Parsing memory structure with reconsolidation

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Abstract

Neural populations in the basolateral amygdala (BLA) have been shown to be an essential substrate for associative fear memories. However, it remains unclear whether distinct associative memories are mediated by independent or overlapping populations of BLA neurons. The focus of this dissertation is to describe efforts undertaken to use reconsolidation to advance this issue. I used the fact that initiation of reconsolidation is dependant on the reactivation of a particular memory. Therefore, if two memories are stored by the same overlapping population within the BLA, then interference through blockade of reconsolidation of one should lead to impairments in both. Conversely, if the two memories are stored independently of each other within the BLA, then blockade of reconsolidation of one memory should leave the second intact. I have investigated this with two protocols that each result in two distinct fear memories. Using a single tone-shock, I investigated the relationship of auditory and contextual fear memory. My findings demonstrate a complex functional interaction between these two memories. I designed a novel 2-tone protocol with which I attempted to use the selective blockade of reconsolidation to test a model of fear memory acquisition. This attempt illuminated the need for further knowledge regarding the boundary conditions of reconsolidation. I also describe an unbiased approach to detect molecular mechanisms unique to either consolidation or reconsolidation. One candidate from this screen was validated for its role in consolidation in the BLA. The experiments described in this dissertation provide a unique view of how fear memory representations are organized in the amygdala and how manipulations of reconsolidation can be used to understand the structure of memory.

Résumé

Les populations neuronales situées dans l'amygdale basolatérale (BLA) sont reconnues comme étant un substrat essentiel aux mémoires associés à la peur. Il n'est pas encore clairement établi si des souvenirs associatifs distincts sont reliés à des populations de neurones de la BLA indépendantes ou se chevauchant. Cette dissertation décritles efforts entrepris pour faire progresser

nos connaissances sur cette dernière interrogation en utilisant la reconsolidation. Pour mon étude, je me suis basé sur le fait que la reconsolidation est dépendante de la réactivation d'un souvenir en particulier. Par conséquent, si deux souvenirs sont emmagasinés par la même population de neurones se chevauchant dans la BLA, alors l'interférence causée par le blocage de la reconsolidation devrait altérer les deux souvenirs. Inversement, si deux souvenirs sont emmagasinés de manière indépendante l'un de l'autre à l'intérieur de la BLA, alors le blocage de la reconsolidation d'un seul souvenir devrait laisser le second intact. J'ai examiné cette hypothèse à l'aide de deux protocoles qui ont chacun produit deux mémoires distinctes associées à la peur. En utilisant une seule association tonalité-décharge électrique, j'ai étudié la relation entre la mémoire auditive et contextuelle associée à la peur. Mes résultats ont démontrés une interaction fonctionnelle complexe entre ces deux types de souvenirs. J'ai conçu un nouveau protocole composé de deux tonalités avec lequel j'ai essayé de bloquer sélectivement la reconsolidation pour tester un modèle d'acquisition de mémoires associées à la peur. Cette expérience démontre le besoin d'en apprendre davantage sur les conditions limitant la reconsolidation. De plus, j'ai décris une approche impartiale pour détecter les mécanismes moléculaires uniques à la consolidation ou à la reconsolidation. Suite au criblage de plusieurs molécules, une molécule candidate a été reconnue pour son rôle dans la consolidation dans la BLA. Les expériences décrites dans cette dissertation amène une perspective nouvelle sur la façon dont les mémoires reliées à la peur sont organisées dans l'amygdale et démontre comment la manipulation de la reconsolidation peut être utilisée pour comprendre la structure de la mémoire.

Preface

The aim of this thesis is to extend the knowledge of how multiple memories interact in the amygdala. Specifically, it focuses on the use of blockades of reconsolidation to demonstrate the complex functional interactions of multiple memories. This thesis contains discussions of three projects that contribute to this aim. The first project investigated the relationship of context and auditory fear memory in the amygdala. Blockades of reconsolidation showed that there is an asymmetrical activation of auditory and context fear memory that cannot be demonstrated using extinction. The second project was designed to investigate the ability of a behavioural manipulation to alter the organization of two auditory fear memories. This design illustrated some limitations to using blockades of reconsolidation to investigate the relationship of multiple memories. The third project is a manuscript in preparation for submission that I contributed to as one of the authors. It details a collaborative project designed to profile the proteomes of consolidation and reconsolidation.

Contribution of Authors

The studies in the second chapter were conceptualized through consultations with my supervisor Dr. Karim Nader. I ran all of the experiments, performed the data analysis, and wrote the chapter. I was responsible for the initial concept behind the studies in the third chapter and the experiments were designed with further consultation with Dr. Nader. I ran all of the experiments, performed the data analysis and wrote the chapter. The fourth chapter is an adaptation of a manuscript that I am submitting for publication in collaboration with Rachel Jeffrey, Christos Gkogkas, Christopher Kent, James Wohlschlegel, Alyson Fournier, Karim Nader and Nahum Sonenberg. The behavioural groups and time points were designed in collaboration between Dr. Nader, Dr. Jeffrey, Dr. Gkogkas and I, based on a pilot that Viginia Migues contributed to with the other authors. Dr. Jeffrey, Dr. Gkogkas and I ran the first set of behavioural experiments and collected brains. The collection and processing of amygdalas for mass spec analysis was done by

Dr. Jeffrey and the mass spec analysis was done by Dr. Wohlschlegel in consultation with Dr. Jeffrey. The validation of the candidates was done by Dr. Jeffrey using reagents provided, previously characterized for their specificity and optimized by Dr. Fournier and Chris Kent. The manuscript was written by Dr. Jeffrey and me with edits provided by the other authors. Chapter 5 serves as the general conclusion for this thesis, and is written exclusively by me.

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Table of Contents

List of Figures

List of frequently used abbreviations

AFC –Auditory Fear Conditioning

AMPAR - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

Aniso -anisomycin

BLA – Basolateral amygdala

cAMP – cyclic adenosine monophosphate

CFC – Contextual fear conditioning

CREB –cAMP response element binding protein

CS – conditioned stimulus

GFP – green fluorescent protein

IEG – immediate early gene

LTM – long-term memory

LTP – long-term potentiation

MAPK – mitogen activated protein kinase

MGN – medial geniculate nucleus

NMDAR – N-methyl –D – aspartate receptor

PKA – cAMP dependent protein kinase

PKC – protein kinase C

PR-LTM –post reactivation long-term memory

PSI – protein synthesis inhibitor

SEM – standard error of the mean

SOFC – second order fear conditioning

STM –short-term memory

US – unconditioned stimulus

Chapter 1:

General Introduction

1.1 Fear memory

Classical fear conditioning occurs when a previously neutral stimulus (termed the conditioned stimulus, or CS) is paired with and aversive stimulus (the unconditioned stimulus, or US). The result is that after this pairing the CS acquires the capacity to elicit behavioural, autonomic and endocrine responses that are characteristically presented in response to danger (Takahashi et al., 2005). A type of classical fear conditioning that is commonly used in memory research in rats is auditory fear conditioning (AFC). In this case, the CS is a discrete sound and the behavioural indicator of the memory is the response to presentation of the tone following conditioning, complete immobility except for respiration, termed freezing (Blanchard and Blanchard, 1969; Fanselow, 1980). AFC provides an extremely useful tool for memory research in that it is rapidly acquired, persistent, phylogenically conserved and involves similar neural circuitry in many vertebrate systems (LeDoux, 2000).

1.2 Anatomy of fear memory

Research in the last few decades has collectively provided a clear picture of the neuroanatomy encoding AFC (Pascoe and Kapp, 1985;Davis, 1992;Fanselow and LeDoux, 1999;LeDoux, 2000). The main result of the acquisition of AFC, like other forms of associative learning, is that the response to the CS is altered after its pairing with the US. Thus, the primary locus of interest is the point at which these two signals converge. The brain structure shown to be this primary locus of convergence is the amygdala (Romanski et al., 1993). The amygdala is composed of many structurally distinct nuclei (Pitkanen, 2000). These nuclei are delineated by differences in cell type or anatomical projections where the primary nuclei involved in fear conditioning are the lateral (LA), basal (BA) and central (Ce) (Pitkanen, 2000). The LA and BA are collectively referred to the basolateral amygdala (BLA).

1.2.1 CS pathways

The brain perceives sound through the ear and this provides information from the surrounding environment that is important for the animal to form a representation of the world it

lives in. Auditory signals from the ear are passed via the inferior colliculus to the medial geniculate nucleus of the thalamus (MGN). These signals are then passed on to the auditory cortex for further sensory processing as well as projecting to the LA (LeDoux et al., 1990; Romanski and LeDoux 1992). The LA also receives subsequent auditory signals from the auditory cortex (LeDoux et al., 1991; Amaral and Insausti 1992; Romanski and LeDoux 1992; Mascagni et al., 1993; Romanski and LeDoux, 1993; Li et al., 1996; McDonald 1998). Other sensory stimuli, such as contextual information from the hippocampus project to the BA (Maren and Fanselow, 1995; Pitkanen et al., 2000).

Neurons in the LA are responsive to auditory stimuli. In contrast to MGN and auditory cortex neurons, BLA neurons have fairly broad tuning curves, except for very high frequencies (Bordi and Ledoux, 1992) meaning that the BLA neurons do not have a preferred frequency which they respond to, but rather are capable of detecting relationships between many different frequencies and other environmental stimuli. The significance of this will be discussed later in this chapter.

1.2.2 US pathways

Much like the auditory signals, nociceptive signals from the peripheral nervous system are passed on to the LA and BA via the posterior thalamus and insular cortex (Shi and Davis, 1999; Brunzell and Kim, 2001; Jasmin et al., 2004; Lanuza Nader and LeDoux, 2004). Recent work has also provided evidence of the importance of the periaquaductal grey (PAG) in relaying to the amygdala US information, and expectation based modifications of US signals (Johansen et al., 2010b). This will be discussed further in section 1.4.6, but in short, the authors found that an unexpected US presentation results in the depolarization of a large population of LA neurons that changed during associative learning. As conditioning progressed, they observed that the decreased magnitude of the US-elicited response was inversely correlated with freezing (Johansen et al., 2010b). This suggests that the expectation of the shock was diminishing the elicited response in the LA. This is not a totally novel finding as both positive and negative unconditioned stimuli have been shown to elicit responses in the amygdala that are altered by expectation (Belova et al.,

2007). However, Johansen and colleagues also demonstrated that this modification in US elicited response in the LA was due to a parallel modification in US elicited response in the PAG (Johansen et al., 2010b). This suggests that PAG afferents to the BLA are a main source of US input.

1.2.3 Output

The Ce receives afferents from the BLA and projects to areas that control the expression of fear responses (Pitkanen, 2000). Autonomic reactions are triggered by direct projections to brainstem and hypothalamus (Kapp et al., 1979; LeDoux et al., 1988). Defensive responses, such as freezing, are triggered by projections to the periaqueductal gray (De Oca et al., 1998), which also trigger an endogenous opioid-mediated analgesic state (Fanselow et al., 1991; Helmstetter and Bellgowan, 1993; Helmstetter and Tershner 1994).

1.3 The LA/BLA is the site of storage of auditory fear memory

Although both auditory and nociceptive signals converge in the BLA, this is not sufficient to declare that the BLA is the site of storage of the fear memory. This convergence could simply suffice to modulate the storage of a memory elsewhere in the brain (Cahill and McGaugh, 1998). However, there are multiple lines of evidence supporting that, at least in AFC, the site of fear memory storage is the BLA (Fanselow and LeDoux, 1999; Schafe et al., 2005).

First, lesions of the BLA impair both acquisition and expression of AFC (Gale et al., 2004). Secondly, long-term potentiation (LTP), a physiological protocol which is widely considered to mimic mechanisms involved in natural learning, can be elicited in the BLA both *in vivo* (Racine and Milgram, 1983; Clugnet and LeDoux, 1990) and in slices (Chapman et al., 1990; McKernan and Shinnick-Gallagher, 1997; Huang and Kandel, 1998). Additionally, molecular mechanisms underlying LTP are necessary for AFC (Bauer et al., 2001) and the physiological responses in the BLA following AFC mimic those produced by LTP protocol in vivo (Quirk et al., 1995).

Recently another line of evidence has provided further support for the BLA being the site of associative fear memory storage. There has been increased interest in the role of a constitutively

active protein kinase C isoform, protein kinase Mζ (PKMζ), in the maintenance of long-term memory (Sacktor et al., 1993; see Sacktor 2011 for review). This kinase has been shown to maintain LTP and LTM by stabilizing GluR2 subunit containing AMPA receptors at the post-synaptic membrane (Ling et al., 2006; Yao et al., 2008; Migues et al., 2010). In vivo blocking of PKMζ activity with zeta pseudosubstrate inhibitory peptide (ZIP) results in the erasure of long term memory in various brain regions (Pastalkova et al., 2006; Shema et al., 2006; Serrano et al., 2008; Kwapis et al., 2009; Migues et al., 2010; von Kraus et al., 2010) including the erasure of associative fear memory in the BLA (Kwapis et al., 2009; Serrano et al., 2008; Migues et al., 2010 but see Parsons and Davis 2011a; Nader 2011; Parsons and Davis 2011b). Along with the above evidence this provides a strong case that the storage of associative fear memory occurs in the BLA. However, in the absence of a method that provides falsifiable predictions regarding the nature of an absence of memory in the BLA (as Hardt et al., 2009 did in the dorsal hippocampus) it is not possible to state this conclusively.

1.4 Encoding of AFC in the BLA

1.4.1 The Hebbian Synapse

A critical change underlying learning and memory is alterations in synaptic transmission (Cajal 1894 see (Hebb, 1949; Jones, 1994; Kandel, 2001). In his influential 1949 book *The Organization of Behaviour* Donald Hebb postulated that when two interconnected neurons fire at the same time, the synapses between them become persistently stronger (Hebb 1949). This phenomenon, coined Hebbian plasticity, is widely accepted to be a neural mechanism for long term memory (Kandel and Spencer, 1968; Levy and Steward, 1979; Hawkins et al., 1983; Kelso et al., 1986; Martin et al., 2000; Sah et al., 2008). One technique used in the lab to study this change in synaptic efficacy is LTP. Brief repetitive stimulations of a synaptic pathway result in long-term enhancement in the responsiveness of this pathway (Bliss and Lomo, 1973). Although this phenomenon was discovered and characterized primarily in hippocampal preparations, it is also

found in the BLA (Clugnet and LeDoux, 1990; Chapman et al., 1990; McKernan and Shinnick-Gallagher, 1997; Huang and Kandel, 1998).

1.4.2 Glutamate receptors and LTP

Because the initial characterization of LTP arose from studies of the Shaffer collateral in the hippocampus, I will briefly review these findings before discussing LTP in the BLA. Glutamatergic synapses (those synapses releasing glutamate as the primary neurotransmitter) express two types of ionotropic glutamate receptors, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR). AMPA receptors, when bound by glutamate become permeable to sodium and potassium and are able to open at resting-membrane potential upon glutamate application. These receptors have rapid kinetics and are primarily involved in basal synaptic transmission. NMDA receptors, on the other hand, are not permeable at resting-membrane potential. NMDAR are blocked by extracellular magnesium which is released by postsynaptic membrane depolarization. Release of the magnesium block results in an increase in permeability to sodium, potassium and calcium through the NMDA receptor. In this manner, the NMDAR can act as a coincidence detector, only being activated when glutamate release is paired with postsynaptic depolarization (Malenka and Bear, 2004). Prevailing models of LTP state that this coincident activity, which causes an increases in intracellular calcium triggers a cascade of biochemical changes which ultimately lead to strengthening of the activated synapse (Bliss and Collingridge, 1993). These biochemical changes result in phosphorylation of AMPARs which increases their conductance and transports new AMPARs to the postsynaptic density (Malinow and Malenka, 2002). The primary manner in which LTP is induced is by applying a high-frequency train of electrical stimulation (ie 100Hz). However, it is also possible to elicit LTP using other protocols such as pairing direct depolarization of the postsynaptic neuron with synaptic activation. The somatically induced action potentials back-propagate into the dendritic tree which is sufficient to release the magnesium block from the NMDAR and permit increases in intracellular calcium (Stuart and Sakmann, 1994).

1.4.3 LTP in the LA

In amygdalar slice preparations or *in vivo* LTP can be induced with high frequency stimulation of auditory thalamus or auditory cortex (e.g. Schafe et al., 2008). It can also be induced by stimulating the internal or external capsule which correspond to thalamic and cortical LA innervation respectively (e.g. (Huang and Kandel, 1998). LA LTP can also be induced by pairing internal or external capsule stimulation with LA pyramidal neuron depolarization (e.g. Shin et al., 2006. This later approach is thought to most closely mimic the manner in which synaptic potentiation is thought to occur in AFC.

The direct application of Hebb's rule to AFC would state that pairing of a weak input from a CS with a strong input from a US should result in the strengthening of the CS input (Blair et al., 2001). There is increasing evidence that this is the case. Unexpected US presentation results in depolarization of LA pyramidal cells (Johansen et al., 2010a). When the CS input occurs in close temporal proximity, the weaker activity at the synapses from the CS paired with the strong depolarization from the US should be sufficient to activate postsynaptic NMDARs and increase intracellular calcium. Research has shown that both thalamic and cortical inputs to the LA are glutamatergic and terminate on synapses containing both AMPA and NMDA receptors (Mahanty and Sah, 1999). NMDA receptors are heterologous, in that they can be formed by different subunits, and alternative splicing of the subunits leads to even more combinations expressed in the neuron (Zukin and Bennett, 1995). Recent evidence suggests that one subunit in particular, the NR2B subunit, appears to play a particularly important role in fear conditioning (Rodrigues et al., 2001; Wang et al., 2009). The convergence of CS and US induced activity results in both a short- and long-term facilitation of neuronal response to the CS alone (Quirk et al., 1995; Quirk et al., 1997; Rogan et al., 1997; Collins and Pare, 2000; Goosens et al., 2003).

1.4.4 US elicited activity in the LA

Recently the necessity of LA pyramidal cell depolarization has been confirmed. Using channelrhodopsins, light activated ion channels that have been genetically inserted into the animal,

combined with behaviour, Johansen et al (2010b) demonstrated that pairing depolarization of LA pyramidal cells with tone presentations resulted in freezing to the tone alone twenty-four hours later. If a US is to act as a teaching signal in the BLA, then as shown by the blocking phenomenon, this signal should be inhibited by expectation rather than simply corresponding to direct sensory input (Johansen et al., 2010b). By tracking electrophysiological responses to US presentations, the role of expectation in BLA response to US has been characterized.

Using single-unit recording in the amygdala during fear conditioning the same group demonstrated that an unexpected shock presentation results in depolarization of a large population of neurons that are broadly distributed throughout the LA (Johansen et al., 2010a). After thorough pairing of a tone with this shock they found an interesting change. When the shock was delivered unexpectedly, they saw a similar response as they did pre-training. However, when they delivered the shock following a presentation of the paired tone, making it expected, the response to the shock was significantly decreased. They also measured the electrophysiological responses to the tone presentation. In agreement with previous work (Repa et al., 2001 Han et al., 2007; Han et al., 2009), they observed that in the conditioned animals approximately one third of the CS responsive neurons CS-evoked responses were enhanced and over half remained unchanged. Importantly, the decrease in US-evoked firing did not depend on an increase in CS-evoked firing. In other words, in the population of neurons that modulated their firing to the expected US, there were both neurons with enhanced and unchanged CS-evoked firing (Johansen et al., 2010a). This is consistent with the role of the US-expectedness as a teaching signal, as described in the blocking phenomenon where, in the presence of a predictive CS, the US is unable to form an association with a novel CS. However, if there is only moderate pairing between CS and US, there is still opportunity for a novel CS to become associated with the US because the US responsive neurons are broadly distributed throughout the network of CS responsive neurons in the LA.

