BEHAVIORAL, MOLECULAR AND NEUROBIOLOGICAL PROCESSES UNDERLYING MEMORY RETRIEVAL

By

James M. Stafford

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CERTIFICATE OF APPROVAL

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This is to certify that the PhD dissertation of

James M. Stafford

has been approved

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Advisor

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Table of Contents

List of Tables

List of Figures

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Abstract

The simple act of retrieving a memory has potential to modify a memory as it is updated in response to a changing environment. For example, longer or repeated re-exposures to a previously reinforced stimulus often result in greater extinction and response loss while brief exposures may strengthen the memory or its expression. However, we know little about the mechanisms that produce these bidirectional changes at behavioral and neurobiological levels of analysis. Therefore, the focus of this dissertation was to use contextual fear conditioning in mice as a vehicle to evaluate the behavioral and brain region-specific transcriptional changes (e.g., histone acetylation and gene expression) that underlie retrieval-induced changes. In Chapter 1, I investigated whether the learning and memory processes engaged by acquisition were similar to those engaged by retrieval. This was studied by administering amnestic agents (e.g., anisomycin), memory-enhancing agents (e.g., the histone deacetylase inhibitor, sodium butyrate-NaB) or behavioral manipulations (e.g., extinction) following contextual fear acquisition and retrieval. Generally, I found that: 1) acquisition was more sensitive to amnestic treatment (e.g., anisomycin) than was retrieval, 2) retrieval processes were more sensitive to enhancement than acquisition and 3) extinction sessions administered immediately following acquisition or retrieval had similar behavioral and molecular effects. Chapter 2 evaluates how the circumstances of memory retrieval such as the duration of fear context exposure and handling would affect the outcome of retrieval. The basic finding from this study was that retrieval was capable of: 1) enhancing fear expression when retrieval contingencies strongly reactivated the memory (e.g., handling) in the absence of explicit extinction (context re-exposure), 2) relatively long

(e.g., those inducing extinction) and very short durations produced response loss dependent on the animal's behavior during the retrieval trial and 3) intermediate retrieval durations left behavior relatively unaffected. I explicitly tested whether certain brain regions were selectively involved in mediating retrieval-induced enhancements and decrements in behavior by administering NaB directly into select brain regions following a retrieval duration that normally produces little effect on behavior (Chapter 3). I found that selectively targeting the neural substrates of retrieval-induced excitatory (e.g., prelimbic) and inhibitory (e.g., infralimbic) processes with NaB shifts the outcome of memory retrieval to generate fear enhancements and decrements (respectively). In addition, manipulations in one brain region (e.g., hippocampus) are capable of driving transcriptional changes in another brain region (e.g., infralimbic cortex) that underlies a more persistent extinction memory. The critical finding from these studies is that retrieval does not engage mutually exclusive processes to effect memory change. Indeed many processes are simultaneously engaged with the most dominant retrieval-processes (e.g., extinction) having a distinct neurobiological and behavioral signature. Together this implies that retrieval makes the original memory susceptible to modification by adding new information to the memory rather than making the original memory directly vulnerable to disruption through a process often referred to as reconsolidation. Future studies will be critical in determining how these pre-clinical findings translate into therapies that rely on memory retrieval to modify pathological memories such as traumatic and drug-associated memories.

Introduction

Significance

Many of the most debilitating psychiatric diseases such as anxiety disorders, substance abuse, and schizophrenia are characterized by pathological learning and memory processes (Corlett, Taylor, Wang, Fletcher, & Krystal, 2010; Peters, Kalivas, & Quirk, 2009). One of the best studied examples is anxiety disorders, including post-traumatic stress disorder (PTSD). A hallmark of PTSD is a failure to inhibit the powerful mental, emotional and physical anguish evoked by environmental stimuli (DSM IV-TR). Current behavioral therapies focus on ways to dampen these reactions by decreasing the ability of the cues to elicit the response in a process called behavioral extinction. This is often achieved by clinical re-exposure to the anxiety-inducing cues requiring the formation of a new inhibitory extinction memory between the cue and the outcome or response (Bouton, 2004; Lattal, Radulovic, & Lukowiak, 2006; Myers, Ressler, & Davis, 2006). However, re-exposure (i.e., memory retrieval) can activate an opposing reactivation process (sometimes called reconsolidation) where the traumatic memory is reactivated, updated and strengthened, potentiating future affective responses to the cue (J. L. Lee, 2008; Rohrbaugh & Riccio, 1970). One additional challenge is that extinction is often incomplete and the cue-induced affective response spontaneously recovers over time (Delamater, 2004). Thus, a major goal of extinction research is to determine combinations of pharmacotherapy and behavioral interventions that enhance extinction memory formation creating a more robust and persistent decrease in cue-induced affective responses (Davis, Barad, Otto, & Southwick, 2006).

Prerequisite to these pre-clinical and clinical approaches to exposure-based therapy is a basic understanding of how memory retrieval can change the expression of a memory at behavioral, neurobiological and molecular levels. As mentioned, there is growing evidence that at a behavioral level, memory retrieval can both increase (e.g., reactivation/reconsolidation) and decrease (e.g., extinction or erasure) memory expression. However, the behavioral conditions, theoretical processes, brain regions and molecular mechanisms that underlie these bi-directional effects remain poorly understood and are the focus of this dissertation.

Behavioral and Theoretical Implications of Memory Retrieval

A contemporary view of memory formation requires that a learning event occurs, the learning event is encoded and then undergoes a time-dependent process where the memory is consolidated into a long-term memory (Abel & Lattal, 2001; McGaugh, 2000). The memory may then be retrieved through a number of processes. Perhaps the best studied is the retrieval process engaged by re-exposure to a previously reinforced stimulus (conditioned stimulus; CS). This cue-induced re-exposure has potential to modify a memory as it is updated in response to a changing environment through a variety of behavioral processes and is generally referred to as retrieval throughout this dissertation. From a very fundamental level there are three possible behavioral outcomes of memory retrieval:

1. Behavior Decreases

Repeated exposure to a CS in the absence of the unconditioned stimulus (US) often results in decreased behavioral expression of the original CS-US association

through a process called behavioral extinction. Seminal studies by Pavlov were among the first to demonstrate that repeated CS exposure leads to response loss within the extinction session itself and on subsequent tests. Importantly, the original response often recovered with the passage of time in a process referred to as spontaneous recovery. This led to early theoretical accounts of extinction that referred to extinction as behavioral inhibition rather than unlearning or erasure of the previously formed CS-US association (Pavlov, 1925; Konorski 1967).

Indeed, research over the past half century has provided evidence that despite an attenuation or extinction of the behavioral response, the original CS-US association remains intact. The most widely accepted evidence comes from; 1) spontaneous recovery effect where behavior recovers following extinction in a time-dependent manner (Robbins, 1990), 2) reinstatement where presentation of the US alone reinstates responsivity to the CS (R. A. Rescorla & Heth, 1975) and 3) renewal of responding when behavioral testing occurs outside the extinction context (Bouton & Bolles, 1979). This has led to a number of theoretical accounts for the behavioral inhibition produced by repeated non-reinforced CS exposure. While some theories focus on unlearning or erasure of the original association (see reconsolidation section below), the preponderance of data indicates that inhibitory relations between stimuli drive many of these effects. Thus extinction is thought to result from new learning —that is, the learning that the CS no longer predicts the presence of the US.

One of the most appealing accounts suggests that the formation of a new inhibitory association between stimuli and some aspect of the original association dampens the expression of the original memory. However, the exact nature of this

inhibitory association is unclear. Early theories suggested that it was in fact an inhibitory CS-US relation that drove extinction (Pavlov 1925; Konorski 1967) while more recent accounts provide evidence for the development of an inhibitory stimulus-response relationship (CS-CR; Rescorla 2001). Others focus on CS/US attention mechanisms where either declining attention to the CS or increased activation of the US representation (thus increasing prediction error) drive extinction (Delamater, 2004). The affective extension of the sometimes opponent processing theory (AESOP), combines elements from many of these theories to suggest that the affective/sensory activation states of the CS and US dictate their ability to enter into inhibitory relations with each other (Brandon, Vogel, & Wagner, 2003).

While providing testable hypotheses, it is difficult to account for every empirical finding with just one theory because each has its own limitations. However, there are some general findings that hold true across most preparations. One of the seminal and most reproducible findings is that long or repeated re-exposure to a previously reinforced CS leads to greater behavioral decrements than shorter durations (Kalish, 1954). Indeed, much of the inhibitory learning that occurs during retrieval relies on the presence of other temporal, discrete and contextual cues that enter into inhibitory association with certain components of the original memory. These additional stimuli are often required to reactivate the extinction memory. In the absence of these extinction cues, the original response often recurs even when the inhibitory learning is conditioned first, leading to the conclusion that this inhibitory extinction learning and/or memory is much more fragile than other excitatory associations (Bouton, 2004; R. A. Rescorla, 2004).

