 1979b). Presentation of the US alone is not sufficient to reinstate responding since a US delivered in an irrelevant context does not result in recovery when the CS is later tested in a different context. Given these observations, it is thought that reinstatement involves conditioning between the context and US that subsequently restores responding to the CS. A number of other characteristics of reinstatement further demonstrate its context dependence. For instance, the strength of the contextual conditioning correlates with the strength of reinstatement (Bouton and King, 1983) and extending exposure to the context in which the US is delivered attenuates reinstatement (Bouton and Bolles, 1979b). In addition, the effect of the US context conditioning is specific to extinguished CSs and not all conditioned CSs

(Bouton, 1984).

A third recovery phenomenon well documented in the extinction literature is spontaneous recovery: with the passage of time, responding to the CS returns (Pavlov, 1927). One explanation for spontaneous recovery put forth by Bouton (1993b; 2004) is that extinction may be sensitive to both changes in physical context as well as its temporal context (but see Rescorla, 2004a). In support of this view, a cue presented intermittently throughout extinction can attenuate recovery if it is presented again at the test (Brooks and Bouton, 1993). Thus, not unlike renewal, when the CS is tested outside its temporal context, the conditioned response returns.

In summary, recovery phenomena demonstrate that extinction involves new learning. Further, extinction memory, unlike the original excitatory conditioning, is context-dependent insofar as a shift in context, whether it be physical (renewal) or temporal (spontaneous recovery), results in recovery of original responding. In the next section, we will see how these observations have driven the field in the identification of the neural circuitry and molecular mechanisms involved in extinction.

1.5 Neurobiology and Neurocircuitry of Fear Extinction

Extinction, like excitatory conditioning involves a distributed network of structures to support learning, memory storage, and retrieval. The primary focus of this thesis is on extinction of fear memories, thus discussion in this section will be primarily limited to research on fear extinction. The mechanisms involved in the acquisition and consolidation of conditioned fear, although not definitive, are well established. In comparison, very little is known about the mechanisms of fear extinction. Currently, there is a general consensus that fear extinction involves three main structures: the

amygdala, hippocampus, and ventromedial prefrontal cortex (vmPFC) (Quirk et al., 2000; Milad and Quirk, 2002; Santini et al., 2004; Myers and Davis, 2006; Herry et al., 2010). Each of these structures is thought to serve different functional roles in acquisition, consolidation, and long-term storage. The strong interest in understanding the neural mechanisms responsible for the context specificity of extinction has led to identification of the neural structures and circuits involved in extinction as well as renewal.

1.6 Acquisition of Fear Extinction

The amygdala is necessary for the storage of fear memories and appears to be the site where CS-US associative memories permanently reside (Gale, 2004; Pape and Pare, 2010). Additionally, distributed plasticity within the amygdala mediates initial conditioning (Wilensky et al., 2006; Zimmerman et al., 2007). Given the importance of the amygdala in conditioning, it is not surprising that research initially focussed on the involvement of this structure in extinction acquisition and consolidation. One of the first studies to understand the neural mechanisms of extinction was conducted by Falls et al. (1992) who investigated whether the *N*-methyl-D-aspartate (NMDA) receptors in the amygdala, critical for synaptic plasticity in excitatory conditioning, might also be involved in extinction. Using a fear-potentiated startle (FPS) paradigm, rats were infused with D,L-2-amino-5-phos-phonovaleric acid (AP5), an NMDA receptor (NMDAR) antagonist, into the amygdala prior to extinction. AP5 blocked extinction learning, suggesting that NMDA receptors are necessary for acquisition.

In line with these results, a partial agonist of NMDA, D-cycloserine (DCS), infused into the amygdala has been shown to facilitate extinction of FPS (Walker et al., 2002) and conditioned freezing (Ledgerwood et al., 2003). Although some of the early

studies are limited by the inability to disentangle acquisition and consolidation processes in the FPS paradigm, it is still clear that blocking NMDA receptors in the lateral nucleus (LA) of the amygdala impairs extinction of feared CRs (Sotres-Bayon et al., 2007). Further, mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) signalling is required in the basolateral nucleus of the amygdala BLA for extinction acquisition (Herry et al., 2006). Thus, it appears that glutamatergic synaptic plasticity in the amygdala is necessary for extinction learning; however, whether activity is occurring at γ-aminobutyric-acid (GABA) interneurons or at neurons in the intercalated (ITC) cell masses remains unclear.

Most of the aforementioned studies did not distinguish between subnuclei of the amygdala or focussed primarily on the LA. The basal nuclei (BA) of the amygdala has also been implicated in extinction acquisition. It has been found that following extinction training, there is an increase in the immediate early gene c-*fos* – a neuronal marker of cellular activity – within the BA (Herry and Mons, 2004). Although pre-training lesions of the BA have no effect on extinction acquisition (Sotres-Bayon et al., 2004), local inactivation of the BA using the $GABA_A$ receptor agonist muscimol at the time of extinction acquisition blocks extinction learning (Herry et al., 2008). The same inactivation given after learning was ineffective at blocking retrieval and expression of extinction, indicating that the BA is important for extinction learning, but not for storage of the trace (Herry et al., 2008). The advantage of the inactivations is the reversibility of the treatment compared to the permanence of pre-training lesions plus the compensatory response of other structures assuming the function of the lesioned area.

Studies involving *in vivo* unit recordings have shown that conditioning-induced

neuronal activity in the BA to a CS is significantly decreased following extinction (Quirk et al., 1995) while a different subset of neurons showed persistent firing with repeated presentation of a non-reinforced CS (Repa et al., 2001; Herry et al., 2008). Conversely, a third subset of neurons show increased responding during extinction with repeated presentations of the CS (Herry et al., 2008) which has led to the classification of 'fear neurons' and 'extinction neurons' within the BA that distinguish between high and low fear states activating the correct pathway and determining the appropriate behavioural response. Two different types of projection cells have also recently been identified in the BA that differ in their relationship to inhibitory interneurons (Popescu and Paré, 2011). Activation of approximately 15% of these projection cells results in the activation of BA interneurons, which in turn inhibit the remaining portion of the BA projection neurons. This interplay between glutamatergic and GABAergic BA neurons may account for the activation of either "extinction" or "fear" in regulating the behavioural outcome.

Recently, there has been some evidence that the infralimbic region (IL) of the vmPFC may be involved in extinction acquisition. Inactivations of the IL impaired within-session extinction as well as retention the following day (Sierra-Mercado et al., 2011). This is in contrast to previous reports indicating that IL lesions or inactivations did not affect initial learning of extinction (Quirk et al., 2000; Quirk and Mueller, 2008). The reason for this discrepancy may be a result of the lack of discrimination between mPFC subregions in early papers. Interestingly, activation of the IL by the $GABA_A$ antagonist picrotoxin (Ptx) appears to "prime" the system, leading to enhanced extinction acquisition on subsequent days (Thompson et al., 2010). Further studies are warranted to confirm the role of the IL in the acquisition of extinction.

1.7 Consolidation and Expression of Fear Extinction

Extinction, like initial learning, requires a period of consolidation subsequent to acquisition. Data to support the involvement of the amygdala in the consolidation and acquisition of extinction are less clear. In the BLA, brain-derived neurotrophic factor (BDNF), a growth factor involved in activity-dependent synaptic plasticity, is not involved in the acquisition of fear extinction, but it is necessary for the consolidation as measured by retention the following day (Chhatwal et al., 2006). Similarly, blocking L-type voltage-gated calcium channels (LVGCCs) in the BLA after extinction learning impaired fear extinction memory when assessed 24 hours later (Davis and Bauer, 2012). Much more attention has been given to the involvement of the vmPFC in extinction consolidation and expression.

The first implication of the involvement of the vmPFC in extinction expression was shown by Morgan et al. (1993) after pre-training lesions caused deficits in extinction learning across days. More recently it has been shown that the infralimbic cortex (IL), a subregion of the vmPFC, is likely the key player in the consolidation and retrieval of extinction. Both lesions and inactivations of the IL block consolidation and retrieval of fear extinction (Quirk et al., 2000; Lebron et al., 2004; Sierra-Mercado et al., 2006; Laurent and Westbrook, 2009). Activity in the IL is also associated with extinction consolidation and retrieval. Successful recall of extinction is accompanied by an increase in c-*fos* expression in the IL (Knapska and Maren, 2009). Further, recordings from IL neurons show CS-evoked responses during extinction retrieval (Milad and Quirk, 2002) and electrical stimulation of the IL but not the prelimbic cortex (PL) leads to more robust extinction (Vidal-Gonzalez et al., 2006; Maroun et al., 2012). Following

extinction recall, neuronal bursting in the IL has been suggested to increase activation of amygdala inhibitory cells that subsequently gate fear expression (Santini et al., 2001; Chang et al., 2010).

Additional studies have found that molecular cascades important for consolidation of conditioned fear are also important for consolidation of extinction and occur within the IL. Just as conditioned fear depends on the action of NMDAR (Collingridge and Bliss, 1987; LeDoux, 2000), blocking NMDA in the period immediately following extinction acquisition impairs later retention of extinction (Burgos-Robles et al., 2007; Laurent and Westbrook, 2008; Sotres-Bayon et al., 2009). Impairments in extinction memory are also evident after IL infusions of β-adrenergic receptor antagonists (Mueller et al., 2008), protein synthesis inhibitors (Santini et al., 2004; Mueller et al., 2008), and a competitive antagonist of cyclic adenosine monophosphate (cAMP) (Mueller et al., 2008). Thus, like initial learning, evidence suggests that a calcium-mediated cascade within the IL triggers downstream protein kinases and protein synthesis to establish long-term extinction memory. Similarly, in FPS inhibiting cannabinoid CB1 receptors within the IL impair later extinction retention (Lin et al., 2009) and both dopamine D1 and D2 receptor antagonists infused into the IL prevent successful recall of conditioned fear extinction learning a day later (Hikind and Maroun, 2008; Mueller et al., 2010). An upstream mediator of these receptors is BDNF, an important mediator in the formation of long-term memory. Not surprisingly, both epigenetic and genetic studies have revealed a critical role for BDNF in the IL for successful extinction retention (Bredy et al., 2007; Yu et al., 2009; Soliman et al., 2010). Together, these studies highlight the importance of the IL in mediating extinction consolidation and subsequent retrieval as well as promoting

successful suppression of fear.

The suppression of fear and concurrent expression of extinction is thought to be mediated by input from the IL to the intercalated (ITC) cell clusters of the amygdala. There is considerable evidence to support a role for these GABAergic neurons in the extinction of fear (Royer and Pare, 2002; Paré et al., 2004) and both selective lesions (Likhtik et al., 2008) and pharmacological inhibition of inputs to the ITC cells (Jüngling et al., 2008) result in extinction impairments. In line with these observations, stimulating the IL increases c-*fos* expression in the ITC (Berretta et al., 2005). Amano et al. (2010) further demonstrated that extinction results in increased inhibition of neurons in the medial central nucleus (CeM) of the amygdala as a result of BL inputs to the ventrally located ITC cells; this requires IL activity which drives the extinction-related plasticity in the amygdala. The inhibitory projections from the ITC cells to the CeM (Pare and Smith, 1993; Royer et al., 1999) are important because the CeM is the main output of the amygdala for the expression of conditioned fear responses.

1.8 Contextual Modulation and the role of the Hippocampus

The hippocampus is important for processing contextual information (Hirsh, 1974; Good and Honey, 1991; Fanselow, 2000; Rudy and O'Reilly, 2001); accordingly, it has been suggested that this structure plays a role in the context-dependent modulation of extinction. As previously outlined in this introduction, the context serves to resolve the ambiguous meaning of the CS following extinction learning; the CS predicts shock in one context, but not in another. Early studies using preconditioning lesions to either the fornix or hippocampus indicated the importance of these structures in spontaneous recovery and reinstatement but not renewal (Wilson et al., 1995; Frohardt et al., 2000). In

contrast, a more recent study found that lesions of the dorsal hippocampus (DH), impair renewal of fear (Ji and Maren, 2005). Zelikowsky et al. (2012) have since shown that the timing of lesions to the DH determine whether renewal of responding is observed: rats with pre-training lesions renew their fear to the extinguished CS, while rats with post-extinction lesions fail to show any renewal. In line with these findings, local inactivations with the $GABA_A$ agonist muscimol in the DH eliminate renewal after extinction normally observed with a change in context (Corcoran and Maren, 2001; 2004). Additionally, inactivation of the DH prior to extinction impairs extinction acquisition and leads to renewed fear in the extinction context itself (Corcoran et al., 2005). Infusions of muscimol into the DH (Maren and Hobin, 2007) have also been shown to disrupt the context-specific spike firing in the amygdala that has been previously observed (Hobin et al., 2003). Thus, interfering with hippocampal function disrupts the context specificity of extinction.

Interestingly, the ventral hippocampus (VH), and not the DH, has been implicated in the expression of auditory fear (Maren and Holt, 2004; Sierra-Mercado et al., 2011). In addition, the VH is necessary for the relay of contextual information to the amygdala (Pitkänen et al., 2000) and sends projections to the mPFC (Hoover and Vertes, 2007). Thus, it is not surprising that it has also been implicated in extinction. During renewal the VH sends projections onto neurons in the amygdala (Herry et al., 2008) and a disconnection between the VH and the BA prevents renewal of fear (Orsini et al., 2011). Inactivation of the VH during extinction training also impairs extinction recall (Sierra-Mercado et al., 2011). The VH, which projects directly to the PL, IL, and BLA, can modulate fear responses and activity in the VH may be necessary for the inhibition of

fear.

1.9 The Circuit of Fear Extinction

Although this is in no way an exhaustive coverage of the literature on the neural mechanisms underlying fear extinction, it does emphasize the importance of three main mediating structures: the amygdala, the hippocampus, and the medial prefrontal cortex. Based on the current literature a model of fear extinction circuitry is emerging. Part of the difficulty in determining the circuitry is that the interactions between structures during extinction and fear recovery are not well understood.

IL and PL have opposing effects on fear expression; whereas the IL is associated with suppression of fear and expression of extinction, the PL is involved in the expression of fear (Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2009; Sierra-Mercado et al., 2011) and renewal of fear after extinction (Knapska and Maren, 2009; Orsini et al., 2011). The connectivity of the IL and PL also differ. The IL projects to the lateral division of the central nucleus (CeL) (McDonald, 1998) and ITC neurons while the PL projects to the BA (Vertes, 2004). Thus, expression of extinction and recall of fear may be dependent on a balance in the activity of the IL and PL (Burgos-Robles et al., 2009).

During extinction the CS activates neurons in the IL which sends glutamatergic projections to the ITC neurons (Berretta et al., 2005; Amir et al., 2011). The ITC neurons in turn inhibit neurons in the CeM (Royer and Pare, 2002; Quirk et al., 2003). A second pathway may involve a subpopulation of neurons in the basal amygdala (BA) shown to preferentially respond to an extinguished CS (Herry et al., 2008). These “extinction” neurons could drive inhibitory responding by either activating ventral ITC neurons that inhibit CeM output neurons or by directly interacting with the central nuclei. Recently,

similar "extinction" neurons have been identified in the lateral nucleus of the amygdala (LA) (Knapska et al., 2012). These neurons, mainly innervated by input from the IL, then indirectly inhibit the CeM through the BA or ITC cells. A final proposed pathway involves CeL inhibition of the CeM. CeM neurons are under the inhibitory control of the CeL (Ciocchi et al., 2010; Haubensak et al., 2010) and both retrieval of extinction and renewal of responding lead to increased c-*fos* expression in the CeL (Knapska et al., 2012). Further, the BLA-CeL pathway has been shown to mediate expression of anxiety by gating out from the CeM (Tye et al., 2011). Although the CeL receives input from both the IL and PL, activity in the CeL that corresponds to extinction or fear renewal does not reflect preferential innervation from the IL or PL respectively (Knapska et al., 2012). Therefore, the CeL appears to play a role in extinction through indirect connections with the IL and PL through the BA and ITC cells.

As reviewed above, the hippocampus plays an important role in gating extinction expression (Ji and Maren, 2007). Both the DH and VH are involved in renewal despite substantial differences in connectivity and function (Fanselow and Dong, 2010). Recent work has focussed more on the involvement of the VH in mediating fear extinction. This is likely due to the dense reciprocal connections shared with the BLA (Pikkarainen et al., 1999; Pitkänen et al., 2000; Ishikawa and Nakamura, 2006) and direct projections to the PL (Ishikawa and Nakamura, 2006; Cenquizca and Swanson, 2007; Hoover and Vertes, 2007). For example, during fear renewal a subpopulation of both BA and LA active neurons (i.e., "fear" neurons) receive preferential innervation from the VH (Herry et al., 2008; Knapska et al., 2012). This is consistent with the observation that inactivation of the VH attenuates fear responding (Sierra-Mercado et al., 2011) and prevents fear renewal

(Hobin et al., 2006). Conversely, the VH also projects to the IL and may modulate behaviour through this pathway (Hugues and Garcia, 2007). Indeed Hugues and Garcia (2007) show that low-frequency stimulation of the VH impairs memory for extinction and inactivation of the VH during extinction acquisition leads to poor extinction retention (Sierra-Mercado et al., 2011). Similarly, the DH is both important for the renewal of fear (Ji and Maren, 2005) and the expression of extinction (Corcoran et al., 2005).

Despite a preliminary understanding of the neural circuitry involved in fear extinction, the exact contribution of various subregions (i.e., VH and DH) and neuronal subpopulations remains unclear. Extinction necessitates both potentiation and depotentiation of synapses within the vmPFC, amygdala, and hippocampus that results in a context-dependent suppression of fear. Future research will likely be directed towards to further understanding the mechanisms through which this circuit functions.

1.10 Summary

Fear extinction is a learning process, driven by changes in the contingency between the CS and US, that allows for adaptations to changes in the environment. As Pavlov first observed, extinction reflects inhibition of the original trace rather than erasure and involves the formation of a separate long-term memory. In Chapter 2, the extent to which extinction reflects mechanisms of new learning or unlearning will be examined in detail through a series of experiments that specifically address the timing of extinction. In line with accumulating evidence, results suggest that even immediate extinction of contextual fear involves new learning.

A number of recovery phenomena are observed after extinction, which further suggest that extinction does not involve erasure. Spontaneous recovery, renewal, and

reinstatement provide examples of how extinction training results in context-dependent suppression of fear behaviour. Chapter 3 (Archbold et al., 2013) further explores the temporal dynamics of spontaneous recovery.

One major advance in the last decade has been the discovery and identification of the molecular mechanisms and neural circuits that mediate different phases of extinction such as acquisition and consolidation. Recent papers point to the necessity of activity in the amygdala, hippocampus, and vmPFC for extinction memory. The IL and hippocampus are particularly important for successful consolidation and recall of extinction. Nevertheless, there is currently no direct evidence to show which structures or mechanisms might be necessary for long-term storage. Advances have recently been made towards understanding the neurobiology of long-term maintenance of memory for fear (Pastalkova et al., 2006; Kwapis et al., 2009; Migues et al., 2010) in addition to other types of memory (Shema et al., 2007; Hardt et al., 2010b; He et al., 2011). In Chapter 4, by combining these findings with the current model of extinction neurocircuitry we explore the long-term maintenance of extinction. Understanding the mechanisms involved in the maintenance of extinction give us insight into how this memory might also be lost. In Chapter 5, we explore the involvement of decay-driven forgetting in recovery from extinction. Taken together, the studies presented in this thesis provide further insight into extinction learning and the suppression of fear.

Chapter 2

Evidence for the Persistence of Contextual Fear Memories Following Immediate Extinction

2.1 Preface

As more is learned about the mechanisms involved in the establishment of fear memories, there is increased interest in whether behavioural and pharmacological manipulations can eliminate them. That intrusive fear memories are characteristic of some psychiatric illnesses strengthens the desire to find a process to eliminate the memory or weaken the impact on day-to-day functioning. Extinction results in behavioural responding akin to erasure of the original memory; however, it is generally accepted that extinction involves new learning since the original memory can eventually be recovered (Bouton, 2004). Nevertheless, what if under certain conditions extinction training was able to eliminate fear recovery? Recently, Myers et al. (2006) demonstrated that the time at which extinction training was delivered determined whether any recovery phenomena (i.e. spontaneous recovery, renewal, and reinstatement) were observed. Specifically, extinction training given immediately after initial fear learning resulted in a permanent reduction in fear behaviour.

Absence of recovery is taken as an indication that the memory has been erased, but it cannot discount the possibility that untested experimental variables may result in renewal, spontaneous recovery, or reinstatement. Thus, the difficulty in establishing whether a memory has been erased arises from the inability to behaviourally discern between a memory that exists but cannot be retrieved and one that is no longer stored. In Chapter 2 this problem is addressed in reexamining whether a short acquisition-extinction interval can erase the original memory.