1.4.5 Integration of inputs in the LA

The thalamic and cortical CS routes to the LA have been referred to as the low road and the high road respectively (Johnson et al., 2009). This refers to the fact that auditory information

requires less synaptic transmissions to reach the LA directly from the thalamus and thus arrives there up to 30 milliseconds (ms) sooner than cortical inputs (Armony et al., 1995; Li et al., 1996; Quirk et al., 1997; Armony et al., 1998). Although the thalamoamydalar pathway is sufficient for simple AFC, the more detailed cortical inputs, regulated by presynaptic inhibition, are thought to be necessary for more detailed fear memories (LeDoux, 2000; Shaban et al., 2006). The interconnectedness of neurons in the superior and inferior dorsal LA, there appears to form a recurrent network which permits the coincident detection of these temporally separate inputs from the thalamus and cortex (Johnson et al., 2008).

However, there remains an irregularity between the theory proposed by Hebb's rule and the evidence of LA neural involvement in AFC memories. Substantial populations of LA neurons are responsive to both somatosensory and auditory stimuli (Romanski et al., 1993). Importantly, the auditory responsiveness of LA neurons is not tuned as the thalamic and cortical neurons are. Rather, LA neurons are responsive to a wide range of frequencies (Bordi and Ledoux, 1992). According to Hebb's rule, the strengthening of synapses and incorporation into the memory trace should occur at each of these points of convergent activity. However, it has been repeatedly demonstrated that this is not the case. Rather, only 20-30% of these neurons are incorporated (Repa et al., 2001; Rumpel et al., 2005; Han et al., 2007; Han et al., 2009; Zhou et al., 2009; Johansen et al., 2010a). A recent series of experiments has begun to provide a possible explanation for why this may be the case (Han et al., 2007; Han et al., 2009; Zhou et al., 2009). It is necessary to discuss this within the context of not only the memory storage, but also the retrieval of a fear memory.

1.5 Retrieval

Associations are formed in the manner discussed above (section 1.4.1). A retrieval cue, or stimulus, activates a unit of an associative network. Based on associative strength this activation will spread to other units of the network. The activation of a neural network can be visualized using immediate early genes (IEG) (Guzowski et al., 2005). These are a family of genes which are rapidly transcribed following neural stimulation (Lanahan and Worley, 1998)

including *c-fos* (Morgan et al., 1987), activity regulated cytoskeleton-associated protein (Arc) (Lyford et al., 1995; Guzowski et al., 1999), and *zif268* (Cole et al., 1989). By labeling either the mRNA or protein product of these genes one can visualize the population of neurons that have been recently active. Using these tools it has been shown that when an animal is presented with a conditioned tone approximately three times more LA neurons are active than home cage controls (Hall et al., 2001b). This increase in neuronal population activity, in a structure critical for the storage of AFC, is thought to represents the memory trace (Guzowski et al., 1999; Reijmers et al., 2007).

However, IEG activity at retrieval is not direct evidence that these neurons are the same neurons that were active during training. A recent experiment has used a genetic approach to investigate this issue. Reijmers et al (2007) created a TetTag mouse that allows for temporal induction of a c-fos activated transgenic tag that can be visualized. When this transgene is turned on (by removing doxycycline from the animals diet) neural activity (through c-fos activation) results in the production of tau-LacZ (LAC) which remains elevated for a period of at least five days after the transgene is turned off by returning doxycyline to the diet. The authors trained animals with AFC with the transgene on. This resulted in neurons in the LA involved in the acquisition of this memory being labeled with LAC. Then, with the transgene off, they gave the animals a LTM test and labeled cells expressing *zif268*. By quantifying the cells that expressed both LAC and *zif268* they were able to determine that fear conditioning and fear memory retrieval activate the same population of neurons.

1.6 Neural selection during encoding

As I have mentioned above, not all of the neurons that are activated by both CS and US become part of the memory trace. This was first suggested by data from Rumpel et al (2005). They demonstrated that although approximately seventy percent of LA pyramidal neurons are responsive to the CS and the US (Romanski et al., 1993; Repa et al., 2001; Johansen et al., 2010a), disrupting synaptic plasticity in only twenty percent of LA neurons disrupted conditioned freezing (Rumpel et al., 2005). This was confirmed by Repa et al (2001) who recorded from one hundred

CS responsive LA neurons and saw potentiation in CS evoked potentials in only twenty-four. These findings have been expanded further by recent work lead by Sheena Josselyn's group at the University of Toronto (Han et al., 2007; Han et al., 2009). They made this advance using herpes simplex virus (HSV) vectors expressing cAMP response binding protein (CREB) in approximately twenty percent of LA neurons. CREB is a transcription factor that has been shown to be critical for long-term synaptic plasticity (Bartsch et al., 1998). The infection of approximately twenty percent of LA neurons was due to the infection rate of the HSV vector that they used, however this corresponds to the size of the sub-populations seen in previous experiments to underlie the AFC memory trace (Repa et al., 2001).

These neurons, in addition to having an increase in CREB levels were labeled with green florescent protein (GFP). After AFC they found that the infected neurons were more likely than their uninfected neighbours to be labeled with Arc following a LTM test, implying that they were involved in the memory trace. If they infected the neurons with GFP alone, the infected neurons no more likely than their uninfected neighbours to be part of the memory trace. Importantly, the size of the memory trace (how many Arc positive neurons) remained constant between their different treatments (Han et al., 2007). This suggests that there is a competitive selection amongst the LA neurons that receive common inputs and that CREB levels will bias this population of neurons to become part of the memory trace. The implication is that the neurons that are recruited by this selection undergo long-term synaptic plasticity whereas those that are not selected remain unpotentiated (Han et al., 2007; Josselyn, 2010).

The Josselyn group further expanded on this seminal finding by infecting twenty percent of LA neurons with a HSV vector co-expressing CREB-GFP and inducible diphtheria toxin (DT) receptors (Han et al., 2009). Since mice do not endogenously express DT receptors, injecting these mice with DT will only induce apoptosis (the result of DT receptor activation) in neurons infected by the HSV vector. They found that LA neurons in animals with increased CREB and expressing DT receptors showed the same selection bias as in the previous study. When they gave systemic injections of DT to these animals they showed a marked deficit in freezing. When they gave DT to trained animals that were either infected with only GFP and expressing DT receptors

or infected with CREB-GFP and not expressing DT receptors injections of DT did not produce this deficit. After giving DT to animals with increased CREB and expressing DT receptors, they were able to subsequently re-train these animals demonstrating that the deficit in freezing was not due to nonspecific impairments to the LA. They also showed that deleting the CREB infected neurons prior to training did not impair acquisition of AFC (Han et al., 2009). One very important implication of these last findings is that although the increased CREB levels can bias a population of LA neurons, specifically ablating these neurons did not eliminate the ability of the rest of the LA to acquire a subsequent auditory fear memory.

This research into the selection effect (Han et al., 2007; Han et al., 2009; Zhou et al., 2009) are the first to show conclusive evidence regarding a possible mechanism for why there is a population of neurons that receive convergent input from the CS and US but only a subset of this population undergo long-term synaptic plasticity.

1.7 Multiple memory interaction

Up until this point, I have restricted my discussion to general encoding and storage of memories in the LA. However, the issue of storage for tone and context fear memory raises an interesting question: how do multiple memories interact in the LA? General consensus is that the unit of memory is the synapse (Kandel and Spencer, 1968; Levy and Steward, 1979; Hawkins et al., 1983; Kelso et al., 1986; Martin et al., 2000; Sah et al., 2008 but see Zhang and Linden, 2003; Govindarajan et al., 2011). Considering each neuron has on the order of a thousand synaptic connections one neuron is capable of being part of multiple memory traces.

1.7.1 Consolidation & Reconsolidation

> "…long-lasting trace of an experience is not completely fixed, consolidated, or coded at the time of the experience. Consolidation requires time, and under at least some circumstances the processes of consolidation appear to be susceptible to a variety of influences both facilitating and impairing-for several hours after the experience. "
>
> (McGaugh, 1966)

> "Learning always occurs in the presence of specific cues and a contextual environment, and to the extent that these cues and context occur again the memory will be reinstated…Under both conditions the memories are open to disruption by amnesic agents."
>
> (Lewis, 1979)

Consolidation is defined as a window of time after learning in which the memory can be impaired or enhanced by pharmacological or other manipulations (McGaugh, 1966). This is represents a shift from a labile, or sensitivity to disruption, short-term memory to a stable long-term memory. The prototypical experiment demonstrating a consolidation effect is as follows. A memory is acquired, for example, AFC. Immediately before or following the acquisition session an amnesic agent is applied to the animal. The retention of the memory is then tested at both short and long time points. Short tem memory (STM) test are typically done approximately one to three hours following the acquisition while long-term memory (LTM) tests are typically done twenty-four or more hours after acquisition. In order for a memory deficit to be defined as a consolidation deficit STM must be shown to be intact, and LTM impaired. If STM is also impaired then the interpretation is that rather than a deficit in consolidation, a deficit in acquisition has been induced.

The common actions of pharmacological amnesic agents and blockers of LTP have been one of the stronger lines of evidence suggesting that LTP is the cellular mechanism underlying memory (Martin et al., 2000). One of the more common classes of pharmacological amnesic agents is the protein synthesis inhibitors (PSI, (Davis and Squire, 1984); ie aniso, rapamycin, cycloheximide). This protein synthesis dependant process is referred to as 'cellular consolidation' (Dudai, 1996).

Although consolidation theory was the dominant theory of memory processing for the later half of the last century, there is mounting evidence that the strong view of consolidation (that a memory trace is only labile once) is not the most parsimonious model of memory. One major line of evidence that weakened the consolidation view of memory was the observation of cue-induced amnesia. Using electro-convulsive shock (ECS), Misamin et al (1968) showed that contrary to consolidation theory, memory could be impaired after the consolidation time window had closed. First, they replicated the established finding that ECS immediately following acquisition impaired performance in both STM and LTM tests. Other groups of animals received

ECS the following day. These animals either received a reminder session in which they presented the animals with the CS or were undisturbed prior to ECS. They found that animals that had been reminded showed a significant deficit in memory compared to the non reminded animals (Misanin et al., 1968). This showed that lability is not exclusive to post-acquisition time points. Rather, the retrieval of a memory can induce subsequent periods of lability.

1.7.2 Using reconsolidation to study the structure of memory

While this data did not make an immediate impact on the field of memory, it has enjoyed a renaissance in the last fifteen years (Nader et al., 2000). Although initially controversial (e.g. McGaugh, 2004; Biedenkapp and Rudy, 2004; Alberini, 2005) cue-induced amnesia has been renamed reconsolidation (Rodriguez et al., 1993; Przybyslawski and Sara, 1997; Nader et al., 2000). As detailed above, the main characteristic that makes reconsolidation an attractive tool in the investigation of multiple memories in the BLA is that the impairments are reactivation specific. One use of this property was demonstrated in an investigation of second-order fear conditioning (SOFC) (Debiec et al., 2006). First order fear conditioning is, as has been described previously, the pairing of a CS and a US. In addition to eliciting unconditioned responses, a CS also acquires reinforcing properties of the US (Estes 1943). This allows the CS to act as a US for subsequent neutral cues. In a SOFC experiment the first cue (CS1) has been paired with the US. In a subsequent session, the CS1 is paired with a second cue (CS2). After this second conditioning session, presentation of CS2 alone will elicit unconditioned responses (Gewirtz and Davis, 2000).

Two possible interpretations of this finding are 1) the presentation of CS1 in the second session activates a representation of the US which CS2 associates directly with or 2) CS1 acquires reinforcing properties of the US and CS2 associates directly with CS1. These two interpretations would result in either two memories (CS1 → US & CS2 → US) or a single serial memory (CS2 → CS1 → US). Two tests that can be used to differentiate these two models are US devaluation, and extinction. In a devaluation experiment, an appetitive US (such as food) is paired with a CS. After the CS – food association is formed, the animals is either satiated, or the food is paired with

illness, then the cue that was directly associated with the food no longer elicits the same behavioural response. However, in second-order conditioning, CS2 does not show this decrease, suggesting that CS1 but not CS2 is directly associated with the US (Holland and Rescorla, 1975).

If the response to CS2 is dependant on the CS1 → US association, then reduction in responding to CS1 should result in a parallel decrease in responding to CS2 (Rizley and Rescorla, 1972). One way to behaviourally reduce response to a CS is to repeatedly present it in the absence of the US. This extinction training results in a gradual decrease in conditioned responses. It has been shown that rather than erasing the CS – US association, extinction is thought to form a new CS – noUS association (Bouton, 2004; but see Quirk et al., 2010 for a review of new data suggesting that under some conditions extinction can erase fear memory). Although there is some conflicting data in different behavioural paradigms (e.g. conditioned suppression, (Rizley and Rescorla, 1972); appetitive Pavlovian conditioning, (Holland and Rescorla, 1975), extinction of CS1 in SOFC has been shown to reduce freezing to CS2 whereas extinction of CS2 has no effect on CS1 (Debiec et al., 2006).

It has been shown that the acquisition of SOFC requires activity and plasticity in the BLA (Parkes and Westbrook, 2010). On a cellular level, the CS1 → CS2 association should be stored in neurons that receive convergent activation from both CS1 and CS2. After the first session of conditioning presentation of CS1 would elicit a potentiated response (Quirk et al., 1997), and pairing CS2 with that activity would lead to long-term synaptic plasticity. Debiec at al (2006) investigated what component of the SOFC memory trace is reactivated in response to presentation of CS1 or CS2. Earlier work had shown that like in the original cue-induced amnesia, post consolidation impairments using PSI is a function of memory retrieval. If the memory is not reactivated, then PSI either systemically (Suzuki et al., 2004), or at the site of memory storage (Nader et al., 2000) will have no effect. Thus blocking reconsolidation with PSI in the amygdala can indicate what aspect of a particular memory has been reactivated. They found that when aniso was infused into the BLA following CS2 presentation, animals were impaired in freezing to CS2 but not CS1. They also found that when they infused aniso after CS1 presentation the animals

were impaired to both CS1 and CS2 (Debiec et al., 2006). This supports the idea that SOFC results in a serial association.

Two subsequent papers from the same group have also used reconsolidation deficits to investigate the structure of memory in the BLA (Doyere et al., 2007; Debiec et al., 2010). The first paper looked at the effect of blocking reconsolidation of one auditory fear memory when an animal had previously acquired two distinct auditory fear memories. The training session consisted of two distinct auditory stimuli (pure 1 kHz tone & complex frequency modulated sound) that were paired with foot shocks. A day later one of the two tones were reactivated along with an intra-BLA infusion of UO126, a mitogen-activated protein kinase kinase (MEK 1/2) inhibitor which has been shown to block both consolidation (Schafe et al., 2000) and reconsolidation (Duvarci et al., 2005). They found that while there was a deficit in freezing to the reactivated tone, the non-reactivated tone elicited intact freezing (Doyere et al., 2007).

The second paper investigated the effect of blocking reconsolidation following US presentation, which like in the previous study, had been used to condition multiple tones (Debiec et al., 2010). They found that when they gave intra-BLA infusions of aniso following a brief foot shock this disrupted freezing to both CSs. When they conditioned two CSs, one with a foot shock, and the second with an eyelid shock, reactivating one US and giving intra-BLA aniso specifically impaired freezing to the CS paired to that US (Debiec et al., 2010). This finding is consistent with earlier work showing reactivation of one memory induced lability of other memories acquired with the same reinforcer. Specifically, animals were trained on 3 operant tasks simultaneously; two were reinforced with the same reinforcer (e.g. food) and the third with another reinforcer (e.g. shock). Reactivation of a food reinforced memory caused both food, but not the shock reinforced memories to undergo reconsolidation (Robbins and Meyer, 1970). These findings have started to shed light into the how multiple AFC memories interact in the BLA. More importantly, they have demonstrated that reconsolidation impairments can be a powerful tool in investigations of memory structure.

An advantage that reconsolidation impairments have is that manipulation can be spatially restricted to the brain region of interest. Fear conditioning results in plasticity in many areas

including the dorsal hippocampus (Doyere et al., 1995), medial prefrontal cortex (Maxwell et al., 1994; Burgos-Robles et al., 2009) thalamus (Lennartz and Weinberger, 1992; Han et al., 2008), and primary (Bakin and Weinberger, 1990;) and secondary sensory cortex (Sacco and Sacchetti 2010). Manipulations of a fear memory such as extinction result in changes in many of these regions (Herry et al., 2010; Pape and Pare, 2010; see section 2.4.5 for further discussion). This makes it more difficult to make inferences regarding memory structure from the perspective of a single brain region.

1.9 Aims of this dissertation

The purpose of my research is to investigate the interactions of multiple memories in the BLA using reconsolidation. On a very general level, I used a similar approach as (Debiec et al., 2006; Doyere et al., 2007; Debiec et al., 2010). The experiments in chapter 2 and 3 are composed of three phases: acquisition, manipulation and testing. In the acquisition phase animals received fear conditioning that result in two fear memories. In the manipulation phase, one of the two memories will be manipulated using either extinction or pharmacological disruption of reconsolidation. In the testing phase, LTM tests assess the effect that the manipulation phase had on the two memories. It is important to note that the LTM test of the manipulated memory is required to confirm that the manipulation was effective, and the LTM test of the non-manipulated memory will be used to assess the relationship of the two memories.

The model that we are testing is that two fear memories could either have independent or overlapping representations in the BLA. There are a number of variables that could conceivably alter the manner in which two fear memories interact in the BLA. The manipulations and parameters in the acquisition phases of these experiments are designed to test some of these variables.

Chapter 2 discusses my investigation into the relationship of an auditory and context fear memory. Unlike two auditory memories, auditory and context fear can be acquired simultaneously with a single US presentation. I used reconsolidation deficits and extinction to investigate how these two memories interact in the BLA.

Chapter 3 discusses my investigation into the structure of two pure tone fear memories. As Doyer et al (2007) demonstrated, multiple AFC memories can be independently reactivated US. However, the recent work lead by Sheena Josselyn's group (Han et al., 2007; Han et al., 2009) suggests that there may be conditions which could alter the relationship of multiple memories. I attempted used blockades of reconsolidation to determine if a behavioural manipulation could alter the relationship of two auditory fear memories.

Chapter 4 will discuss a collaboration that attempted to use a proteomic approach to identify candidate mechanisms that are dissociated between consolidation and reconsolidation. This study led to the identification for a role of 14-3-3 proteins in mammalian memory consolidation.

Chapter 5 will provide an overall discussion of the work presented in this dissertation.

Chapter 2:

How context and tone memories interact in the BLA

2.1 Introduction

The purpose of my research is to investigate the interactions of multiple memories in the BLA using reconsolidation. The first pair of fear memories that I investigated was an auditory fear memory and a contextual fear memory. Following a single tone -shock pairing animals will acquire fear responses to both the training context as well as the tone in a neutral context (Stiedl and Spiess, 1997). A wide variety of parameters could effect how two memories interact within a single memory system (i.e. cue similarity, relative predictability, passage if time between learning, etc.). The advantage of investigating these two memories is that they can be acquired simultaneously. This simultaneity eliminates one potential parameter, time between learning, thus simplifying the relationship between them.