2) Behavioral Increases

It has long been appreciated that response loss is not the only consequence of retrieval. In fact, Pavlov (1927) was among the first to show in animals that presentation of a CS alone could enhance subsequent responding to that CS. His findings were similar to early human studies where CS presentations during a period where the organism was in an aroused state or where other stimuli conferred new excitatory information about the CS could enhance subsequent CS-responding (first part Pavlov Lecture III, Thorndike & Woodworth, 1901).

Recent studies have begun to shed light on the behavioral processes that produce behavioral enhancements following retrieval. One recently re-visited idea is that CS re-exposure reactivates a memory and induces a period of reconsolidation where the original memory needs to be consolidated back into a stable state. During this period of reconsolidation, the memory is labile and can be directly strengthened or impaired by any number of manipulations. A reconsolidation account would therefore suggest that enhanced behavioral performance following retrieval occurs by a strengthening of the original memory. However, beyond synaptic or systems strengthening, reconsolidation theory makes no specific predictions about what processes, behavioral or otherwise, are engaged to produce enhancements in memory expression (Tronson & Taylor, 2007).

For theoretical purposes, it is important to note that reconsolidation is believed to appropriately describe post-retrieval processes because it was discovered using criteria similar to those that underlie consolidation theory. Namely, 1) amnestic agents (e.g.,

protein synthesis inhibition) and memory disruptive manipulations (e.g., new learning) must be administered in a temporally limited post-learning or post-retrieval window to produce amnesia, and 2) agents/manipulations that enhance memory must also be administered in a temporally limited post-learning/retrieval window (McGaugh, 2000; Nader & Hardt, 2009). The reconsolidation account thus assumes that retrieval causes the original memory itself to undergo a time-limited consolidation process once again. This theory is appealing and can describe many recent findings but remains controversial due to conflicting evidence and a lack of specific falsifiable predictions about how reconsolidation changes memory and behavioral expression of that memory (see *Unified Retrieval Theory* section below).

Besides extinction (detailed above), there are a number of well described learning processes induced by retrieval, all of which presumably require consolidation into a stable state. Second-order conditioning is one such process that allows for the presentation of neutral stimuli in the presence of a CS to confer some associative strength to the neutral stimulus thus increasing behavioral expression on subsequent tests (Helmstetter, 1989; R.A. Rescorla, 1984). This type of second-order learning is similar across organisms. In fact, stimulus sampling theory provides a similar account in humans suggesting that certain stimuli present during training do not necessarily enter into association with the original memory because they were not accurately sampled during the original learning. However, when these unassociated stimuli are presented again in the presence of cues that reactivate the original memory (e.g., retrieval) they enter into association with original memory thus increasing the number of associative elements and overall memory strength. Early in the session this strengthening

preferentially occurs despite some underlying extinction in the associated elements as they are entering into new associations with previously unassociated stimuli (Estes, 1997).

A growing body of literature indicates that short memory retrieval trials cause a memory to be strongly reactivated thus enhancing or maintaining behavioral expression. Early descriptions describe retrieval-induced increases in subsequent behavioral expression as “paradoxical” because non-reinforced CS exposures were thought to primarily drive response loss (Rohrbaugh & Riccio, 1970; Rohrbaugh, Riccio, & Arthur, 1972). Importantly, these and more recent studies used fear conditioning procedures with very brief fear CS re-exposures (and sometimes multiple brief re-exposures) to produce these retrieval-induced enhancements (Inda, Muravieva, & Alberini, 2011). Recent accounts suggest that strengthening of the original memory via reconsolidation is responsible for this enhancement. An alternative explanation is that the organism is reinforced for making the fear response. For example, the brief CS presentations only allow for expression of the fear response and no extinction of that behavior. The animals then learn that when they make the fear response in the presence of the CS it is not followed by the aversive US (foot shock), therefore reinforcing the fear response in the presence of the CS. Importantly, this sort of retrieval-induced enhancements are not limited to fear memories. In fact, many cognitive processes have been used to describe these phenomena such as hypermnesia and memory distortion (Roediger & Thorpe, 1978; Estes, 1997).

3) Behavior does not change

Retrieval often leaves behavior unaffected. However, a lack of behavioral change does not necessarily mean that the memory trace itself is not changing. Indeed it has long been appreciated that performance does not always reflect the content of learning or memory (R. A. Rescorla, 1988). A strong possibility is that retrieval actually engages multiple processes (e.g., extinction or second-order conditioning) that compete for dominance as the animals try to update their memory in response to a changing environment. In this case, when the learning contingencies or the state of the animal reflects those of the original learning, there is no dominant retrieval process engaged and no net change in the expression of the memory.

Towards a unified, hypothesis generating theory of memory retrieval

The idea that multiple learning and/or mnemonic processes are simultaneously engaged by retrieval forms the theoretical framework for my dissertation work. This framework relies heavily on the reproducible finding that long or repeated CS presentations often induce behavioral extinction whereas short durations can enhance memory expression or leave the memory vulnerable to amnestic treatment (Duvarci & Nader, 2004; Eisenberg & Dudai, 2004; Inda et al., 2011; Mamiya et al., 2009). My basic hypothesis is that both inhibitory and excitatory processes are simultaneously engaged by memory retrieval. For the sake of simplicity, we will assume that the retrieved memory trace referenced is an excitatory CS-US relationship. Early in a retrieval trial, excitatory processes are more strongly engaged as the excitatory properties of the CS-US association are in heightened state of activation increasing the probability that new, excitatory information can be added to the memory trace. While not

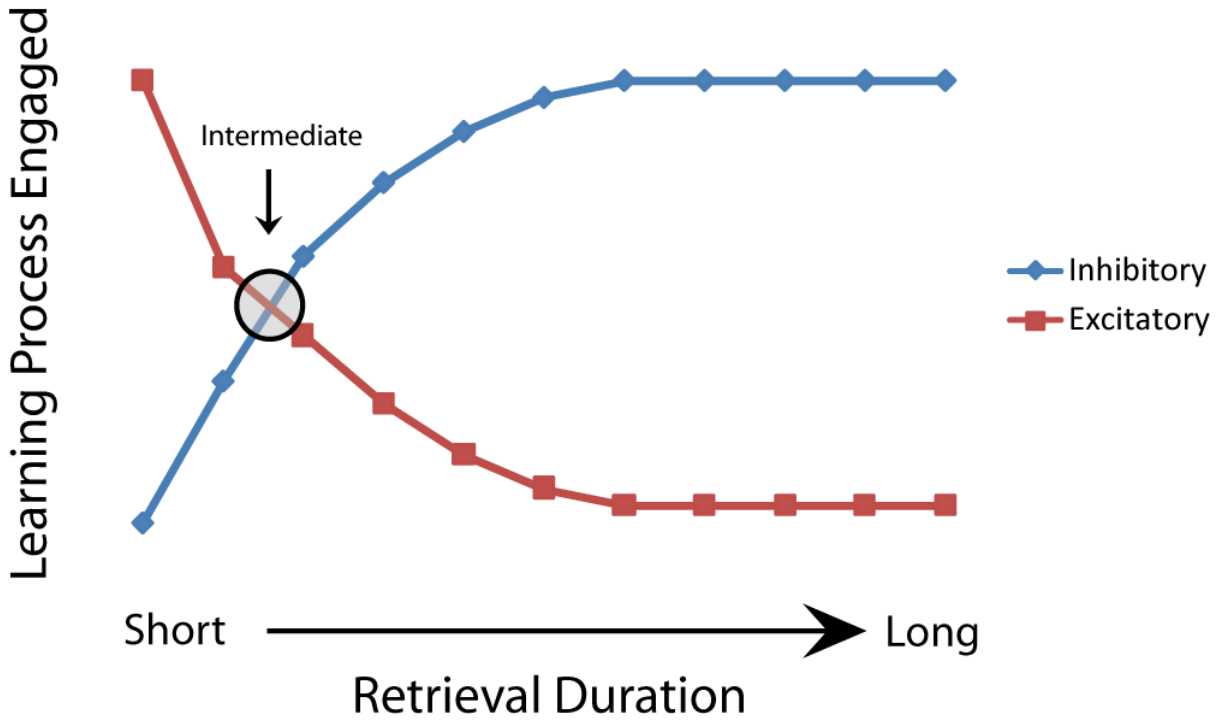


Figure 1. Theoretical model of retrieval process. Early in a retrieval trial learning processes are engaged that confer new, excitatory information to a memory or components of that memory (e.g., CS or US). As the retrieval trial continues, inhibitory learning occurs as the organism learns that the previously conditioned stimuli are poor predictors of the occurrence of the US.

as salient early in the session, inhibitory processes are also engaged as the organism evaluates whether the present retrieval contingencies accurately reflect the predictive value of the original memory. As the retrieval trial continues, the excitatory properties of the memory wane and the inhibitory processes become the most salient as the organism learns that the CS no longer predicts the US. One major prediction of this theory is that there is an intermediate point in retrieval where both inhibitory and excitatory processes are equally engaged resulting in no net modification of the original memory or response. This retrieval theory is outlined in Figure 1.