Consolidation of memories within the hippocampus depends on a series of neurochemical events: activation of *N*-methyl-D-aspartate receptors (NMDAR) and

subsequent entry of calcium initiates signalling cascades that ultimately lead to gene expression, protein synthesis, and synaptic modifications. Surprisingly, a number of studies have now shown that hippocampal dependent memories require NMDAR during the first instance of learning but not the second (Bannerman et al., 1995; Sanders and Fanselow, 2003; Hardt et al., 2009). Thus, if a memory is stored, whether or not it is behaviourally expressed, learning a second instance of a similar task (i.e., fear conditioning in a novel context) should no longer require NMDAR and will not be affected by pharmacological disruption of NMDAR.

By exploiting this finding, determining whether a behavioural manipulation has erased the original memory was possible. In these experiments we trained rats in a contextual fear conditioning paradigm. Extinction was conducted either 15 minutes after initial learning or after the standard delay of 24 hours. A few days later rats then received a second instance of contextual fear in a novel context. Prior to this second learning an NMDAR antagonist, AP5, was infused into the dorsal hippocampus (DH). If memory for the first instance of contextual fear is intact, than these infusions should not prevent learning. However, if the memory for the first contextual fear has been erased due to extinction, then AP5 should prevent rats from learning to fear a second novel context.

Regardless of whether rats underwent immediate or delayed extinction, learning to fear a second context was not impaired by blocking NMDAR activity. These results argue against the idea that immediate extinction of contextual fear involves a process of erasure. Thus, the findings presented in Chapter 2 are consistent with the view that extinction involves new learning that inhibits the original, rather than a process of unlearning.

Archbold GE, Bouton ME, Nader K (2010) Evidence for the persistence of contextual fear memories following immediate extinction. Eur J Neuro 31:1303-1311.

2.2 Abstract

Evidence suggests that extinction, the suppression of a learned fear response to a Pavlovian signal that is produced by exposure to the signal alone after conditioning, is a consequence of new inhibitory learning. However, it has been proposed that extinction given immediately after conditioning reflects memory 'erasure'. Using contextual fear conditioning, we examine the nature of extinction further using a novel behavioral paradigm that probes for the absence or presence of a memory. Rats received a context paired with one of three different shock intensities (either 0.8mA, 1.2mA, or 1.6mA), and then received extinction either immediately (15 mins) or after a delay (24 hrs). Spontaneous recovery was roughly equivalent in the immediate and delayed extinction groups when they were tested 24 hr after extinction. To further test the status of the original memory trace, we exploited the effect that only the first, but not second learning of contextual fear requires NMDA receptors (NMDAR) in the dorsal hippocampus (Sanders and Fanselow, 2003). Here we use this property of second learning to determine if memory of an immediately extinguished fear also persists. Rats received bilateral infusions of the NMDAR antagonist AP5 (2-amino-5-phosphonopentanoic acid) into the dorsal hippocampus prior to training in a novel second context. Memory for the second learning is not affected by NMDAR blockade in either group, suggesting that the extinguished memory is not erased, but inhibited. Overall, the results provide little

evidence that extinction conducted immediately after conditioning destroys or erases the original memory trace.

2.3 Introduction

Conditioned fear responses may be suppressed by extinction training during which the conditioned stimulus (CS) is presented in absence of the unconditioned stimulus (US). Evidence suggests that extinction does not "erase" the original trace but is mediated by new learning that inhibits expression of the original memory (Rescorla, 1997; Bouton, 2004) since return of the extinguished behavior is observed in tests of spontaneous recovery (Pavlov, 1927), renewal (Bouton and King, 1983), and reinstatement (Rescorla and Heth, 1975; Bouton and Bolles, 1979b).

Recent findings suggest that extinction might reflect memory erasure when given immediately following training (Myers, 2006). Specifically, immediate extinction of the fear-potentiated startle response abolished its spontaneous recovery, renewal, and reinstatement. Furthermore, the conditioning-induced increase in α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluR1, a proposed molecular correlate of associative fear memory (Rumpel et al., 2005; Yeh et al., 2006), decreased to levels of naïve controls with immediate extinction (Mao, 2006).

In contrast, Maren and Chang (2006) were unable to show the same level of attenuation in conditioned freezing in spontaneous recovery tests after immediate extinction. In conditioned suppression and appetitive conditioning, Woods and Bouton (2008)found immediate extinction generated more, rather than less, spontaneous recovery than extinction conducted 24 hrs after conditioning. Lack of erasure following immediate extinction has similarly been found in different fear paradigms and species (Cain et al., 2005; Alvarez et al., 2007; Schiller et al., 2008).

In the present study we further compared the extent to which immediate versus delayed extinction would "erase" the original trace. In addition to assessing spontaneous recovery after immediate and delayed extinction, we tested the persistence of an extinguished memory using a novel paradigm that makes positive predictions for amnesia being a storage impairment. Within the amnesia literature, it has been argued that a disadvantage of using recovery of performance to measure memory are the alternative interpretations of recovery consistent with initial behavioral impairments caused by memory erasure (Gold and King, 1974; Nader and Wang, 2006; Squire, 2006). These interpretations might also apply to recovery from extinction, the dominant paradigm used to assess existence of an extinguished memory. Thus, to circumvent many of the issues surrounding recovery from amnesia we used a test which makes positive predictions for the absence of a memory induced by amnesic agents (Hardt et al., 2009).

This test exploits the property that the first spatial or contextual learning is blocked by N-methyl-D-aspartate (NMDA) receptor antagonists such as DC-2-amino-5-phosphonovaleric acid (AP5), while a second spatial or contextual learning is not (Bannerman et al., 1995; Saucier and Cain, 1995; Sanders and Fanselow, 2003). We predicted that if immediate extinction of the first learning erases the memory, then second learning should be blocked by AP5 infusions. Conversely, if immediate extinction inhibited an existing memory, then second learning should not be blocked by AP5. We found that after immediate or delayed extinction of first learning, second learning was unaffected by AP5. This is consistent with the view that extinction is the inhibition of an existing memory.

2.4 Methods

2.4.1 Subjects

Adult male Sprague-Dawley rats (Charles River Laboratories, Québec), initially weighing 275-300g, were housed individually in plastic Nalgene cages and maintained on 12 h light/dark cycle (lights on at 7am, off at 7pm) with food and water available *ad libitum*. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care and Use Committee.

2.4.2 Surgery

Rats were anesthetized (3.33 mg/kg xylazine; 55.55 mg/kg ketamine; 0.27 mg/kg medetomidine hydrochloride), and then mounted into a Kopf stereotactic frame. Stainless steel guide cannulas (22 gauge; Plastics One) were implanted bilaterally aiming at the dorsal hippocampus (DH). The coordinates were 3.6 mm posterior to bregma, 3.10 mm lateral to the midline, and 2.40 mm ventral to the skull surface based on the rat atlas by Paxinos and Watson (2005). Three jewelry screws implanted in the skull and acrylic cement were used to stabilize the cannula. Obturators inserted in the guides prevented blocking. After surgery, an intra-musclar injection of analgesic (Buprenorphine, .324 mg/kg) was given. An intra-peritoneal injection of Antisedan (7.5mg/kg) suspended anaesthesia. Animals were given seven days to recover from surgery.

2.4.3 Infusions

Rats were infused with either DC-2-amino-5-phosphonovaleric acid (AP5; Sigma-Aldrich) dissolved in saline (0.9% NaCl) or normal physiological saline (VEH) adjusted to pH 7.4. Injector cannulas (22 gauge), extending 0.5 mm beyond the guide cannulas when inserted, were connected to 5-µl Hamilton syringes with polyethylene tubing. AP5

(2.5 μg/μl) or VEH were infused over 5 min at a rate of 0.4μl/min. Injectors were left in place for an additional 1 minute to allow the drug to diffuse away from cannula tip. The final dose of AP5 was 10 μg per rat (5 μg/side). Both dose and volume have previously been shown to impair context learning (Sanders and Fanselow, 2003).

2.4.4 Apparatus

Two different conditioning contexts were used. To limit generalization between them, they varied along several dimensions, i.e., shape, illumination, scent, visual and tactile features, and ambient sound. Context A was a box (29 cm X 25 cm X 25 cm), manufactured by Med Associates (St. Albans, VT), with aluminum sidewalls. Two lights were on the right wall and one on the left. The lights flashed in alternation at a rate of 1 cycle/second. A plastic insert was placed into the box to create a curved back wall. A black-and-white-striped wallpaper (stripes 2.5cm wide) was placed on the front wall. The floor, slanted (7°) with respect to the wall, was a stainless steel grid (rod radius 1mm, spaced .5cm apart) that delivered the footshock. Wood-chip bedding barely covered the grid. Peppermint scent was sprayed onto the curved plastic wall before the animal was put in the box. A fan provided a constant background noise. A digital camera was mounted on the ceiling and videotaped the sessions for later analysis.

Context B was a box (30 cm X 26 cm X 33 cm), manufactured by Coulbourn Instruments, placed into a soundproof chamber. One wall was made of stainless steel and had a light bulb providing dim light. The other walls were made of colorless Plexiglas. The floor was a level stainless steel grid (bar radius 2.5mm, spread 1cm apart). Vanilla scent was sprayed into one corner of the box before the animal was put into it. A digital cameral in front of the box recorded the sessions.

In all experiments the first fear conditioning took place in Context A. In Experiments 2 and 3 a second conditioning session was performed in Context B.

2.4.5 Behavioral Procedures

Contextual fear conditioning. Rats were placed in the conditioning chamber for four and a half minutes. After three minutes they were given two 0.8 second shocks (Exp. 1: 0.8, 1.2, or 1.6 mA; Exp. 2: 0.8 mA; Exp. 3: 0.8 mA), spaced 30 s apart. Rats were removed from the chamber one minute following the last footshock.

Extinction training. Extinction training consisted of a single 30 min continuous exposure to the training context in the absence of any foot shocks and was conducted either 15 min (immediate) or 24 hours (delay) following training.

Fear memory testing. Freezing to the conditioning context was measured by returning the rat to the context for 5 min in the absence of shocks.

Experiment 1. On Day 1, rats were placed in Context A and given two shocks (both shocks were either 0.8, 1.2, or 1.6 mA). Then half of the animals in each group received immediate extinction training, the other half delayed extinction training. Twenty-four hours after the extinction session, rats were returned to Context A to measure freezing to the conditioning context.

Experiment 2. Rats underwent the same conditioning and extinction procedures as in Experiment 1, but this time the shock intensity did not vary (all animals received shocks of 0.8 mA). Five days after the initial training, animals received an infusion of either AP5 or VEH (Immed/AP5, Immed/VEH, Delay/AP5, Delay/VEH). Immediately following the infusion, rats were fear conditioned to Context B. Memory for the second fear conditioning was tested twenty-four hours later by returning animals to Context B.

Experiment 3. Immediately before conditioning in Context A, rats received an infusion of AP5. Then, 15 minutes after conditioning, fear memory to Context A was extinguished (immediate extinction). As in Experiment 2, five days later animals received an infusion of either AP5 or VEH immediately before fear conditioning in Context B. As in Experiment 2, rats were returned to Context B twenty-four hours later to assess contextual fear memory.

2.4.6 Behavioral Data Analysis

Freezing behavior, defined as immobilization except for respiratory movements (Blanchard and Blanchard, 1969) was used as the dependent measure of fear and was scored manually using a 1/100 sec chronograph. Post training freezing was observed for a duration of 30 seconds, 30 seconds following shock presentation. In the extinction sessions, animals were observed over two 30 second blocks at five minute intervals and the time spent freezing was recorded. Freezing during test was measured within 30 s blocks continuously for five minutes. An experimenter blind to the experimental condition scored freezing behavior. Only rats with histological indication that both injectors targeted the DH were included in the analysis.

Histology. Animals were transcardially perfused with physiological saline, followed by 10% formalin-saline. The brains were then cryosectioned at 50μm thickness and stained with formal-thionin to identify cannula placement.

2.5 Results

2.5.1 Training strength does not moderate the likelihood of spontaneous recovery following immediate or delayed extinction

The aim of Experiment 1 was to examine whether recovery of performance, measured by freezing upon return to the conditioning context, would be observed when

extinction was conducted shortly after contextual fear conditioning (immediate extinction), as opposed to when extinction occurred one day after training (delayed extinction). Absence of performance recovery might suggest that extinction induced actual memory loss. We further studied whether manipulating shock intensity at training would affect the likelihood of performance recovery. Three groups of rats received two shocks varying in intensity between groups. These groups were further subdivided into animals that received extinction immediately (15 min) after training and those that received extinction after a 24 hour delay.

Figure 1B shows extinction of freezing for the three shock intensities. A 2 (Extinction Timing) × 3 (Shock Intensity) × 7 (Extinction Block) repeated measures ANOVA revealed a significant main effect of Extinction Timing ($F_{1,47} = 6.747$, $P = .013$) and a significant within subjects effect of Block ($F_{6,282} = 69.484$, $P < .001$). There was also an Extinction Timing × Extinction Block interaction ($F_{6,282} = 4.542$, $P < .001$). *Post hoc* analyses indicated a significant effect of extinction timing at blocks 3 and 4, indicating that the delayed groups were faster to extinguish than the immediate groups, only for the high (1.6mA) and low (0.8mA), not for the intermediate (1.2mA) shock-intensity condition.

As can be seen in Figure 1B, it appears that although extinction was slightly faster in the delayed groups, this did not affect the amount of recovery seen in either group across shock intensity conditions. A 2 (Extinction Timing) × 3 (Intensity) × 2 (Block) repeated measures ANOVA, comparing the last block of extinction with the first block of the test, confirmed this impression. The analysis revealed a main effect of Block ($F_{1,47} = 72.528$, $P < .001$), indicating an increase in freezing with return to the conditioned

context. There was no main effect of Extinction Timing ($F_{1,47}$ = 1.604, P = .212) or intensity ($F_{2,47}$ = .964, P = .389). However, the interaction of Extinction Timing × Block almost reached significance ($F_{2,47}$ = 3.896, P = .054). Although both immediate and delayed extinction groups demonstrated significant recovery from the last block of extinction to the first of the test session, the delayed groups showed a trend towards greater recovery. However, this trend was nonsignificant and appears to be due in part to anomalously high freezing at the end of extinction in the 1.2mA immediate extinction group.

A 2 (Extinction Timing) × 3 (Shock Intensity) × 5 (Test Block) repeated measures ANOVA of the test session (Figure 1C) did not show a main effect of extinction timing ($F_{1,47}$ = .065, P = .8) or shock intensity ($F_{2,47}$ = 1.334, P = .273). There was a significant effect of test Block ($F_{4,188}$ = 12.045, P < .001) and a significant Extinction timing × Block interaction ($F_{4,188}$ = 3.208, P = .014), indicating some within test session extinction and less recovery in the immediate groups.

The equivalent recovery seen in all groups between the end of extinction and the beginning of testing is traditionally interpreted as evidence that the original trace persisted—perhaps equivalently—after both immediate and delayed extinction. However, recovery cannot necessarily be treated as evidence against the hypothesis that immediate extinction leads to some "erasure" of the original fear conditioning memory. Indeed, in the memory consolidation literature it has been argued that recovery of performance over time is also consistent with loss of the initial behavioral response as a result of partial memory erasure (Nader and Wang, 2006; Squire, 2006). Thus, in Experiment 2 we use a

method which can make positive predictions for the persistence or absence of the original memory.

2.5.2 Immediate extinction of the first contextual fear conditioning does not render the second contextual fear learning dependent on DH-NMDAR

It is possible that spontaneous recovery from amnesia may occur even if the initial behavioral impairment results from impaired storage of the memory. Memory erasure may never be complete, and this leaves the possibility open that a residual memory can change and lead to increased performance on test. One mechanism might be pattern completion of the residual trace, where cues present during test may build or generate a pattern of activity that more closely resembles that of the original trace producing spontaneous recovery of performance (Nader and Wang, 2006). Recently, a computational model has been proposed which includes such a process (Amaral et al., 2008). Other theoretical mechanisms include an alternate storage process that develops slowly over days and is not disrupted by pre-training memory blockade (Squire and Barondes, 1972), or other strengthening of the residual trace (Gold et al., 1973). If immediate extinction has erased the memory, but erasure is not 100%, there may be a residual memory that could undergo the performance enhancing processes just described. Thus, the fact that immediate extinction in Experiment 1 led to spontaneous recovery that was almost indistinguishable from recovery seen after delayed extinction could be consistent with the idea that extinction leads to actual memory erasure.

In order to circumvent many of the interpretive shortfalls of the recovery of performance paradigms, we tested if immediate extinction eliminates fear memories using a novel paradigm developed to test the for memory erasure in which retrieval and storage impairment views make different predictions (Hardt et al., 2009). Thus, we can

positively test for the absence of a memory. If the original memory survives extinction, then learning to fear a second context should not depend on NMDAR in the DH (Sanders and Fanselow, 2003). Conversely, if the original memory is erased, then learning to fear a second context should require the same NMDAR dependence as in the first learning. Thus, the second learning is treated as a first learning. To test these predictions we repeated the training and extinction of experiment 1 with DH cannulated rats. Five days after conditioning rats were infused with either AP5 or VEH before training in a second context. Twenty-four hours later they were returned to the second context to test for memory retention.

We predicted that if extinction erased the first learning then memory for the first conditioning should not be available during the second fear learning, and, as a consequence, the second conditioning should be blocked by AP5 in the DH, a *positive prediction* for the absence of a memory. However, if extinction inhibits the first memory, then the memory for the first learning persists, and the second learning should no longer require NMDAR in the DH. Consequently, an infusion of AP5 should be ineffective at blocking acquisition of the second memory. Animals received a first conditioning session followed by extinction immediately (15 min) or 24 hours after training. Five days later, an infusion of AP5 or vehicle was given immediately prior to training in a second context. Memory for this context was tested 24 hours later.

As shown in Figure 2B, animals in all groups showed an equivalent reduction in freezing throughout the extinction session ($F_{6,156} = 16.898$, $P < .001$) and no main effects of Drug ($F_{1,26} = .042$, $P = .839$) or Extinction Timing ($F_{1,26} = 1.915$, $P = .178$) were found. The Extinction Timing × Block interaction seen in Experiment 1 was not

replicated ($F_{6,156}$ = 1.021, P = .414). Thus, there was no difference between groups in the rate of extinction.

In figure 2C a 2-way ANOVA on second training performance showed a significant effect of drug (Veh vs. AP5) ($F_{1,26}$ = 5.805, P = .023), and no effect of extinction timing or Drug by Extinction Timing interaction (all $F_{1, 26} < 1.5$, p>0.25). Second training performance is freezing behavior measured 30 seconds after shock termination. Infusions of AP5 did not appear to eliminate freezing since freezing in drug treated animals is still relatively high. AP5 infusions may increase motor activity in the one minute following exposure to the shock stimulus.

A 2-way ANOVA on Test performance showed no significant Drug effect, Extinction Timing, or Drug by Extinction Timing interaction (all $F_{1, 26} < 1.5$, p>0.25). A 2 (Drug) × 2 (Extinction Timing) × 2 (Second Training vs Avg Test) repeated measures ANOVA did not reveal a main effect Drug ($F_{1,26}$ = 1.896, P = .180) or Extinction Timing ($F_{1,26}$ = 1.245, P = .275). There was a significant effect of Second Training vs Average Test ($F_{1,26}$ = 10.676, P = .003) and a Drug × Second Training vs Avg Test interaction ($F_{1,26}$ = 6.853, P = .015). *Post hoc* pair-wise t-tests showed that a decrease in freezing from Post CS to Avg Test was only apparent in the groups administered vehicle (t_{14} = 3.917, P = .002). The results of this experiment are consistent with the prediction that if immediate extinction fails to erase the memory, then conditioning in a second context will be NMDAR independent in the DH. Treatment with AP5 prior to the second learning does not affect freezing behavior on the test.

Unlike Experiment 1, no test of spontaneous recovery was conducted. Thus, to ascertain whether behaviour on this test would be equivalent across groups and whether

performing this test changed the behaviour in a second training, rats were exposed for 5 minutes to the context 24 hours following extinction training. In all groups, animals showed minimal recovery that was roughly equivalent (Figure 3). A 2-way ANOVA on the test of spontaneous recovery showed no significant effect of Drug, Extinction Timing, or Drug by Extinction Timing interaction (all $F_{1, 18} < 1.5$, $p>0.25$).

2.5.3 With the present methods, AP5 in the DH is sufficient to block the first but not second learning

To confirm that the AP5 infusions used in the previous experiment were sufficient to block learning, we infused AP5 at the same dose and concentration into the DH prior to the first learning, which requires NMDAR (Sanders and Fanselow, 2003). Experiment 3 followed a similar protocol as Experiment 2. All rats were infused with AP5 before the first contextual fear conditioning and then underwent immediate extinction. Five days after training rats received either AP5 or VEH before the second conditioning session. If AP5 blocks the second learning under these conditions in which memory of the first learning has not been stored as a consequence of the AP5 infusions administered before it, then animals given AP5 before both conditioning sessions should show impaired freezing during test.