It is generally agreed that the BLA is responsible for the learning and consolidation of auditory and contextual conditioned fear (Fanselow and LeDoux, 1999; LeDoux, 2000; Goosens and Maren, 2001). However, the relationship of auditory and contextual fear memories in the BLA is not clear. Some findings have suggested that tone and context fear memories are spatially distinct in the BLA. Reijmers et al (2007) showed that the populations of neurons that correlate with the expression of contextual and auditory fear memories are spatially distinct. Specifically, the number of neurons active during both fear conditioning and LTM test in the LA correlated with amount of freezing elicited by the tone and in the BA correlated with the amount of freeing elicited by the context. These results support previous work done using lesions and temporary inactivation of specific amygdalar nuclei. Pretraining electrolytic lesions of the LA but not BA have been shown to impair AFC (Nader et al., 2001) and pretraining ibotinic lesions of the BA, which spare fibers of passage, attenuated CFC but had no effect on simultaneously acquired AFC (Onishi and Xavier, 2010). Contrary to this, post-training lesions of the BA has been shown to block the expression of AFC when the BA was intact during training (Anglada-Figueroa and Quirk, 2005). However, after this BA lesion, these animals were able to reacquire AFC (consistent with Nader et al 2001) indicating that in an intact brain the BA is involved in AFC acquisition but the LA is sufficient in the absence of the BA (Anglada-Figueroa and Quirk, 2005). Reversible inactivation using lidocaine (a sodium channel blocker) has been used to further

dissociate the roles of the LA and BA in the acquisition of AFC and CFC. Calandreau et al (2005) showed that, like with pretraining lesions, pretraining inactivation of the LA, but not the BA block the acquisition of AFC.

The effect of pretraining LA and BA inactivation on CFC is dependant on how the contextual fear memory is acquired. Calandreau et al (2005) used two different acquisition protocols, paired and unpaired. In the paired protocol, the tone coterminates with a foot shock, while in the unpaired protocol the tone and shock are explicitly unpaired. The paired protocol results in freezing to both tone and context, while the unpaired protocol results in freezing to the context only. In the paired protocol the context is learned as a background cue whereas in the unpaired protocol the context is learned as a foreground cue (Phillips and LeDoux, 1994). When the BA is inactivated during acquisition of either protocol, an impairment in CFC is observed (Calandreau et al., 2005). When the LA is inactivated during the acquisition of the unpaired protocol, an impairment in CFC is observed as well. However, when the LA is inactivated during the acquisition of the paired protocol an enhancement in CFC is observed. This indicates that changes in acquisition parameters can alter the manner in which the BLA processes the relevant cues. From these collective findings, it appears that a paired protocol acquisition of AFC and CFC result in spatially discrete representations; AFC primarily in the LA and CFC primarily in the BA.

However, there are also findings that support AFC and CFC being stored in a less spatially discrete manner. Hall et al (2001) looked at zif268 (an immediate early gene product which correlates with neural activity) expression in the LA and BA (as well as other areas) during auditory and context LTM tests following either a paired or unpaired acquisition protocol. They found that after the paired training, the auditory LTM test elicited increases in both LA and BA activity. Similarly, after the unpaired protocol, the context LTM test elicited a similar pattern of activity in both nuclei (Hall et al., 2001a). Another finding that supports the idea that auditory and contextual fear memories have overlapping representations is work by Han et al (2009). They show that if a population of neurons in the lateral nucleus are biased towards inclusion into an auditory fear memory trace, and subsequently selectively ablated, both auditory and contextual

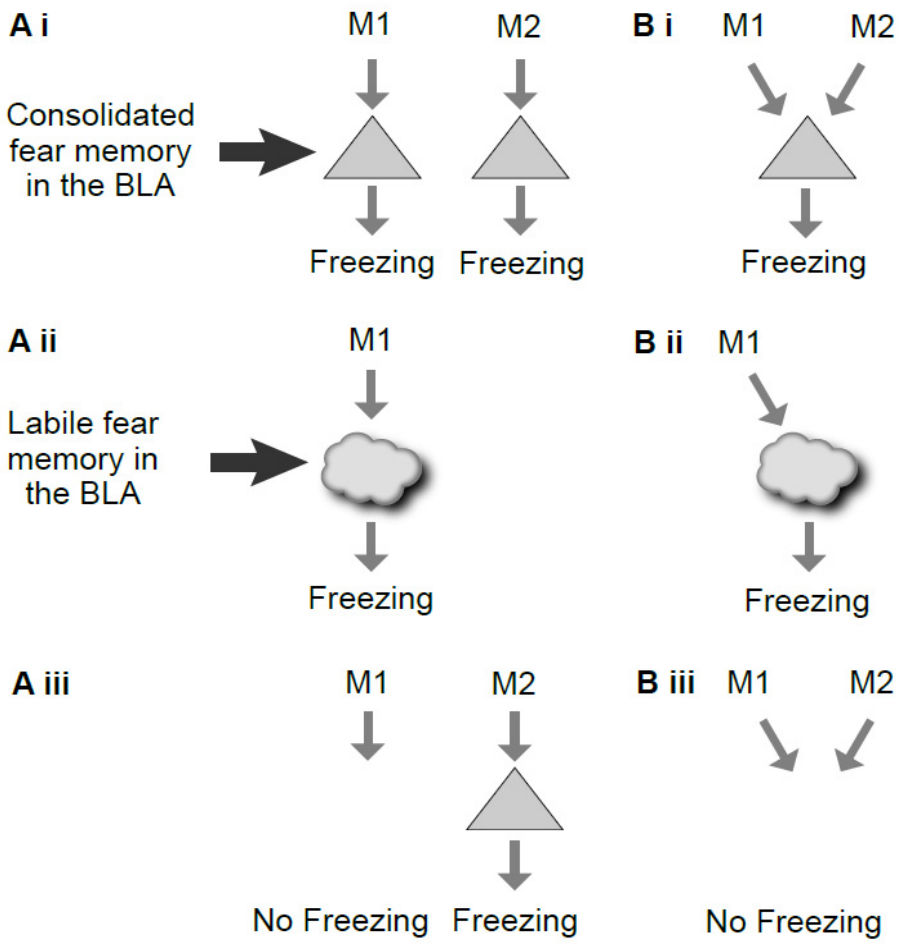
fear memories are impaired. These findings suggest that both cued and context fear memories rely on overlapping populations of neurons in the BLA.

2.1.1 Aims of this chapter

The main goal of the experiments in this chapter was to use reconsolidation as a tool to determine if AFC and CFC share overlapping or independent representations in the BLA. Following the reactivation of a memory trace, it can become temporarily labile, sensitive to amnesic agents such as anisomycin, and requires reconsolidation to retain stability (Nader, 2003; Dudai, 2004; Alberini, 2005; Tronson and Taylor, 2007; Lee, 2009; Nader and Einarsson, 2010). These processes are reactivation dependant such that non-reactivated memory traces are insensitive to amnesic agents (Nader et al., 2000). Therefore, if these two fear memories are stored independently within the BLA then reactivation of one memory followed by an intra-amygdala amnesic agent should block reconsolidation of only the reactivated memory. The non-reactivated memory should be impervious to the amnesic agent and therefore persist on a future retention test (Fig.2.1A). Conversely, if two fear memories share an overlapping representation in the amygdala, then blocking reconsolidation of one memory should lead to impairment in the second memory even though it was not explicitly reactivated. This effect should be observed regardless of which memory is reactivated (Fig.2.1B).

In the first experiment I examined the effect of reactivating either the contextual or auditory fear memory acquired from a single tone-shock pairing and administration of the amnesic agent anisomycin into the BLA. My findings show that blockade of contextual reconsolidation also blocked tone fear yet blocking reconsolidation of auditory fear memory spared contextual fear conditioning.

Figure 2.1



1.1: Testing the structure of memories in the BLA by blocking reconsolidation. **Ai,** Memories with independent fear representations within the BLA would be mediated by a largely independent population of neurons such that two different auditory fear memories, M1 and M2, would each be mediated by relatively non-overlapping populations of BLA neurons. **Aii,** If M1 and M2 have independent representations in the BLA, then when M1 is reactivated only the M1 fear representation will return to a labile state and be blocked by post-reactivation disruption of reconsolidation such as with anisomycin. If the M2 fear representation within the BLA was not reactivated it will remain impervious to the amnesic treatment. **Aiii,** During PR-LTM presentation of M2 will elicit its fear representation that will in turn elicit freezing behavior. **Bi,** A second possibility is that they share largely overlapping fear representations within the BLA, such that M1 and M1 are mediated by overlapping populations of neurons within the BLA. **Bii,** If M1 and M2 share overlapping fear representations within the BLA, reactivation of M1 will cause the fear representation that is common to both memories to return to a labile state. This common representation will be impaired by the amnesic agent. **Biii,** During the PR-LTM test M1 and M2 presentation should elicit impaired freezing.

2.2 Materials and methods

2.2.1 Subjects

Adult Sprague-Dawley rats (275-325g at surgery) were housed individually and maintained on a 12/12 light/dark cycle (lights on at 7am) with food and water provided *ad libitum*. All procedures were in accordance with the Canadian Council of Animal Care guidelines, and were approved by McGill University Animal Care and Use Committee.

2.2.2 Behavioral Apparatus

Conditioning and tone testing were conducted in different chambers (Med-Associates; Coulbourn Instruments) Rats were conditioned in a Plexiglass rodent conditioning chamber (chamber A) lit by a cue light near the top of the chamber, a metal grid floor and vanilla scent. A distinct chamber (chamber B) was used for auditory fear memory reactivation and testing to minimize context generalization. This chamber contained a red house light positioned just outside the chamber, a white Plexiglas floor, black and white stripped walls and a peppermint scent. A micro video camera was used to record behavior for analysis. Freezing behavior (Blanchard and Blanchard, 1969) (the complete absence of movement) was scored by an experimenter blind to the experimental conditions of the animals with Freeze-View (Actimetrics).

2.2.3 Behavioural Protocol

Animals were habituated to chamber B for 20 min daily for two days prior to training. Fear conditioning consisted of rats placed in chamber A and after a 2 min delay presented with a 30 s, 5kHz; 75dB tone which co-terminated with a 1 s 1.5mA foot shock. The rats were left in the chamber for 1 min after the termination of the US. Rats were then returned to their cages.

2.2.3.1 Experiment 1

To reactivate a memory, 24 h after conditioning, rats were either placed in the training context for 90s (context reactivation) or placed in chamber B and given a single 30s tone presentation (tone reactivation). Microinjections into the BLA of either anisomycin or vehicle were administered immediately following this session.

2.2.3.2 Experiment 2

To extinguish a memory, 24 hrs after conditioning, rats were either placed in context A for 30 min (context extinction) or placed in context B and after a 2 min delay given 16 30s tone presentations (60s ISI; tone extinction). No extinction groups were either left undisturbed in the colony room or placed in chamber B for 30 min.

24 h after the reactivation/extinction session all rats received two long term memory tests (LTM 1 and LTM 2) during which freezing elicited by either the training chamber or tone in the chamber B was measured. Context and auditory tests were counterbalanced between LTM 1 and LTM 2 and rats were returned to their home cage for 24 h between tests. No effect of testing order was observed (data not shown). Context LTM consisted of 5 min exposure to Chamber A. Auditory LTM consisted of a 2min delay followed by three non-reinforced tone presentations (60 s ISI). Context elicited freezing was scored continuously during the 5 min context exposure and tone elicited freezing was scored continuously during the tone presentation and averaged across the three tones.

2.2.4 Surgery and histology

The methods and infusions were identical to those previously described (Nader et al., 2000). Briefly, under deep anesthesia (3.33 mg/kg xylazine; 55.55 mg/kg ketamine; 0.27 mg/kg medetomidine hydrochloride), rats were implanted bilaterally with 22-gauge stainless steel cannulas into the lateral amygdala. Coordinates were 3.0 mm posterior, 5.2 mm lateral and 8.0 mm ventral of bregma. Anisomycin (62.5µg/0.5µl/side) was injected at a rate of .25µl/min, with sterile saline as the vehicle. Following infusions, injectors were left in place for an additional

minute to allow for diffusion away from the cannula tip. Anisomycin (Sigma-Aldrich) was dissolved in equimolar HCl, diluted with sterile saline and adjusted to pH 7.4 with NaOH. Following the completion of the experiment animals were sacrificed and their brains removed and placed in 20% sucrose in formal saline. Cannula placements were checked by examining cryostat (Microm) sliced 50-µm brain sections under a light microscope. Animals were excluded from analyses if the injector tip was not within the boundary of the BLA (Paxinos and Watson, 2005) or showed signs of tissue damage.

2.2.5 Statistics

Freezing during the two LTM tests were compared using repeated measures ANOVA (SPSS) followed by Fischer LSD *post hoc* analyses in the event of a significant interaction. Animals were excluded from analyses if they showed signs of nonspecific freezing (>30% in 30s) prior to the onset of the first tone in the auditory LTM test.

2.3 Results

2.3.1 Blocking reconsolidation of contextual fear memories impairs the retention of auditory fear conditioning

To determine whether AFC and CFC share overlapping or independent representations in the BLA, I trained rats using a single tone-shock from which rats acquire both auditory and contextual fear memories. In order to determine the effect of blocking reconsolidation of the contextual fear memory on the auditory fear memory, 24 hrs after training, half of the trained animals were returned to the training chamber for a 90 s reactivation of their contextual fear memory. No tone was presented during this time. This was followed immediately with bilateral BLA infusions of anisomycin or vehicle

Two counterbalanced LTM tests, separated by 24hrs, were used to measure contextual- and auditory- elicited freezing. A repeated measures ANOVA (Test x drug) demonstrated a significant main effect of test ($F_{(2,14)} = 8.973$, $p = 0.010$) drug ($F_{(1,14)} = 5.137$, $p = 0.040$) and test by drug interaction ($F_{(1,14)} = 4.598$, $p = 0.050$). Post hoc analysis showed that anisomycin and

vehicle groups demonstrated comparable levels of freezing during reactivation (vehicle =50.59% (SEM =12.92, n = 6), aniso =40.48% (SEM =7.84, n = 10) ($t_{(14)}$ = 1.286; p= 0.2192)). Freezing elicited by the training context in the anisomycin group (11.91% (SEM = 3.43)) was significantly lower that freezing in the vehicle group (45.86% (SEM = 9.37) $t_{(14)}$ = 4.317; $p < 0.001$). This demonstrates that reconsolidation of the contextual fear memory was impaired. Although the auditory stimulus was never presented during the reactivation, freezing induced by the tone CS was also impaired during LTM test (vehicle = 75.84% (SEM = 8.67), aniso = 41.31 (SEM = 9.26) $t_{(14)}$ = 4.391; $p < 0.001$) (Fig.2.2B). This suggests that context fear memory reactivation is able to elicit lability of a simultaneously acquired auditory fear memory.

2.3.2 Blocking reconsolidation of auditory fear conditioning has no effect on the retention of contextual fear conditioning

Take in isolation, the context reactivation data suggests that context and tone are represented by overlapping populations of neurons in the BLA. In order to test this, 24 hrs after training the other half of the trained animals were returned to the non-trained chamber for a single 30 s tone presentation which we used to reactivate their auditory fear memory this was followed immediately with bilateral BLA infusions of anisomycin or vehicle.

Two counterbalanced LTM tests, separated by 24hrs, were used to measure contextual and auditory elicited freezing. A 3 x 2 repeated measures ANOVA (test x drug) demonstrated a significant main effect of test ($F_{(2,21)}$ = 9.024, p = 0.007) but not drug ($F_{(1,21)}$ = 1.348, p = 0.26) and a significant test by drug interaction ($F_{(1,21)}$ = 6.459, p = 0.019). Post hoc analysis showed that while both anisomycin and vehicle groups demonstrated comparable levels of freezing to the tone during reactivation (vehicle = 59.96% (SEM = 8.05, n = 15), aniso = 69.83% (SEM = 9.67, n = 8) $t_{(21)} < 1$), freezing elicited by the tone in the anisomycin group (22.54% (SEM = 10.00)) was significantly lower that freezing in the vehicle group (56.02% (SEM = 8.21); $t_{(21)}$ = 2.776; p= 0.011). This demonstrates that reconsolidation of the auditory fear memory was impaired. However, freezing induced by the training context was not impaired during LTM test (vehicle = 30.26% (SEM = 6.24), aniso = 27.70 (SEM = 9,63) ($t_{(21)} < 1$) (Fig.2.2C). This indicates that

reactivation of an auditory fear memory is not capable of eliciting lability of a simultaneously acquired contextual fear memory.

I have shown here that the impairment in freezing following a blockade in tone fear memory reconsolidation is restricted to the tone memory while a blockade in context fear memory reconsolidation results in impairments to both context and tone elicited fear (Fig. 2.3).

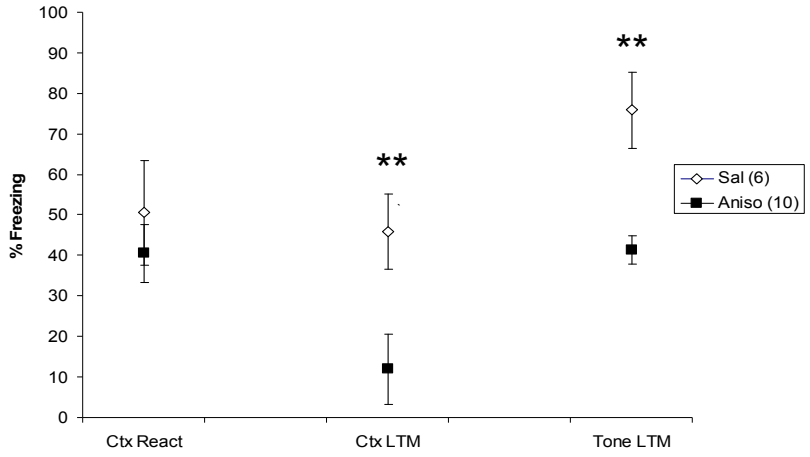
Figure 2.2

A

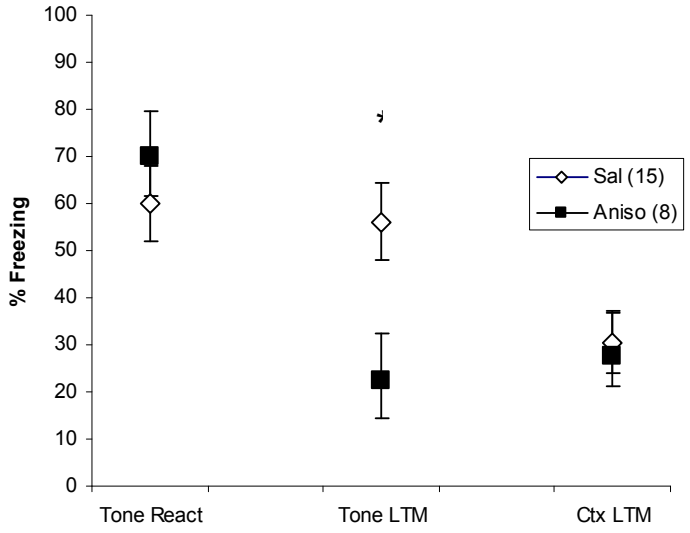
Train	24 hrs	*React*
Tone A - Shock	→	**1x Tone A**
Tone A - Shock	→	**90s Context**

B

Aniso/Veh

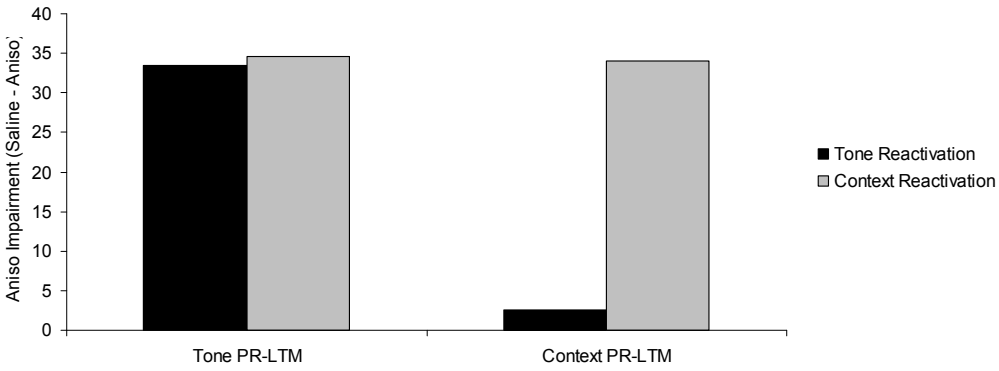


C



2.1 A) Schematic of Behavioural protocol used in reconsolidation experiment. **B)** Blocking reconsolidation following context reactivation impairs freezing to context and tone. Animals were tested for freezing elicited by the training context and tone in a counterbalanced manner. Freezing to both the training context and conditioned tone were impaired by bilateral anisomycin injections to the BLA following a brief re-exposure to the training context. **C)** Blocking reconsolidation following conditioned tone reactivation impairs freezing to tone only. Freezing to the conditioned tone but not the training context were impaired by bilateral anisomycin injections to the BLA following a brief re-exposure to the conditioned tone. Data represent Mean + SEM. * $p < 0.05$, **$p < 0.01$.