There are numerous inhibitory or excitatory learning mechanisms that could be taking place during retrieval. However, my hypothesis is that either internal or external

stimuli (e.g., affective states or environmental cues, respectively) add some associative value to the original memory. In the case of inhibition where the CS loses predictive value, these cues would function to dampen the representation of either components or the entire CS-US representation thus driving subsequent response loss. Conversely, these cues would add new excitatory information to individual components or the entire CS-US representation thus driving increased responding on subsequent tests. This theoretical account of retrieval processes gains some validity from theories which suggest that the development of conditioned inhibition requires numerous or prolonged stimulus exposure as a stimulus being conditioned as an inhibitor can obtain second-order excitatory properties if there are relatively few presentations. The implication here is the same; the conditioning of excitatory valence and inhibitory valence to a cue are not mutually exclusive (Cunningham, 1981; Yin, Barnet, & Miller, 1994).

Importantly, there is an underlying assumption that each of these excitatory or inhibitory retrieval-induced learning processes induces a period of time-dependent memory consolidation. During this “consolidation window” the excitatory or inhibitory process can be modified by any number of manipulations including amnestic agents, memory-enhancing agents and behavioral interference. Such modulation can dampen a dominant learning process or memory trace and allow the other competing process to gain salience. For example, an excitatory process such as second-order conditioning may be disrupted via pharmacological manipulation early in retrieval thus unmasking the concurrent inhibitory extinction processes resulting in decreased behavior on subsequent tests.

This account distinguishes itself from accounts suggesting that retrieval may require the original memory to reconsolidate. There is a period of post-retrieval memory lability assumed by my model, however, it is only lability of the new learning induced by retrieval and not the lability of the original memory *per se*.

In this way, the assumption that retrieval induces mutually exclusive and opposing extinction or reconsolidation processes can also be avoided. Part of the justification for staying away from reconsolidation based theories is that deficits in memory reconsolidation look behaviorally identical to enhancements in extinction (e.g., persistent, decreased behavioral expression) making disambiguation of these processes difficult. In addition, it is inappropriate to compare reconsolidation and extinction processes because reconsolidation describes a memory process with no specific accompanying behavioral mechanisms whereas extinction describes specific behavioral phenomena that may engage memory consolidation processes. Therefore, comparisons between a mnemonic and behavioral process is like comparing apples to oranges, making the testing of any specific hypothesis regarding differences between reconsolidation and extinction difficult if not impossible.

This more unified, competing retrieval-processes account provides a way of viewing retrieval that relies on descriptions of behavioral processes that engage memory processes. In doing so, it forms a hypothesis driven mechanism to examine the behavioral, neurobiological and molecular underpinnings of retrieval processes. The major experimental vehicle for this investigation will be fear conditioning, in which a mouse learns that a particular environment or discrete cue (conditioned stimulus; CS) is paired with a shock. When re-exposed to the CS (e.g., during retrieval), mice show

freezing behavior and a heightened state of autonomic arousal which are correlated with symptoms of PTSD in humans (DSM-IVTR). This makes it an ideal model for studying basic processes with translational implications.

Neural substrates of fear learning and memory

Exciting work is beginning to illuminate the neural substrates of retrieval-induced enhancements and decrements in performance. Much of what is already known comes from studies of fear retrieval. It has long been appreciated that the hippocampus and medial prefrontal cortex interact with each other and ultimately the amygdala to regulate fear expression and inhibition.

The amygdala itself gained appreciation as a critical component in regulating the fear response because it receives numerous direct afferents from sensory systems (e.g., olfactory bulb) and projects to circuits that control the behavioral fear response (i.e., periacqueductal grey) leaving it well poised to quickly adapt the vertebrate response to changing environmental stimuli. Current models of the function of the amygdala in fear learning, memory and response indicate a complex amygdalar network that critically depends on amygdala subregion and cell-type. Central Medial (CeM) output is largely governed by direct excitatory inputs from the central lateral nucleus (CeL) “on” neurons (e.g., PKCδ-) and basolateral nucleus (BLA) as well as inhibitory inputs from CeL “off” (e.g., PKCδ+) neurons and intercalated cells [ITCs; (Palomares-Castillo et al., 2012)]. The central medial subregion is largely responsible for the fear response as it directly projects to the periacqueductal grey, lateral hypothalamus and paraventricular nucleus of the hypothalamus (Johansen, Cain, Ostroff, & LeDoux, 2012).

The BLA is thought of as a brain region that receives converging direct or indirect (routed through the LA) sensory and cortical input to modulate fear memories and the fear response. In fact, there is evidence that the BLA is involved in generating inhibitory extinction memories as well reactivating strong fear representations to generate excitatory retrieval processes such as second-order conditioning (Davis, Walker, & Myers, 2003; Duvarci, Mamou, & Nader, 2006; Parkes & Westbrook).

An area of increasing investigation is the critical role of the ITCs in regulating the expression and inhibition of the fear response. Indeed, distinct ITC populations interact heavily with the BLA and lateral amygdala (LA) as well as with each other. For example, the paracapsular ITC nucleus (ImP) receives strong projections from the LA and BLA. In turn, it forms inhibitory projections to the CeL as well as the main ITC nucleus (IN). The IN itself forms inhibitory projection to the CeM (Busti et al., 2011; Manko, Geracitano, & Capogna, 2011; Whittle, Hauschild, Lubec, Holmes, & Singewald, 2010). Interestingly, the ImP cluster appears to be active following fear expression and fear extinction while the IN is active selectively following extinction suggesting that these distinct populations may serve distinct roles in regulating the fear response.

Amygdalar activity is largely modified from cortical inputs. Perhaps the best studied of these are the direct and often functionally distinct inputs of the infralimbic (IrL) and prelimbic cortices (PrL). There is strong evidence from rats and cats that the IrL has direct afferents to the LA, BLA and ImP. This IrL connectivity to the amygdala plays a central role in the generation of fear extinction (Palomares-Castillo et al., 2012; Pare & Duvarci, 2012). A similar physiological and behavioral role for these projections is beginning to be appreciated in mice(Gutman et al., 2012; Knapska & Maren, 2009;

Whittle et al., 2010). In contrast, the PrL projects directly to the LA, ImP and CeL and is thought to be critically involved in generating the fear response (Quirk & Mueller, 2008; Vertes, 2004). The PrL and IrL also may inhibit one another allowing for specific generation of either excitatory or inhibitory effects on behavior (Miller & Marshall, 2004). The dorsal anterior cingulate cortex (dACC) also likely plays a role in modulating fear expression and inhibition given its projections to the amygdala, however, its role is less well understood (Vertes, 2004).

The hippocampus has long been appreciated as a central player in regulating contextual control over fear memory formation and fear extinction memories. One current systems level account speculates that the formation of a contextual fear extinction memory is associated with hippocampal signaling to the IrL, which in turn activates GABAergic cells in the amygdala (i.e., intercalated cells, interneurons), leading to decreased central amygdala firing and behavioral response inhibition (see Figure 2; reviewed in Quirk and Mueller, 2008; Palomares-Castillo et al., 2012; see also Knapsa & Maren, 2009). Some evidence for the selectivity of this circuit comes from the observations that the dorsal hippocampus (CA1) may have direct projections to the IrL but sparse projections to the PrL and dACC (Hoover & Vertes, 2007; Gutman et al., 2012). In addition, projections from the dorsal hippocampus to the ventral hippocampus have been shown to project to certain amygdala subregions suggesting that direct hippocampal-amygdala circuits may modulate fear expression (reviewed in Gross and Canteras, 2012).