The results are shown in Figure 4. A 2 (Drug) × 7 (Extinction Block) repeated measures ANOVA confirmed extinction (Figure 4B) reduced freezing in all animals ($F_{6,66} = 19.737$, $P < .001$). As seen in Figure 3C, a 2 (Drug) × 2 (PostCS/Avg Test) repeated measures ANOVA showed a main effect of Drug ($F1_{,11} = 9.212$, $P = .011$). There was also a Drug × PostCS/Avg Test interaction such that both drug conditions froze less during the test session, but the decrease from Post CS to Test was greatest in the

AP5/AP5 group. A comparison between groups confirmed that the AP5/AP5 animals froze less than the AP5/VEH animals during the test indicating that AP5/AP5 animals were unable to acquire the second learning ($F_{1,11}$ = 24.159, P <.001). Based on these results, we suggest that the AP5-induced memory erasure represents a storage deficit. In contrast, immediate extinction did not cause the second learning to be blocked by AP5 which implies that memory for the first learning is stored and is not erased.

We conducted a further test to show that AP5 given before training that is not followed by extinction results in a memory for a second fearful context that is NMDAR-dependent in the dorsal hippocampus. The retention test given after the second contextual fear conditioning (Figure 4C) suggests that blocking NMDA receptors impairs contextual learning of a second context if memory for the first learning has been disrupted ($F_{1,9}$ = 15.857, p <.01). This was a replication of the findings reported by Hardt et al., (2009).

2.6 Discussion

It has been suggested that extinction conducted immediately after training may erase the memory trace (Myers, 2006). However, this effect may not be true for all paradigms (Maren and Chang, 2006; Woods and Bouton, 2008). We used two approaches to test for the persistence of a contextual fear memory. The first was to determine whether a context-fear association that had undergone immediate extinction would be resistant to recovery with the passage of time (spontaneous recovery). Secondly, we employed a novel approach for testing the persistence of a memory that exploits the finding that a second similar contextual memory does not require NMDAR in the DH (Bannerman et al., 1995; Saucier and Cain, 1995; Sanders and Fanselow, 2003). The present study addressed the efficacy of a short-delay (immediate) extinction

intervention in contextual fear conditioning using both traditional assays for the presence of a memory as well as a novel procedure. The latter was included as there may be some difficulty in the interpretation of traditional assays (Nader and Wang, 2006; Squire, 2006).

Animals that received immediate extinction did not differ in the amount of recovery compared to animals given the more traditional 24 hour delay between training and extinction suggesting that by that measure immediate extinction does not erase the original trace. This finding is in contrast to that observed in fear-potentiated startle (Myers, 2006). Our data are consistent with the recovery observed following immediate extinction of an auditory fear memory (Maren and Chang, 2006), both CER and appetitive conditioning (Woods and Bouton, 2008) and startle reflex in humans (Alvarez et al., 2007). The results were consistent over several shock intensities. Nor did we observe a greater resistance to extinction during immediate extinction (see for example Maren and Chang, 2006). This is consistent with Woods and Bouton (2008), who additionally found greater recovery in the immediate group despite lower fear during extinction compared to the delayed group as well as greater recovery with immediate extinction of appetitive conditioning. Thus it is clear that the level of fear observed at the beginning of extinction does not predict the effect of immediate extinction in the various paradigms.

Given that the absence of spontaneous recovery, renewal or reinstatement cannot be taken as conclusive evidence for the loss of memory, it is possible that the impaired fear response observed after immediate extinction reflects a retrieval deficit rather than memory erasure. Successful recovery following delayed extinction is traditionally

interpreted in that way; for example, the original memory is usually thought to be inhibited rather than permanently eliminated (Myers and Davis, 2002; Bouton, 2004; Delamater, 2004). In the present study we found evidence for spontaneous recovery in both the immediate and delayed groups. However, recovery alone may not be sufficient to indicate a return of the *original* memory. As we noted earlier, spontaneous recovery may be a result of a slowly developing alternate memory storage process that can occur despite impairment of the original trace (Squire and Barondes, 1972). Alternatively, a residual trace remaining after incomplete erasure may be reconstructed by a process such as pattern completion allowing for subsequent expression of the behavior (Nader and Wang, 2006; Amaral et al., 2008). Thus, to ascertain whether the observed recovery was due to expression of the original CS-US association rather than an alternate process, it was necessary to include another test that could make positive predictions for the absence of memory.

Sanders and Fanselow (2003) found that pre-training animals to fear one context rendered learning to fear a second context no longer susceptible to disruption by intra-DH AP5. In other words, a first learning appears to result in a second learning that no longer requires NMDAR in the DH. Therefore, we used the AP5-sensitivity of second learning to assess whether memory of a first learning has survived extinction. We extinguished the first learning and then infused either AP5 or VEH before the second learning. In both the Immediate and Delayed groups, animals that received AP5 were able to acquire the second learning. Thus, immediate extinction of a contextual fear memory did not render subsequent fear conditioning NMDAR-dependent in the DH. To confirm that memory erasure for the first learning would result in an NMDAR-dependent second learning, we

induced impairment for the first learning with pretraining infusions of AP5. Animals that received infusions of AP5 prior to the second learning showed no evidence of a learned context-fear association when tested twenty-four hours later. This demonstrates that NMDAR were only required for second learning if the memory for the first learning had been erased. Importantly, this is consistent with the requirement of NMDAR for a second contextual fear learning after amnesia for the first learning was induced by anisomycin (Hardt et al., 2009).

Considering that extinction itself involves learning, it could be argued that contextual information acquired during this process could take the place of first learning. This predicts that all experiments in which extinction was performed should demonstrate second learning that was AP5 independent. First, as shown in Figure 4, AP5 administered prior to second learning blocked second learning in animals that had immediate extinction following a pre-training infusion of AP5. Even if AP5 was on board at the time of immediate extinction, this remains consistent with the effect observed by Sanders and Fanselow (2003). Second, we have unpublished data indicating that a context exposure alone is not sufficient to make training to fear a second context independent of NMDAR in the DH (Finnie et al., 2007). Furthermore, one could argue that the residual freezing behaviour was a confound in the second learning paradigm. However, the same amount of residual freezing induced by either anisomycin or extinction does not result in a second contextual fear memory that engages the same mechanisms as in the first learning (Hardt et al., 2009). This rules out the possibility of residual performance being a confound to our findings.

Given that NMDAR antagonists interfere with learning and memory, it may appear anomalous to have post-shock freezing in both Experiments 2 and 3, as well as significant amounts of freezing during immediate extinction session 15 min post training. However, AP5 does not appear to affect associative post-shock freezing within three minutes of shock administration (Kim et al., 1991; 1992). Furthermore, in a delayed match-to-place Morris watermaze task, rats infused with AP5 in the DH 30 minutes prior to trial 1 demonstrated similar escape latencies and savings as the control animals if the second intertrial interval between the first and second trial was 15 seconds. Thus, the freezing observed by AP5 treated animals in this experiment, when returned to the context 15 minutes following conditioning, can be explained since there is a short duration where behavioral expression of initial learning is insensitive to AP5.

A further explanation for the discrepancy between the results of this study and the results of Myers et al. (2006) is that we used freezing as a behavioral measure of fear rather than potentiated startle. Whereas in contextual fear the measure of interest can be viewed as the suppression of active behavior, acoustic startle measures the enhancement of a response. However, it is worth noting that Alvarez et al. (2007) more recently reported a similar failure to show erasure of the fear memory following its immediate extinction in human fear-potentiated startle.

It could be argued that our extinction protocols were simply not sufficient to reduce responding sufficiently to impair the memory. In our experiments, extinction was not complete. However, it should be noted that Myers et al. (2006) did not observe complete absence of post-extinction responding as well. In their study, none of the experiments that these authors present does extinction conducted 10 minutes following

training generate startle responses to the light CS that are equivalent to the startle response in the absence of the light CS.

In summary, the present experiments produced no evidence to suggest that immediate extinction of memory for context is able to erase the original trace in both the traditional paradigms or in a new paradigm that can make positive predictions for the absence of a memory. Our results support the hypothesis that extinction whether conducted shortly after training or after a longer delay reflects inhibition by new learning (Rescorla, 1997; e.g. Bouton, 2004). This supports previous findings in which immediate extinction did not result in memory loss (Maren and Chang, 2006; Alvarez et al., 2007; Schiller et al., 2008; Woods and Bouton, 2008).

2.7 Figures

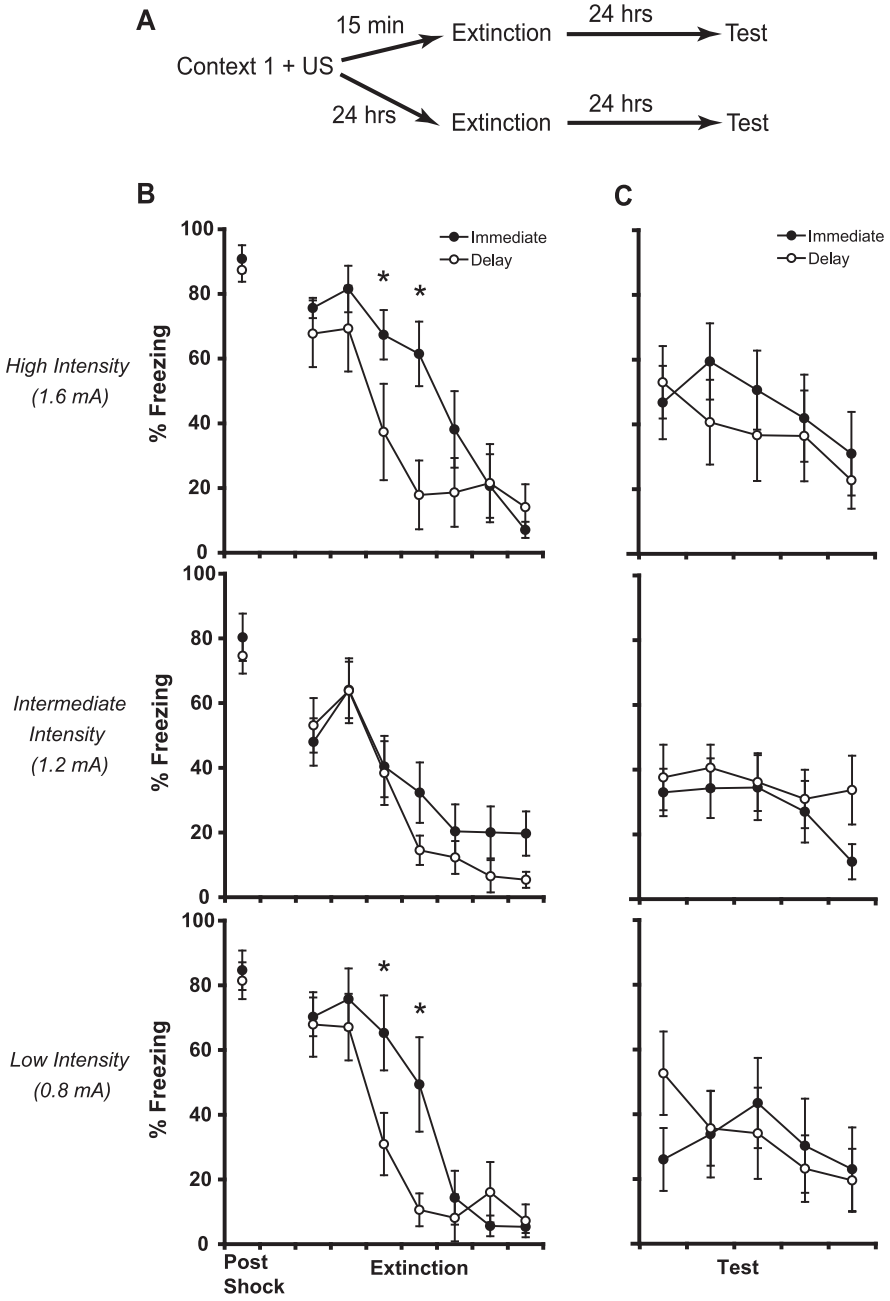

Figure 1. The effect of immediate and delayed extinction as a function of shock intensity and its effect on extinction and spontaneous recovery. Top panel (A) illustrates the experimental procedure. Left panel (B) shows mean (±*SEM*) freezing during immediate and delayed extinction after contextual fear conditioning with the high intensity shock

(1.6 mA; Immediate $n = 8$, Delay $n = 7$), intermediate intensity shock (1.2 mA; both Immediate and Delay $n = 12$), and low intensity shock (0.8 mA; both Immediate and Delay $n = 7$). Post shock data points represent the last 30 seconds of the conditioning session. Extinction data points are the average of two 30 second sample periods taken at 5 minute intervals over the course of the 30 minute extinction session. * $P < 0.05$. Right panel (C) shows mean (±*SEM*) freezing behavior of the test conducted 24 h later for the high intensity shock (1.6 mA), intermediate intensity shock (1.2 mA), and low intensity shock (0.8 mA). The level of recovery in each group for both time points was roughly equivalent ($F_{1,47} = .065$, $P = .8$). Test data points represent the average of two consecutive 30 second sampling periods made over the five minute test session.

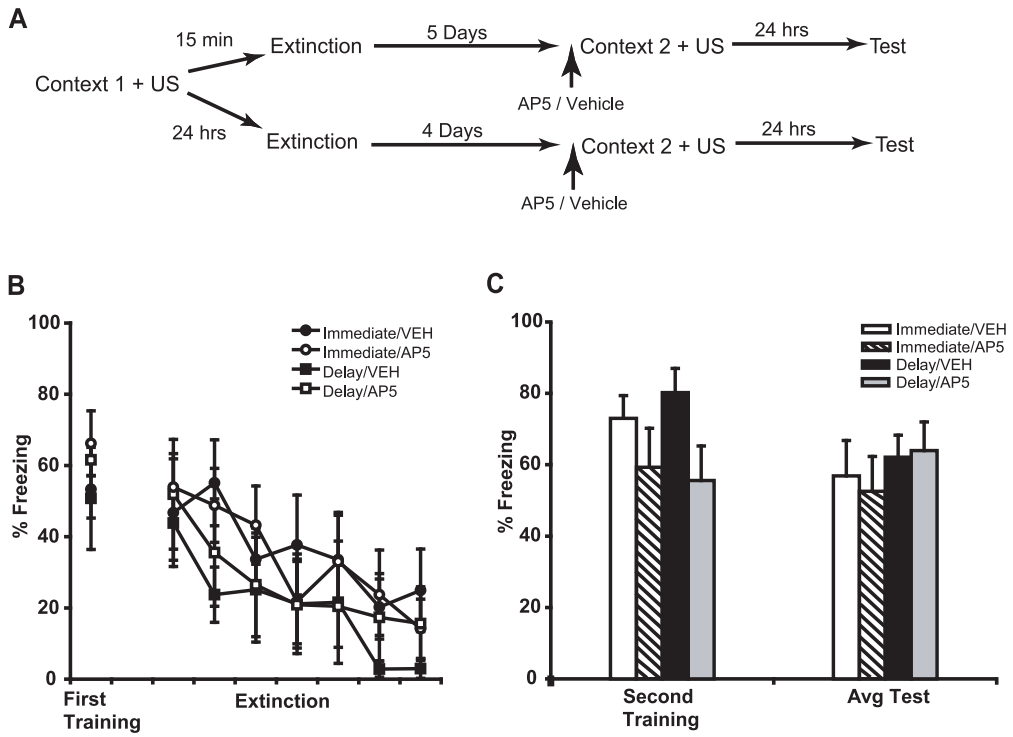

Figure 2. NMDAR blockade by AP5 does not impair second learning after successful immediate or delayed extinction. Top panel (A) illustrates the experiment procedure. Right panel (B) shows post training and extinction freezing levels. All animals received the first contextual fear conditioning followed by either an immediate (15 minutes) or delayed extinction (24 hours) session. All points are means ± *SEM*. Left panel (C) shows mean (± *SEM*) freezing levels following conditioning in a second context and the subsequent memory retention test. Either AP5 or VEH was infused before the second training in a novel context (Immediate/VEH, $n = 7$; Immediate/AP5, $n = 8$; Delay/VEH, $n = 8$; Delay/AP5, $n = 7$). Animals were then tested in this context for memory retention 24 hours later. The test score is the average time spent freezing over the five minute test session.

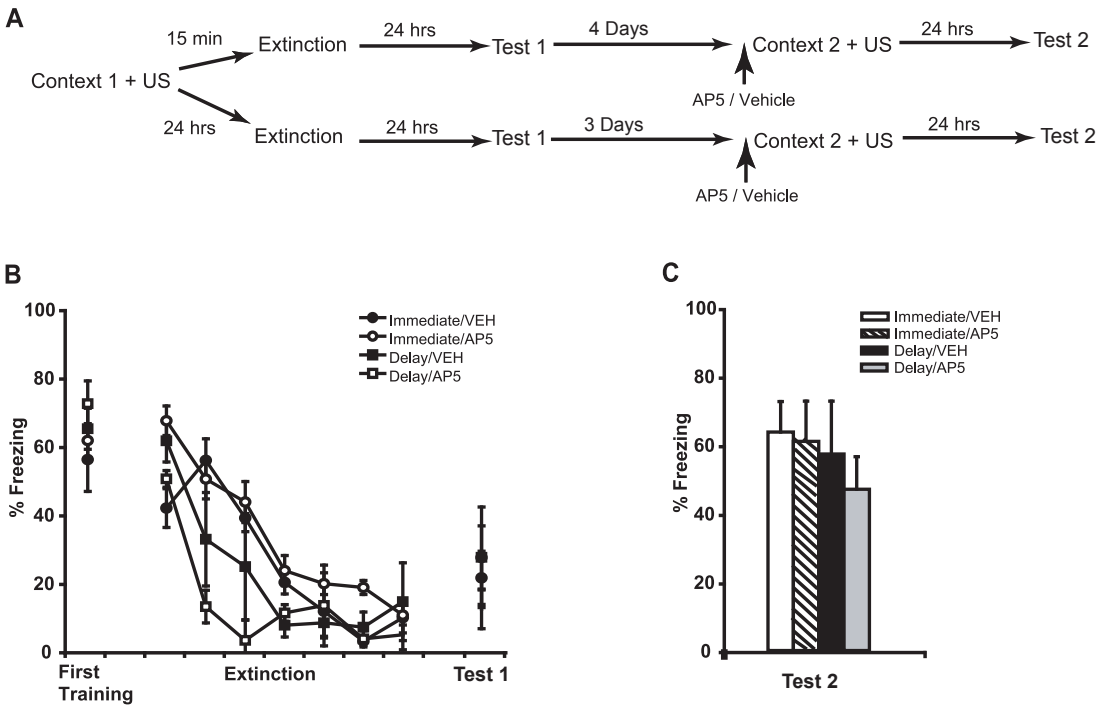

Figure 3. NMDAR blockade by AP5 does not impair second learning after successful immediate or delayed extinction with low and equivalent levels of spontaneous recovery. Top panel (A) illustrates the experiment procedure. Right panel (B) shows post training, freezing during extinction, and freezing on a test of spontaneous recovery. All animals received the first contextual fear conditioning followed by either an immediate (15 minutes) or delayed extinction (24 hours) session. All points are means ± *SEM*. Left panel (C) shows mean (± *SEM*) freezing levels for the retention test given 24 hours after second training. Either AP5 or VEH was infused before the second training in a novel context (Immediate/VEH, $n = 6$; Immediate/AP5, $n = 6$; Delay/VEH, $n = 5$; Delay/AP5, $n = 5$).