Figure 2.3



2.2 Summary of anisomycin deficits in freezing to tone and context. Data represents the difference in percent freeing between the vehicle and anisomycin groups. Aniso following context reactivation resulted in similar impairments in freezing to the context and to the tone. Conversely, Aniso following tone reactivation resulted in an impairment in freezing to tone only.

While this data doesn't fit either of the proposed models outlined previously (Fig. 2.1) the asymmetrical pattern of impairments does provide tantalizing clues as to how these two memories interact in the BLA. One possible explanation for these asymmetrical impairments is that context and tone and shock are organized in a higher-order association. Debiec et al (2006) and colleagues found that blocking reconsolidation of a second order auditory memory impaired only that memory, while blocking reconsolidation of a first order auditory memory impairs freezing to both first and second order memories. The data presented here follow the same pattern of impairments. Blocking reconsolidation of the auditory fear memory impairs only freezing to the tone, while blocking reconsolidation of the contextual fear memory impairs freezing to both stimuli. If these two fear memories are organized in a higher order association, our data suggest that following a single tone-shock pairing the tone is associated with the context which is associated with the shock (Fig. 2.6A). If this is the case, the auditory and contextual fear memories should share other characteristics with higher-order memories. The effects of extinction on second order associated memories have been shown to be asymmetrical as well; extinction of a second order auditory fear memory results in reductions in freezing to the extinguished tone only, while extinction of the first order auditory fear memory results in reduction in freezing to both tones. If context and tone are organized in a higher order association as predicted above, then extinction of the context should impair freezing to the tone, and extinction of the tone should have no effect on context freezing.

In the next experiment I examined the effect of extinguishing either the contextual or auditory fear memory acquired from a single tone shock pairing. My findings suggest that following a single tone shock pairing tone, context and shock are not serially associated.

2.3.3 Asymmetrical effects result from extinction of context and tone

In order to determine if context, tone and shock are organized in a serial association 24hrs after training, animals were either placed back into the training context for 30 min (context extinction condition), were placed in a neutral context and presented with 16 30s tones (tone

extinction condition) or remained in the colony room (no extinction condition). 24 and 48 hrs later all animals were tested for context and tone elicited freezing in a counterbalanced manner.

A repeated measure ANOVA (test x ext) showed a significant main effect of test ($F_{(1,30)}$ = 26.172, $p < 0.001$), extinction condition ($F_{(2,30)} = 14.700$, $p < 0.001$) and a significant text x extinction condition interaction ($F_{(2,30)} = 8.831$, $p = 0.001$). Compared to rats that had received no extinction, both rats that were extinguished to the training context and to the tone showed a decrease in freezing to the training context (no ext = 53.14% (SEM = 12.06, n = 7); context ext = 18.64% (SEM = 6.42, n = 14) $t_{(19)} = 3.655$, $p = 0.001$; tone ext = 12.95% (SEM = 5.72, n = 12) $t_{(17)}$ = 4.144 $p < 0.001$). Conversely, compared to rats that had received no extinction, rats that were extinguished to the tone showed a decrease in freezing to the tone but rats extinguished to the training context did not (no ext = 79.44% (SEM = 8.58, n = 7); tone ext = 16.24% (SEM = 7.55, n = 12)) $t_{(17)} = 6.516$ $p < 0.001$; context ext = 69.55% (SEM = 7.35, n = 14)) $t_{(19)} = 1.047$, p = 0.308) (Fig.2.4 B).

2.3.4 Reduction in training context freezing is not due to context generalization

One interpretation of these results is that the animals in the tone extinction group were being extinguished both to a discrete cue (the tone) as well as a context, and that this context extinction was being generalized to the training context. To test this hypothesis, 24hrs after training I extinguished two groups of rats in chamber B to either 16 30s tones (tone extinction) or 30 min in the context (control context extinction). 24 and 48 hrs later all animals were tested for context and tone elicited freezing in a counterbalanced manner.

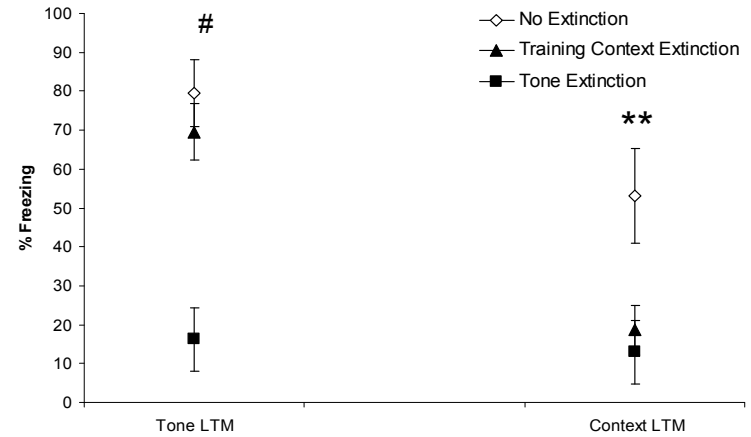
Like in the previous experiment tone extinguished animals showed a significant decrease in freezing compared to the non-training context extinction in both tone LTM (tone ext = 16.70% (SEM = 6.90, n = 12); control context ext = 60.39% (SEM = 8.22, n = 14); $t_{(24)} = 5.267$ $p < 0.001$) and context (tone ext = 12.50% (SEM = 3.61); control context ext = 38.52% (SEM = 8.14); $t_{(24)}$ = 2.873 $p = 0.009$) (Fig.2.4 C) LTM tests. This suggests that the reductions in freezing to the training context are not due to generalizations of context extinction acquired during tone extinction sessions.

Figure 2.4

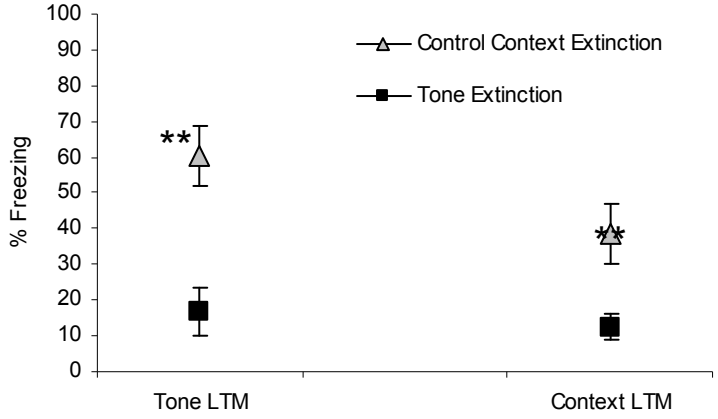
A

Train	24 hrs	*Extinction*
Tone - Shock	→	**16 x CS**
Tone - Shock	→	**30' Training Context**
Tone - Shock	→	**No Extinction**
Tone - Shock	→	**30' Control Context**

B

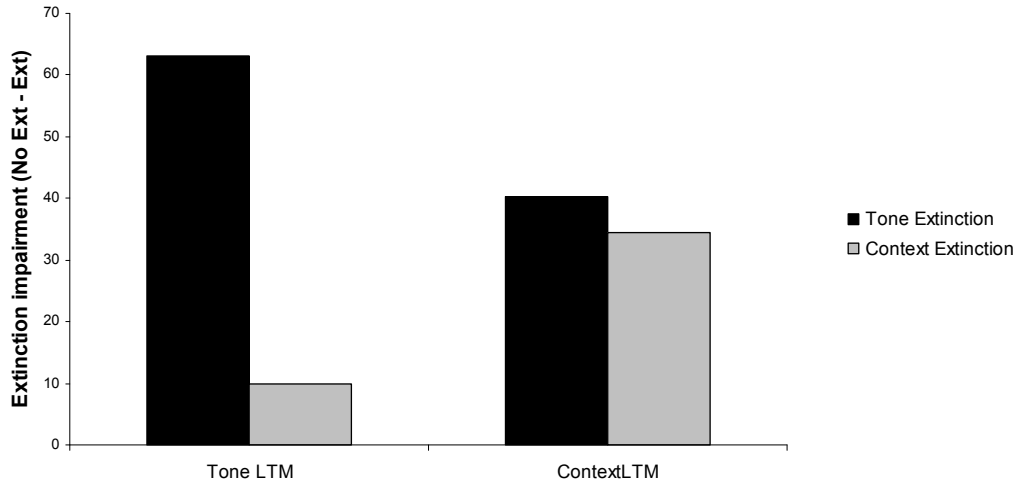


C



2.3 A) Extinction of tone and of context have asymmetrical effects. Extinction of the training context results in decreases in freezing only to that context. Conversely, extinction of the tone results in decreases in freezing to both the tone and training context. No extinction animals remained in the colony room. ** $p < 0.01$ both tone and context extinction compared to no extinction, # $p < 0.01$ tone compared to no extinction. **B)** Tone extinction effects on context freezing are not due to generalization of context extinction. Extinction of a control context did not cause attenuation in freezing to either tone or training context. ** $p < 0.01$ compared to tone extinction. Data represent Mean + SEM

Figure 2.5



2.4 Summary of attenuation in freezing to tone and context following tone or context extinction. Data represent the difference between the no extinction (home cage) and extinction (tone or context) groups. Tone extinction resulted in decreases in freezing to both tone and context. Conversely, context extinction resulted in decreases in freezing to context only.

2.4 Discussion

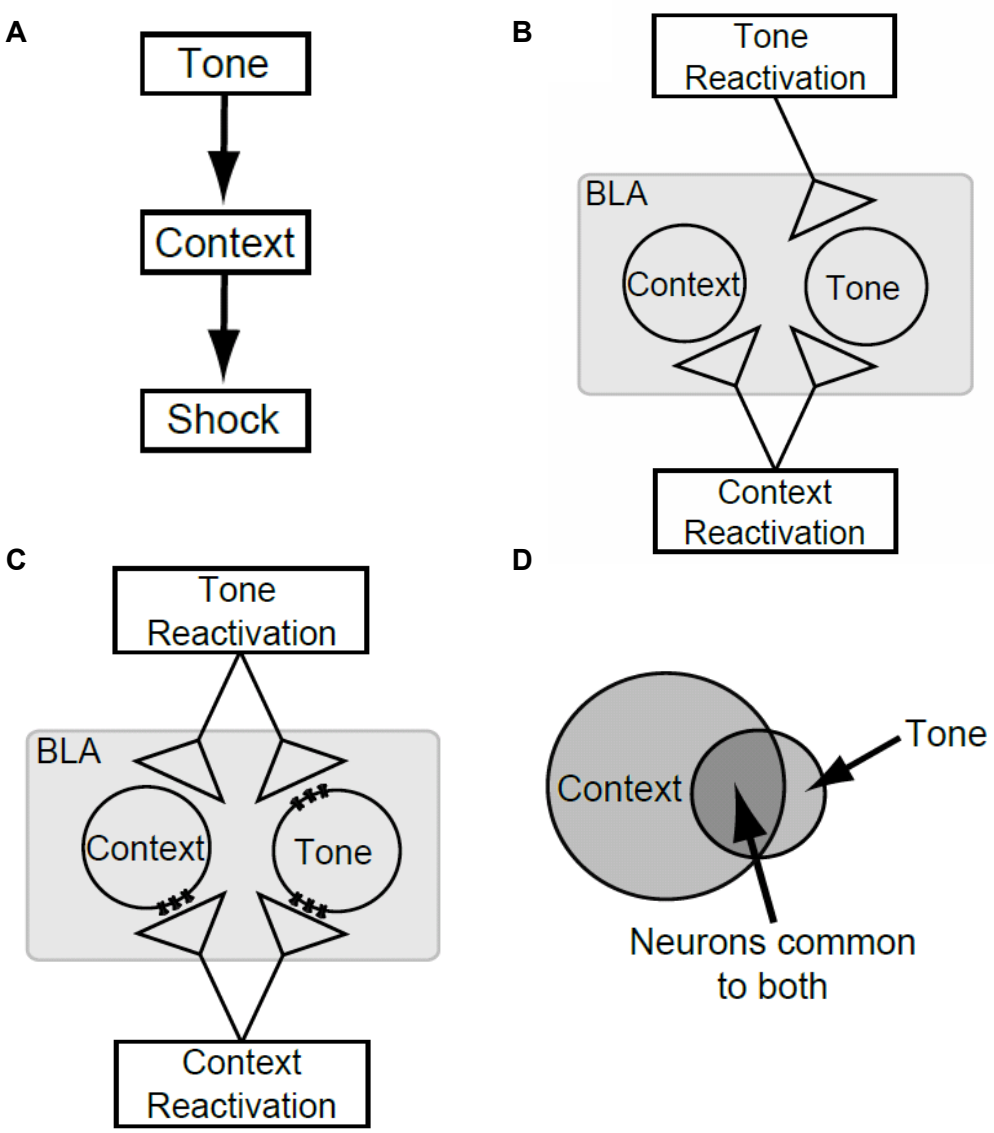
Exploiting the fact that reconsolidation is reactivation dependent, we predicted that if two fear memories have independent representations within the BLA, then reactivation of one memory should return the reactivated, but not the non-reactivated, memory to a labile state. In turn, amnesic agents should block reconsolidation of the reactivated memory and preserve performance of the non-reactivated memory. Conversely, if two memories are mediated by overlapping representations within a memory system, then reactivation of one should return the common representation to a labile state which should be blocked by an amnesic agent and impair performance of both memories (Fig. 2.1). Here we applied this logic to fear conditioning in which a single shock can condition both contextual and auditory fear which are commonly used for consolidation and reconsolidation studies (i.e. Kim and Fanselow, 1992; Schafe et al., 2000; Nader et al., 2000; Debiec et al., 2002).

If auditory and contextual fear memories are in fact stored in independent circuits within the amygdala, then this would predict that blockade of reconsolidation of one memory would not affect the expression of the other. Partially consistent with this hypothesis, we showed here that reactivation of a conditioned auditory fear memory followed by anisomycin injected into the BLA decreases freezing to the tone, but not to the context. This would suggest that contextual fear reconsolidation is not induced by the retrieval of auditory fear memory. However, when reconsolidation was blocked following reactivation of a contextual fear memory, the decrease in freezing is seen in response to both tone and context. This would suggest that the tone fear memory shares common elements and/or networks with the contextual fear representation within the BLA. The data presented here suggests that although auditory and contextual fear memories are not represented independently in the BLA, they are also not completely overlapping either. The use of a reactivation dependant manipulation allows the complex functional interaction of these two memories to be demonstrated.

2.4.1 Higher-order association model

There are at least four possible models of how the representations of these memories could interact to produce the asymmetrical reactivations found here. The first possibility that we considered was that the tone, context and shock were organized in a high-order association. This was considered due to the similarity of impairments between the experiments reported here and by Debiec et al (2006). Following second order fear conditioning Debiec et al (2006) found that that blocking reconsolidation of a second order auditory memory impaired only that memory, while blocking reconsolidation of a first order auditory memory impairs freezing to both first and second order memories. This asymmetry is similar to my findings here. In order to test whether tone, context and shock were organized in a serial association following a single paired tone shock training (Fig.2.6 A) we extinguished the tone or context freezing and tested the effect on the non-extinguished CS.

Figure 2.6



2.5 Possible memory structures underlying asymmetrical reactivations. **A)** Tone, context and shock representations are associated in a serial manner such that Tone is associated with context which is associated the foot shock. **B)** Mechanisms underlying context reactivation innervate and elicits reconsolidation in both tone and context fear memory representations in the BLA. Whereas mechanisms underlying tone reactivation only elicit reconsolidation in the tone memory representation in the BLA. **C)** Retrieval mechanisms underlying tone and context reactivation innervate both representations. However, molecular mechanisms required for eliciting reconsolidation are lacking in the tone reactivations innervations of the context fear memory representation in the BLA. **D)** Context and tone fear memory are represented by overlapping populations of neurons in the BLA. However, the size of the population is greater for the context fear memory than for the tone fear memory.

I found that extinction of context freezing had no effect on tone elicited freezing which suggests that tone, context and shock are not organized in a serial association. I also found that extinction of tone freezing attenuated both tone- and context-elicited freezing. In sum, blockade of reconsolidation and extinction of auditory and context fear memories result in opposite patterns of freezing impairments (Fig. 2.3 & 2.5). This is the opposite pattern of results as was found by Debiec et al. (2006). This suggests that the two memories that those authors investigated and the two investigated in the experiments described in this thesis are organized in a different manner. I will discuss the difference between my reconsolidation impairment and extinction resuts further after presenting the remaining possible explanations for my asymmetrical reconsolidation findings.

2.4.2 Asymmetrical parallel connectivity model

A second possibility is that cells that encode the contextual memory may, through either local inter-neurons or extra-amygdala structures, activate the cells within the LA that store the tone fear representation, while the cells mediating the tone representation might not have any excitatory connections with the contextual memory that is stored independently of it (Fig.2.6 B). Temporary inactivation of the LA during acquisition of AFC results in the *enhancement* of CFC (Calandreau et al., 2005). This suggests that in the absence of this treatment the auditory fear memory is inhibiting the contextual fear memory. It is then possible that reactivation of the auditory fear memory would not reactivate the context fear memory and only auditory fear memory would undergo reconsolidation in the BLA and be susceptible to anisomycin. Conversely, it has been shown that a specific population of BA neurons are involved in the context specific activation of auditory fear memory (Herry et al., 2008). This data supports the possibility that when the contextual memory is reactivated, both the contextual and auditory fear memories within the BLA are reactivated and undergo reconsolidation. Anisomycin would impair this reconsolidation and lead to impairments in both contextual and auditory fear conditioning.