An exciting possibility is that the outcome of fear retrieval is modified by a variety of behavioral processes (e.g., extinction) that engage the mPFC, hippocampus, and

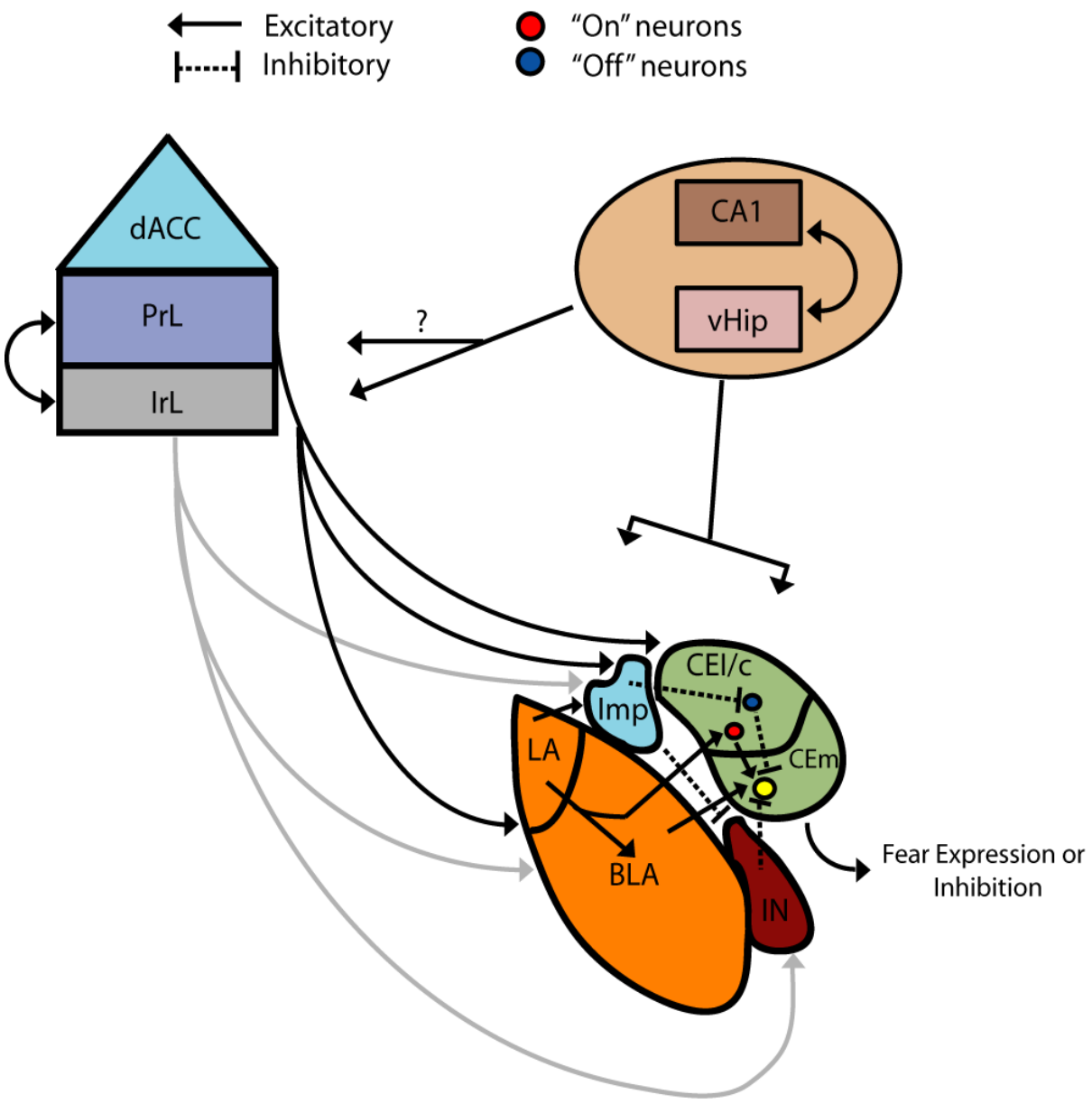


Figure 2. Circuits involved in contextual fear memory retrieval. Generally, the hippocampus (CA1 and ventral portions) provides information about the context to various amygdala and medial prefrontal cortex (mPFC). The mPFC, contains opposing brain regions such as those that tend to drive fear expression (dorsal anterior cingulated-dACC, prelimbic cortex-PrL) or inhibit fear expression (infralimbic cortex-IL) through their effects on various amygdala subregions. Generally, the lateral (LA) and basolateral (BLA) nuclei of the amygdala engage excitatory networks in the central amygdala leading to fear expression. Certain local networks within the central lateral (CeL) dampen central medial (CeM) firing and thus decrease output (e.g., "on" neurons). Intercalated nuclei are also capable of activating (paracapsular island-ImP) or inhibiting (main island-IN) the CeM either directly or through these On/Off neuronal circuits in the CeL.

amygdala interact to produce change in memory expression. Understanding how these complex retrieval processes interact with these neural circuits is a major thrust of my dissertation work.

The involvement of histone acetylation and associated transcriptional changes in memory formation and retrieval

Recent research has shown that the generation of persistent memories relies on long-lasting changes in neural structure and function. A challenge for the field is understanding these cellular and molecular changes that underlie memory formation. What is known is that for a persistent memory to form, a diverse array of receptor systems and signaling cascades must converge on the genome to induce changes in gene expression, and, in turn, long-term functional changes associated with memory consolidation. These transcriptional mechanisms include modifications of DNA as well as proteins involved in regulating the expression of genes required for memory formation. Most recently, the field of epigenetics has identified a critical role for chromatin modifications, such as histone acetylation (HA) and methylation as well as DNA methylation in new memory formation. There has been some recent speculation that these epigenetic marks represent a direct storage mechanism for a behavioral memory as these same epigenetic marks code for the memory of cell fate. For example, Miller et al. (2010) showed that 30 days after fear memory formation, medial prefrontal DNA methylation marks on the promoter of the memory suppressor gene calcineurin were positively correlated with fear memory expression. When this DNA methylation was pharmacologically removed, the fear behavior decreased. This

suggests that the prefrontal methylation signature may be a long-term biochemical mark for maintaining or suppressing a fear memory.

However, many of these marks (e.g., HA) are much less persistent following memory formation, suggesting that these more transient epigenetic marks are not necessarily long-term signatures of an engram. Modifications such as HA are thought to lead to persistent changes in neuronal function underlying memory formation by changing the expression of genes critical for cellular memory formation [i.e., long-term potentiation; (Barrett & Wood, 2008)]. My dissertation work specifically focuses on understanding how the chromatin modification, HA impacts behavioral and transcriptional processes involved in memory retrieval.

HA in new memory formation

What is known about the role of HA in memory comes largely from studies of new memory formation. Acetylation of histones typically occurs at lysine (K) residues on histone tails. While we know little about how memory modulates acetylation at specific K residues HA genome-wide, we do know that, generally, the formation of new memories is associated with increases in HA. These increases in HA, in turn allow for the recruitment of transcriptional machinery and the expression of genes important in memory formation (reviewed in Peixoto & Abel, 2012). A few specific K residues such as H3K9/14 and H4K8 are known to be intimately tied to gene expression and subsequent memory formation. Interestingly, increases in acetylation at one of these residues does not always predict increases in acetylation at other residues suggesting that memory formation may be tied to specific acetylation events rather than affecting

global acetylation (e.g., Barrett et al., 2011). Furthermore, not all genes are affected by increases in acetylation. Surprisingly, only a few genes are known to be changed by specific memory-related acetylation events (e.g., BDNF, c-Fos, NR4a1/2), further indicating that acetylation selectively changes molecular substrates underlying memory formation (Barrett et al., 2011; Guan et al., 2009; Lubin, Roth, & Sweatt, 2008; Mahan et al., 2011; McQuown et al., 2011; Vecsey et al., 2007). An important theme emerges from these studies of acetylation and memory; memory signaling cascades likely converge on the nucleus to generate specific histone acetylation patterns that in turn lead to the expression of genes and proteins required for long-term memory formation.

Intimately tied to these memory processes are the enzymes that either add acetyl groups (histone acetyltransferases; HATs) or remove acetyl groups (histone deacetylases; HDACs). Seminal studies showed that either genetic or pharmacological inhibition of HDACs during memory consolidation leads to specific increases in acetylation, gene expression and subsequent memory expression. In contrast, genetic or pharmacological inhibition of HATs during consolidation leads to specific decreases in acetylation, gene expression and memory suppression. The exact isozymes involved in these processes are beginning to be appreciated. Specifically, it was recently discovered that preventing the removal of H4K5/12ac or H2BK12ac via HDAC2 inhibition enhances memory as does preventing the removal of H4K8ac by HDAC3 (Guan et al., 2009; McQuown et al., 2011). It is also known that the addition of H3K14ac, H4K8ac and H2BK12ac via the HAT, CREB binding protein (CBP) is required for memory formation and gene expression underlying memory formation (Barrett et al., 2011; Korzus, Rosenfeld, & Mayford, 2004; Vecsey et al., 2007). While other HDACs

and HATs (e.g., p300/pCAF) likely play an important role, their specific involvement is less clear (Oliveira, Wood, McDonough, & Abel, 2007).