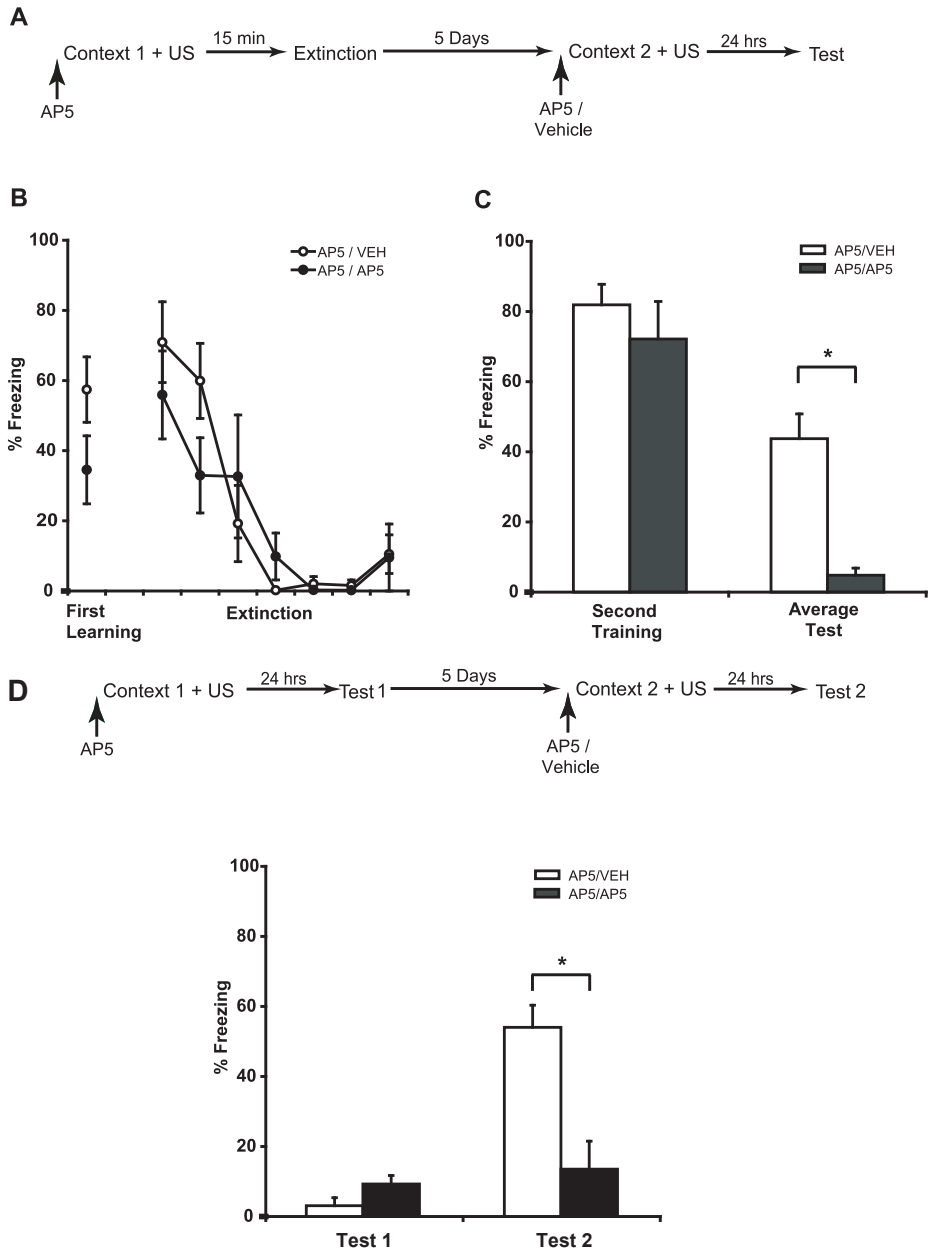

Figure 4. NMDAR blockade by AP5 impairs second learning if AP5 is given prior to first learning and extinction. Top panel (A) illustrates the experimental procedure. Prior to initial fear conditioning and immediate extinction all animals were infused with AP5. Training and extinction in the first context is shown in the left panel (B). Either AP5 (n = 6) or VEH (n = 7) was infused before the second training in a novel context. Memory retention was then tested 24 hours later (C). NMDAR blockade by AP5 impairs second learning if AP5 is given prior to first learning in the absence of extinction training. Prior

to initial fear conditioning all animals were infused with AP5 and either AP5 (n = 5) or VEH (n = 5) before the second training in a novel context. Test of memory retention was tested 24 hours after each conditioning session (D). All points are means ± *SEM*. * $P <$ 0.05.

Chapter 3

Temporal Dynamics of Recovery from Extinction Shortly After Extinction Acquisition

3.1 Preface

In Chapter 2 (Archbold et al., 2010) as in previous studies (Maren and Chang, 2006; Alvarez et al., 2007; Schiller et al., 2008; Woods and Bouton, 2008), it has been shown that extinction training delivered shortly after initial learning does not lead to erasure of the original memory. Collectively, these results lend further support for the idea that extinction is new learning. Thus, a separate memory is formed that inhibits the first.

The review of the neurobiology of extinction in Chapter 1 outlined a number of similarities shared between fear extinction and initial fear learning. Indeed, the molecular mechanisms of extinction resemble those that underlie the formation of new memories. Although extinction has been given the distinction of new learning, few studies have examined short- and long-term phases of a fear extinction trace. Within much of the extinction literature the earliest standard retention test for auditory fear extinction occurs 24 hours after extinction learning. These tests of extinction retention are used to measure "spontaneous recovery" of the conditioned responses with a delay following extinction.

Interestingly, in conditioned taste aversion (CTA), expression of extinction does not develop until two hours after extinction learning (Berman et al., 2003). Thus, conditioned responding does not follow the predicted development of recovery. This raises some important questions regarding extinction of auditory fear. Is there recall of extinction shortly after extinction learning? If not, what might cause this recovery? We set out to determine the time course of extinction retention, measured by spontaneous recovery, shortly after extinction of auditory fear. We show that extinction retention does not follow the typical monotonic function since there is significant recovery of

conditioned responding between one and four hours after extinction learning. Further we show that this is not due to an absent phase of memory (i.e., no short term memory), but rather a result of physiological changes that occur in response to acquisition of extinction.

The results of these studies are important for understanding the mechanisms involved in recovery. Moreover, they contribute to our current knowledge of the extinction process and the control of behaviour by two often competing internal representations of learned fear associations: fear of the tone and no fear of the tone.

Archbold GE, Dobbek N, Nader K (2013) Temporal dynamics of recovery from extinction shortly after extinction acquisition. Learn Mem 20:395-398.

3.2 Abstract

Evidence suggests that extinction is new learning. Memory acquisition involves both short-term (STM) and long-term (LTM) memory components; however, few studies have examined early phases of extinction retention. Retention of auditory fear extinction was examined at various time points. Shortly (1-4 h) after extinction acquisition spontaneous recovery was high compared to longer delays (8-24 h). Recall of a consolidated extinction trace was also impaired if it was preceded 1 h by extinction of a novel CS; propranolol did not attenuate this effect. These results suggest poor extinction retention reflects a retrieval impairment caused by the aversive experience of extinction training.

3.3 Introduction

In Pavlovian fear conditioning pairing a neutral stimulus with an unconditioned aversive stimulus (US) results in conditioned learning, whereby the previously neutral stimulus comes to elicit fear responses. Conversely, the conditioned stimulus (CS) repeatedly presented in the absence of the US, leads to reduction of the fear behaviour known as extinction (Pavlov, 1927). While there has been some debate over the exact nature of extinction, it is widely accepted that this phenomenon is new learning rather than unlearning of the original association (Rescorla, 1997; Bouton, 2004). Return of the extinguished behavior is observed in tests of spontaneous recovery (Pavlov, 1927), renewal (Bouton and King, 1983), and reinstatement (Rescorla and Heth, 1975; Bouton and Bolles, 1979a).

Both behavioural (McGaugh, 1966) and molecular (Kandel, 2001) studies suggest that following learning there are two distinct phases of memory: short-term memory (STM) and long-term memory (LTM). STM, lasting on the order of minutes to hours, involves covalent modifications of preexisting proteins, while LTM is much less transient and involves protein synthesis, new gene expression, and changes in synaptic structure. Disruption of protein synthesis following learning leads to impairments in long term memory retention while sparing short term memory (Davis and Squire, 1984). Short-term memory for fear extinction is often considered to be the within-session responding during extinction training, whereas long-term memory for fear extinction, is measured by successful extinction recall usually tested upwards of 24 hours (Quirk, 2002). Tests of long-term extinction memory are frequently used to measure spontaneous recovery, the return of the original trace that occurs with the passage of time; more recovery is

observed with increasing delays between extinction and test (Pavlov, 1927; Ellson, 1939; Burdick and James, 1970; Robbins, 1990). Surprisingly, few studies have tested memory for fear extinction within 24 hours of extinction acquisition.

Berman et al. (2003) explored the time course of an extinguished conditioned taste aversion (CTA) memory, testing retention of extinction at intervals as short as thirty minutes following extinction. They found that the extinguished behaviour was not evident shortly after extinction, but became apparent with a delay of two hours. The authors further determined that the absence of extinction shortly after extinction training was likely due to generation of a short-term aversive trace that blocked the immediate development of extinction. These results are inconsistent with the idea that recovery of the original memory increases with time. Further, it remains unclear whether extinction of an auditory fear memory will exhibit a similar pattern of recovery. Here we addressed this question and further attempted to determine a mechanism mediating our findings.

3.4 Methods

3.4.1 Subjects

Adult male Sprague-Dawley rats (Charles River Laboratories, Québec) initially weighing 300-325g, were housed individually in plastic Nalgene cages. The rats were maintained on a 12 h light/dark cycle (lights on at 7am, off at 7pm) with food and water available *ad libitum*. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care and Use Committee.

3.4.2 Apparatus

Two conditioning contexts were used: one for fear conditioning and one for testing. Generalization between contexts was minimized by varying a number of dimensions such as shape, illumination, scent, visual and tactile features, and ambient sound. The training context, Context A, was a box (29 cm X 25 cm X 25 cm), manufactured by Med Associates (St. Albans, VT), with aluminum sidewalls and equipped with a stainless steel grid floor (bar radius = 2.5 mm, spread = 1 cm apart). Two lights were on the right wall and one on the left. The lights flashed in alternation at a rate of 1 cycle/second. Context B, used for extinction and testing, had a curved plastic back wall and black-and-white-striped wallpaper (stripes 2.5cm wide) placed on the front wall. The floor was an opaque panel of Plexiglas. In both contexts a digital camera was mounted on the ceiling and videotaped the sessions for later analysis.

3.4.3 Behavioral Procedures

Rats were habituated to the training context for 20 min one day prior to conditioning. On the day of conditioning rats were placed in Context A for a total of 4.5 minutes. In Experiments 1, 3, and 4 rats received two tone presentations co-terminating with shock (0.8mA, 0.5s shock; 5000 Hz, 75dB, 20s tone). In Experiment 2 animals were fear conditioned to both a tone and a white noise (2 kHz, 75 dB, 20s hiss). Training of each stimulus took place in separate sessions on consecutive days. The presentation order of stimuli was counterbalanced such that half the animals received tone as CS1 and white noise as CS2. Extinction was conducted in Context B and consisted of 16 non-reinforced stimulus presentations in a 30 minute session. Test sessions involved three stimulus presentations in Context B. Injections (i.p) of propranolol (5.0 mg/kg) were given to rats

in their home cage in Experiment 3. dl-Propranolol hydrochloride was dissolved in saline (0.9%) to provide a final concentration of 1.0 mg/ml.

In Experiment 1 rats were trained in Context A with two tone-shock pairings. Three days following conditioning, rats received one extinction session in Context B and were tested in the same context for retention of extinction (i.e. the inhibition of conditioned responding) 1, 4, 8, or 24 hours later.

In Experiment 2 rats were conditioned on two consecutive days to two different stimuli (CS1 and CS2). Three days following conditioning of CS2, CS1 was extinguished in Context B. The next day animals received an extinction session of CS2. Retention of CS1 extinction was then tested either one or 24 hours after termination of CS2 extinction.

The behavioural procedures of Experiment 3 and 4 were identical to Experiment 1. However, in Experiment 3, animals were injected with propranolol 20 minutes prior to the retention test. In Experiment 4, injections of propranolol were given 20 minutes prior to extinction. Further, only the 1 hour group was tested.

3.4.4 Data Analysis

Memory was evaluated by manually measuring the duration of freezing (immobilization except for respiratory movements) during the tone presentation with a stopwatch.

3.5 Results

We first determined whether extinction of an auditory fear memory is expressed shortly after extinction acquisition by comparing levels of freezing to a tone at different time points following extinction. Rats were divided into four groups and conditioned

using two tone presentations co-terminating with shock (0.8mA, 0.5s shock; 5 kHz, 75dB, 20s tone). Three days later all groups received16 non-reinforced stimulus presentations over 30 minutes in Context B. Memory for extinction was tested in Context B 1, 4, 8, or 24 hours (Figure 1A) after extinction training. Extinction produced roughly equivalent within-session reductions in freezing. Figure 1B shows that recovery was greater in groups tested 1 and 4 hours after extinction than the groups tested at 8 and 24 hours. A 4 (Group) x 2 (Block) mixed ANOVA comparing the last two-trial block of extinction with the test confirmed a main effect of Group, $F_{(3, 45)} = 5.41$, $P < 0.05$ and a main effect of Block, $F_{(1, 45)} = 50.54$, $p < 0.001$. There was also a Group x Block interaction, $F_{(3,45)} = 4.65$, $P < 0.05$, indicating that freezing was higher in groups tested after a short delay. Overall, the results suggest that extinction behavior is absent at short-term time points after extinction acquisition, but develops with longer delays between extinction training and test. Consistent with this, in rats that had received two spaced extinction sessions, an extinction-test interval of two minutes resulted in more recovery of freezing to a feared context than an interval of 24 hours (Morris et al., 2005a).

The transient recovery may reflect a breakdown between phases of memory that support performance (i.e., intermediate-term memory) or a retrieval impairment due to physiological fluctuations produced by the extinction session. In a second experiment we tested whether the absence of extinction behavior was a specific property of the CS being extinguished or would generalize to other previously extinguished CSs. Animals were fear conditioned to both a tone and a white noise (2 kHz, 75 dB, 20s hiss) during separate sessions on consecutive days, the presentation order counterbalanced such that half the animals received tone as CS1 and white noise as CS2. Both stimuli were then

extinguished in separate extinction sessions on successive days. Expression of extinction of CS1 was tested either one hour or 24 hours following the extinction of CS2 (i.e. 25 or 48 hours after extinction of CS1) (Figure 2A). At test, 25 hours had elapsed since extinction of CS1 and we might predict that the expression of extinction would be intact; however, the results in Figure 2B demonstrate that this was not the case. Recent extinction of a second CS is sufficient to reinstate responding to a previously extinguished CS. A 2 (Group) x 2 (Block) mixed ANOVA on the last two trial block of S1 extinction and test session revealed main effects of Group, $F_{(1, 23)} = 10.20$, $P < 0.05$, and Block, $F_{(1, 23)} = 8.19$, $P < 0.05$. Importantly, the Group x Block interaction was also significant, $F_{(1, 23)} = 5.58$, $P < 0.05$, indicating that there was greater recovery of fear amongst animals that were tested for freezing to CS1 an hour after extinction of CS2 compared to those tested 24 hours after extinction of CS2.

These results were similar to the first experiment: a test given shortly after extinction led to fear recovery. However, in this experiment return of responding to the CS was not a result of a deficit in short-term or intermediate-term extinction memory. Indeed, expression of extinction may be impeded by changes in the physiological state of the animal that occur during extinction training.

Adrenergic transmission has been implicated in the formation and persistence of emotional memories (Izquierdo and Medina, 1995; Cahill et al., 2000). Similarly, propranolol, a β-adrenergic receptor antagonist, has been used to reduce fear and anxiety in both humans and rats (Gorman and Dunn, 1993; Kent et al., 2002). Morris et al. (2005b) discovered that a brief exposure to a feared context was sufficient to reinstate freezing to an extinguished CS, but only if the exposure occurred two minutes before the

CS test and not 24 hours prior. The reinstatement of freezing following exposure to a feared context appears to be dependent on β-adrenergic activation as treatment with propranolol attenuated this effect.

In a third experiment we set out to address whether adrenergic activity was mediating the transient recovery from extinction. Behavioural procedures of Experiment 3 were identical to Experiment 1; however, 20 minutes prior to the CS1 retention test, animals were injected intraperitoneally with 5 mg/kg of propranolol or vehicle (Figure 3A). All results are shown in Figure 3B. A three-way mixed ANOVA of Test (1hr vs 24hr) x Drug (Prop vs Veh) x Block (last extinction vs test) revealed no main effect of drug or testing time and no interaction between the two. Analysis did indicate a significant main effect of Block $F_{(3, 28)} = 19.38$, $P < 0.0001$ and significant interaction of Block x Test, $F_{(3, 28)} = 14.10$, $P < 0.001$. Overall, these results indicate that under these conditions treatment with propranolol was unable to prevent the transient recovery observed shortly after extinction.

This is in contrast to previous findings in which propranolol was able to block reinstatement of extinguished fear following exposure to a dangerous context (Morris et al., 2005b). Nonetheless, in these experiments propranolol was given prior to the context exposure which reinstated freezing to an extinguished CS. Consequently, we determined whether propranolol given before extinction was able to block increases in adrenergic activity that might develop during extinction which would effect the short interval retention test. Examining only the one hour group, propranolol was injected 20 minutes prior to extinction acquisition (Figure 3C). Results indicated no differences between treatment groups as both groups showed significant recovery during the 1 hour retention

test after extinction acquisition as indicated by a significant effect of block, $F_{(1,14)} = 4.59$, $P < 0.0001$. Even at this time point propranolol was unable to prevent recovery of the original fear conditioning.

3.6 Discussion

The present experiments suggest that memory for auditory fear extinction does not have the same temporal pattern of behavioural expression as predicted by our current understanding of spontaneous recovery. Although significant within-session (short-term) extinction was observed, memory for extinction was absent at 1 and 4 hour time points. Good extinction retention was again observed in the 8 and 24 hour test groups. This is consistent with the profile of behaviour following CTA extinction (Berman et al., 2003) and context fear extinction (Morris et al., 2005a). Generally, the amount of spontaneous recovery is considered a function of the time between extinction and test, such that greater recovery is observed with longer retention intervals. The present results suggest that spontaneous recovery follows a nonmonotonic function that resembles the U-shaped retention curve first described by Kamin (1957).

The "Kamin effect" has been widely demonstrated (Gerber and Menzel, 2000; Sutton et al., 2001; Rudy and Wright-Hardesty, 2005; McNally et al., 2008). It was initially thought to be a result of two memory systems. One system was responsible for immediate retention, losing strength rapidly after acquisition. A second system, requiring time for consolidation, gradually assumed responsibility for memory retention and expression. Any transient lapse in memory retention was thought to reflect a discontinuity between these two systems. A second interpretation has also been proposed suggesting that the observed lapse in memory is a result of a retrieval impairment due to a

discrepancy in the internal state of the animal at intermediate intervals compared to immediate or long-term intervals (Klein and Spear, 1970; Klein, 1972).

Discontinuous expression of extinction may reflect absence of the memory or the inability to retrieve the extinction memory and respond accordingly. Berman and colleagues (2003) note that extinction itself may be aversive and lead to hormonal and neurochemical changes that promote reinstatement of fear. Indeed, we found that testing a previously extinguished CS (CS1) an hour after extinction of a second CS (CS2) elicited spontaneous recovery. If however the CS1 extinction retention test occurred 24 hours after extinction of CS2, there was little recovery. The absence of extinction was not specific to a recently extinguished CS and instead generalized to a previously extinguished CS; thus, the aversive experience of extinction may be sufficient to reinstate freezing to a different previously extinguished CS. These results are consistent with the observations that a temporally recent pre-exposure to a feared context can reinstate extinguished auditory fear memory (Morris et al., 2005a) and exposure to a different malaise associated taste is sufficient to reinstate an extinguished conditioned taste (Berman et al., 2003).

Interestingly, in an appetitive task in which the CS is not an aversive stimulus, memory for extinction is observed five hours following extinction training (Brooks and Bouton, 1993). Thus, the 'lapse' of extinction at a short retention interval may not be a characteristic of all extinction memories. Also contrary to our findings, Quirk (2002) showed that 30 minutes after extinction of an auditory fear memory, extinction retention was successful. Discrepancies in the results may be due to stark differences in

behavioural protocols or they may indicate that impaired extinction recall does not occur until after 30 minutes.

It is well established that context can influence responding to a CS after extinction (Bouton et al., 2006). Beyond the modulatory effect of the physical context, there is evidence that the emotional state of the animal can serve as a 'context' and lead to the return of the fear responding (Richardson et al., 1984). It has previously been shown that administration of propranolol prior to a brief period spent in a feared context, prevents reinstatement of freezing to an extinguished CS that would have otherwise occurred (Morris et al., 2005b). Based on this finding, we hypothesized that the adrenergic system, in response to the aversive experience of being reexposed to the feared CS during extinction, might be mediating the transient spontaneous recovery. Propranolol was administered twenty minutes prior to the retention test in both 1 hour and 24 hour groups. Additionally, we administered propranolol 20 minutes prior to the extinction session and then tested for retention at the 1 hour time point. Under both conditions propranolol was unable to prevent the transient recovery of freezing observed in the 1 hour group. These results suggest that blocking β-adrenergic activity is unable to attenuate the effects of emotional arousal resulting from the recent experience of extinction. This is in contrast to previous findings in which propranolol was able to block reinstatement of extinguished fear following exposure to a dangerous context (Morris et al., 2005b).

The currently established neurocircuitry for fear extinction memories involves the basolateral amygdala (BLA), the central amygdala (CeA), the ventral medial prefrontal cortex (vmPFC), and the hippocampus (HC). The infralimbic cortex (IL) appears to be the critical site in the vmPFC involved in extinction consolidation and retrieval (Laurent

and Westbrook, 2009; Sierra-Mercado et al., 2011). Inputs to the intercalated (ITC) cells in the amygdala from the IL are also necessary for extinction (Likhtik et al., 2008) and activity in the ITC subsequently suppresses activity in CeA neurons (Quirk et al., 2003; Amano et al., 2010). Given that prelimbic (PL) activity impairs extinction and increases fear expression (Vidal-Gonzalez et al., 2006) while IL activity suppresses fear after extinction (Quirk et al., 2006), it is reasonable to suspect the transient recovery from extinction we observe soon after acquisition may result from disruptions in IL activity and ITC projections to the CeA necessary for fear suppression.