2.4.3 Bidirectional circuit model

Another possibility that builds on the interpretation above is that both the auditory and contextual representations in the amygdala are spatially distinct but can bidirectional activate the other representation via inter-neurons or extra-amygdala pathways (Fig.2.6 C). Recent work by Herry et al (2008) supports this idea that there could be bidirectional connections between tone and context representations in the BLA. These authors found a population of neurons in the BA that were active in response to the CS+ (paired tone) but not CS- (unpaired tone) following AFC. Additionally, they showed that following extinction a distinct population of neurons was active in response to the extinguished CS+. The activation of these two population of BA neurons were shown to be context specific (training vs extinction context) and have distinct connectivity with the hippocampus (HC) and medial prefrontal cortex (mPFC). The fear neurons had afferents from the HC and efferent to the mPFC while the extinction neurons had bidirectional connectivity with the mPFC. They also demonstrated that expression of AFC is intact when BA neuronal activity was silenced using gama-aminobuturic acid subtype A ($GABA_A$) receptor antagonist muscimol. However, as discussed previously (section 2.4.2) this does not rule out the possibility that BA fear neurons are capable of activating the tone representation in the LA (and eliciting lability). One test that Herry et al (2008) did not perform that would have spoken more directly to this proposed model would have been to measure the activity of these BA fear neurons when the animal is re-exposed to the training context 24hrs following training. They do demonstrate what appears to be a slight increase in baseline activity in both fear and extinction neurons when testing for fear renewal in the training context compared to baseline activity in the extinction context (Herry et al 2008 Fig.4c, d). However, without the proper statistical test it is impossible to elaborate on this. Obviously, the most direct test of this hypothesis would be to record from neurons in the LA which show enhanced CS+ elicited responses and show that their activity is also increased in response to the training context alone. And importantly, these fear neurons in the BA show clear activation in response to tone presentation (Herry et al., 2008) which provides some evidence for bidirectional activation of the auditory and contextual fear memory representation.

However, in order for fear memories to undergo reconsolidation within the BLA, previous work from the Nader lab shows that NR2B subunit containing NMDA receptors (NR2B-

NMDAR) must be activated during fear memory reactivation to elicit lability (Ben Mamou et al., 2006; Wang et al., 2009). If ifenprodil (a NR2B-NMDAR inhibitor) is bilaterally infused in the BLA prior to memory reactivation, anisomycin has no effect on the stability of the memory (Ben Mamou et al 2006). NR2B-NMDAR can be down-regulated due to behavioural manipulations as well. Previous work has shown that strong training can result in a down-regulation of NMDAR currents and subunit phosphorylation in the LA (Zinebi et al., 2003). If a rat receives ten tone-shock pairings in a single session, the auditory fear memory will become insensitive to anisomycin following subsequent reactivation (Wang et al 2009). It has been shown that this insensitivity is temporally restricted, and is due to the down-regulation of NR2B-NMDAR in the BLA (Wang et al 2009). It is possible that context reactivation actives the tone memory by synapses that contain NR2B-NMDAR. Thus the auditory memory would undergo reconsolidation when the training context is presented. In contrast, if the contextual memory was activated by the tone representation via synapses that do not contain NR2B-NMDAR, then the contextual memory would not undergo reconsolidation and therefore be insensitive to anisomycin. This would explain how reactivation of the tone memory only induces a deficit in tone freezing.

2.4.4 Asymmetrical overlapping populations model

One last possibility is that the two memories are mediated by overlapping populations of neurons within the BLA; however, the number of neurons that mediate the contextual fear memory may be greater than in the tone memory (Fig.2.6 D). For example, the contextual fear memory is represented by a larger population of neurons than the auditory fear memory with a significant portion of the auditory fear memory being mediated by neurons common to both. There is some indirect evidence that this may be true. Hall et al (2001) measured zif268 positive cells (a marker of neuronal activity) in the LA and BA in response to either context LTM or auditory LTM tests. Although they did not directly test this, it appears that in both the LA and BA context LTM activates more neurons than auditory LTM (Hall et al 2001 Fig.2f,g & 3f,g). In this situation blocking reconsolidation of the auditory fear memory would impair the entire auditory fear representation in addition to a small portion of the contextual fear representation. Because

memories are thought to be represented by distributed network of neurons (Perez-Orive et al., 2002) the remaining population mediating the contextual fear representation may be sufficient to elicit freezing to the context indistinguishably from normal levels (French, 1999). Conversely, if reconsolidation of contextual fear memory is blocked, all of the context fear representation as well as a large majority of the auditory fear representation is impaired. This could lead to impairments in freezing to both modalities.

One technique that could further test these three models is cellular compartment analysis of temporal activity by Fluorescence in situ hybridization (CatFISH). In this technique takes advantage of a property of Arc mRNA. Shortly following neural activity Arc mRNA is seen as dense intranuclear clusters of florescence and 20 minutes later it is seen in the cytoplasm. The activity history of a neuron can be determined by detecting these two distinct patterns of fluorescence. If a neuron is active both 5 minutes and 40 minutes prior to fixation it will show both patterns of fluorescence (Guzowski et al, 2001). This approach can also be used by visualizing two different mRNA, Arc and Homer 1a which have different temporal activation profiles (Vazdarjanova and Guzowski, 2004).

To use this technique to test the models proposed based on the reconsolidation impairment data reported here, 24hrs after receiving a single tone co-terminating with a foot shock animals would be exposed to a brief reminder of the conditioned tone and training context separated by 40 minutes (in a counterbalanced order). Shortly following the second reactivation animals would be sacrificed. Cell counts showing intranuclear clusters, cytoplasmic staining and both would be collected from the BLA. CatFISH analysis provides cell counts for the following categories of neurons: total activated by tone, total activated by context, total activated by both, activated uniquely by tone (excludes double labeled cells) and activated by context (excludes double labeled cells). The bidirectional circuit model would elicit the most distinct profile. The prediction would be that if both tone and context activate the same populations but differ in their ability to elicit lability is that the total number of neurons activated by the tone or the context would be equal to neurons activated by both and there would be very few neurons unique to either tone or context. The asymmetrical parallel connectivity and asymmetrical overlapping models

make the same prediction regarding CatFISH results. Both predict that there would be a greater total number of neurons activated by context than by tone and that most of the tone neurons would be double labeled while there would be a significant population of unique context neurons. The difference between the two models is that the spatial relationship of the tone and context populations. Asymmetrical parallel connectivity model predicts that the context and tone representations are spatially independent while the asymmetrical overlapping model predicts that the two representations are spatially overlapping. By noting the relative location of the context reactivation elicited neurons in relationship to the tone reactivation and double labeled neurons could help to dissociate between these two models.

2.4.5 Extinction as a tool for parsing out memory structure

Extinction, the repeated presentation of the CS in the absence of the US, results in a decline of CS-evoked fear responses (for review see Bouton, 2004). Extinction was used here as a secondary tool to investigate the organization of auditory and contextual fear memories. The primary motivation in using extinction was to test whether the symmetry of reconsolidation impairments between my results and Debiec et al (2006) would be reflected in the attenuation in freezing following extinction. In second order fear conditioning CS1 and CS2 are explicitly organized in a serial association (Rizley and Rescorla, 1972). I wanted to test if the pattern of reconsolidation impairments that I saw was indicating that the two CSs in my paradigm (tone and context) were implicitly organized in a serial association. If a tone → context → shock association was produced by my training protocol, then extinction of context freezing should also attenuate freezing to the tone, and extinction of the tone should have no effect on context freezing.

Although extinction is typically thought of as being context specific, this is not entirely true. One demonstration of context specificity is shown in the renewal paradigm. In the ABA design the animal is conditioned in context A, extinguished in context B and tested in context A. This is contrasted with animals in an AAA design in which training, extinction and testing all occur in the same context. Context dependence of extinction is demonstrated by showing that animals extinguished in context B demonstrate more fear to the tone in the final test than animals

extinguished in context A (Bouton and King, 1983). However, this appears to be specific to discrete CSs as animals that are extinguished in context B have similarly reduced fear to context A as animals extinguished in context A (Bouton & King 1983). Thus, the finding that tone extinction attenuates training context freezing (Fig.2.5) is consistent with other evidence that supports the model of extinction as context dependant.

2.4.6 Reconsolidation vs. extinction as tools to study memory interactions

The attenuation in freezing that I found with extinction was exactly opposite of the prediction based on the hypothesis of serial association (Fig. 2.3 & 2.3). This highlights why the development of reconsolidation impairments as a tool to investigate the organization of memories is necessary. Although they are both elicited by the presentation of a non-reinforced CS there are a number of differences between the attenuation of freezing due to extinction and blockades of reconsolidation. Most important for this discussion is the fact that while reconsolidation impairments are thought to erase the original memory (Hardt et al., 2009) extinction is thought to be a combination of depotentiation of the original memory and the formation of a new inhibitory memory (Herry et al., 2010; Pape and Pare, 2010).

The formation of an extinction memory is thought to have two components; formation of a new inhibitory memory, and the destabilization of the existing fear memory. The extinction memory itself is thought to be acquired and consolidated in the infralimbic cortex of the mPFC (Milad and Quirk, 2002; Burgos-Robles et al., 2007). This region innervates the intercalated cell masses in the amygdala (Likhtik et al., 2008) which sends GABAergic projection to the central nucleus of the amygdala (Royer et al., 1999)which results in the dampening of fear expression (Quirk et al., 2003). The mPFC also projects to the BA which has been shown to be involved in context specific switching of a tone from an extinguished state to a fear eliciting one (Herry et al 2008). The destabilization of the existing fear memory has been shown, like the initiation of lability in reconsolidation experiments, to require NR2B-NMDAR (Sotres-Bayon et al., 2007; Dalton et al., 2008). Extinction has also been shown to increase levels of calcineurin, a protein phosphatase, and dephosphorylate a number of kinases required for consolidation (Lin et al.,

2003). Long term depression (LTD) or depotentiation by exocytosis of AMPA receptors has been postulated to be a mediator of extinction (Kim et al., 2007; Dalton et al., 2008). These findings support the idea that extinction involves both the inhibition of the fear response as well as some degree of depotentiation or reorganization of the original memory trace in the BLA.

In this chapter I showed that blocking reconsolidation can be used to investigate the complex functional interaction of two BLA dependant associative fear memories. My findings also suggest that while extinction is the mechanism that is typically used by the brain to reduce unnecessary fear responses, its multi-component nature, and reliance on multiple brain systems makes it a poor candidate for investigations of memory structure.

Chapter 3:

Using reconsolidation to examine memory allocation

3.1 Introduction

In the previous chapter, I showed that blockade of reconsolidation can be used to demonstrate the complex functional interaction of auditory fear memory and contextual fear memory in the BLA. In this chapter I will discuss my efforts to use reconsolidation blockades to test a recent model of allocation of fear memory acquisition in the BLA.

It has been repeatedly demonstrated that although seventy percent of LA pyramidal neurons are responsive to auditory (CS) and somatosensory (US) input (Romanski et al., 1993; Repa et al., 2001; Johansen et al., 2010b) only approximately 20-30% of these neurons are subsequently incorporated into an auditory fear memory trace (Repa et al., 2001; Rumpel et al., 2005; Han et al., 2007; Zhou et al., 2009; Han et al., 2009; Johansen et al., 2010b).

Recent work, which was reviewed in section 1.6, has suggested that there is a competitive selection amongst the LA neurons that receive convergent CS-US input and that this can occurs through CREB levels biasing certain neurons in this population to become part of the memory trace (Han et al., 2007; Zhou et al., 2009; Han et al., 2009). The implication is that neurons that are recruited by this selection undergo long-term synaptic plasticity whereas those that are not selected remain unpotentiated (Han et al., 2007; Josselyn, 2010).

Based on these findings Silva and colleagues have proposed a model of how related memories can be allocated to overlapping populations of neurons so that activation of one component increases the likelihood of retrieval of related components (Won and Silva, 2008; Silva et al., 2009). They propose that events, such as memory acquisition, which increase activity in plasticity-related pathways such as CREB, and which decay over time. If a subsequent event occurs prior to the decrease in these pathway its allocation will be biased towards the neural population activated by the first event. If a subsequent event occurs following the return to baseline levels of these pathways, its allocation will be unaffected (Silva et al., 2009). Additionally, this model posits that parallel to these excitatory pathways, there could also be inhibitory pathways that enhance the probability of allocating subsequent memories to independent populations (Won and Silva, 2008).

Previous experiments using reconsolidation impairments have shown that two auditory fear memories can have independent representations (Doyere et al., 2007; replicated in Debiec et al 2010). Because the reactivation of a memory has been shown to increase the activity in many plasticity-related pathways (Tronson and Taylor, 2007) the model of memory allocation posited by Silva and colleagues (Won and Silva, 2008;Silva et al., 2009) predicts that the reactivation of a memory prior to the encoding of a subsequent memory may bias the encoding of a subsequent memory.

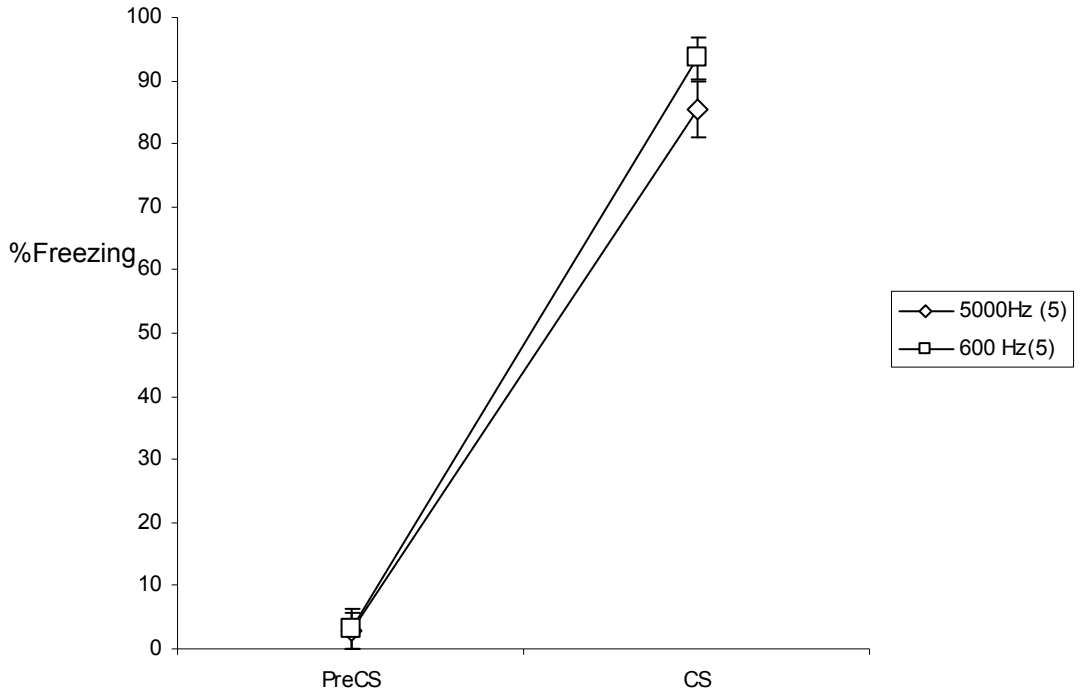
The main goal of the experiments in this chapter is to use the general experimental design from the previous chapter to test the allocation model. Each experiment contained three phases: acquisition, manipulation and testing. On day 1 of the acquisition phase Tone A fear memory was learned. On day two all rats acquired a second fear memory, Tone B, but half of the rats had Tone A reactivated prior to the acquisition of Tone B (Fig 3.1 A).

Figure 3.1

A

24 hrs		45 min			Group Name
Tone A - Shock	→	Tone A	→	Tone B - Shock	React
Tone A - Shock	→	-	→	Tone B - Shock	No React

B



3.1 A) Schematic of the two-tone acquisition protocol designed to test the allocation model of memory encoding. **B)** 24 hrs following a single pairing with a mild foot shock rats freeze equally to both tones used in this protocol (5000 & 6ooHz)

In the manipulation phase, the Tone A fear memory will be altered. If the reactivation of Tone A prior to the acquisition of Tone B was able to bias the allocation of the Tone B fear memory, I predicted that subsequent manipulation of the Tone A fear memory would have an effect on the retention of Tone B fear memory.

In the first experiment I examined the effect of extinguishing Tone A on Tone B elicited freezing and found that the manipulation in the acquisition phase did in fact have an effect on the Tone B fear memory. Subsequent experiments were aimed at using reconsolidation to test whether the change in the Tone B fear memory was due to a change in the relationship of Tone A and Tone B fear memories. These experiments show a limitation in using reconsolidation as a tool, and we were unable to examine the modification of memory allocation in this system.

3.2 Materials and Methods

3.2.1 Subjects

Adult Sprague-Dawley rats (275-325g at surgery) were housed individually and maintained on a 12/12 light/dark cycle (lights on at 7am) with food and water provided *ad libitum*. All procedures were in accordance with the Canadian Council of Animal Care guidelines, and were approved by McGill University Animal Care and Use Committee.

3.2.2 Behavioral Apparatus

Conditioning and tone testing were conducted in different chambers (Med-Associates; Coulbourn Instruments) Rats were conditioned in two different configurations of Plexiglass rodent conditioning chambers (chambers A & A') in the same experimenting room. These configurations were different enough that animals conditioned in chamber A did not freeze to chamber A' 24 h later (data not shown). Chamber A was lit by a cue light near the top of the chamber, had a metal grid floor, clear plexiglass walls, background fan and a vanilla scent. Chamber A' was lit by a flashing light in the ceiling in addition to a cue light near the top of the chamber, had a metal grid floor white and black and white spotted walls and the tray below the grid was whipped with 70% ethanol scent. In all experiments a distinct chamber (chamber B) was used for auditory fear

memory extinction, reactivation and testing to minimize context generalization. This chamber contained a red house light positioned just outside the chamber, a white Plexiglas floor, black and white stripped walls and a peppermint scent. The tones used in fear conditioning had frequencies of 5000 and 600Hz. These two frequencies were counterbalanced between Tones A and B in all experiments. Rats are able to equally associate these two frequencies with a foot shock as demonstrated by freezing 24 hrs later (Fig 3.1 B). A micro video camera was used to record behavior for analysis. Freezing behavior (the complete absence of movement) (Blanchard and Blanchard, 1969) was scored by an experimenter blind to the experimental conditions of the animals with Freeze-View (Actimetrics).

3.2.3 Behavioural Protocol

All experiment consisted of four phases: habituation, acquisition, manipulation, and testing. Each experiment had different parameters for each phase, as outlined below.

3.2.3.1 Habituation

Animals were habituated to chamber B for 20 min daily for two days prior to training.

3.2.3.2 Acquisition

3.2.3.2.1 Experiment 1-3:

Fear conditioning of Tone A (Train A) consisted of rats placed in chamber A and after a 3 min delay presented with two 30s, 75dB tones (120s ISI) which co-terminated with a 1s 0.7mA foot shock. The rats were left in the chamber for 4 minutes after the termination of the final foot shock, and then returned to their cages.

On day 2 of the acquisition phase all rats were placed in chamber B. After 2 min one group of rats had Tone A memory reactivated (React A) by presenting one 30s 75dB Tone A, the remaining rats remained undisturbed in the chamber. 60s after the termination of the tone (or an equivalent time in non reactivated animals) animals were removed from the chamber and returned to their cages.