The known roles of specific HAT/HDAC isozymes, acetylation at specific residues and their effects on specific genes are summarized in Figure 3A. Much of what is known about the specific molecular players in HA and new memory formation (that is not associated with a drug of abuse) come largely from studies of the hippocampus. There have been a few other studies outside the hippocampus, however, the direct effects on specific acetylation events on gene expression in some of these brain regions remains elusive (Monsey, Ota, Akingbade, Hong, & Schafe, 2011).

HA in memory retrieval processes

HA is also important for memory retrieval processes, however, its role is much less clear and the literature is mixed perhaps due to the numerous potential processes engaged by retrieval. Some of the literature is in line with what is known about new memory formation. For example, increased acetylation of H3K14ac and H4K5/8/12ac are generally associated with increased BDNFexon I/IV and c-Fos expression following fear extinction in the mPFC (Bredy et al., 2007; Stafford, Raybuck, Ryabinin, & Lattal, 2012). Likewise, pan-HDAC inhibitors administered systemically and directly to the IrL enhance extinction (Stafford et al., 2012).

The involvement of HATs on acetylation, gene expression and memory retrieval in the mPFC is much less clear. Inhibition of the HATs CBP and p300 following weak extinction appear to enhance extinction while their activation prevents extinction (Marek et al., 2011). In contrast, inhibition of the HAT, pCAF impairs extinction while its

activation enhances extinction (Wei et al., 2012). Together this suggests that CBP/p300 are important for mediating consolidation of excitatory post-retrieval processes while pCAF is involved in inhibitory post-retrieval processes. However, the seemingly opposing roles of these enzymes in mediating response loss lacks some molecular clarity as no specific changes in their presumed target, HA were evaluated.

HDAC inhibition in the hippocampus adds another level of complexity as there have been demonstrations that hippocampal HDAC inhibition enhances extinction (Lattal et al., 2007; Stafford et al., 2012) and others showing that inhibition of HDAC1 blocks extinction. This study showed that repeated non-reinforced CS presentations increased recruitment of HDAC1 and decreased H3K9ac at the c-Fos/EGR2 promoter thus decreasing their expression and engaging response inhibition (Bahari-Javan et al., 2012). Unfortunately, this study was without proper control groups that did not receive retrieval, making it unclear whether these behavioral effects were on extinction memory or some other non-specific response-loss processes.

The effect of retrieval on HA-associated gene expression following retrieval are also mixed but are generally in line with current accounts of post-retrieval processes. For example, while increases in HA and BDNF IV/c-Fos have been observed in the mPFC following extinction, there are other demonstrations that zif268 decreases expression in response to extinction (Bredy & Barad, 2007; Stafford et al., 2012; Wei et al., 2012). In the hippocampus, repeated non-reinforced CS presentations decreases c-Fos and EGR2 expression and HA while brief CS exposures increase HA and zif268 expression (Lubin et al., 2007; Bahari-Javan et al., 2012). Increased gene expression in the mPFC is not surprising as this brain region is believed to be critical for extinction

memory formation. The decreased mPFC zif268 following repeated CS re-exposure and increased hippocampal zif268 following brief CS-presentations are generally in line with the suggestion that zif268 is engaged following retrieval processes that are reminiscent of reconsolidation [e.g., brief CS exposures; (J. L. Lee, Everitt, & Thomas, 2004). In the hippocampus, it is not necessarily surprising that there is decreased c-Fos and EGR2 expression following repeated CS exposures as repeated CS exposures often decrease transcription in this brain region (Radulovic & Tronson, 2010).

While there is growing knowledge about how HA is involved in mediating transcriptional changes and the outcome of retrieval (see Figure 3B for a summary), we know little about how brain-region specific changes in HA and subsequent gene expression dictate the outcome of memory retrieval. This is a fundamental question that provides me with a vehicle by which to understand the behavioral, neurobiological and molecular underpinnings of retrieval-induced enhancements and decrements in performance.

Overarching Goals of Dissertation

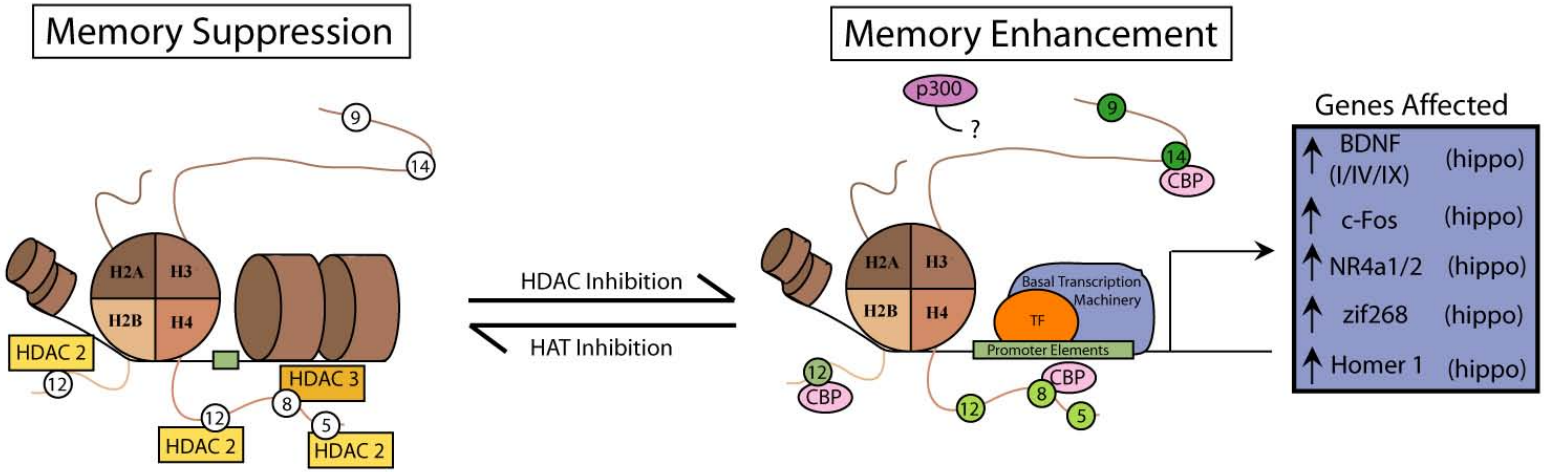
The major goal of this dissertation is to understand how memory retrieval changes memory expression from a behavioral, neural systems and molecular (e.g., HA) level. The specifics goals of these investigations are outlined in each chapter. The general approach to understanding of retrieval from multiple levels of analysis will be to ask 3 fundamental questions:

1. Are the learning and memory processes that occur following retrieval similar to those that occur during acquisition?

2. How do the conditions of memory retrieval (e.g., duration) affect the behavioral expression of memory?
3. How do pharmacological manipulations of brain-region specific histone acetylation change the outcome of memory retrieval?

Answering these questions will require overcoming a host a theoretical, procedural and technical hurdles in examining the behavioral, neural systems and molecular mechanisms involved in retrieval. The goal is that by conquering some of these challenges I will be able to provide insight into the basic processes underlying behavior change with the ultimate goal of using this information to overcome pathological memories.