Exposure to stressors results in a number of neurochemical changes within the mPFC. Extinction of eyelid conditioning causes an increase in the extracellular levels of dopamine and noradrenaline in the mPFC that decrease slowly in the two hours following (Hugues et al., 2007). Either of these in addition to other neuromodulators may be involved inhibiting activity in the IL. Further determining the neurochemical changes that accompany IL activation will help elucidate the mechanisms involved in the transient recovery from extinction.

In conclusion, our results suggest that auditory fear extinction is not expressed shortly after extinction training. Poor extinction recall, as measured by spontaneous recovery, follows the nonmonotonic function characteristic of the "Kamin effect". This impairment in retention does not appear to be a result of a storage failure nor is it mediated solely by β-adrenergic activity. Understanding the mechanisms and neural circuitry involved in the formation and expression of extinction remains to be fully understood. Future studies directed in this area will likely shed light on the interplay

between the expression of fear and the expression of fear extinction and may account for the transient recovery from extinction.

3.7 Figures

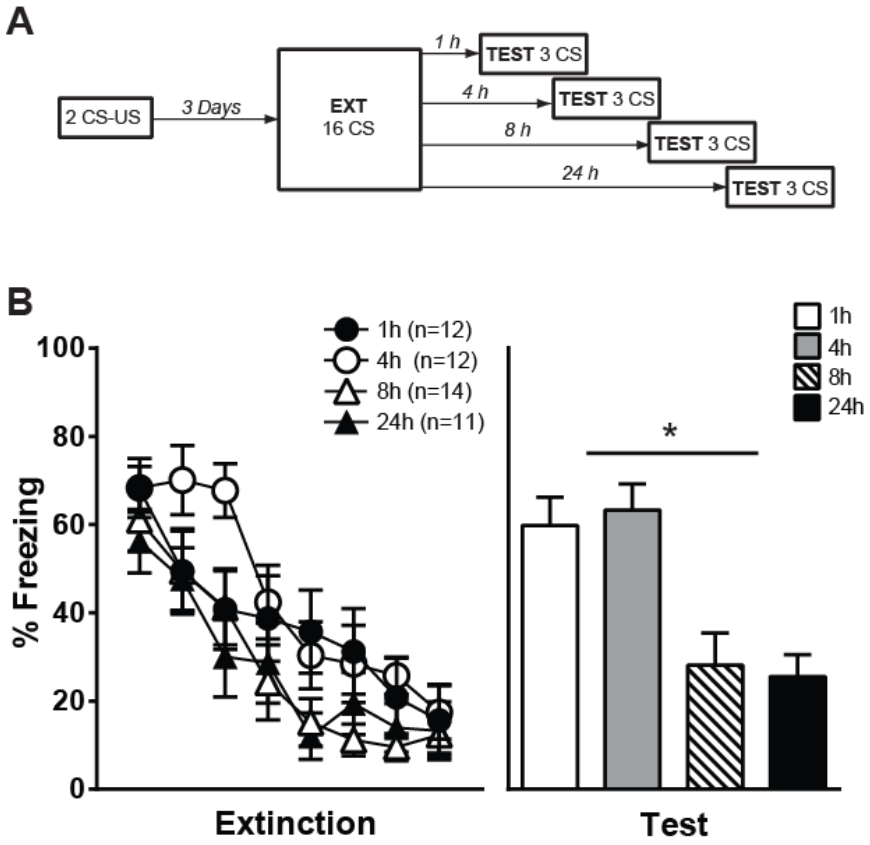

Figure 1. Test for extinction retention at various time points after extinction acquisition. ***A,*** Experimental design. ***B***, Extinction acquisition. Mean (± SEM) freezing to the tone averaged over two 20 sec stimulus presentations. All animals acquired comparable extinction. Test of extinction memory retention represents mean (± SEM) freezing to the tone averaged over 3 presentations. Animals were divided into four groups and tested 1, 4, 8, or 24 hours after extinction acquisition, respectively. Rats in the short delay groups (1h and 4 h) exhibited high levels of freezing compared to the last block of extinction and to the longer delay testing groups (*$P < 0.05$).

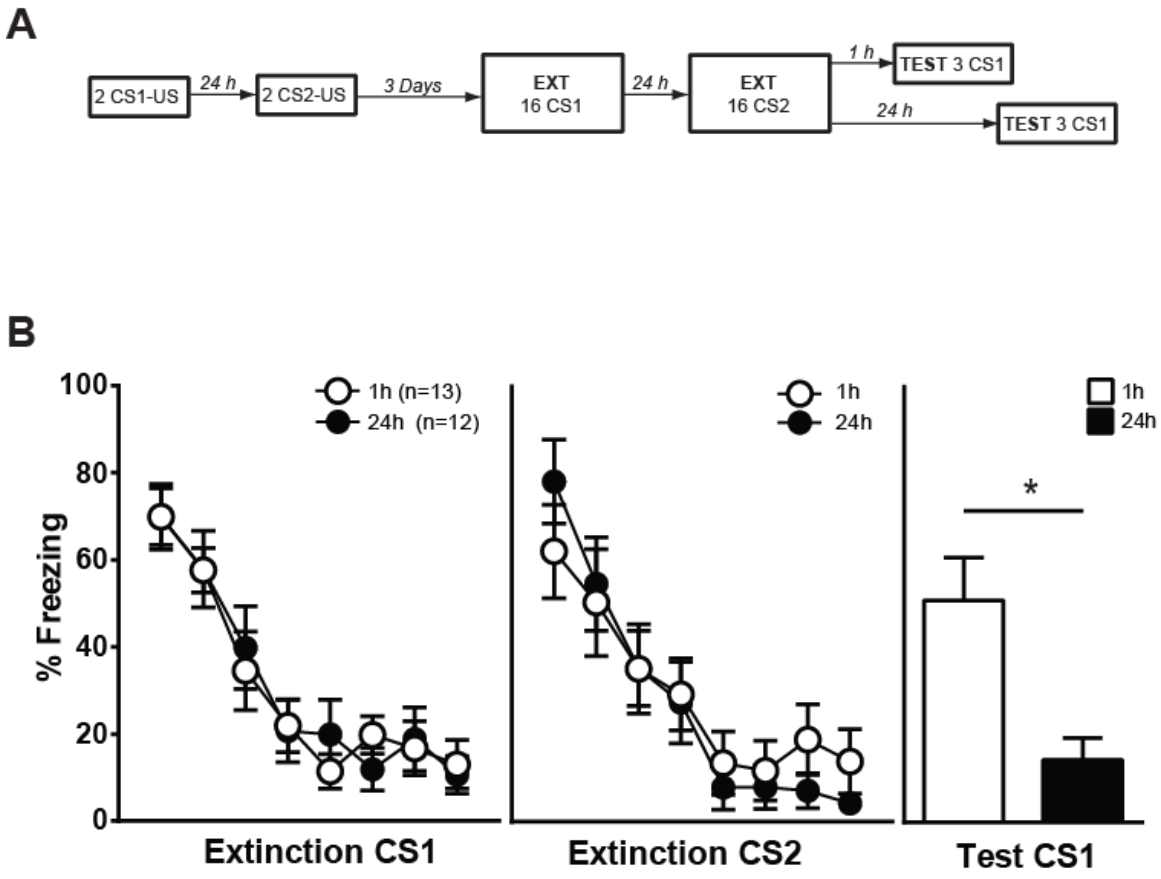

Figure 2. Recent extinction training of a second CS is sufficient to reinstate responding to a previously extinguished CS. ***A***, Experimental design. ***B***, Extinction acquisition for two conditioned stimuli, CS1 and CS2. Points represent mean (± SEM) freezing to the tone averaged over two 20 sec stimulus presentations. Extinction was comparable between groups for both stimuli. Extinction retention for the first CS was tested 1 or 24 hours after CS2 acquisition. Test represents mean (± SEM) freezing to the tone averaged over 3 presentations. Animals tested 1 h after cessation of CS2 extinction exhibited significantly more freezing to CS1 than animals tested 24 h later (*$P < 0.05$).

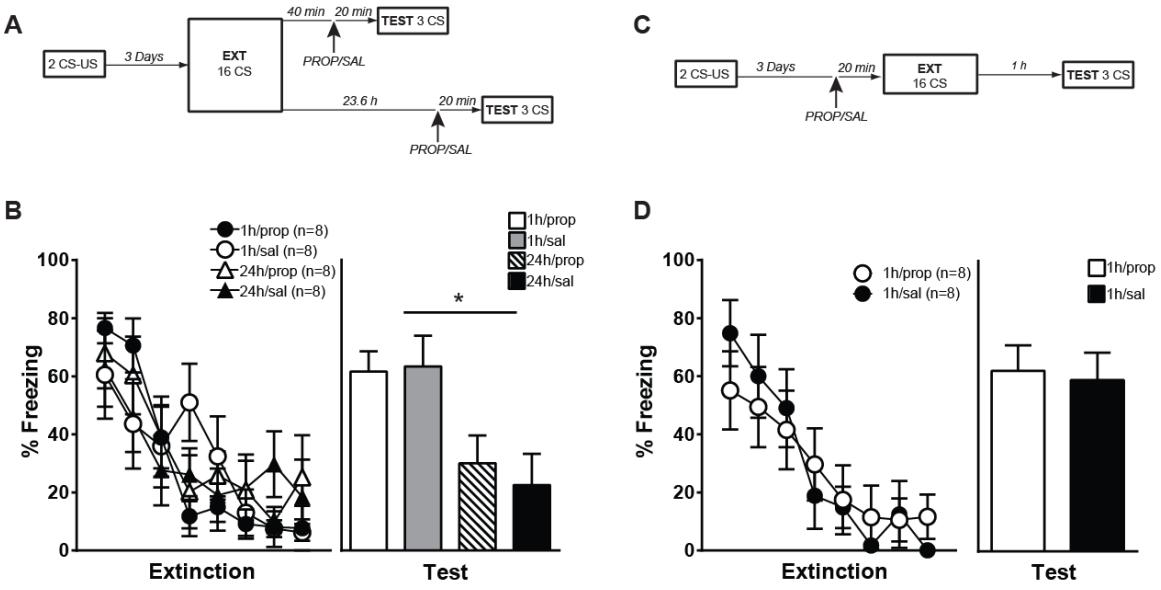

Figure 3. Propranolol does not attenuate the recovery of freezing to the CS tested shortly after extinction. ***A***, Experimental design. ***B***, Extinction and average test for propranolol treatment 20 minutes prior to test. Points represent mean (± SEM) freezing to the tone averaged over two 20 sec stimulus presentations. Extinction was comparable across all groups. Propranolol was ineffective at reducing recovery of freezing in the 1 h group during the retention test (*$P < 0.05$). ***C.*** Experimental design. ***D.*** Propranolol given before extinction was unable to prevent recovery of freezing in the 1 hour test group (*$P < 0.05$).

Chapter 4

The Interaction Between N-Ethylmaleimide-Sensitive Factor and GluA2 is Involved in the Maintenance of Fear Extinction Memory

4.1 Preface

In Chapter 2 (Archbold et al., 2010) and reviewed in Chapter 1, it has been shown that extinction involves new learning rather than erasure. New learning is understood to involve activity-dependent changes in synaptic strength and connections between neurons, physiologically represented by long-term potentiation (LTP) (Bliss and Collingridge, 1993). Although memory can persist for a lifetime, many of these synaptic processes are transient and are no longer involved after consolidation. How then are memories maintained? Answers to this question point to the involvement of α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor (AMPAR) trafficking, an important player in both synaptic plasticity and stabilization (Malinow, 2003; Anggono and Huganir, 2012).

The experiments reported in this chapter were motivated by the discovery that maintenance of both auditory fear and object location memories, in the amygdala and DH respectively, involve the regulation of GluA2-containing AMPAR trafficking by the constitutively active isoform of protein kinase C, protein kinase Mzeta (PKMζ) (Migues et al., 2010). PKMζ has also been implicated in the maintenance of several other forms of memory across a number of different brain regions (Pastalkova et al., 2006; Shema et al., 2007; Kwapis et al., 2009; Hardt et al., 2010b). In addition PKMζ appears to mediate maintenance of LTP by regulating the N-ethylmaleimide sensitive factor (NSF) interaction with GluA2 (Yao et al., 2008). This interaction prevents binding of other endocytotic promoting molecules thus allowing for stablization of AMPARs at the synapse.

Recently, peptides shown to block the binding of NSF to GluA2 have impaired the maintenance of object location memory in the dorsal hippocampus (Migues et al., 2012). This result implies that the NSF-GluA2 interaction may be required for the maintenance of other memories. In Chapter 4, we examine the necessity of this interaction in maintaining extinction memory. One day after successful extinction retention, rats were infused with an interference peptide Tat-pep-R845A, known to specifically target NSF-GluA2 binding, into the IL or DH. Rats were then tested for extinction recall one day later. We found that Tat-pep-R845A into the IL led to significant spontaneous recovery compared to the scrambled peptide control. Infusions of Tat-pep-R845A into the DH led to attenuation of renewal when rats were returned to the original training context. Together these results suggest that auditory fear extinction memory is maintained, at least in part, by the IL and DH and requires stabilization of GluA2 at the synaptic membrane by way of the NSF-GluA2 interaction.

Archbold, GE, McKelvey, K, Hardt, O, Nader, K (2013) The interaction between N-ethylmaleimide-sensitive factor and GluA2 is involved in the maintenance of fear extinction memory. *In preparation.*

4.2 Abstract

In fear conditioning, a conditioned stimulus (CS, e.g., tone) is paired with an unconditioned stimulus (US, e.g., foot shock), such that the animal expresses fear to the CS alone. The animal can learn to suppress this fear response during extinction when the CS is repeatedly presented without the US. The infralimbic cortex (IL) has been implicated in the acquisition and consolidation of extinction memory. Accordingly, changes in synaptic plasticity within the IL may be responsible for long-term maintenance of an extinction trace. Persistent NSF-GluA2 dependent trafficking of AMPA receptors to the synapse is involved in the maintenance of L-LTP and long-term memory storage in other memory paradigms involving other brain structures. To assess the possible role of NSF-GluA2 dependent AMPAR trafficking in maintaining extinction memory within the IL we infused a specific NSF-GluA2 interaction inhibitory peptide, Tat-pep-R845A, directly into the IL. Infusions of Tat-pep-R845A led to a significant impairment in extinction retention compared to animals treated with the scrambled control peptide. Attenuating AMPAR endocytosis by infusing the peptide Tat-GluA2$_{3Y}$ into the IL one hour before infusions of Tat-pep-R845A prevented the recovery of fear one day later and upheld the expression of extinction. These results suggest that GluA2/NSF interaction is involved in a pathway that maintains long-term memory by regulating GluA2-containing

AMPAR trafficking. Further, these results confirm that extinction does not erase the original fear memory, but rather leads to acquisition of memory that inhibits expression of the fear response.

4.3 Introduction

In classical conditioning a conditional stimulus (CS) is associated with an unconditional stimulus (US), whereby the CS comes to elicit a conditioned response (CR). Extinction training, during which the CS is presented repeatedly in absence of the paired US, results in a reduction in conditioned responding. It is well established that extinction does not "erase" the original trace; rather, it is mediated by new learning that inhibits expression of the original memory (Rescorla, 1997; Bouton, 2004) as return of conditioned responding is observed in tests of spontaneous recovery (Pavlov, 1927), renewal (Bouton and King, 1983), and reinstatement (Rescorla and Heth, 1975; Bouton and Bolles, 1979a).

Although it is generally accepted that extinction involves new learning and associated neural plasticity, the mechanisms involved in the maintenance of such an inhibitory association are poorly understood. The amygdala, hippocampus (HC), and medial prefrontal cortex (mPFC) appear to be differentially involved in the acquisition, consolidation, and retrieval of extinction (Sierra-Mercado et al., 2011). In particular, the infralimbic (IL) region of mPFC is important for extinction consolidation and retrieval; lesions and inactivations of the IL prior to extinction impair subsequent retrieval (Quirk et al., 2000; Lebron et al., 2004; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011) and inactivation of the IL prior to extinction retention leads to fear recovery (Laurent and Westbrook, 2009). Additionally, the dorsal hippocampus (DH) is implicated in the context specificity of extinction (Corcoran and Maren, 2004; Zelikowsky et al., 2012) and DH inactivation prior to an extinction test attenuates renewal (Corcoran and Maren, 2001; Corcoran et al., 2005), the return of responding that occurs when the extinguished CS is

tested outside the extinction context. The DH may provide the contextual information necessary to differentiate the two opposing outcomes associated with the CS: CS leads to shock and CS leads to no shock. Still, there is currently no general agreement on a site or mechanism whereby the brain retains extinction and very few studies have examined the maintenance of extinction memories. Previous data suggest the IL is a likely candidate site for this process (Quirk et al., 2000), but the DH may also be involved given its role in spatial processing and storing contextual representations (Fanselow, 2000).

The constitutively active kinase PKMζ has been implicated in the maintenance of a number of forms of memory in many brain systems (Serrano et al., 2005; Pastalkova et al., 2006; Shema et al., 2007; Migues et al., 2010; Hardt et al., 2010b) including that of drug related cue extinction in the IL (He et al., 2011). Although there has been some debate regarding the ubiquity of PKMζ (Volk et al., 2013), its involvement in regulating GluA2 containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) at the synapse (Ling et al., 2006; Yao et al., 2008; Migues et al., 2010) highlights an important mechanism in maintaining memory. The trafficking protein N-ethylmaleimide-sensitive factor (NSF) appears to be particularly important for stabilizing GluA2-containing AMPARs in the post-synaptic membrane (Braithwaite et al., 2002; Lee et al., 2002). Disruption of the NSF-GluA2 interaction interferes with AMPAR synaptic transmission (Nishimune et al., 1998; Song et al., 1998) and causes a reduction in the rate of AMPAR insertion (Araki et al., 2010). Recently, our lab has shown that the maintenance of object location memory is impaired when the NSF-GluA2 interaction is disrupted (Migues et al., 2012). Based on these results we tested whether the IL maintains memory for auditory fear extinction and whether maintenance of this inhibitory

memory was dependent on the interaction of NSF-GluA2. We further tested if contextual information maintained in the DH was involved in renewal of fear responding.

4.4 Materials and Methods

4.4.1 Subjects

Adult male Long Evans rats (Charles River Laboratories, Québec) initially weighing 275-300g, were housed in pairs in plastic Nalgene cages with environmental enrichment. The rats were maintained on a 12 h light/dark cycle (lights on at 7am, off at 7pm) with food and water available *ad libitum*. All experiments were carried out during the light phase. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care and Use Committee.

4.4.2 Surgery

Rats were anesthetized (3.0 mg/kg xylazine, 50.0 mg/kg ketamine and 0.175 mg/kg DexDormitor) and then mounted into a Kopf stereotactic frame. Stainless steel double-guide cannulas (22 gauge; Plastics One) were implanted aiming at the infralimbic cortex (IL) (A/P 2.5, D/V -4.40, 0.6 from midline) (Paxinos and Watson, 2005). In experiment 3 stainless steel guide cannulas (22 gauge; Plastics One) targeting the dorsal hippocampus (DH) (A/P -3.6, D/V -2.4, +/-3.1 from midline) were implanted. Three jewelry screws implanted in the skull and acrylic cement were used to stabilize the cannula. Obturators inserted in the guides prevented blocking. After surgery, an intra-musclar injection of analgesic (Carprofen, 5 mg/kg) was given. An intra-peritoneal injection of Antisedan (7.5 mg/kg) suspended anesthesia. Animals were given 7 days to recover from surgery and handled daily during the recovery period. At the end of the

experiment, cannula placement was checked by examining 50-μm brain sections stained with formal-thionin under a light microscope.

4.4.3 Drug Infusions

To interfere with the NSF-GluA2 interaction we used a peptide that mimics the NSF-binding site on GluA2, pep-R845A. All peptides used in these experiments were fused to Tat protein to allow for membrane permeability. Tat-pep-R845A peptide (Biotin-YGRKKRRQRRRKAMKVAKNPQ, Anaspec), tat-Scr-pep-R845A (Myr-RLYRKRIWRSAGR-OH, Anaspec) was dissolved in ACSF. $GluR2_{3Y}$(TAT(47–57)-^{869}YKEGYNVYG^{877}), and Scr-$GluR2_{3Y}$ (TAT(47–57)-AKEGANVAG) were dissolved in 100 mM Tris-saline (pH 7.2). They were infused into the IL (1 μl per hemisphere) via a microinjector (28 gauge) connected to a Hamilton syringe with plastic tubing at a rate of 0.25 μl per min. The injector remained connected for an additional min to allow for drug diffusion away from the tip of the cannula.