45min after the end of the reactivation session, rats were fear conditioned to Tone B (Train B). This timing was selected so as to optimize the up regulation of plasticity related

pathways following reactivation. CREB phosphorylation in the BLA has been shown to be elevated at both 30 and 60 min post reactivation (Mamiya et al., 2009). Rats were placed in chamber A' and after a 3 min delay presented with a 30s tone B which co-terminated with a 1s 0.7mA foot shock. The rats were left in the chamber for 4 min after the termination of the foot shock and then returned to their cages.

3.2.3.2.2 Experiment 4:

The acquisition phase of experiment 4 consisted of conditioning Tone A as described above. On day 2 of the acquisition phase all rats were left undisturbed in their home cages.

3.2.3.2.3 Experiment 5:

The acquisition phase of experiment 5 consisted of conditioning Tone A and Tone B as described above. Rats were not placed in chamber B nor reactivated to Tone A prior to conditioning Tone B.

3.2.3.3 Manipulation

3.2.3.3.1 Experiment 1:

To extinguish the Tone A freezing, 24 and 48 hrs after the completion of the acquisition phase, rats were placed in chamber B for 30 min and given sixteen unreinforced 30 s tone presentation.

3.2.3.3.2 Experiment 2-5:

To reactivate Tone A memory, 24 h after completions of the acquisition phase, rats were placed in chamber B and given a single 30s Tone A presentation. In experiments 2, 4 & 5 microinjections into the BLA of either anisomycin or vehicle were administered immediately following this session. In experiment 3 microinjections into the BLA of either UO126 or vehicle were administered 1 hr prior to this session. The timing of these injection have been previously shown to interrupt reconsolidation of auditory fear memory in the BLA (e.g. Nader et al., 2000; Duvarci et al., 2005)

3.2.3.4 Testing

The testing phase began 24h after the manipulation phase. All rats received two long term memory tests (LTM 1 and LTM 2) during which freezing elicited by either tone in chamber B was measured. Tone A and Tone B tests were counterbalanced between LTM 1 and LTM 2 and rats were returned to their home cage for 24 h between tests. No effect of testing order was observed (data not shown). LTM consisted of 3 non-reinforced tone presentations. Tone elicited freezing was scored continuously during the tone presentation and averaged across the three tones.

3.2.4 Surgery and histology

The methods and infusions were identical to those previously described (Nader et al., 2000). Briefly, under deep anesthesia (3.33 mg/kg xylazine; 55.55 mg/kg ketamine; 0.27 mg/kg medetomidine hydrochloride), rats were implanted bilaterally with 22-gauge stainless steel canulas into the lateral amygdala. Coordinates were 3.0 mm posterior, 5.2 mm lateral and 8.0 mm ventral of bregma. Anisomycin (Sigma-Aldrich, 62.5µg/0.5µl/side) was dissolved in equimolar HCl, diluted with sterile saline and adjusted to pH 7.4 with NaOH and was injected at a rate of .25µl/min, with sterile saline as the vehicle. UO126 (1µg/0.5µl/side) was dissolved in 50% DMSO and injected at a rate of .25µl/min. Following infusions, injectors were left in place for an additional minute to allow for diffusion away from the cannula tip. This dose of UO126 has been show to impair fear memory consolidation (Schafe et al., 2000) and reconsolidation (Duvarci et al., 2005) in the LA. Following the completion of the experiment animals were sacrificed and their brains removed at placed in 20% sucrose in formal saline. Cannula placements were checked by examining cryostat sliced (Microm) 50-µm brain sections under a light microscope. Animals were excluded from analyses if the injector tip was not within the boundary of the BLA (Paxinos and Watson, 2005) or showed signs of tissue damage.

3.2.5 Statistics

Freezing during the two LTM tests were compared using repeated measures ANOVA (SPSS) followed by Fischer LSD *post hoc* analysis in the event of a significant interaction. Comparisons between drug and vehicle groups were performed prior to analyses including the

acquisition phase manipulations. Animals were excluded from analyses if they showed signs of nonspecific freezing (>30% in 30s) prior to the onset of the tone in the first auditory LTM test.

3.3 Results

3.3.1 Extinction of Tone A reveals that manipulations in the acquisition phase have an effect on Tone B freezing

In order to determine if the reactivation of a previously acquired memory could bias the allocation of a subsequently acquired memory, all animals were fear conditioned in chamber A to Tone A. 24 hrs later half of the rats were re-exposed to a 30 s presentation of Tone A in chamber B while the remaining rats were exposed to chamber B alone. 45 min following their removal from chamber B all animals were conditioned in chamber A' to Tone B. This acquisition protocol was used in experiments 1 – 3 (see figure 3.1A for schematic).

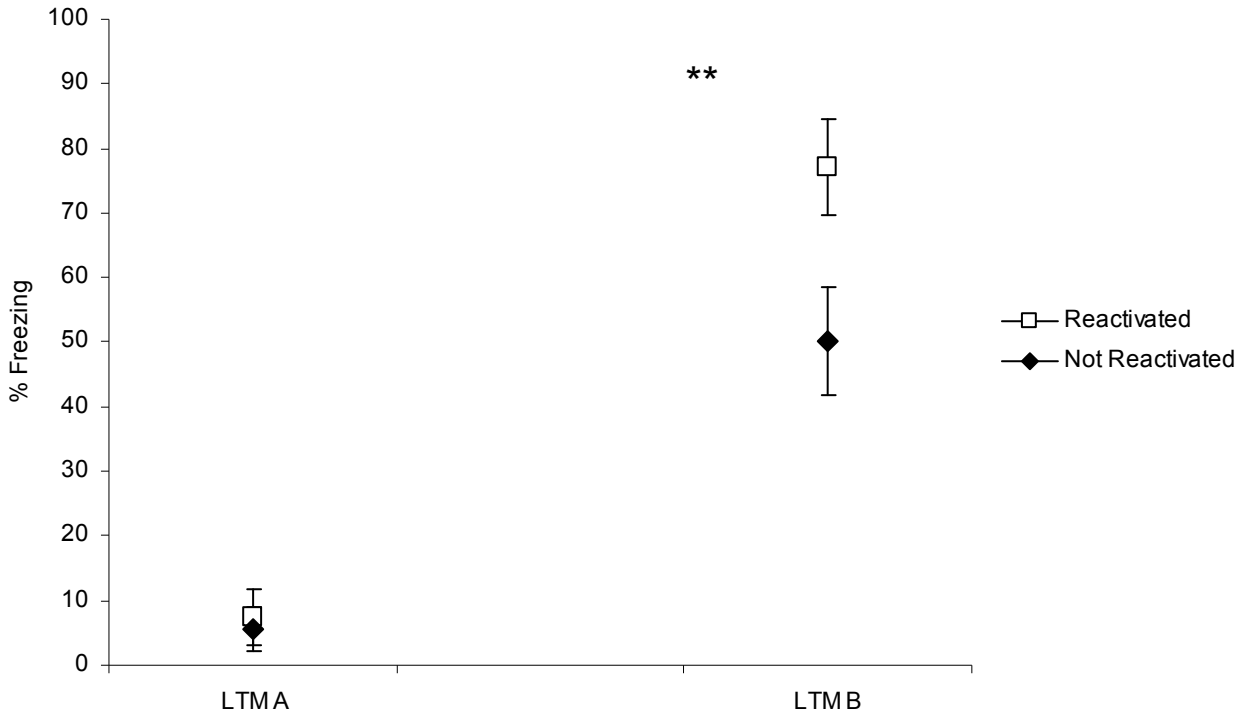
In order to start testing whether the reactivation in the acquisition phase had any effect on Tone B acquisition 24 and 48 hrs after the end of the acquisition phase all rats underwent Tone A extinction session which consisted of 16 unreinforced presentations of Tone A. Following extinction of Tone A, freezing to Tone B was significantly higher in rats that had Tone A reactivated 45 min prior to the Tone B conditioning (React = 77.06 % (SEM = 7.45) n = 13; No React = 53.50% (SEM = 8.31) n = 10; $t_{(21)} = 3.339$ $p = 0.003$) (Fig 3.2 B). This demonstrates that the reactivation of Tone A prior to the encoding of Tone B fear conditioning results in a change in the relationship of Tone A and Tone B. While this data is suggestive of a strengthening of Tone B it could also indicate a change in extinction specificity such that the extinction of Tone A is less generalized to Tone B.

Figure 3.2

A

24 hrs		45 min			Extinction
Tone A - Shock	→	Tone A	→	Tone B - Shock	Tone A
Tone A - Shock	→	-	→	Tone B - Shock	Tone A

B



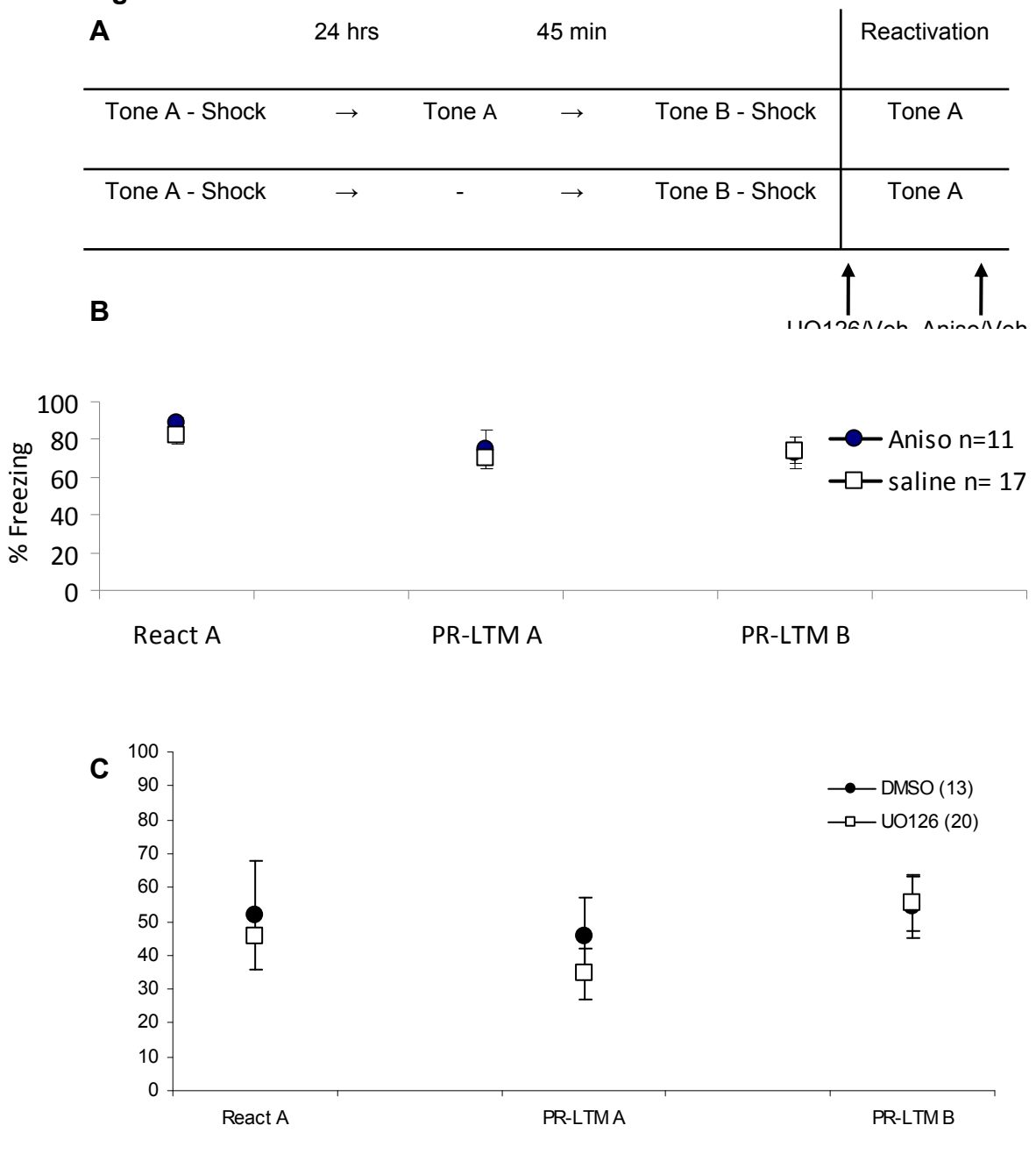
3.2 A) Schematic of Behavioural protocol using extinction to test if the reactivation of tone A prior to Tone B effects the encoding of Tone B. **B)** Reactivation of Tone A prior to training Tone B alters the encoding of Tone B. After the extinction of Tone A, animals that had tone A reactivated prior to training tone B froze more to Tone B. ** $p < 0.01$. Data represent Mean + SEM

3.3.2 Anisomycin infusions following Tone A reactivation have no effect on subsequent Tone A or Tone B freezing

If the reactivation of Tone A prior to the encoding of Tone B changes the relationship of the two tones as predicted by the allocation model, then blocking the reconsolidation of Tone A should effect Tone B more in the react group than in no-react animals. In experiment 2 I tested this hypothesis by placing all rats in chamber B 24 hrs after the end of the acquisition phase and presented one 30s unreinforced Tone A presentation. Immediately after this Tone A reactivation session, rats were bilaterally infused with anisomycin or its vehicle. Subsequent PR-LTM tests showed no effect of post reactivation anisomycin on either Tone A or Tone B. A repeated measure ANOVA showed no effect of test ($F_{(2,26)} < 1$) or drug ($F_{(1,26)} < 1$) and no interaction ($F_{(2,26)} < 1$) (Fig 3.3 B).

These results are unable to shed light on the relationship of Tone A and Tone B because there is no evidence that we were able to block reconsolidation with anisomycin of Tone A. It is known from previous work in the Nader lab that auditory fear memories can become insensitive to amnesic agents following reactivation after different behavioural or pharmacological manipulations (Ben Mamou et al 2006; Wang et al 2009). In experiment 3 I tested if lability of Tone A is not being elicited by the reactivation of Tone A in the manipulation phase. To do so I replicated the same experiment as above, but used UO126 in place of anisomycin to disrupt reconsolidation.

Figure 3.3



3.3 A) Schematic of Behavioural protocol using blockade of reconsolidation to test if the reactivation of Tone A prior to Tone B effects the encoding of Tone B. Anisomycin or vehicle was injected into the BLA immediately following reactivation. UO126 or vehicle was injected into the BLA 60 min prior to reactivation. Neither anisomycin nor UO126 were effective in impairing post-reactivation freezing. **B)** Bilateral anisomycin injections to the BLA did not result in an impairment in freezing to either tone. **C)** Bilateral UO126 injections to the BLA did not result in an impairment in freezing to either tone. Data represent Mean + SEM

UO126 is an inhibitor of mitogen-activated kinase (MEK1/2 Duncia et al., 1998; Favata et al., 1998) that has been shown to impair both consolidation (Schafe et al., 2000) and reconsolidation (Duvarci et al., 2005) of auditory fear memories. If reconsolidation is not impaired by a different pharmacological agent acting on a distinct molecular mechanism, this would strengthen the conclusion that lability of Tone A is not being elicited by the reactivation of Tone A following the acquisition phase.

3.3.3 UO126 infusions following Tone A reactivation have no effect on subsequent Tone A or Tone B freezing

UO126 or its vehicle (50% DMSO in water) was bilaterally infused into the BLA 60 min prior to the manipulation phase reactivation of Tone A. Subsequent PR-LTM tests showed no effect of pre reactivation UO126 on either Tone A or Tone B (Fig 3.3 C). A repeated measure ANOVA confirmed no effect of test ($F_{(2,30)} < 1$) or drug ($F_{(1,30)} < 1$) and showed no interaction ($F_{(2,30)} < 1$). This data suggests that the acquisition phase of these experiments is changing the Tone A fear memory such that a 30s unreinforced tone presentation does not induce lability.

3.3.4 The attenuation of induction of lability of the Tone A fear memory is caused by the acquisition of the Tone B fear memory

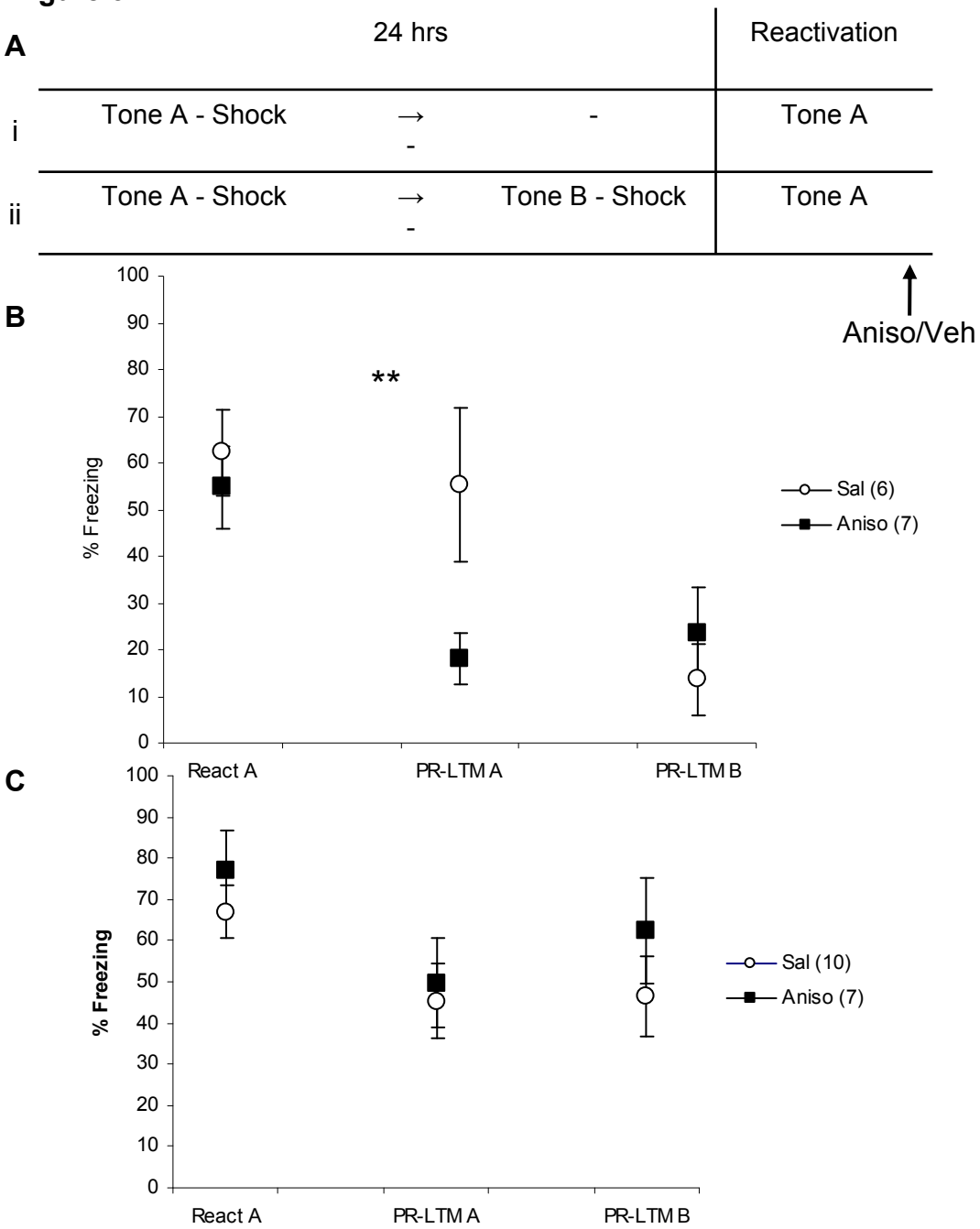
In order to determine which of the three aspects of the acquisition protocol (Train A, React A or Train B) is responsible for subsequent tone presentation not inducing lability. I conducted two control experiments. In experiment 4 I reduced the acquisition protocol to conditioning Tone A only. Two days following Train A all rats had Tone A fear memory reactivated by a 30s unreinforced presentation of Tone A and received bilateral infusions of either aniso or vehicle (Fig 3.4 Ai).