A) Histone Acetylation in Memory Consolidation Processes



B) Histone Acetylation in Memory Retrieval Processes

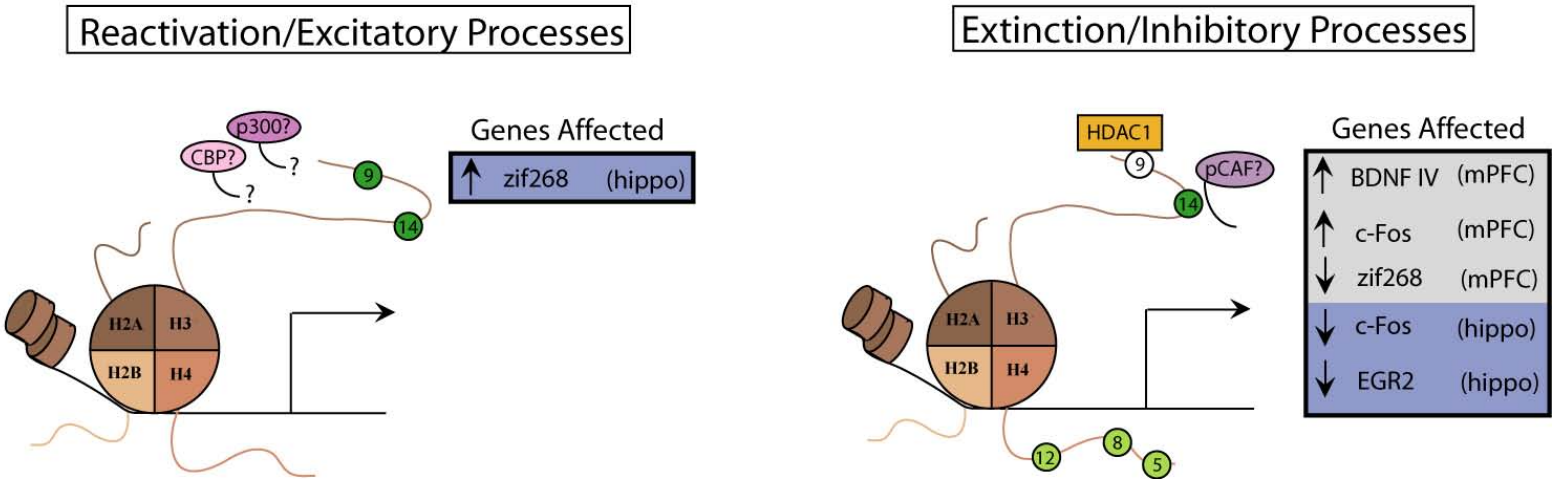


Figure 3. Known roles of histone acetylation in memory formation, retrieval and the expression of genes underlying these processes. Studies that directly link histone acetylation to changes in brain-region specific gene expression are summarized. Briefly, the numbered circles on the histone tails represent lysine residues with green circles indicating acetylated residues and open circles representing unacetylated residues. **Panel A** shows that HDAC2 and HDAC3 are associated with a hypoacetylated state, decreased HAT occupancy and decreased gene expression and memory suppression. Pharmacological blockade of HDACs facilitates a hyperacetylated state, increased HAT occupancy, increases in specific genes and enhancements in memory consolidation. Increases in acetylation and gene expression are also associated with a variety of retrieval processes (**Panel B-** reactivation/extinction). However, there have been some reports that the binding of certain HATs can impair extinction memory formation while increased HDAC occupancy and decreased acetylation at certain genes may actually enhance extinction memory formation.

Chapter 2: Are the learning and memory processes that occur during retrieval similar to those that occur during acquisition?

General Introduction

There is substantial evidence in a variety of behavioral preparations that memories can be disrupted before they are completely formed, leading to the suggestion that memories are consolidated in a time-dependent manner (reviewed in McGaugh & Roozendaal, 2009). The act of memory retrieval can also induce any number of processes that require a time-dependent period of memory consolidation (e.g., extinction, second-order conditioning; reviewed above). However, we know little about whether the learning processes engaged by new memory acquisition and those engaged by retrieval are similarly susceptible to manipulations of consolidation. Understanding whether retrieval induces a period of consolidation that mimics post-acquisition consolidation is critical to theories that rely on reconsolidation accounts to explain retrieval-induced behavior changes. What is known about post-acquisition and post-retrieval processes comes from indirect comparisons of separate experiments examining one process or the other; there have been few attempts to compare these deficits directly in a single experiment. As a consequence, there are a number of very basic issues about these differences—including the relative size and the relative persistence of these effects— that remain unknown.

A challenge in using results from separate experiments to make general conclusions about post-acquisition and post-retrieval consolidation processes is that this

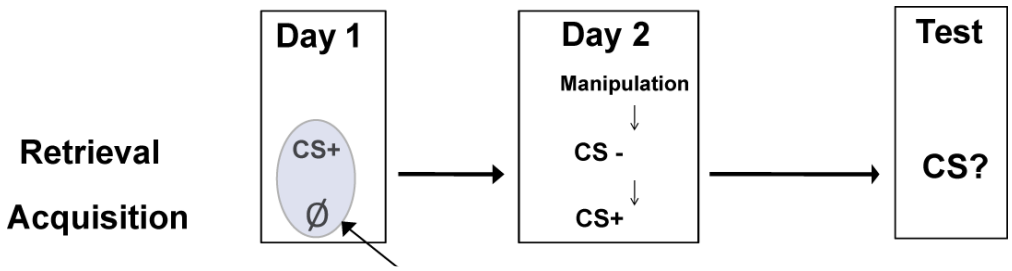


Figure 4. Comparing Acquisition and Retrieval Processes. Studies comparing post-acquisition and-retreival processes often do no match experiences of the acquisition and retrieval groups prior to manipulation and test

necessarily involves comparisons between groups that are not matched on factors that may influence the behavioral effects. These factors, such as familiarity with the stimulus, the expression of behavior, and the internal state of the animal both before and after the amnestic manipulation all may influence the size and persistence of the deficits (Biedenkapp & Rudy, 2004; Estes, 1997; Hinderliter, Webster, & Riccio, 1975). For example, in many preparations, manipulations designed to affect post-acquisition consolidation occur immediately after an animal's first experience with the behavioral treatment (e.g., contextual fear conditioning), whereas post-retrieval manipulations most frequently occur after the animal's second experience (e.g., re-exposure to the conditioning context; See Figure 4). Thus, any comparison between acquisition and retrieval processes is often confounded with the animal's previous overall history with the conditioned stimulus and with the different levels of behavioral response evoked prior to the deficit. This makes it difficult to determine whether group differences at behavioral and molecular levels are due to differences in specific memory processes or to other differences in experience or performance. By closely matching the experiences of different groups of animals, one can be more confident that behavioral and molecular differences reflect different memory processes [see (S. H. Lee et al., 2008), for a related approach].

Another important issue that remains unresolved in comparisons between post-acquisition and retrieval processes is whether the behavioral deficit that is observed soon after amnestic treatment persists across longer retention intervals. The majority of studies examining post-acquisition consolidation deficits have found that these deficits persist across long retention intervals. Many studies also show persistent post-retrieval deficits, but many others show reversal of these deficits [reviewed in (Amaral, Osan, Roesler, & Tort, 2008)]. Some attempts to account for these discrepancies suggest that post-retrieval deficits are sometimes smaller than are consolidation deficits, which may increase the likelihood that the deficit would reverse with time (e.g., Duvarci & Nader, 2004). This is a reasonable hypothesis, but again, there have been few direct examinations of the differences in size and persistence of these deficits from common starting points in behavior.

The overall goal of the studies described in this chapter was to overcome some of these challenges using contextual fear by closely matching experiences prior to manipulations of post-acquisition and -retrieval processes. This allows for the evaluation of whether post-acquisition and post-retrieval processes are equally affected by pharmacological disruptions (Experiments 1-4) or pharmacological enhancements (Experiments 5-7). In Experiments 8-10, we take a different approach to examine whether behavioral manipulations during the post-acquisition and post-retrieval window can disrupt behavior.

Do pharmacological *disruptions* in post-acquisition and retrieval processes produce similar behavioral effects? (Experiments 1-4)

NOTE: *This study was previously published (Stafford & Lattal, 2009).*

The purpose of this Experimental Series was to closely match the experiences of groups receiving amnestic treatment (systemic or intrahippocampal injections of the protein synthesis inhibitor anisomycin) following initial contextual fear conditioning and retrieval of the context-shock memory. This serves two purposes. First, by matching experiences and levels of behavior prior to the deficit, we can make direct comparisons between groups that received conditioning or retrieval immediately prior to anisomycin injections. Second, by matching the size of the anisomycin-induced deficits in post-acquisition- and retrieval-processes, we can determine how the size of the original deficit is related to the amount of behavioral recovery after a long retention interval. If size of the deficit is a primary factor influencing recovery, then groups matched in size of initial deficit should show equal levels of recovery, regardless of whether the deficit was induced post-acquisition or retrieval.

Method

Subjects

A total of 281 male C57BL/6 mice ranging in age from eight to eleven weeks old were used in the experiments. All mice were either bred at Oregon Health & Science University (OHSU) or obtained from Jackson Laboratory (Bar Harbor, ME). The OHSU colony originated from C57BL/6J breeders periodically replaced with C57BL/6J mice acquired from Jackson Laboratory. Each polycarbonate cage housed four mice which hung in a Thoren rack. Animals were allowed free access to lab chow and water during all experiments. Subjects were maintained on a 12-h light/dark cycle (lights on at 0600 h). The laboratory temperature remained at 21 ± 1°C. All experiments were performed

during the animal's light cycle. Protocols were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) "Principles of Laboratory Animal Care."