4.4.4 Apparatus

Two conditioning contexts were used: one for fear conditioning and one for extinction and testing. Generalization between contexts was minimized by varying a number of dimensions such as shape, illumination, scent, visual and tactile features, and ambient sound. The training context, Context A, consisted of a box (30 cm × 26 cm × 33 cm), manufactured by Coulbourn Instruments, placed into a sound attenuating chamber. One wall was made of stainless steel and had a light bulb providing dim light. The other walls were made of colorless Plexiglas. The floor was a level stainless steel grid (bar radius 2.5mm, spread 1cm apart). Vanilla scent was sprayed into one corner of the box before the animal was put into it. A digital camera in front of the box recorded the sessions. Room lights were off and illumination was dimmed.

Context B, used for extinction and testing, was a box (29 cm × 25 cm × 25 cm) manufactured by Med Associates (St. Albans, VT). The box consisted of a curved white Plexiglas back wall and front wall covered by black-and-white-striped wallpaper (stripes 2.5cm wide). The floor was an opaque panel of Plexiglas. Peppermint scent was sprayed onto the curved plastic wall before the animal was put in the box. A ventilation fan provided a constant background noise. A digital camera was mounted on the ceiling and videotaped the sessions for later analysis.

4.4.5 Behavioral Procedures

Rats were habituated to the extinction context for 20 min for two consecutive days prior to conditioning. On the day of conditioning rats were placed into the box in Context A for a total of six minutes. After two minutes, three tones (5 kHz, 75dB) were presented for 30 seconds and each tone co-terminating with a foot shock (0.9 mA, 1s shock). Tones were separated by a 60 s interstimulus interval (ISI). Following the final tone, rats were given an additional 30s in the context before being removed from the box and transported back to the colony room.

Twenty-four hours after conditioning, rats underwent three consecutive days of extinction training in Context B. In each session, following a two minute acclimation period, they received 12 tone-alone presentations, each one separated by a 60 sec ISI. Rats remained in the boxes for an additional 60 sec before being removed to their homecage and returned to the colony room. Retention tests of extinction memory were conducted 1 and 3 days after the final extinction session. In experiments 1 and 2 rats were returned to Context B and after 2 minutes in the context, presented with 3 tones (60 sec ISI). The animals remained in the context for a further 60 sec before being removed and returned to the colony room. In experiment 3 the second retention test occurred in

Context A (ABA renewal) but tone presentations remained the same. Peptide infusions occurred in the colony room on the day between retention tests.

4.4.6 Data Analysis

Memory was evaluated by manually measuring the duration of freezing (immobilization except for respiratory movements) during the 30s tone presentation with a stopwatch. Baseline freezing (PreCS) to the context was also scored for 30s prior to CS onset. During extinction tone presentation were divided into 6 blocks averaging 2 tone presentations. Retention tests were presented as the average of 3 tone presentations. Data were statistically analyzed using repeated measures ANOVAs. Tukey-Kramer HSD post-hoc comparisons were performed for significant results.

4.5 Results

4.5.1 Disrupting the NSF-GluA2 interaction in the IL impairs extinction retention

The aim of the first experiment was to determine whether the IL maintains an extinction memory and if its maintenance was dependent on the interaction between NSF and GluA2. If the interaction is important for the memory maintenance, then disrupting it should impair expression of extinction memory. To investigate this, rats were fear conditioned and were given three days of extinction training. They were then tested for extinction retention (mean freezing ±SEM during extinction and test are shown in Figure 1). One day after the first test, rats were infused into the IL with Tat-pep-R845A, an inhibitory peptide that targets the NSF-GluA2 interaction, or with the inactive scrambled control peptide. On the following day, memory for extinction was again tested. Extinction acquisition was comparable for both Scr (n= 6) and pep-R845A (n=7) treated rats over the three days (Fs < 1) and both groups demonstrated robust extinction across

each separate day (Ext 1 $F_{(5,7)} = 5.24$, $P = .025$; Ext 2 $F_{(5,7)} = 8.20$, $P = .007$; Ext 3 $F_{(5,7)} = 4.72$, $P = .03$). Importantly, there was no significant interaction between treatment and extinction block over the three days of extinction acquisition (Ps > .05).

Comparison of test 1 and test 2 revealed a significant main effect of treatment ($F_{(1,11)} = 6.23$, $P = .029$) (Figure 1C). There was also a significant main effect of test time as well as a significant interaction (test: $F_{(1,11)} = 9.24$, $P = 0.01$; treatment X test: $F_{(1,11)} = 11.18$, $P < 0.01$). Post-hoc comparisons revealed there were no differences between groups at Test 1 ($P > .05$) but the pep-R845A infused animals froze significantly more than the Scr group at Test 2 ($P = 0.01$). These results suggest that inhibiting the NSF-GluA2 interaction in the IL after memory consolidation has been completed leads to a loss of extinction memory. These findings are consistent with the hypothesis that the interaction of NSF and GluA2 is necessary for memory maintenance.

4.5.2 Preventing AMPAR endocytosis blocks the effects of pep-R845A

It has been proposed that NSF serves to maintain GluA2-containing AMPA receptors at the synapse (Braithwaite et al., 2002); consequently, disrupting the NSF-GluA2 interaction should result in a decrease in GluA2 containing AMPARs. In the previous experiment we showed that administration of pep-R845A in the IL prevents extinction memory retention. To confirm that disrupting the NSF-GluA2 interaction with pep-R845A prevents insertion and recycling of AMPAR, we infused the synthetic peptide $GluA2_{3Y}$ which contains a short terminal sequence of GluA2 and has been shown to block endocytosis in the hippocampus, nucleus accumbens, prefrontal cortex, and amygdala (Brebner et al., 2005; Fox et al., 2007; Van den Oever et al., 2008; Migues et al., 2010). This experiment was almost identical to the first, with the exception of the additional infusion of $GluA2_{3Y}$ or the inactive scrambled version of it, one hour prior to infusing

pep-R845A (Figure 2A). Thus, we compared four groups: GluA2$_{3Y}$+ pep-R845A (GluA2$_{3Y}$+pepR; n= 8), GluA2$_{3Y}$+Scr pep-R845A (GluA2$_{3Y}$+Scr; n= 6), Scr GluA2$_{3Y}$+pep-R845A (Scr+pepR; n= 9), and Scr GluA2$_{3Y}$+Scr pep-R845A (Scr+Scr; n= 6).

Extinction acquisition for all four groups was comparable (no main effect of treatment and no significant interaction between treatment and extinction block, *P*s > .05) and animals in all groups showed a significant decrease in freezing to the tone across the three extinction sessions (Ext 1 $F_{(5, 21)}$ =14.73, *P* <.0001; Ext 2 $F_{(5, 21)}$ =18.74, *P* <.0001; Ext 3 $F_{(5, 21)}$ = 5.45, *P* < .01; Figure 2B). As shown in figure 2C, a comparison of test 1 to test 2 revealed a significant main effect of treatment ($F_{(3, 25)}$ = 17.21, *P* <.0001), test ($F_{(1,25)}$= 12.49, *P* = .0016), and a significant interaction ($F_{(3,25)}$ = 21.99, *P* < .0001). Post hoc comparisons showed no significant differences in freezing levels between groups on Test 1. Post-hoc comparisons revealed that during test 2 Scr+pepR treated rats froze significantly more than rats in the GluA2$_{3Y}$+pepR, GluA2$_{3Y}$+Scr, and Scr+Scr groups (*P*s <.0001 for all three comparisons). There was no significant difference in freezing between the GluA2$_{3Y}$+pepR, GluA2$_{3Y}$+Scr, and the Scr+Scr groups (*P*s > .05). Consistent with the previous experiment, rats given Scr-GluA2$_{3Y}$ prior to pep-R845A had impaired extinction retention (i.e., froze more) one day after drug administration; however, this impairment was attenuated if animals first received an infusion of the active GluA2$_{3Y}$ peptide. When given in conjunction with the scr-pep-R845A, GluA2$_{3Y}$ had no evident behavioural effect in comparison to the rats treated with both inactive peptides. Overall, these results suggest that blocking AMPAR endocytosis prevents the impairment caused by disrupting the NSF-GluA2 interaction. This further suggests that the NSF-

GluA2 interaction serves to maintain extinction memory in the IL by promoting synaptic expression of GluA2-containing AMPARs at the synapse.

4.5.3 Disrupting the NSF-GluA2 interaction in the DH attenuates renewal of extinction

Plasticity in the DH is necessary for the storage of context information (McClelland et al., 1995; Whitlock et al., 2006). After extinction, the inhibition of fear is particularly sensitive to changes in context. Presenting the extinguished tone outside the extinction context results in renewal of fear responding. The hippocampus is a likely candidate in mediating this effect. Until now, however, there is no direct evidence demonstrating that information stored in the hippocampus is involved in renewed responding to an extinguished CS. We thus set out to investigate the possibility that memory maintained in the DH is required for renewal. As in the first experiment, rats were infused with either pep-R845A (n = 15) or a scrambled control (n=13) one day after the first extinction retention test. On the following day, rats were returned to the initial training context and given three CS presentations. Freezing behaviour during all extinction sessions was equivalent for both groups (Fs < 1; Figure 3B) and there was no significant interaction between treatment X and extinction block (Ps > .05). Both groups also showed significant decreases in freezing across extinction blocks (Ext 1 $F_{(5,22)}$ =15.19, P < .0001; Ext 2 $F_{(5,22)}$ = 6.39, P < .001; Ext 3 $F_{(5, 22)}$ = 4.86, P = .004; Figure 3B). A comparison between freezing during the first retention test and the renewal test revealed a significant main effect of treatment ($F_{(1,26)}$ =7.84, P = .01), a significant main effect of test ($F_{(1,26)}$ = 145.14, P < .0001), and a significant interaction ($F_{(1,26)}$ = 12.03, P = .002) (Figure 3C). Post hoc tests showed no significant difference in freezing between the groups on test 1 (P > .05), but the extent of renewal was significantly less in rats

treated with pep-R845A as compared to the rats that received the scrambled control peptides ($P = .001$). Importantly, differences between the two groups were not due to differences in context fear as baseline freezing in the training context was not significantly different between the groups ($P > .05$). These data indicate that disrupting the interaction between NSF and GluA2 in the DH attenuates renewal. Moreover, these findings demonstrate that memory maintained in the hippocampus is important for the context-dependency of fear memories after extinction.

4.6 Discussion

Understanding how memories, particularly those of an inhibitory nature, persist over time is poorly understood. The aim of this study was thus to characterize molecular mechanisms that are involved in maintaining fear extinction memory. We show that the IL is critically involved in maintaining memory for auditory fear extinction. Disrupting the interaction between NSF and GluA2 within the IL using the peptide pep-R845A that specifically targets NSF, attenuates expression of extinction memory when tested 24 hours after peptide infusion. This impairment can be prevented if rats are first treated with $GluA2_{3Y}$, a synthetic peptide that blocks AMPAR endocytosis. Taken together, these results suggest that extinction memory maintenance relies on, at least in part, post-synaptic stability of GluA2-containing AMPARs within the IL; NSF is an important mediator in the incorporation and stabilization at these synaptic sites. These findings highlight the importance of the association between NSF and GluA2 as a mechanism critical for the long-term persistence of memory.

Further, the maintenance of the context specificity of extinction memory involves the DH. Disrupting the interaction between NSF and GluA2 within the DH attenuated

renewal of responding when animals were returned to the original training context. These results strongly support a role for the DH in maintaining the contextual dependency of extinction memory. Notably, our results point to the continued trafficking of AMPARs to the synapse as a mechanism for memory maintenance that is preserved across both the IL and DH, two structures implicated in the long-term storage of extinction.

The role of the IL in consolidation and expression of extinction memory has been established (Quirk, 2002; Lebron et al., 2004; Laurent and Westbrook, 2009) and reports on the involvement of the IL in extinction acquisition are beginning to emerge (Sierra-Mercado et al., 2011). Nevertheless, the importance of the IL in the maintenance of extinction has yet to be fully explored. A recent report demonstrated a requirement for PKMζ within the IL in the maintenance of extinction memory for morphine reward related cues in conditioned place preference and conditioned place aversion (He et al., 2011). Our data are consistent with these observations, extending the involvement of the IL in extinction memory maintenance to include the persistence of fear extinction.

The DH is important for the encoding and retrieval of contextual information and has been implicated in spatial learning (Moser et al., 1993; 1995; Fanselow, 2000). Given the sensitivity of extinction memory to shifts in context (Bouton, 2004), a number of papers have sought to address the role the DH plays in extinction. Both lesions and inactivations of the DH impair renewal (Corcoran and Maren, 2001; 2004; Corcoran et al., 2005; Zelikowsky et al., 2012). Consistent with these results, we show that disrupting the NSF-GluA2 interaction in the DH leads to a marked impairment in the renewal of responding that normally occurs when animals are returned to the training context. This suggests that contextual representations maintained within the DH are important for the

contextually driven recovery from extinction. Surprisingly, there is a discrepancy in the literature with respect to the involvement of the DH in ABA renewal. ABA renewal involves training in context A, extinction in context B, and a return to context A for testing. Corcoran and Maren (2004) found that post-extinction inactivation of the DH did not affect ABA renewal. In contrast, Zelikowsky et al. (2012) found that post-extinction lesions of the DH did attenuate ABA renewal. Our results are consistent with the effect of DH lesions. One explanation for the observed differences may be due to differences in protocols. As Zelikowsky et al. (2012) point out, Corocoran and Maren (2004) use a long continuous tone during extinction retention in contrast to the discrete tone presentations delivered during extinction which may change the involvement of hippocampus.

Previous work has pointed to the involvement of PKMζ in the long-term maintenance of memories (Pastalkova et al., 2006; Kwapis et al., 2009; Migues et al., 2010; Hardt et al., 2010b). A proposed mechanism whereby PKMζ supports long-term memory maintenance is through the NSF-GluA2 interaction, leading to persistent expression of GluA2 at the synapse (Yao et al., 2008; Migues et al., 2010; Sacktor, 2011). Specifically blocking the NSF-GluA2 interaction has been shown to interfere with the maintenance of AMPAR-mediated transmission at hippocampal synapses (Nishimune et al., 1998; Song et al., 1998; Lüthi et al., 1999; Noel et al., 1999). Dynamic trafficking of AMPA receptors regulates synaptic plasticity, synaptic strength, and subsequent learning and memory. It has been suggested that NSF likely interacts with GluA2 to stabilize AMPARs at the surface of the postsynaptic membrane, preventing AMPAR internalization (Braithwaite et al., 2002). Recently, it has been reported that disrupting the NSF-GluA2 interaction in the DH after consolidation is complete results in impaired

memory for object location and contextual fear (Migues et al., 2012). The necessity of the NSF-GluA2 interaction in maintaining extinction memory is therefore in line with previous work investigating PKMζ and memory maintenance.

We infused the peptide 48 hours after the last extinction training and 24 hours after the first retention test. This timing of treatment fell outside the standard window of extinction consolidation (Santini et al., 2004) ensuring that the treatment was not interfering with consolidation of the extinction memory, but rather targeting its maintenance. The disruption of memory maintenance that we observed is in stark contrast to a previous paper examining the role of NSF-GluA2 in auditory fear conditioning (Joels and Lamprecht, 2010). When pep-R845A was delivered to the lateral amygdala (LA) 24 hours after fear conditioning, no impairment was observed when tested 48 hours later. These authors concluded that disrupting the NSF-GluA2 interaction does not disrupt the long-term maintenance of an auditory fear memory. One reason for the discrepancy between these findings and our data may be a consequence of memory strength. Memory strength has been shown to moderate the effects of drugs (Walker and Davis, 2000).

Interestingly, higher levels of freezing are correlated with greater expression of GluA2 in the amygdala (Migues et al., 2010). Under these circumstances, the treatment parameters used by Joels and Lamprecht (2010) may be insufficient to disrupt memory maintenance because fear memory of this strength is not sensitive to the amount of pep-R845A infused. Alternatively, expression of some AMPARs at amygdala synapses may be maintained by mechanisms independent of NSF. As a result, disrupting the NSF-GluA2 interaction may, under some conditions, be ineffective.

Since we only probe for the absence of extinction memory at an early time point, we cannot exclude the possibility that the observed effects are transient. Ideally, to verify that we are disrupting memory maintenance, we would test at a time point well beyond the treatment. Unfortunately, given that the nature of extinction memory involves a loss of expression over time (spontaneous recovery), it is not possible to behaviourally show whether the effect of pep-R845A is a permanent impairment – the return of fear expression due to the peptide infusion could be attributed to spontaneous recovery. Previously reported data, using pep-R845A in the hippocampus, show a disruption of contextual fear memory for at least 10 days after infusion (Migues et al., 2012); thus, it is possible that the loss of extinction memory lasts beyond one day and might indeed be a permanent impairment.

Further, we cannot discount the involvement of other brain structures in maintaining extinction memory. The currently established neurocircuitry for fear extinction memories involves the amygdala, ventral hippocampus (VH), and prelimbic cortex (PL) of the mPFC in addition to the IL and DH. While the data suggest that the IL is critical for the maintenance of extinction memory, given that recovery of freezing after the pep-R845A infusions is not complete, it is likely that a network of structures supports extinction memory maintenance. A subpopulation of neurons in the basal amygdala (BA) preferentially respond to an extinguished CS (Herry et al., 2008). These "extinction" neurons may suppress fear even in the absence of input from the IL, by inhibiting intercalated cell masses that in turn inhibit the medial division of the central amygdala, the main output from the amygdala involved in conditioned fear responding.

In conclusion, we have demonstrated that the NSF-GluA2 interaction in the IL is

critical for the maintenance of fear extinction memory. Interfering with the NSF-GluA2 interaction causes a marked impairment in extinction retention and this impairment can be prevented if AMPAR endocytosis is blocked. Furthermore, the NSF-GluA2 interaction is necessary in the DH for the context-dependent retrieval of fear after extinction. Future studies may reveal the contributions of other structures in the extinction circuit towards the persistence of extinction memory and provide further clues as to how the brain regulates fear memory after extinction.

4.7 Figures

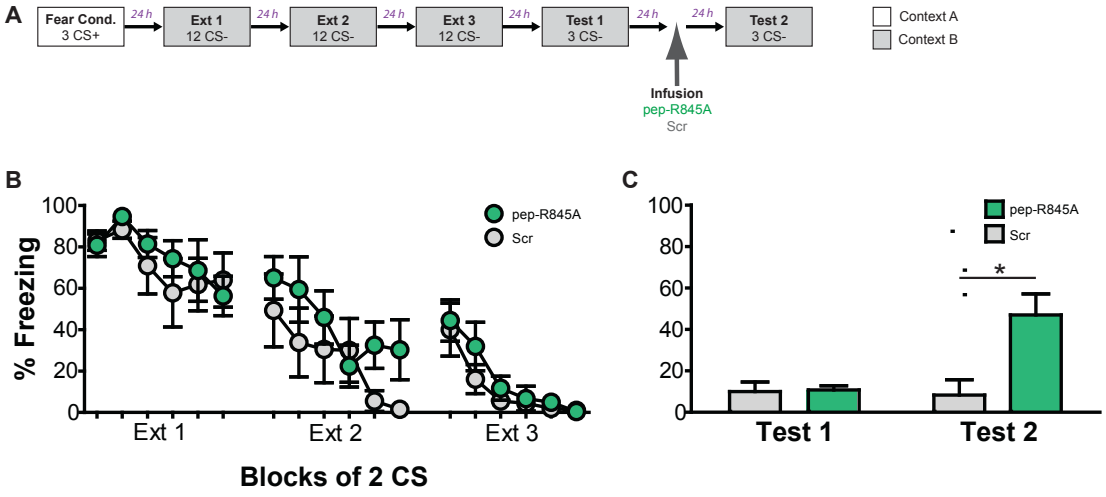

Figure 1. Infusions of pep-R845A into the infralimbic cortex (IL) impair retention of extinction memory. *(A)* Behavioural protocol. *(B)* Mean percentage of freezing (±SEM) over 3 days of extinction Data are presented as blocks of the average of 2 tone-alone trials. *(C)* Mean percentage of freezing (±SEM) during the two retention tests consisting of 3 tone-alone presentations. Infusions of pep-R845A or an inactive scrambled control (Scr) were infused 24 hours after the first test and one day prior to the second test. Pep-R845A infusions led to a significant recovery of freezing at Test 2. **P* < .05.