A repeated measure ANOVA showed a significant main effect of test ($F_{(1,22)} = 12.111$, $p < 0.001$), and a test x drug interaction ($F_{(1,11)} = 6.530$, $p = 0.027$). *Post hoc* analyses showed that during PR-LTM tests rats infused with aniso froze significantly less to Tone A than rats infused with saline (aniso = 18.05% (SEM = 5.45); veh = 55.18% (SEM = 16.48); $t_{(12)} = 3.466$, $p = 0.005$)

while there was no difference in freezing to Tone B (aniso = 23.41% (SEM = 9.75); veh = 13.58 (SEM = 7.60); $t_{(7)} < 1$) (Fig 3.4 B).

This data confirms that auditory fear memories are susceptible to disruption by anisomycin following one tone-shock pairing conditioning session. This is not a novel finding (eg Nader et al 2000) but is necessary to show where in the acquisition protocol the Tone A memory qualitatively changes. In experiment 5 I reintroduced Train B but not React A to the acquisition protocol and tested whether this was sufficient to impair the induction of lability of the Tone A fear memory. 24 hrs after conditioning to Tone A all rats were conditioned to Tone B. One day later all rats had Tone A fear memory reactivated by a 30s unreinforced presentation of Tone A and received bilateral infusions of either aniso or vehicle (Fig 3.4 Aii). A repeated measure ANOVA showed no significant main effect of drug ($F_{(1,14)} = 1.668$, $p = 0.217$) or interaction ($F_{(1,14)} < 1$) (Fig 3.4 C).

Figure 3.4



3.4 **A)** Schematic of Behavioural protocols used to determine if the attenuation of induction of lability of the Tone A fear memory is caused by the acquisition of the Tone B fear memory. **Ai)** Protocol used to confirm that reconsolidation can be elicited following only training of Tone A. **Aii)** Protocol used to test if reconsolidation of Tone A can be elicited following the additional training of Tone B. **B)** Bilateral anisomycin injections to the BLA following reactivation of Tone A resulted in a subsequent impairment in freezing to tone A. Additionally, the training of Tone A did not result in freezing to Tone B. ** $p < 0.01$. **C)** 3.12: Training Tone B 24hrs following the training of Tone A resulted in bilateral anisomycin injections to the BLA immediately following reactivation of Tone A not having any effect on freezing to either tone. Data represent Mean + SEM

This data suggest that a second conditioning session 24 hrs changes the first memory in a manner that attenuates the induction of lability following reactivation.

3.4 Discussion

In this series of experiments I set out to test the allocation model of BLA fear memory acquisition (Silva et al 2009). It predicts that the reactivation of a memory may bias the encoding of a subsequently acquired memory. I developed an acquisition protocol that this model predicted could alter the relationship of two otherwise independent auditory fear memories to having overlapping representations in the BLA.

In the first experiment in this chapter I demonstrated that extinction of Tone A revealed a difference in the Tone B fear memory due to the manipulation in the acquisition phase. Specifically, animals that had the Tone A memory reactivated prior to the acquisition of the Tone B fear memory froze more to Tone B after the extinction of Tone A. As demonstrated in chapter 2, extinction is a problematic tool for investigating the specific organization of memories due to the number of processes involved. Because extinction involves the formation of a new memory, it is unclear if the changes in Tone B elicited freezing are due to alteration of the original encoding, or changes in the specificity of Tone A extinction. In order to specifically investigate if there was a change in neuronal allocation during encoding of Tone B in the BLA, I attempted to block reconsolidation of Tone A. I found that neither inhibiting protein synthesis (using anisomycin) after, nor inhibiting the MAPK pathway (using UO126) before reactivation of Tone A were effective in attenuating subsequent freezing to Tone A or Tone B. I determined that this inability of a 30 s tone reactivation to convert a stable Tone A fear memory into a labile one was due to the acquisition of a subsequent auditory fear memory. This effect occurred in the absence of the reactivation manipulation in the acquisition phase. This finding highlights the fact that understanding the boundary conditions(Nader and Einarsson, 2010) that modulate the reactivation of memories is critical if blocking reconsolidation is to be used effectively to understand the organization of memory.

One possible interpretation of this finding is that the Tone A fear memory is being reactivated during the Tone B conditioning session. It has been shown that presentation of the US can reactivate a fear memory much like presentation of the CS (Debiec et al., 2010). It has also been suggested that one result of reconsolidation can be to strengthen a memory to the point where subsequent reactivations do not convert the memory to a labile state (Inda et al., 2011). Recent work from Satoshi Kida's lab has demonstrated that animals that receive a fear memory reactivation have a stronger memory 24hrs later if the memory is allowed to reconsolidate. They also found that while inhibition of protein synthesis in the BLA blocked reconsolidation and produced a memory impairment, inhibiting protein synthesis in the medial prefrontal cortex (mPFC) or hippocampus resulted in the original memory remaining intact, but the memory enhancement being reversed (S. Kida, personal communication). This suggests that the strengthening of a BLA dependant memory following reconsolidation is driven by other memory systems. This is consistent with the findings of Wang et al (2009) which show that the inhibition of the induction of lability in the BLA is due to activity of the dorsal hippocampus. Thus, following the Tone B conditioning session the Tone B memory is consolidating while the Tone A memory is reconsolidating. This reconsolidation of the Tone A fear memory during the acquisition of the Tone B fear memory could explain why it becomes insensitive to amnesic agents after 30 s tone reactivation 24hrs later.

It is possible that this boundary condition is not insurmountable. One way to possibly get around the boundary of reconsolidation encountered here would be to delay the attempt to elicit lability. Previous work in the Nader lab showed that when a strongly trained memory is reactivated 24hrs after acquisition it cannot become labile. However, if the reactivation is delayed 30 days then the memory will once again become labile and sensitive to disruption (Wang et al 2009). Thus, even in the situation outlined in this chapter it may still possible to block reconsolidation to investigate the organization of multiple fear memories. A more comprehensive understanding of the neural systems and molecular mechanisms of reactivation and reconsolidation will enable the improvement of the use of this behavioural paradigm as a tool.

Chapter 4

14-3-3 proteins are upregulated during the formation of mammalian long-term memory

Connecting text

As shown in chapter 3 understanding the boundary conditions of reconsolidation, and the behavioural parameters (i.e. delay after training) that could surmount these boundary conditions, is one approach that will enhance the scope of questions that can be investigated using blockades of reconsolidation. Another parameter that can be investigated is the molecular mechanisms that are involved in the induction of lability and reconsolidation. While there have been some advances in this area (i.e. Lee et al 2005; Ben Mamou et al 2005; Tronson & Taylor 2007; Wang et al 2009) much of this work is based on testing mechanisms that have been previously identified as playing a role in consolidation. While this candidate approach has yielded some interesting results, an unbiased approach has the potential to uncover previously unknown mechanisms specific to either consolidation or reconsolidation of memory.

In this chapter I present an adaptation of a manuscript (in preparation) investigating such possible mechanisms. Using MudPIT mass spectrometry done by James Wohlschlegel at UCLA, we screened amygdala lysates from animals undergoing either consolidation or reconsolidation of and auditory fear memory. One candidate from this screen was validated for its role in consolidation using Western blots. I directly contributed to many behavioural experiments reported in this chapter. For a detailed breakdown of the contributions to this work please see the *Contribution of Authors* section.

4.1 Abstract

Formation of long-term memory (LTM) requires new protein translation, transcription and activation of numerous signaling pathways at the synapse and throughout the neuron. While select protein products have been identified for their role in LTM, many remain undiscovered. Additionally, identification of proteins specifically involved in consolidation or reconsolidation is of interest for studies of memory and psychiatric disorders. In order to elucidate new and/or unique proteins translated after LTM consolidation and reconsolidation, we performed associative fear conditioning in rats in concert with a proteomics screen. One candidate that we validated, the 14-3-3 family of proteins, are abundant in the brain and serve as central regulators to a wide number of cellular processes including PKA, MAPK and PKC signaling pathways. However, the role of 14-3-3 proteins in mammalian learning and memory remains unclear. We find that 14-3-3 proteins are up-regulated after consolidation of LTM but not after reconsolidation.

4.2 Introduction

4.2.1 Memory on a molecular level

Following synaptic activation, cellular processes required for learning and memory form a complex and dynamic molecular signature. These numerous processes constitute memory consolidation, the stabilization of a memory trace after the initial acquisition (Dudai, 2004). Synaptic plasticity, the capacity of neurons to change the strength of their connections with experience, provides a cellular mechanism for LTM and is driven by molecular changes comprising consolidation. While this stabilization can lead to a memory lasting years, it can also be susceptible to disruption during the retrieval process, where the memory once again undergo a re-stabilization known as reconsolidation, which also relies on cellular and molecular processes (Nader et al., 2000).

Elucidation of specific molecular pathways have provided valuable understanding and insight into the physiology of learning and memory in addition to therapeutic strategies for neurodegeneration and other mental disorders (Yin and Yang, 1994, Sutton and Carew, 2000,

Kandel, 2001, Alberini, 2009). Additionally, discerning differences between consolidation and reconsolidation will further treatment of memory and post-traumatic stress disorder (PTSD), as knowledge of specific pathways involved will allow for more precise treatment without adverse cognitive side-effects (Lee et al., 2004). While many pathways have been thoroughly studied for their role in plasticity, new focus on scaffolding and regulatory proteins has allowed for a deeper understanding of changes at the synapse and in the neuron during LTM (Kennedy et al., 2005, Bramham et al., 2008, Hotulainen and Hoogenraad, 2010, Wang et al., 2011).

4.2.2 14-3-3 proteins

The 14-3-3 (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation proteins) family of proteins consists of seven isoforms in mammals: β/α, ε, η, γ, τ/θ, ζ/δ, and σ/stratifin encoded by seven distinct genes (Bridges and Moorhead, 2005). These proteins are highly conserved molecular chaperones that directly bind their target signaling proteins, including kinases, phosphatases and membrane receptors (Fu et al., 2000). 14-3-3s have been implicated in regulation of metabolism, signal transduction, cell-cycle control, protein trafficking, and apoptosis (An et al., 2002, van Heusden, 2005). 14-3-3s act as homodimers and heterodimers through recognizing specific phospho-serine/ phospho-threonine motifs (RSXpSXP and RXY/FXpSXP) on their target proteins, thereby regulating activity, interaction with other binding partners, and subcellular localization of the proteins to which they bind (Yaffe et al. 1997; Muslin and Xing, 2000; Xing et al., 2000; Nagata-Ohashi et al., 2004;Jones et al., 1995).

4.2.3 14-3-3 proteins and the brain

While highly abundant in the brain, much remains unknown about the depth of character that 14-3-3s play in neuronal physiology. There is evidence that they are expressed at differential levels in clinical samples and may play an important role in progression of neurodegenerative diseases such as Parkinson's Disease, Alzheimer's disease, Multiple Sclerosis, and other disorders such as schizophrenia, tumorigenesis and alcoholism (Ikeda et al., 2008; Mackay et al., 2011; Ling et al., 2010; Li et al., 2011; Wang et al., 2009a). In screening for complex gene interactions

in schizophrenia, Ikeda et al. used heterozygous 14-3-3 epsilon knockout mice and to show in addition to addition to schizophrenic susceptibility some memory defects in radial arm maze (Ikeda et al., 2008). In neurons, 14-3-3 proteins are involved in cell signaling processes, interacting with PKC, MAPK, L-type voltage gaited calcium channel and components of dopamine synthesis (Dai and Murakami, 2003, Davidson et al., 2005, Li et al., 2007). In a recent paper, Kent et al. identified members of the 14-3-3 family in axon growth cone preparations (Kent et al., 2010). This work showed direct binding of 14-3-3s to PKA, indicating regulation of cAMP effectors in neurons. Control of the cAMP pathway is of particular interest in synaptic plasticity, learning and memory (Castellucci et al., 1982). Indeed, work from Skoulakis and Davis showed in Drosophila that flies mutant in *leonardo*, a gene highly homologous to mammalian 14-3-3 epsilon, exhibit deficits in olfactory memory (Skoulakis and Davis, 1996).

4.2.4 Objective of the current work

In a proteomic screen using multi-dimensional protein identification technology (MudPIT) mass spectrometry combined with chromatography, we identified numerous proteins regulated after memory consolidation. The 14-3-3 family of proteins were identified and validated as proteins up-regulated after memory consolidation in the amygdala at timepoints during memory consolidation. Based on the role of 14-3-3s in regulation of PKA activity at distal sites of the neuron during development, we were interested in the possibility that 14-3-3s could also regulate these same signaling pathways in adult plasticity. We found that numerous isoforms were up-regulated in the amygdala during memory consolidation of auditory fear conditioning (AFC).

4.3 Materials and Methods

4.3.1 Subjects

Adult Sprague-Dawley rats (275-325g at surgery) were housed individually and maintained on a 12/12 light/dark cycle (lights on at 7am) with food and water provided *ad libitum*. All procedures were in accordance with the Canadian Council of Animal Care guidelines, and were approved by McGill University Animal Care and Use Committee.

4.3.2 Behavioural Apparatus

Conditioning and tone testing were conducted in different chambers (Med-Associates; Coulbourn Instruments). Rats were conditioned in a Plexiglass rodent conditioning chamber (chamber A) lit by a cue light near the top of the chamber, a metal grid floor and vanilla scent. A distinct chamber (chamber B) was used for auditory fear memory reactivation and long term memory testing to minimize context generalization. This chamber contained a red house light positioned just outside the chamber, a white Plexiglas floor, black and white stripped walls and a peppermint scent. A micro video camera was used to record behaviour for analysis. Freezing behaviour (the complete absence of movement) (Blanchard and Blanchard, 1969) was scored by an experimenter blind to the experimental conditions of the rats with Freeze-View (Actimetrics).

4.3.3 Behavioural Protocol and tissue collection

Rats were habituated to both chambers A and B in a counterbalanced order for 20 min daily for two days prior to training. Fear conditioning consisted of rats placed in chamber A and after a 2 min delay presented with a 30s, 5kHz; 75dB tone which co-terminated with a 1s 1mA foot shock. The rats were left in the chamber for 1 min after the termination of the US. Rats were then returned to their cages.

Rats in the consolidation groups were sacrificed 0, 15, 90 or 240 min following the conclusion of the conditioning session. Rats in the reconsolidation groups were placed in chamber B 24h following conditioning. Following a 2 min delay a 30s 5KHz 75dB tone was presented. The rats were left in the chamber for 1 min before being returned to their cages. Rats in the reconsolidation group were sacrificed 0, 15, 90 or 240 min following the conclusion of the reactivation session. All rats were anesthetized (3.33 mg/kg xylazine; 55.55 mg/kg ketamine; 0.27 mg/kg medetomidine hydrochloride) and sacrificed by rapid decapitation. Whole brains were dissected from the rats and snap frozen in liquid nitrogen and stored at -80° C. Amygdalas were dissected using cryostat sectioning and a stainless steel needle with a 1mm thick opening. Tissue

samples were digested in modified RIPA buffer, and clarified through centrifugation at 10,000g for 15min.

4.3.4 Mass spectrometry

The extracted proteins were precipitated by the addition of trichloroacetic acid and then digested by the sequential addition of lys-C and trypsin proteases (Motoyama and Yates, 2008). The digested peptide samples were then fractionated online using sequential strong-cation exchange and reversed-phase chromatography and eluted directly into an LTQ-Orbitrap mass spectrometer (Thermofisher) where MS/MS spectra were collected. Data analysis was performed using the SEQUEST and DTASelect2 algorithms and peptide identifications were filtered using a false positive rate of less than 5% as estimated using a decoy database strategy. Normalized Spectral Abundance Factor (NSAF) values were calculated as previously described (Florens et al., 2006).

4.3.5 Statistics

Freezing during training session and reactivation session were each analyzed using one-way ANOVAs followed by Fisher LSD *post hoc* analyses (SPSS).

4.3.6 Antibodies

Antibodies used include: Beta-actin monoclonal (Sigma),14-3-3 isoform specific rabbit polyclonals (Santa Cruz), Arc monoclonal (Santa Cruz).

4.3.7 SDS-PAGE and Western Blots

Amygdala samples were boiled in Laemmli sample buffer and proteins were resolved by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes, blocked in TBS-T 5% BSA and probed with primary antibodies, HRP-conjugated secondary antibodies and detected by chemiluminescence.

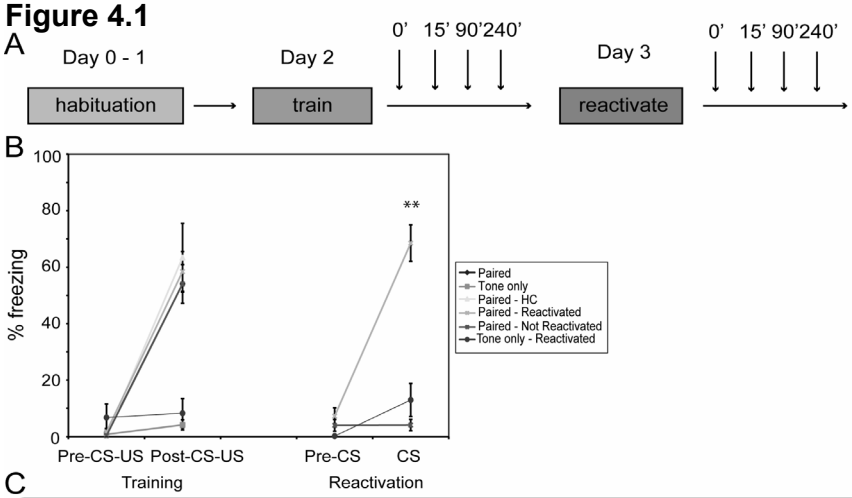
4.4 Results and Discussion

4.4.1 Proteomic profiling of memory consolidation and reconsolidation

In order to elucidate new and/or unique proteins translated after LTM consolidation and reconsolidation, we performed associative auditory fear conditioning (AFC) in rats in concert with a proteomics screen. Figure 4.1A shows schematic of behavioural paradigm used to generate our proteomic database at different timepoints during memory consolidation and reconsolidation. Rats in the consolidation group (paired, paired - HC, paired – reactivated & paired not reactivated) received a tone-shock pairing and exhibited heightened freezing response post-CS compared to rats that received tone only (tone only & tone only - reactivated) (paired = 54.09% (SEM =6.45, n = 21); paired HC = 63.38% (SEM = 12.15, n = 5); paired – reactivated = 58.43 (SEM = 7.04, n = 20); paired not reactivated = 54.05% (SEM = 6.86, n = 22); tone only = 4.17% (SEM = 1.80, n = 19); tone only reactivated = 8.31% (SEM = 5.16, n = 19) $F_{(5, 99)} = 17.336$, $p < 0.001$; paired vs paired – HC, paired reactivated, paired not reactivated all n.s.; paired vs tone only & tone only- reactivated both $p < 0.001$) (Fig 4.1B). On day 2, rats in the reconsolidation groups (paired-reactivated) received a 30s presentation of the tone. Paired-reactivated rats froze significantly more during this time than control rats (paired- not reactivated & tone only reactivated) (Paired – reactivate = 64.19% (SEM = 7.77) paired not reactivate = 2.19% (SEM = 1.2) tone only – reactivate = 19.03% (SEM = 6.94) $F_{(2, 58)} = 43.016$, $p < 0.001$, paired-reactivated vs paired not-reactivated & tone only-reactivated both $p < 0.001$) (Fig 4.1B).