Cannulations and Histology

Isoflurane (2-5% in air) was used to anesthetize mice throughout the cannulation procedure. After sedation, mice were mounted on a stereotactic apparatus designed for use in mice (Kopf; Tujunga, CA). A small piece of the scalp was removed and the skull was then conditioned using Ketac conditioner (3M ESPE; Seefeld, Germany), an abrasive that facilitates adherence of the glue to the skull. After 2 min, the conditioner was rinsed off the skull using 1X phosphate buffered saline (PBS). Holes were drilled bilaterally above the hippocampus (AP-1.7mm, ML ± 1.5mm). Cannulae guides (Plastics One, Inc., Roanoke, VA) were then inserted into the holes and glued to the skull using Ketac dental cement (3M ESPE). Before the surgery, stylets were inserted into each cannulae to ensure that the cannulae holes did not get clogged. The injectors (28 ga) extended 0.5 mm below the cannula guide into the brain (2.0 mm total length). Forty-five min prior to surgery mice were given 5mg/kg Rimadyl (Pfizer; Exton, PA) to manage post-operative pain. The entire cannulation surgery was performed under aseptic conditions and lasted ~20 minutes per mouse. After the surgery, mice were individually housed.

After the behavioral experiments were completed, brains were removed and flash frozen in methyl butane chilled on dry ice for storage in a -80°C freezer. Brains were later sectioned on a cryostat. Slices were stained using cresyl violet and evaluated to verify correct cannula track position (Figure 9).

Of the 107 mice cannulated, 68 were used in the final analysis. This was due to various factors such as incorrect guide cannulae placement, guide cannulae coming loose, and health issues following surgery. Group sizes listed in the text include only mice with functional cannulae correctly placed in the hippocampus.

Injections

For systemic experiments, anisomycin (Sigma Aldrich, St. Louis MO) was dissolved in 10% w/v β-cyclodextrin solution (Sigma Aldrich, St. Louis MO) for which 1X PBS was the solvent. This solution was used because β-cyclodextrin facilitates the water solubility of anisomycin, which is water insoluble at the concentrations needed for adequate dosing. Anisomycin or vehicle was administered subcutaneously (*sc*) at a dose of 75mg of anisomycin per kg mouse bodyweight (7.5mg/mL solution) immediately and 2 hr after the session to increase the duration of protein synthesis inhibition (Alberini, 2008; Lattal & Abel, 2004). Vehicle doses were an equivalent per kg dose of the 10% w/v β-cyclodextrin PBS solution.

For the intrahippocampal experiments, anisomycin was diluted in PBS and then dissolved in 1M HCl. The pH was adjusted back to ~7 using NaOH. PBS was added to reach the appropriate concentration for infusion (160 µg anisomycin per µL PBS). The vehicle solution consisted of equal amounts of HCl and NaOH as in the anisomycin solution. Mice received bilateral intrahippocampal injections (.25 µL per side) of either anisomycin (40 µg) or vehicle from a 5.0 µL Hamilton syringe (Reno, NV) operated by a Harvard Apparatus Pump II Dual Syringe micropump (Natick, MA). Injections were administered over 1 min at a rate of .25 µL per min. Injectors were left in place for an additional 30 s to ensure diffusion of the solution into the brain. Each side was injected

individually, one occurring immediately after the other. During the infusion animals were allowed to walk freely. Animals were only briefly restrained to remove the stylets and to insert/remove the injectors. The entire microinjection procedure took a total of 6 min per mouse.

Apparatus

Fear Conditioning. A 21.5 cm diameter Plexiglas chamber measuring 23 cm in height was placed on a grid floor. The grid floor consisted of stainless steel rods 3.2 mm in diameter placed .5 cm apart (Coulbourn Instruments product H10-35M-08; Allentown, PA). A .35 mA scrambled shock was delivered through the floor by a shock generator (Coulbourn Instruments product H24-61). An infrared activity monitor was fixed to the top of each chamber to record freezing (Coulbourn Instruments product H24-61). This context (CTX) was illuminated throughout the experimental session with the house light. The chamber was cleaned with water before each subject was placed in the CTX. The CTX apparatus was placed inside a sound attenuating chamber (Habitest Isolation Cubicle; Coulbourn Instruments product H10-24). Infrared and video camera based behavioral records were kept during all sessions.

There were 4 of these conditioned fear chambers in the experimental procedure room (down the hall from the mouse colony) which allowed all 4 animals in a group cage to be run simultaneously. Assignment to these chambers was counterbalanced across experimental groups.

Behavioral Procedures

General Experimental Design. Each experiment consisted of habituation, reinforced and nonreinforced context exposures, and testing (described below). The

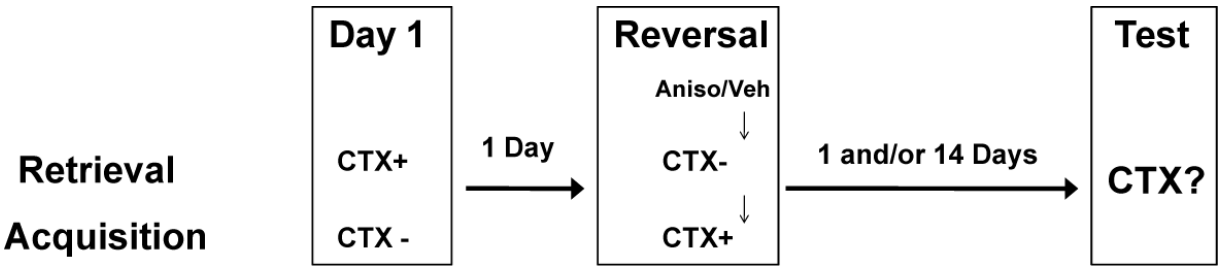


Figure 5. Experience Matching Approach. Total experience with the context (CTX) prior to drug infusion and testing was equated by pre-exposing the acquisition group to the CTX on Day 1.

goal of these experiments was to match the behavioral performance and CTX exposure before testing groups getting post-acquisition (Acquisition) and post-retrieval (Retrieval) anisomycin for differences in the magnitude and persistence of their respective memory deficits. By also equating performance in the vehicle groups, we could directly compare the effects of anisomycin in the Acquisition and Retrieval groups. To accomplish this, the following general experimental design was used throughout all experiments in this chapter (depicted in Figure 5): On Day 1 the Acquisition group received the CTX paired with shock (CTX+) while the Retrieval group was exposed to the CTX in the absence of the shock (CTX). On Day 2 (reversal) the conditions were reversed such that the Acquisition group received a CTX+ experience while the Retrieval group received a CTX- (no shock) experience. This reversal procedure ensured that all groups received the same amount of total context exposure and the same number of shocks. Immediately after the reversal session, mice were assigned to either anisomycin or vehicle groups. Group assignments assured that half the animals received anisomycin, while the other half received vehicle. Assignment into drug groups were based on levels of freezing behavior during reversal to ensure that both drug groups had reached a common level of performance prior to testing. Depending on the experiment, mice

were tested on Day 3 and/or Day 17 by being placed in the CTX in the absence of shock (CTX-).

Habituation. All animals used in the systemic experiments were handled for ~ 1 min per day in the experimental procedure room for 3 d prior to the first conditioning CTX exposure. During handling, mice in the systemic experiments were given injections of .25 mL of 1X PBS to habituate them to injections. Mice used in the cannulated experiment were given at least 3 d to recover from surgeries (e.g., Huang, et al. 2007; Lattal, Barrett & Wood, 2007; Vecsey et al., 2007). After recovery, mice were brought to the experimental room where they were handled and scruffed under environmental conditions similar to those present during drug infusion (e.g., microinfusion pump activated for background noise). The next day, mice were placed under light anesthesia and had their hippocampal stylets removed and dust caps attached to the guide cannula. Later that same day mice were again scruffed with light pressure being put on their cannulae to simulate the drug infusion experience. The following day experiments began.

Each day animals were brought into the experimental procedure room 1 h prior to the experimental session so they could acclimate to the ambient environment.

Conditioning. On the day of conditioning (CTX+) mice were placed into the context conditioning apparatus and received a 2 s .35mA footshock after 2.5, 5, 9, and 11.5 minutes. Mice were removed 30s after the final shock. This occurred on Day 1 for the Retrieval group and Day 2 for the Acquisition group. This CTX + procedure was consistent throughout all of the following experiments.