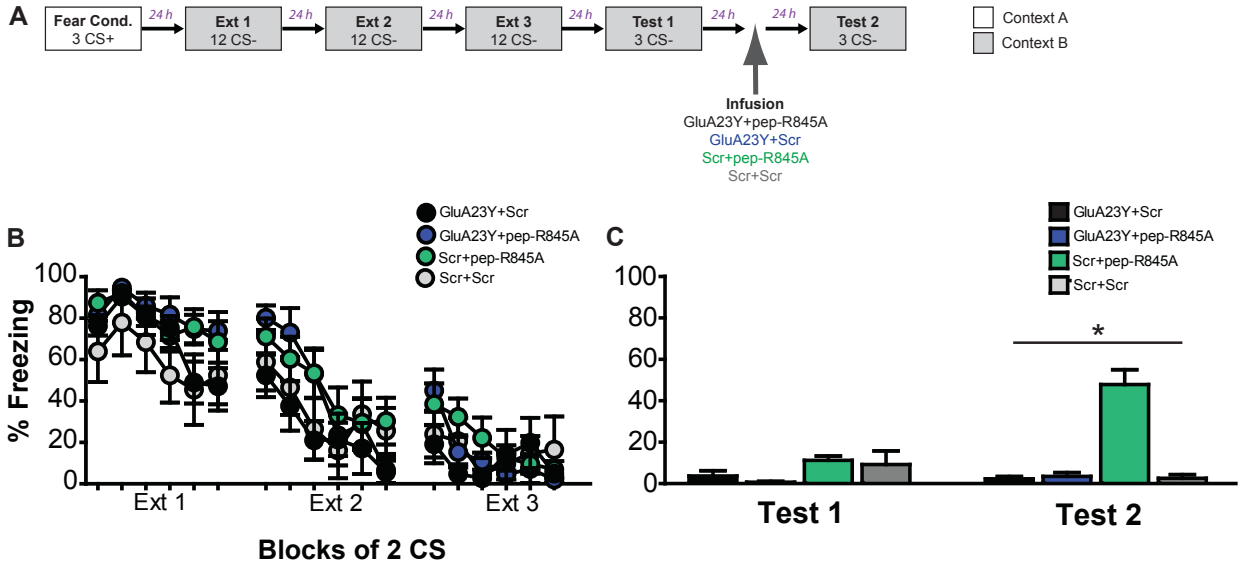

Figure 2. Blocking AMPAR endocytosis with GluA2$_{3Y}$ counteracts the effects of pep-R845A in the infralimbic cortex (IL) on extinction retention. *(A)* Behavioural protocol. *(B)* Mean percentage of freezing (±SEM) over 3 days of extinction. Data are presented as blocks of the average of 2 tone-alone trials. *(C)* Mean percentage of freezing (±SEM) during the two retention tests. Tests are the average of 3 tone-alone presentations. As in the previous experiment, infusions occurred 24 hours after Test 1 and 24 hours prior to Test 2. Infusions of GluA2$_{3Y}$ and its scrambled control were delivered 1 hour before the infusions pep-R845A or the scrambled version. GluA2$_{3Y}$ prevented the recovery caused by pep-R845A; however, significant recovery was observed if pep-R845A was preceded by an infusion of scrambled peptide. *$P < .05$.

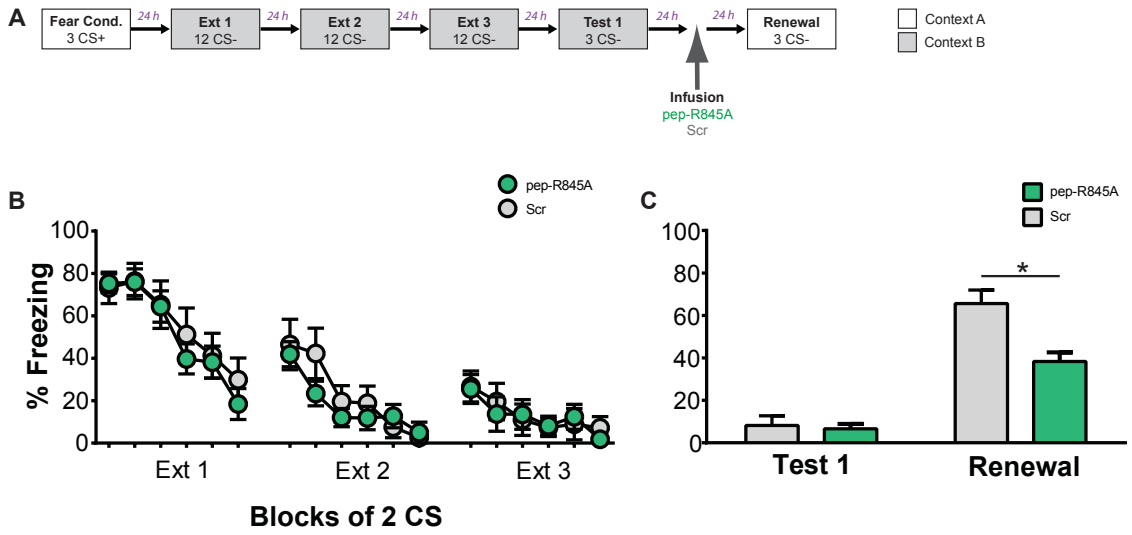

Figure 3. Infusions of pep-R845A into the dorsal hippocampus (DH) attenuate renewal. *(A)* Behavioural protocol. *(B)* Mean percentage of freezing (±SEM) over 3 days of extinction. Data are presented as blocks of the average of 2 tone-alone trials. *(C)* Mean percentage of freezing (±SEM) during the extinction retention and renewal test. Tests are the average of 3 tone-alone presentations. Infusions of pep-R845A into the DH were given 24 hours after the first retention test. 24 hours after the infusions, rats were returned to the training context for a test of renewal. pep-R845A led to a significant impairment in renewal of responding compared to the rats treated with scrambled peptide. *$P < .05$.

Chapter 5

Spontaneous Recovery: Forgetting of Extinction

5.1 Preface

Chapter 4 explored the mechanisms involved in the maintenance of extinction memory. The data suggest that the stabilization of GluA2-containing AMPARs at the synapse in both the infralimbic cortex (IL) and the dorsal hippocampus (DH) is necessary for the long-term persistence of auditory fear extinction and its context-specific retrieval. Removal of GluA2-containing AMPARs from the synapse led to spontaneous recovery. Could this same process be responsible for the recovery of responding to the extinguished CS that occurs over time?

Previous theoretical and behavioural accounts have offered alternative explanations for spontaneous recovery. In one such explanation, spontaneous recovery shares a number of similarities with associative interference. Information about the CS learned during conditioning might be expected to compete with information learned about the CS during extinction (Spear, 1971). Thus, during the retention test there is retrieval interference between the outcomes trained during the different phases. Along these same lines, Spear (Spear, 1973; 1976) has argued that memory expression is dependent on the similarity between cues presented at training and those presented at retrieval. Whether the CS elicits the outcome associated with initial conditioning or the outcome learned in extinction may specifically depend on how well the context during the test resembles the contexts of conditioning and extinction (Spear, 1978; Bouton, 1993b). With time, the test context comes to resemble that of conditioning which interferes with activation of the extinction representation.

Currently held theories of extinction combine these ideas and suggest that spontaneous recovery is more a context-dependent retrieval process. Thus, forgetting in

the form of retrieval failure occurs because there are insufficient training cues present at retrieval to elicit behaviour. A discrepancy in cues available at training and retrieval often results from a change in context. Bouton (1993a) adds that time is an important component of context. Imposing a long retention interval after extinction is likely to change the temporal component of context through internal and external cues. Thus, a change in temporal context (i.e., the passage of time) is akin to a change in physical context.

The underlying assumption of these previous models is that the extinction trace persists beyond recovery from extinction. In contrast, decay theory offers an alternate fate for the extinction trace that is not the result of interference or absent retrieval cues: the memory is gradually lost or removed. Recently, Hardt et al. (2013) have proposed an active mechanism responsible for decay: the internalization of GluA2-containing AMPA receptors from the synapse. If the IL is important for the maintenance of extinction memory, then does a loss of this memory lead to recovery of responding to the CS? Here we investigate whether a process of active decay in the IL contributes to spontaneous recovery.

Archbold GE, Hardt O, Nader K (2013) Spontaneous recovery: Forgetting of extinction. *In preparation*

5.2 Abstract

The first evidence that memory persists following extinction came from Pavlov's observation that over time responding to the conditioned stimulus (CS) recovers. Since then, a number of explanations have been put forth to account for this behavioural phenomenon. Although the prominent view posits that spontaneous recovery results from an inability to retrieve the extinction memory at test, recent research investigating the mechanisms involved in the long-term memory maintenance offers an alternative explanation. The regulation of GluA2-containing postsynaptic AMPA receptor trafficking has been shown to be important for the maintenance of memory in the hippocampus, amygdala, and neocortex. In the infralimbic cortex, internalization of GluA2-AMPA leads to recovery of responding to an extinguished CS. Here we explored whether a loss of synaptic GluA2 over time is responsible for spontaneous recovery. Following extinction we found that blocking GluA2 endocytosis with the $GluA2_{3Y}$ peptide in the infralimbic cortex prevented spontaneous recovery. These results are consistent with emerging data that propose GluA2-AMPA internalization is a neurobiological mechanism for forgetting by decay. Furthermore, the data support a role in spontaneous recovery for decay-driven forgetting within the infralimbic cortex.

5.3 Introduction

The observation that previously extinguished responses can return with the passage of time led to Pavlov's initial assertion that extinction involves an active inhibitory learning process (Pavlov, 1927). During extinction, the conditioned stimulus presented alone results in learned inhibition that counteracts the initial association between conditioned stimulus and reinforcement; however, over time this inhibition is attenuated. Pavlov further believed that although both initial conditioning (excitation) and subsequent inhibition were susceptible to decay, spontaneous recovery, the aforementioned return of responding, resulted from differential decay rates. For this to be true, inhibition would have to dissipate more rapidly than excitation.

Liberman (1944) also suggested that spontaneous recovery involved forgetting of the "negative learning" he thought occurred during extinction and that this learning was forgotten more rapidly than conditioning. However, unlike Pavlov, Liberman believed the mechanism of forgetting to be interference rather than decay. The predominant view of forgetting is focused on a mechanism of interference. In extinction the CS first elicits an excitatory response (freezing in fear conditioning) before inhibiting this response as a result of extinction. The earlier excitatory association may interfere with successful recall of the later acquired inhibitory association; this produces the observed forgetting of extinction in spontaneous recovery.

More recent theories of spontaneous recovery are based on the idea that time acts as a contextual cue (Bouton, 1993b). Over long retention intervals after extinction the temporal component of the context changes resulting in impaired retrieval of extinction. Thus, despite Pavlov's initial belief that recovery from extinction was a consequence of

decay, explanations for spontaneous recovery have predominantly assumed that 'forgetting' of extinction came down to interference of previously learned information or a retrieval failure due to a change in the temporal context.

Despite this strongly held view, few studies have actually investigated the fate of extinction, either behaviourally or neurobiologically, once initial learning has recovered. The possibility that extinction is forgotten through decay has not been irrefutably disproved. Interestingly, there is some evidence to suggest asymmetric forgetting of reinforced and non-reinforced associations occurs even after experimentally controlling for interference. Hendersen (1978) compared the length of retention of conditioned fear to conditioned fear inhibition. While there was no evidence of any forgetting in conditioned fear, there was significant attenuation of conditioned inhibition over the same long-term retention interval. This is consistent with Pavlov's idea that inhibitory processes are inherently more fragile than excitatory ones and raises the possibility that extinction is more easily forgotten than fear conditioning due to a more rapid decay process.

Other explanations for spontaneous recovery exist and whether forgetting of extinction is due to decay cannot be discerned using behavioural assays alone. In the present study, we were interested in determining whether differential rates of forgetting, through a process of decay, are characteristic of fear learning and fear extinction. Although behaviour can suggest a system involving more rapid decay of extinction, this does not necessarily translate to loss of memory at the neural level.

However, recent advances in our understanding of the neurobiology of memory maintenance point to a process of decay-driven forgetting. Preventing internalization of

GluA2-containing AMPA receptors has been implicated in the maintenance of memory (Migues et al., 2010). Blocking the action of PKMζ, a kinase that prevents GluA2 endocytosis, results in memory loss (Pastalkova et al., 2006; Migues et al., 2010; Hardt et al., 2010a; He et al., 2011). Similarly, disrupting the NSF-GluA2 interaction, thereby allowing for internalization of GluA2-containing AMPARs, interferes with long-term memory maintenance (Migues et al., 2012; see also Chapter 4).

Importantly, blocking PKMζ or disrupting NSF-GluA2 does not lead to the loss of memory maintenance if GluA2/AMPAR endocytosis is first prevented with Tat-GluA2$_{3Y}$, a cell-permeating peptide derived from the C-terminal of GluA2. Our colleagues have recently exploited this finding to test whether preventing GluA2 internalization can prevent forgetting of object location memory (Hardt et al., 2013 – personal communication). Twice daily infusions of Tat-GluA2$_{3Y}$ into the dorsal hippocampus of rats over 13 days prevented the loss of memory typically observed after two weeks.

Based on our previous findings (Chapter 4), the infralimbic cortex (IL) plays a critical role in the maintenance of extinction. Consequently, forgetting processes in the IL may account for spontaneous recovery. Here we use an infusion protocol similar to that of Hardt et al. (2013) to determine whether internalization of GluA2-containing AMPARs in the IL is responsible for spontaneous recovery and decay of the extinction trace.

5.4 Materials and Methods

5.4.1 Subjects

Adult male Long Evans rats (Charles River Laboratories, Québec) initially weighing 275-300g, were in pairs in plastic Nalgene cages with environmental

enrichment. The rats were maintained on a 12 h light/dark cycle (lights on at 7am, off at 7pm) with food and water available *ad libitum*. All experiments were carried out during the light phase. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care and Use Committee.

5.4.2 Surgery

Rats were anesthetized (3.0 mg/kg xylazine, 50.0 mg/kg ketamine and 0.175 mg/kg DexDormitor) and then mounted into a Kopf stereotactic frame. Into each rat a stainless steel double-guide cannula (22 gauge; Plastics One) was implanted aiming at the infralimbic cortex (IL) (A/P 2.5, D/V -4.40, 0.6 from midline) (Paxinos and Watson, 2005). Dental cement was applied to stabilize the implant. An obturator inserted in the guide prevented blocking. Three jewelry screws implanted in the skull and acrylic cement were used to stabilize the cannula. After surgery, an intra-musclar injection of analgesic (Carprofen, 5 mg/kg) was given. An intra-peritoneal injection of Antisedan (7.5 mg/kg) suspended anesthesia. Animals were given 7 days to recover from surgery and handled daily during the recovery period. At the end of the experiment, cannula placement was checked by examining 50-μm brain sections stained with formal-thionin under a light microscope.

5.4.3 Drug Infusions

$GluA2_{3Y}$(TAT(47–57)-^{869}YKEGYNVYG^{877}), and Scr-$GluA2_{3Y}$ (TAT(47–57)-AKEGANVAG) were dissolved in 100 mM Tris-saline (pH 7.2). Peptides used in these experiments were fused to Tat protein to ensure membrane permeability. They were infused into the IL (1 μl per hemisphere) via a microinjector (28 gauge) connected to a Hamilton syringe with plastic tubing at a rate of 0.25 μl per min. The injector remained

connected for an additional min to allow for drug diffusion away from the tip of the cannula. Infusions took place twice a day (morning and afternoon) for 8 days.

5.4.4 Apparatus

Two conditioning contexts were used: one for fear conditioning and one for extinction and testing. Generalization between contexts was minimized by varying a number of dimensions such as shape, illumination, scent, visual and tactile features, and ambient sound. The training context, Context A, consisted of a box (30 cm × 26 cm × 33 cm), manufactured by Coulbourn Instruments, placed into a sound attenuating chamber; 4 identical boxes were used. Within each box, one wall was made of stainless steel and had a light bulb providing dim light. The other walls were made of colorless Plexiglas. The floor was a level stainless steel grid (bar radius 2.5mm, spread 1cm apart). Vanilla scent was sprayed into one corner of the box before the animal was put into it. A digital camera in front of the box recorded the sessions. Room lights were off and illumination involved a red light.

Context B, used for extinction and testing, was a box (29 cm × 25 cm × 25 cm) manufactured by Med Associates (St. Albans, VT); 4 identical boxes were used. Each box consisted of a curved white Plexiglas back wall and front wall covered by black-and-white-striped wallpaper (stripes 2.5cm wide). The floor was an opaque panel of Plexiglas. Peppermint scent was sprayed onto the curved plastic wall before the animal was put in the box. A ventilation fan provided a constant background noise. A digital camera was mounted on the ceiling of each box and videotaped the sessions for later analysis. Room lights were on.

5.4.5 Behavioral Procedures

Rats were habituated to the extinction Context B for 20 min for two consecutive days prior to conditioning. On the day of conditioning rats were placed into the box in Context A for a total of six minutes. After two minutes, three tones (5 kHz, 75dB) were presented for 30 seconds each co-terminating with a foot shock (0.9 mA, 1s shock). Tones were separated by a 60 s interstimulus interval (ISI). Following the final tone, rats were given an additional 30s in the context before being removed from the box and transported back to the colony room.

Twenty-four hours after conditioning, rats underwent three consecutive days of extinction training in Context B. In each session, following a two minute acclimation period, they received 12 tone-alone presentations separated by a 60 sec ISI. Rats remained in the boxes for an additional 60 sec before being removed to their homecage and returned to the colony room. A retention test of extinction memory was conducted 1 and 9 days after the final extinction session. Rats were returned to Context B and after 2 minutes in the context, presented with 3 tones (60 sec ISI). The animals remained in the context for a further 60 sec before being removed and returned to the colony room. Peptide infusions occurred in the colony room on the days between retention tests.

5.4.6 Data Analysis

Memory was evaluated by manually measuring with a stopwatch the duration of freezing (immobilization except for respiratory movements) during the baseline (Pre-CS) and during tone presentations. Extinction was divided into blocks involving the average of two tone presentations. Freezing during test was taken as the average of three tone presentations. Data were statistically analyzed using repeated measures ANOVAs.

Tukey-Kramer HSD post hoc comparisons were performed following significant F values.

5.5 Results

5.5.1 Preventing GluA2-containing AMPAR endocytosis prevents spontaneous recovery

To examine whether forgetting of extinction may underlie spontaneous recovery, rats were infused with the interference peptide $GluA2_{3Y}$ or its scrambled version. One day after the first extinction retention test, twice-daily microinfusions into the IL for a period of 8 days commenced. A second test of extinction retention was one day after the final infusions. Mean freezing (±SEM) for the three sessions of extinction and the two retention tests are displayed in Figure 1.

All animals showed less than 20 % baseline freezing to the context during the initial 120 s acclimation period. Repeated measures ANOVA on extinction revealed significant extinction learning across blocks for each extinction session (Ext 1 $F_{(5,14)} = 16.01$, $P < .0001$; Ext 2 $F_{(5,14)} = 10.19$, $P < .001$; Ext 3 $F_{(5,14)} = 4.11$, $P = .017$) . Extinction was comparable for both groups as there was no main effect of treatment (Fs < 1) and no treatment X block interaction (Fs < 1) across all three extinction days.

A repeated measures ANOVA performed on the two extinction retention tests revealed a significant effect of treatment ($F_{(1,18)} = 12.08$, $P = .003$) and a significant effect of test ($F_{(1,18)} = 46.81$, $P < .0001$). There was also a significant interaction of treatment X test ($F_{(1,18)} = 11.93$, $P = .003$). Post-hoc analyses indicated that there were no differences between the two groups on the first test ($P > .05$), but rats receiving twice daily infusions of the scrambled peptide froze significantly more than those receiving twice daily infusions of $GluA2_{3Y}$ ($P = .002$).

Overall, these results suggest that following extinction the loss of synaptic GluA2-containing AMPARs in the IL leads to spontaneous recovery. Animals that received infusions of $GluA2_{3Y}$ do not show the same return of fear.

5.6 Discussion

Presenting a conditioned stimulus (CS) in the absence of reinforcement leads to extinction of the fear response. Nevertheless, with time the fear response recovers, suggesting that the initial memory is not lost but instead actively inhibited by a new memory. This reemergence of fear and concurrent attenuation of the inhibitory trace is referred to as spontaneous recovery (Pavlov, 1927). However, to date, the neurobiological mechanisms responsible for spontaneous recovery remain unclear. Previous theoretical views have offered both interference and retrieval failure as potential explanations. Recent work investigating the role of synaptic GluA2-containing AMPARs in the maintenance of memories points to another possibility. Here we sought to determine whether spontaneous recovery was a result of decay-driven forgetting involving internalization of GluA2-containing AMPARs.

Preventing AMPAR endocytosis in the IL led to good extinction recall 9 days after the last extinction session. In contrast, animals treated with an inactive scrambled peptide over the same interval showed spontaneous recovery, as indicated by significantly more freezing. These results are consistent with work from our laboratory that implicates GluA2 in the maintenance of extinction memory (Chapter 4). They also extend this work by demonstrating that over time GluA2 is removed from synaptic sites and this removal leads to recovery of fear responding to the extinguished CS.

These data support the view that an active process of decay-driven forgetting is responsible for spontaneous recovery. They are also in line with Pavlov's (1927) original assertion that inhibition and excitation have differential rates of decay. Here we show that as memory for extinction decays the original excitatory fear memory remains intact and drives fear-responding.