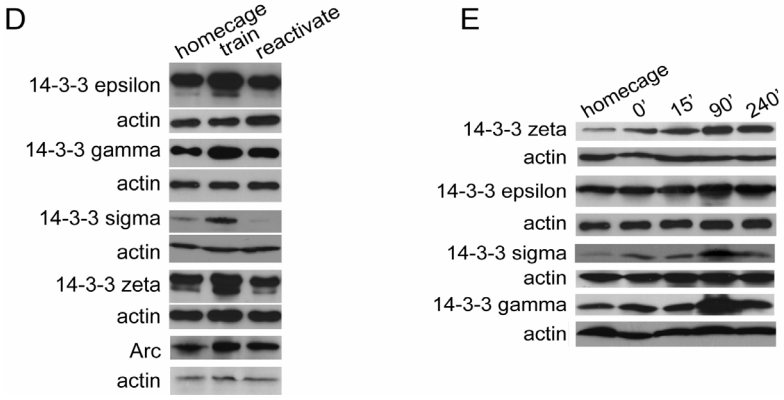
Rats in each group were sacrificed at timepoints after training or reactivation (Fig 4.1A). Using proteins precipitated from microdissected amygdala at 90 minutes after training (LTM consolidation) and 90 minutes after retrieval 24h later (reconsolidation) we ran triplicate samples in MudPIT mass spectrometry to compare proteomic profiles with homecage control rats. While many expected hits were present, some obvious proteins involved in consolidation (and reconsolidation) were absent or did not quantitatively change across different samples. This is likely due to the nature of working with a highly complex sample, consisting of high levels of membrane-associated peptides and lipid content, both of which pose a challenge for mass spectrometry in brain tissue (Liao et al., 2009). We validated by Western blot analysis select candidates meeting our exclusion criteria of being detected in all of the triplicate samples and having a 20% deviation from homecage control (Fig 4.1 and data not shown).

Figure 4.1



C

Locus ID	L	MW	pI	Paired	React	Control	Description
P63101	245	27771	4.8	1104.9	1106.3	867.2	14-3-3 protein zeta/delta
P62259	255	29174	4.7	1013.4	868.3	844.1	14-3-3 protein epsilon
[+]group	244	2773	9.5	703.8	581.7	572.4	Isoform 1 of 14-3-3 protein theta
[+]group	245	27970		630.0	149.4	520.4	Isoform short of 14-3-3 protein beta/alpha
P61982	247	28303	4.9	571.2	508.8	467.5	14-3-3 protein gamma
P68510	246	28212	4.9	590.5	503.7	489.4	14-3-3 protein eta



4.1 A) Schematic of behavioral protocol and timecourse of brain collection after training (for consolidation) or reactivation (for reconsolidation) of auditory fear conditioning. **B)** Increased freezing is seen following paired tone-shock presentation and 24hrs later compared to rats that received tone only during training. Freezing was scored 30 seconds prior to tone onset and the final 30 seconds of the training session. Freezing was also scored 30 seconds prior to tone onset and during tone presentation (or equivalent time). **C)** Mass spec analysis shows increases in peptide frequency and coverage of 14-3-3 isoforms in paired group (consolidation) at 90 minutes after training compared with Control (homecage) or 90 minutes after reactivation. Table denotes SwissProt accession number (LocisID), length of peptide (L), molecular weight (MW) isoelectric point (pI), peptide counts from paired, reactivated and home cage control animals, and description of members of the 14-3-3 family of proteins identified. **D)** Samples were analyzed by SDS-PAGE and Western blot analysis and show 14-3-3 isoforms, and Arc as a control, are increased at the 90 minute timepoint after training. **E)** Timecourse of expression by Western blot analysis for isoforms of 14-3-3 zeta, epsilon, sigma and gamma shows increases in expression of 14-3-3 isoforms by the 90 minute timepoint. All Westerns were repeated in triplicate and densitometry analysis was done normalizing to actin (*$p<0.05$, **$p<0.01$ means±S.E.M. for all figures).

4.4.2 14-3-3 proteins are up-regulated during memory consolidation

Due to the previously demonstrated role of 14-3-3s in regulation of PKA activity (Kent et al., 2010), we were interested in the possibility that 14-3-3s could regulate memory consolidation through these same signaling pathways. The 14-3-3 family of proteins were identified as proteins up-regulated after memory consolidation in the amygdala in our proteomic screen (Fig 4.1C). To validate the proteomic data we used Western blot analysis for specific isoforms of 14-3-3. While all isoforms tested showed varying increases 90 minutes after paired training, four were significantly up-regulated in densitometry levels compared with homecage controls after normalization to actin: epsilon (2.2 fold increase, $p<0.005$), sigma (2.4 fold increase, $p<0.05$), zeta (1.65 fold increase, $p<0.05$), and gamma (2.4 fold increase, $p<0.01$) (Fig 4.1D). No increase in 14-3-3 immunoreactivity by Western analysis was seen in reconsolidation groups (Fig 4.1D). Arc (Activity-regulated cytoskeleton-associated protein/Arg3.1), another protein whose expression increases after neuronal activity (Guzowski et al., 1999), is also seen up-regulated during consolidation using the same samples (1.7 fold increase, $p<0.01$, Fig 4.1D).

The increase that we observe at the 90 minute timepoint correlates with up-regulation of other plasticity-related genes such as c-fos and Arc and is consistent with a timeframe of a change in transcription (Dragunow et al., 1989, Curran and Morgan, 1995, Lonergan et al., 2010). In order to further examine the timing of 14-3-3 expression, a timecourse containing samples of amygdala 0, 15, 90 and 240 minutes after training were analyzed by Western blot. A clear increase in expression of select 14-3-3 isoforms is seen peaking at the 90 minute timepoint (Fig 4.1E). One

possible explanation for our observed up-regulation of 14-3-3s is given by evidence of a cyclic adenosine monophosphate (cAMP) response element (CRE) in the 5′-upstream region of one isoform of 14-3-3 (Muratake et al., 1996). It is possible that other isoforms are similarly regulated and also contain CREs. Transcriptional regulation upon activation of the adenylate cyclase pathway during memory formation occurs by a family of CRE-binding proteins (Dash et al., 1990). During LTM formation, cAMP response element-binding protein (CREB) and phosphorylated CREB (pCREB) may bind to this CRE along with others and trigger increased production of 14-3-3 isoforms.

The work in this chapter demonstrates a correlation between the consolidation of an auditory fear memory and levels of 14-3-3 proteins. Future work should aim to demonstrate a causal link between 14-3-3 proteins and consolidation. One such approach would be to measure the effect on consolidation of inhibiting 14-3-3 activity. A recent paper has used the peptide R18 to specifically inhibit 14-3-3 activity (Kent et al. 2010). By encoding this peptide in a viral vector and injecting it into the BLA prior to training it could be determined if 14-3-3 activity is required for consolidation of an auditory fear memory. The prediction based on our work is that blocking 14-3-3 activity would impair consolidation.

Other future work should attempt a more direct unbiased approach. Rather than attempting to quantify every protein in the amygdala, as we tried here, directly measure only the proteins that are being actively translated during consolidation and reconsolidation. This could be accomplished by making polysome fractions from amygdala during consolidation and reconsolidation. A polysome fraction is a preparation that separates mRNA that are actively being translated. This preparation can then be analyzed by quantitative PCR (qPCR) or microarray to determine the identity of these mRNA (Larsson et al., 2010). Once the actively translated mRNA are identified, the protein that they code for can be inferred.

4.5 Conclusions

We show that 14-3-3 proteins are specifically up-regulated in consolidation after fear conditioning in the amygdala. Future experiments will test which pathway of 14-3-3 interactions

is responsible for this regulation of LTM formation. As a regulator of targets such as PKA and PKC, 14-3-3 isoforms could fine-tune the responsiveness of a synapse or a neuron in its role in plasticity.

4.6 References

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Chapter 5

General discussion and future directions

5.1 General Discussion

In this dissertation I presented experiments that use blockades of reconsolidation to investigate multiple memory interactions and experiments that demonstrate a potential limitation in using blockades of reconsolidation to investigate multiple memory structures which will contribute to our understanding of how memories are stored and interact in the brain.

5.1.1 How context and tone memories interact in the BLA

In the chapter 2 I showed that the relationship of context and auditory fear memory cannot be categorized as either independent, or overlapping. Blocking reconsolidation immediately following tone reactivation has no impact on context elicited freezing. Conversely, blocking reconsolidation following context reactivation impairs freezing to both tone and context. I presented evidence that suggests that this asymmetrical pattern of impairments is not due to tone context and shock being organized in a serial association. However, there are three other models of context-tone interaction that could have resulted in this pattern of results: asymmetrical parallel connectivity, bidirectional circuits and the asymmetrical overlapping populations model.

The asymmetrical parallel connectivity model posits that the contextual representation activates cells in the LA that store the tone fear representation, while the cells mediating the tone representation do not have any excitatory connections with the contextual memory that is stored independently. This is supported by findings that while there are neurons in the BA (an area thought to be involved in the storage of CFC Calandreau et al., 2005) involved in the context modulation of auditory freezing (Herry et al 2008), there are suggestions that the auditory fear representation in the LA is actively inhibiting the context fear representation in the BA (Calandreau et al., 2005). Future experiments to investigate this model should focus on showing that LA neurons that represent the auditory fear memory are activated by context exposure and not vise versa. This could include detecting coincident activity in BLA populations using electrophysiological or multi-event molecular visualization (i.e. Guzowski et al., 2001; Reijmers et al 2007).

The bidirectional circuit model posits that both tone and context fear representations are capable of activating each other. However, these mutual innervations are not symmetrical in terms of ability to elicit lability. For example, if the context → tone synapses have NR2B-NMDAr and tone → context synapses do not, reactivation of context could elicit lability in the context representation, but not vise versa.

The asymmetrical overlapping populations model posits that tone and context are represented by overlapping populations of neurons in the BLA. There is some indirect evidence that context fear is represented by a larger population of neurons than tone fear (Hall et al 2001). This model may in fact be a functional result of the asymmetrical parallel connectivity model described above, and could be tested in a similar manner (i.e. multi-event molecular visualization). If the reactivation of the tone representation is inhibiting the context representation but not vice versa, visualizations of activated neural populations of would appear to support context being represented by a larger population than tone. However, if, as predicted in the asymmetrical parallel connectivity model, the reduced neural population activated by the tone reactivation is due to inhibition of the context reactivation, then blocking inhibitory activity during tone reactivation could permit tone reactivation to induce lability of the context representation.

In the second chapter I also showed that blocking reconsolidation and extinction had opposite effects on context and auditory fear memories. This asymmetry could be used in future experiments aimed at elucidating differences between the mechanisms of reconsolidation and extinction. Re-exposure to a CS can trigger either of these opposing processes and there is much interest in determining what the mechanisms are that control each respective process and how they might interact (i.e Suzuki et al., 2004; Mamiya et al., 2009; de la Fuente et al., 2011; Inda et al., 2011).

If a manipulation is performed surrounding the time of fear memory retrieval, three things could happen; freezing decreases, freezing increases, or freezing remains static. A decrease in freezing can be interpreted either as an impairment in reconsolidation or as an enhancement in extinction. This is typically dissociated by assessing the memory over time using renewal, reinstatement or spontaneous recovery tests (i.e. Duvarci and Nader, 2004). No change in freezing

can be interpreted as either impairment in extinction or an inability to elicit lability. Lastly, an increase in freezing is typically considered evidence of an enhancement of reconsolidation (i.e. Tronson et al., 2006). However, this dissociation that I have shown between extinction and reconsolidation with regard to their effect on related memories could be used additionally to discern between reconsolidation and extinction mechanisms. For example, if a manipulation in the time-period surrounding a tone reactivation results in a decrease in freezing, instead of testing for renewal, reinstatement or spontaneous recovery, one could simply test freezing to the training context. If context freezing is low, the manipulation enhanced extinction, if it is high, the manipulation blocked reconsolidation. However, before this approach can be used, a parametric analysis of various acquisition protocols should be done to test if this effect I showed in chapter 2 is unique to a single tone-shock pairing or if it can be generalized to more AFC protocols.

5.1.2 Using reconsolidation to examine memory allocation

In the third chapter I attempted to use the general design from the second chapter to test a recently proposed model of fear memory allocation in the LA. To do this I reactivated a previously learned CS (Tone A) prior to the acquisition of a second fear memory (Tone B). According to the allocation hypothesis, this manipulation should have biased the allocation of the Tone B memory to the population of neurons that underlies the Tone A memory. Although extinction of Tone A did reveal that reactivation of Tone A prior to acquisition of Tone B caused and increase in Tone B freezing, I was unable to test the allocation model by blocking reconsolidation. I showed that this was due to a change in the Tone A memory that was caused by the acquisition of Tone B. The model that I put forward posits that the foot shock used in train Tone B also acted to reactivate the Tone A memory. US presentation has been shown to be able to elicit reconsolidation in all CSs that it has been associated with (Debiec et al., 2010). One result of reconsolidation is thought to be the strengthening, or enhancement of that memory (Inda et al., 2011), and a strong memory can be resistant to attempts to elicit reconsolidation (Suzuki et al., 2004; Wang et al., 2009; Inda et al., 2011). This model predicts that the Tone A memory is reconsolidating while the Tone B is consolidating. There are two ways that this model could be

tested. The first would be to bilaterally administer anisomycin to the BLA following Tone B conditioning. This model predicts that freezing should be impaired. A more elegant test of this model would be to administer ifenprodil (a NR2B-NMDAR antagonist) to the BLA prior to conditioning of Tone B. While blocking BLA NR2B-NMDAR blocks the induction of lability in previously stabilized fear memories (Ben Mamou et al., 2006) it blocks the acquisition of new fear memories (Rodriguez et al., 2001). Thus pre Tone B conditioning infusions of ifenprodil would result in impaired freezing to Tone B but intact freezing to Tone A.

Interestingly, this memory enhancement of the Tone A memory does not appear to generalize to the Tone B fear memory. I have preliminary data that suggests that the reactivation of Tone B 24 hours after its acquisition (and 48hrs after the acquisition of Tone A) does result in its conversion to a labile state. Additionally Tone A freezing is also impaired by anisomycin following Tone B reactivation (data not shown). The finding that extinction of Tone A did not impair the freezing to Tone B in either the reactivated or the non reactivated groups (Fig 3.2B) suggests that there is no generalization between Tone A and Tone B. One interpretation of this preliminary data would be that the reactivation of Tone A during the consolidation of Tone B resulted in an overlapping representation, such that both Tone A and Tone B form synapses onto the same population of neurons. Because the Tone B fear memory has not undergone additional strengthening (reconsolidation) it remains sensitive to inductions of lability by 30 s tone unreinforced presentations.

The molecular mechanisms that have been shown to be necessary for the induction of lability, and have been shown to be endogenously (as opposed to pharmacologically) manipulated (i.e. NR2B-NMDAR, Wang et al 2009) are synaptically located (as opposed to intracellularly). Thus, two memories on the same neuron could be in different states with respect to the induction of lability (A = blocked induction of lability; B = susceptible to becoming labile). However, the mechanisms of reconsolidation are not necessarily synapse specific (i.e. protein/mRNA synthesis, MAPK, PKA activity) and the experimental manipulation of these mechanisms certainly are not (i.e. Nader et al., 2000; Duvarci et al., 2005; Tronson et al., 2006). Thus, if the induction of lability occurs at synapse B (in this example), but the mechanism of reconsolidation that is being

manipulated is cell wide, impairments could be detected by subsequently testing synapse A. For example, one extrasynaptic mechanism of long-term memory is increases in intrinsic excitability including decreases in action potential threshold and reduced afterhyperpolarization (Zhang and Linden, 2003; Benito and Barco 2010) and the acquisition of this cell wide potentiation has been show to be sensitive to similar parameters as synaptic potentiation (Cohen-Matsliah et al 2007; Lopez de Armentia et al., 2007; Mozzachiodi et al., 2008). Thus, the loss of these cell wide changes would present as a behavioural impairment much like those seen following blockades of reconsolidation. I am not suggesting that *all* effects of reconsolidation blockades are due to cell wide mechanisms, but this would be consistent with the data discussed above.

5.1.3 14-3-3 proteins are upregulated during the formation of mammalian LTM

In chapter 4 I presented a collaboration geared towards finding novel mechanism involved in consolidation, reconsolidation or both. Ultimately, the unbiased quantitative approach was unsuccessful, likely due to the complexity of the tissue samples that was used (Liao et al., 2009). Future iterations of this approach could improve on these results by simplifying the samples that are analyzed. One approach would be to perform nuclear or synaptosomal enrichment preparations to reduce the total amount of peptides being quantified. However, the compatibility of detergents used in these preparations with the mass spec poses a challenge (Liao et al., 2009). An even more effective preparation would be to immunopreciptate proteins of interest (such as receptors) and use an unbiased proteomic approach, as described here, to detect changes in binding partners during consolidation or reconsolidation.

Our intention with using MudPIT mass spectroscopy was to detect changes in protein levels during consolidation and reconsolidation. A more direct approach, rather than attempting to quantify every protein in the amygdala, would be to directly measure only the proteins that were being actively translated during consolidation and reconsolidation. This could be accomplished by making polysome fractions from amygdala during consolidation and reconsolidation. A polysome fraction is a preparation that separates mRNA that are actively being translated. This preparation can then be analyzed by quantitative PCR (qPCR) or microarray to determine the identity of these

mRNA (Larsson et al., 2010). Once the actively translated mRNA are identified, the protein that they code for can be inferred.

5.2 Conclusion

My hope is that the experiments described in this dissertation will aid in furthering the understanding of the organization of fear memory in the BLA. In addition to the insights regarding the organization of fear memories, this work sheds light on differences between reconsolidation and extinction as well as potential aspects of memory acquisition and enhancement that change the way that lability is induced.

The issues discussed here should also be of interest to human clinical practices. There has been great interest in using manipulations of reconsolidation to treat various psychopathologies especially drug addiction (von der Goltz C. et al., 2009; Taylor et al., 2009; Milton and Everitt, 2010) and post traumatic stress disorder (PTSD) (Brunet et al., 2008; Diergaarde et al., 2008; de Quervain and Margraf, 2008) since both drug addiction and PTDS are thought of as disorders of memory (Grillon et al., 1996; Torregrossa et al., 2011). However, before manipulations of reconsolidation can be effectively used clinically, there needs to be a greater understanding of the specificity of memory reactivation. Some previous work has started to look at this issue (Debiec et al., 2006; Doyere et al., 2007; Debiec et al., 2010) but the experiments presented in this dissertation especially the interaction between context and tone fear memory provide a foundation for further work in this area.

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