An alternative explanation for the effects of $GluA2_{3Y}$ might be that the peptide prevents interference from the original fear memory. Given that extinction and renewal of fear have differential patterns of activation (Knapska and Maren, 2009), it seems unlikely that the IL would be the site of interference between the extinction and fear trace. Nevertheless, we cannot explicitly rule out the possibility that $GluA2_{3Y}$ in the IL prevents interference.

In his experiments studying forgetting in conditioned fear inhibition, Hendersen (1978) attempted to rule out any possible proactive interference between a CS that first elicits an excitatory reaction before later becoming a conditioned inhibitor. Even with these controls in place, the results showed that conditioned excitation is well maintained while conditioned inhibition is lost over the same retention interval. These results further point to differential rates of decay for excitatory and inhibitory memories and support our findings.

It is widely accepted that extinction memories are long lasting and persist following spontaneous recovery (Quirk, 2002). Despite near perfect recovery of initial fear two weeks after extinction learning, rats given a second extinction training session at this time showed more rapid extinction reacquisition than during initial extinction learning. This phenomenon is known as "savings" and has been suggested as evidence

for the persistence of memory after extinction (Macrae and Kehoe, 1999; Bouton, 2002). In Quirk's experiments "savings" is used to show that extinction is spared after recovery and is consistent with a retrieval failure explanation of spontaneous recovery; however, it does not discount the possibility of forgetting due to decay. It is probable that extinction does not need to be completely lost before the system favours expression of fear. A residual trace may be sufficient to facilitate reacquisition of extinction.

In conclusion, our results suggest that spontaneous recovery that occurs with time results from forgetting of extinction. Specifically, our results show that this is mediated by the gradual loss of GluA2-containing AMPARs from the synapse in the IL. In contrast, the original fear memory does not appear to undergo these same decay mechanisms at the same rate. This leads to the recovery of fear since the IL can no longer act to suppress it.

5.7 Figures

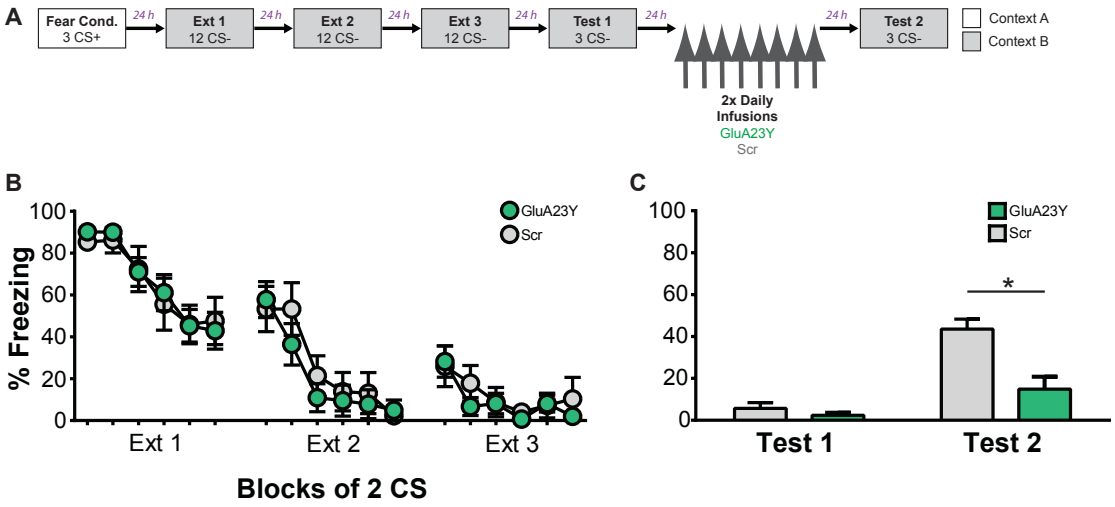

Figure 1. Blocking AMPAR endocytosis in the infralimbic cortex (IL) with $GluA2_{3Y}$ prevents spontaneous recovery. *(A)* Behavioural protocol. *(B)* Mean percentage of freezing (±SEM) over 3 days of extinction. Data are presented as blocks of the average of 2 tone-alone trials. *(C)* Mean percentage of freezing (±SEM) during the two retention tests. Tests are the average of 3 tone-alone presentations. Infusions of $GluA2_{3Y}$ and its scrambled control were delivered twice daily for 8 days. $GluA2_{3Y}$ prevented attenuated the spontaneous recovery. $*P < .05$.

Chapter 6

General Discussion

6.1 Summary

Fear conditioning and fear extinction are two models used to study associative fear learning and fear inhibition respectively. While considerable advances have been made in recent years in establishing the behavioural and neural mechanisms involved in fear learning, these same processes in fear extinction are less well understood.

The focus of the experiments in this thesis was to further characterize fear extinction at both the behavioural and neural level. In this section I will first summarize the results of these experiments and address how they contribute to our current understanding of fear extinction. Based on the results of this thesis, we can draw three main conclusions. The first is that extinction, even conducted immediately after conditioning, can reflect "new learning" rather than "unlearning" (Chapter 2). Secondly, spontaneous recovery of fear does not develop linearly. Instead, spontaneous recovery is observed with both short (1-4 hours) and long (7 days) intervals from the time of extinction. Recovery at these time points appears to involve different mechanisms (Chapters 3 and 5). Finally, mechanisms engaged in the maintenance of initial learning are also necessary for the maintenance of extinction (Chapters 4 and Chapter 5). Extinction maintenance engages the infralimbic cortex (IL) and dorsal hippocampus (DH), two structures that have previously been implicated in extinction memory. Extinction, like initial conditioning, requires stabilization of GluA2-containing AMPA receptors at the synapse for successful retention. With time, removal of GluA2 at synaptic sites results in forgetting of extinction and recovery of fear.

6.2 Early Extinction Is New Learning

In the experiments reported in Chapter 2, we tested whether extinction given at different time intervals after conditioning initiates different fear suppressing mechanisms: "new learning" with longer delays and "unlearning" with shorter ones. As reviewed in Chapter 1, whether extinction can erase memory has been in continual debate. Even if behaviourally a memory appears to have been erased, it may not actually have been lost; rather, it could be inaccessible. Thus, poor recovery following immediate extinction may reflect either a storage or retrieval impairment.

To circumvent the difficulty of discerning between these two possibilities we made use of the finding that encoding of a first instance of contextual fear conditioning requires NMDARs in the DH, but a second instance does not (Sanders and Fanselow, 2003; Hardt et al., 2009). We reasoned that if extinction erased initial contextual fear learning, a second experience of contextual fear would be treated as first learning and again would require NMDA receptors. In contrast to previous work (Myers, 2006), we found that following immediate extinction learning a new context-shock association was NMDAR-independent in the DH, suggesting that a short interval between conditioning and extinction was not sufficient to erase the original context fear memory. This argues against the idea that short intervals between conditioning and extinction produce "unlearning." Instead, they are in line with numerous other studies that have found immediate extinction unable to prevent spontaneous recovery of auditory fear (Maren and Chang, 2006; Schiller et al., 2008) and conditioned emotional response (CER) (Woods and Bouton, 2008) in rats, as well as the skin-conductance response (Schiller et al., 2008) and acoustic startle (Alvarez et al., 2007) in humans.

Newly formed memories undergo a period of consolidation in which they are labile (McGaugh, 2000). Within this time window of lability memories are sensitive to disruption and modification by both pharmacological and behavioural manipulations. This presents an opportunity for the integration of new information into the existing memory (McGaugh, 2000). Why then is extinction training conducted within this time window unable to permanently attenuate recently acquired fear? At a physiological level at least erasure of fear seems possible. Indeed, depotentiation – the reversal of conditioning-induced potentiation – of synapses is possible with low frequency stimulation (LFS) 10 minutes after LTP induction (Lin et al., 2003).

One possible explanation for the inconsistencies in the literature is likely due to procedural differences; fear potentiated startle may preferentially engage "unlearning" mechanisms compared to Pavlovian fear conditioning. Maren and Chang (2006) have also suggested that the amount of fear acquired at learning and the state of the animal (i.e., the level of arousal) at the beginning of extinction learning may help determine whether immediate extinction will cause a permanent suppression of fear. Interestingly, CS-alone presentations given shortly after initial fear learning appear to reflect a context-independent habituation-like process (Chang and Maren, 2009). This idea has been supported by recent work showing that immediate extinction does not involve the typically recruited mPFC circuits normally seen in fear extinction learning (Chang et al., 2010; Kim et al., 2010).

In summary, our findings in Chapter 2 add to a body of literature that suggests immediate extinction does not erase memory. Nonetheless, in light of some evidence to the contrary, the question of whether extinction can involve "new learning" or

"unlearning" remains. Rather than choosing sides in this debate, it will be more useful to ask why under some conditions extinction can reliably cause erasure and how these conditions are mechanistically different from those where initial learning is spared.

6.3 Spontaneous Recovery Can Reflect Different Mechanisms

The goal of the experiments reported in Chapter 3 (Archbold et al., 2013) was to test the retention of fear extinction at short intervals after extinction learning. Commonly, longer retention intervals are used to measure spontaneous recovery (the observation that an extinguished response can recover over time). Previous research in CTA suggests that extinction is not immediately evident following acquisition (Berman et al., 2003). This could reflect an absence of short-term memory for extinction or simply a deficit in extinction recall. We found that auditory fear extinction was not expressed at short retention intervals (1 and 4 hours) following extinction. This was despite very little freezing observed to the last CS-alone presentation during extinction acquisition. In contrast, intervals of 12 and 24 hours produced good extinction retention, as there was very little spontaneous recovery.

Incidentally, the pattern of spontaneous recovery of auditory fear extinction observed in these experiments is congruent with the retention curve described by Kamin (1957) and now known as the "Kamin effect." Two theories have been suggested to account for the observance of this nonmonotonic function: 1) multiple memory traces subserve performance and a discontinuity between two traces results in a lapse in behaviour; 2) there is a discrepancy between internal cues present at the time of training and those present at intermediate retention intervals. The results of these experiments support the latter explanation. Poor recall of extinction at short intervals results from the

aversive experience of extinction itself as testing a previously extinguished CS shortly after extinction of a second CS was sufficient to produce recovery. Based on previous studies that have shown that adrenergic activity regulates memory retrieval following extinction (Morris et al., 2005b), we injected propranolol, a β-adrenergic antagonist, either before extinction or before the retention test. In both cases propranolol was unable to attenuate the spontaneous recovery observed when the retention test followed extinction acquisition by 1 hour.

Expression of extinction is dependent on the IL (Sierra-Mercado et al., 2011) and under stress IL function may be impaired, interfering with extinction performance (Izquierdo et al., 2006; Maroun, 2006; Muigg et al., 2008). It is possible that reexposure to the CS is sufficient to elicit a stress response that alters neural processing in the few hours following fear extinction acquisition. Consequently, fear extinction expression is inhibited. Future studies aimed at reducing the physiological changes that occur after extinction learning may provide additional insight into the mechanisms interfering with successful extinction retrieval.

In Chapter 5, we examined the involvement of decay-driven forgetting in spontaneous recovery. Previous work has shown that regulation of GluA2-dependent AMPAR trafficking is important for maintenance of memory (Migues et al., 2010; 2012). In accordance with these findings, a theory of active forgetting involving the internalization of GluA2-containing AMPA receptors has been proposed (Hardt et al., 2013). Furthermore, blocking the internalization of GluA2-containing AMPA receptors prevents normal forgetting of object location memory in the DH (Hardt et al., 2013 - personal communication). We found that preventing GluA2 endocytosis in the IL with

the interference peptide $GluA2_{3Y}$, infused twice a day for 8 days, attenuated the degree of spontaneous recovery observed during the retention test. This result is consistent with Pavlov's (1927) early assertion that spontaneous recovery is a result of differential decay rates between excitation and inhibition; however, it is counter to current explanations of spontaneous recovery.

It is widely believed that spontaneous recovery reflects the "renewal effect" where a shift in temporal context, as opposed to a physical change in context, determines the return of responding(Bouton, 1993b). By this view, time prevents activation of the extinction trace resulting in failure to retrieve the extinction memory. The results from Chapter 5 show that even with the passage of time extinction memory can be retrieved if AMPAR internalization in the IL is prevented. Given that GluA2-AMPAR internalization has been linked to the decay concept of forgetting (Hardt et al., 2013) a more congruous interpretation of spontaneous recovery is that it results from decay-driven forgetting. Interestingly, a recent model of extinction learning and spontaneous recovery based on the activity of midbrain dopamine (DA) neurons uses forgetting to account for spontaneous recovery (Pan et al., 2008). In this instance, excitatory and inhibitory responses have differential rates of decay, in line with Pavlov's first propositions and the findings observed in this thesis (Chapter 5).

A major assumption underlying differential decay rates is that the extent of learning between both excitation and inhibition is equivalent. In our experiments we cannot be sure that the extinction memory is as well learned as the original fear memory. Although the sum of the CS alone extinction trials greatly outweighs the sum of the reinforced CS trials of conditioning, other factors such as salience may determine the

strength of the memory. Nevertheless, this limitation does not discount the observation that the loss of GluA2-containing AMPA receptors, a proposed mechanism of decay based forgetting, is involved in the recovery of fear.

The findings reported in Chapters 2 and 5 suggest that spontaneous recovery can reflect different mechanisms. Recovery of fear that occurs shortly after extinction acquisition is the result of a retrieval failure mediated by the physiological state of the animal. In contrast, recovery of fear observed with longer time delays occurs because the trace has been forgotten. Spontaneous recovery is already a phenomenon that causes particular difficulty for models of conditioning (Rescorla and Wagner, 1972; Mackintosh, 1975; Pearce and Hall, 1980). The results of these experiments present a further challenge for current theoretical views of spontaneous recovery which assert that all instances of spontaneous recovery occur because of a single process, be it retrieval failure (Spear, 1971; Bouton, 1991), changes in CS processing (Robbins, 1990), or a diminished temporal advantage of the extinction memory (Devenport, 1998). As Rescorla (2004b) notes, no one process is likely responsible for extinction given that there is evidence to support many of the proposed explanations.

6.4 Parallels with Initial Learning: Involvement of AMPARs in Long-term Memory Maintenance

In Chapter 4, we examined whether extinction memory, like initial conditioning, is maintained by regulation of GluA2-containing AMPAR endocytosis. Among the regulators of AMPAR trafficking is the NSF-GluA2 interaction which is important for maintaining GluA2-containing AMPARs at the PSD (Nishimune et al., 1998; Song et al., 1998; Lee et al., 2002). We found that disrupting the NSF-GluA2 interaction in the IL, a

structure highly implicated in the acquisition, consolidation, and retrieval of extinction (Milad and Quirk, 2002; Milad et al., 2004; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011), prevented successful recall of extinction. Our infusions of pep-R845A, the peptide that specifically blocks binding of NSF, were given well after the extinction consolidation window (Santini et al., 2004) and one day prior to the test for extinction retention. As such, it is unlikely that the effects we observed were due to impairments in either consolidation or retrieval. Thus, interfering with the NSF-GluA2 interaction impedes the long-term maintenance of extinction. We further showed that the hippocampus also plays a role in maintaining extinction memory as disrupting the NSF-GluA2 in the DH led to attenuation of fear renewal when animals were place back in the original training context.

These findings are consistent with previous work that has shown the necessity of NSF-GluA2 in the maintenance of hippocampal dependent memories (Migues et al., 2012). Moreover, our results are in line with those of He et al. (2011) who demonstrated the necessity of PKMζ within the IL for the maintenance of extinction of morphine reward related cues. Together these results add to a growing body of literature elucidating the neural circuitry of extinction. Although the IL has been implicated in the storage of extinction memory, due in part to the importance of this structure in consolidation and retrieval (for review see Quirk and Mueller, 2008; Herry et al., 2010), here we directly show that the IL acts as a locus of long-term storage for auditory fear extinction memories.

Both the DH and the ventral hippocampus (VH) are implicated in mediating fear extinction; however, previous studies have used lesions or temporary inactivations to

determine the involvement of these regions. Here we demonstrate that disrupting the maintenance of memory in the DH interferes with the renewal typically observed when the extinguished CS is presented again in the training context. Given that the DH is largely implicated in learning and memory associated with spatial navigation and contextual representations (Fanselow, 2000; Fanselow and Dong, 2010), it stands to reason that pep-R845A, the interference peptide used in these experiments, has erased, at least in part, contextual memory. Attenuation of renewal after disrupting the NSF-GluA2 interaction in the DH may be due to either, or both, the loss of context fear memory or the loss of memory for the extinction context. In this experiment we employ an ABA renewal paradigm: training occurs in context A, extinction occurs in context B, and testing occurs in context A. In this paradigm, fear responding expressed during the test in context A may result from a context-US association formed during training. In order to determine whether loss of memory for the feared context is involved in the attenuation of renewal, infusions of pep-R845A would have to be delivered into the DH prior to the onset of extinction learning.

Although inactivation of either the DH or VH can prevent renewal (Corcoran and Maren, 2001; Hobin et al., 2006), it is becoming more clear that there are dissociations in the contributions to both extinction learning and recall made by these regions. More extensively connected to the amygdala and ventromedial prefrontal cortex (vmPFC), the VH is thought to be important for motivational and emotional behaviour and involved in conditioned freezing (Bannerman et al., 2004). Whether a part of extinction memory is also maintained in the VH has yet to be shown. The VH may act more as a relay of contextual information from the DH to the amygdala and vmPFC rather than acting as a

site of long-term storage. If this is true, then future studies directed at disrupting the NSF-GluA2 interaction in the VH should not prevent renewal. Alternatively, some other aspect of extinction memory may be stored in the VH that might then be lost following infusions of pep-R845A. To explore this possibility, we would need to undertake additional experiments and compare the how responding in both the extinction context and the training context is affected after memory in the VH is disrupted.

Finally, it is conceivable that pep-R845A is not causing its effects by disrupting the NSF-GluA2 interaction, which in turn leads to AMPAR endocytosis. In Chapter 4 we do show that preventing internalization of GluA2-containing AMPARs counteracts the effects of pep-R845A. To further strengthen this argument it will be necessary in future studies to examine levels of postsynaptic GluA2 in the IL after treatment with pep-R845A. A decrease in the level of GluA2 in the synaptic fraction might be expected. Other proteins associated with synapse formation and function may too be affected following the loss of GluA2, a critical receptor in the long-term maintenance of memory.

In addition to showing that memory maintained in the IL and DH is necessary for extinction and the contextual retrieval of extinction memory, the results reported in Chapter 4 support the idea that extinction involves new learning that inhibits the original memory. The amygdala is also among the structures that have been implicated in the formation of the inhibitory extinction memory. Whether it is also involved in the maintenance of extinction, and whether this is dependent on the stabilization of GluA2-containing AMPA receptors remains to be seen. Given that both "fear" and "extinction" neurons have been identified in the basal amygdala (BA) (Herry et al., 2008), it is possible that AMPAR trafficking in these "extinction" neurons may also be responsible

for the persistence of extinction. Although it currently remains impossible to specifically target that stability of GluA2-containing AMPA receptors at the synapse of "extinction" neurons, electrophysiological recordings can uncover the activity of these neurons during recall of extinction. Responding of these neurons during extinction recall, despite the loss of extinction memory in the IL, would suggest that memory for extinction may also be stored by the amygdala. Similarly, it would be interesting to see if eliminating extinction memory in the IL leads to a decrease in responding of other cells that inhibit the CeM, including the ITC cells. Understanding how changes in one structure within the extinction circuitry correlate with the activity in other structures will shed more light on how these structures interact to maintain extinction memory.

6.5 Conclusion

The data reported in this thesis highlight the complexity of fear extinction, emphasizing the fact that extinction is not simply the undoing of learned fear. Although in our case the timing of extinction with respect to initial learning resulted in the recovery of fear, there is evidence that early extinction can involve unlearning mechanisms. Whether or not these mechanisms are engaged may also depend on other contributing factors. The timing of testing for extinction retention can also affect the recovery of fear. Experiments in this thesis show that shortly after fear extinction retention is impaired resulting in spontaneous recovery. This points to a failure of retrieval processes. Conversely, spontaneous recovery that is observed with a long time delay occurs due to the forgetting of extinction memory resulting from decay.

Just as a single process cannot explain all instances of recovery, a single brain region is not responsible for the regulation of extinction. Instead, extinction is mediated

by a network of neural structures including the IL and DH. Although the IL has been implicated in fear extinction (Herry and Garcia, 2002; Milad and Quirk, 2002; Quirk et al., 2006; Burgos-Robles et al., 2007), the experiments in this thesis show that continuous trafficking of synaptic AMPA receptors in the IL is necessary for long-term storage of extinction. The same mechanism at work in the DH is also necessary for the storage of contextual information that appears to gate the retrieval of extinction memory. Thus, ongoing activity in these structures is involved in regulating the inhibition of fear expression. Future research will likely seek to uncover how these and other structures interact. Greater focus on the underlying neurobiological mechanisms of extinction will go far to reveal how the brain learns to suppress previously learned fear.

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