Experiment 1: Long Non-reinforced Exposure (systemic). The habituation, apparatus, general procedure, and systemic drug injection protocols used in this experiment were as described above. On CTX – days mice received a12-min non-reinforced exposure. On Day 1 the Acquisition group received a 12-min CTX-no shock exposure while the Retrieval group received a CTX+ experience. These conditions were reversed on Day 2. Immediately after removal from the CTX mice received a 75mg/kg *sc* dose of anisomycin or vehicle. They received a second identical dose 2 hr after the reversal session. On Day 3, animals were tested by placing them in the CTX in the absence of shock for 12 min.

Experiment 2: Short Non-reinforced Exposure (systemic). Conditioning parameters used in this experiment were identical to those in Experiment 1. However, in this experiment the pre-exposure for the Acquisition Group and retrieval trial for the Retrieval group were shortened to 3 min to minimize extinction during the retrieval trial. Systemic injection and testing procedures were identical to those used in Experiment 1. Mice in this experiment received a second, identical test 14 d later.

Experiment 3: No Retrieval (systemic). To investigate whether anisomycin-induced deficits in the Acquisition group were dependent on memory retrieval or some non-specific action of anisomycin, a group that did not receive the memory retrieval trial was used. This No Retrieval group received the same CTX+ experience on Day 1 as the Retrieval Group. However, on Day 2 when the Retrieval group received a 3 min non-reinforced context exposure, the No Retrieval group was handled for ~10 s. Immediately after their respective Day 2 experiences mice in both groups received either anisomycin or vehicle injections as described in Experiment 1. Mice in the

vehicle and anisomycin No-retrieval groups were matched based on their average freezing levels during Day 1 conditioning. Testing parameters were identical to those used in Experiments 1-3.

Experiment 4a. Short Non-reinforced Exposure (intrahippocampal) with 1-d and 14-d tests. All context exposure and conditioning procedures were identical to those used in Experiment 2 (3-min nonreinforced exposure). Immediately after Day 2 (reversal) mice received bilateral infusions of either anisomycin or vehicle into the hippocampus. Mice were tested the next day and 14 d later, as in Experiment 2.

Experiment 4b. Short Non-reinforced Exposure (intrahippocampal) with only 14-d test. To ensure that the first test (Day 3) did not influence behavior during the second test (Day 17), a group that did not receive the first test was used. The group that received Test 1 and the group that did not receive Test 1 received identical conditioning, reversal, intrahippocampal injections and Test 2 procedures as were used in Experiment 2.

Data Analysis

Behavior. In all experiments fear memory expression was evaluated by measuring freezing behavior. Freezing behavior in the systemic experiments was defined as the absence of detected movement for at least 3 s using the Coulbourn infrared activity monitors (e.g., Boatman & Kim, 2006; Lattal et al., 2007). In the intrahippocampal experiments, freezing (absence of all movement except respiration) was assessed every 8 s by a trained observer who was unaware of group assignments.

Recovery Testing. Spontaneous recovery is sometimes assessed by examining changes in performance from the first test to the second test. These between-test

comparisons are difficult, especially with long test intervals due to the varied and sometimes uncertain influence of time on behavioral performance. As such, our analyses of recovery focus on common test session comparisons between groups, so that any potential nonspecific time-dependent influences on performance will be controlled across all groups (e.g., Rescorla, 2004).

Statistics. Analyses of variance (ANOVAs) were used to evaluate fear acquisition and extinction. Greenhouse-Geisser corrections were used to account for violations of the sphericity assumption in within-subjects measures. ANOVAs were used to evaluate differences during Days 1 and 2, as well as during the test sessions. During Days 1 and 2, effects of context treatment order (unshocked on Day 1, shocked on Day 2 in Acquistion groups; shocked on Day 1, unshocked on Day 2 in Retrieval groups) and 3-min time block were factors. Test differences were evaluated by examining effects of Context Treatment just prior to injections (corresponding to Acquisition or Retrieval groups) and Drug (anisomycin or vehicle). The driving hypothesis of these experiments was that the effects of anisomycin on post-acquisition and -retrieval processes would differ. Therefore, this *a priori* hypotheses were tested with two-tailed Student's t tests. Alpha levels for all comparisons were held at .05.

Results

Experiment 1: Long Non-reinforced Exposure (systemic). In this experiment we used a 12 min non-reinforced context exposure (CTX-) and a 12 min reinforced exposure (CTX+). As can be seen in Figure 6, during Day 1, the Retrieval group increased freezing throughout the reinforced session (CTX+) and the Acquisition group showed low levels of freezing throughout the nonreinforced pre-exposure session

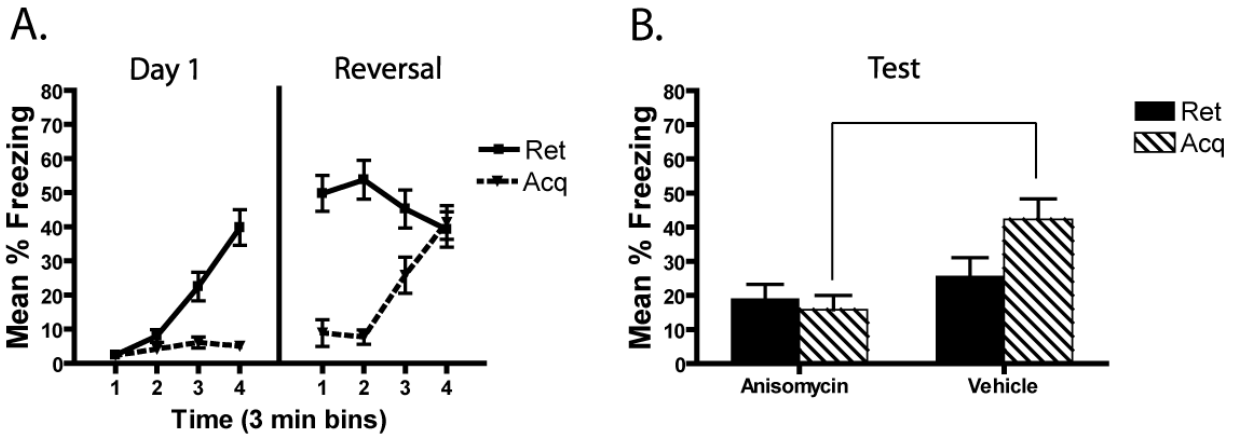


Figure 6. Post-acquisition anisomycin treatment blocks memory expression while anisomycin treatment following a 12 min retrieval trial does not affect performance when tested 1 d later. The right side of Panel A shows that during reversal mice in the Acquisition (CTX+ exposure) and Retrieval (CTX- exposure) groups were brought to the same level of behavioral performance immediately prior to anisomycin and vehicle treatment. B) During a test the next day, the anisomycin-treated Acquisition group showed impaired fear memory expression relative to their vehicle treated controls. Anisomycin did not change the Retrieval group's freezing behavior relative to controls, likely due to extinction in the Retrieval vehicle group.

(CTX), which was confirmed by a reliable two-way interaction between Time Block and Context Treatment (reinforced or nonreinforced; $F(1.6, 67.9) = 33.2$, $p<.001$). The increase in freezing during the CTX+ treatment was reliable in the Retrieval group ($F(1.4, 32.5) = 44.8$, $p =.001$).

During Day 2 (Reversal), mice in the Retrieval group showed some loss of freezing during the nonreinforced context treatment (CTX-), but the Acquisition group increased freezing over the course of the reinforced context treatment (CTX+). There were no interactions or main effect of post-reversal drug treatment group during Days 1 or 2 (all ps >.05). However, there was a reliable interaction between Time Block and Context Treatment during Day 2 ($F(1.6, 67.9) = 33.2$, $p <.001$). This interaction was driven by a decrease in freezing throughout the CTX- session in the Retrieval group ($F(2.5, 58.3) = 9.8$, $p <.001$) and a concurrent increase in freezing throughout the 12 min CTX+ session in the Acquisition group ($F(2.1, 45.9) = 23.3$, $p<.001$). Freezing

levels between the Acquisition and Retrieval groups were statistically equal during the final 3-min block of the Day 2 session ($t(45) = .28$, $p=.78$). These results show that the reversal session was successful at bringing the Retrieval and Acquisition groups to a common level of performance prior to drug treatment and subsequent memory testing.

During the test (Figure 6B), the two anisomycin-treated groups showed similar levels of freezing. Only the Acquisition group appeared to show a memory deficit relative to its vehicle control. However, the Retrieval vehicle group showed very low levels of freezing; thus, extinction in that group may have masked any anisomycin-induced reconsolidation deficit. Interestingly there was no effect of anisomycin on extinction memory consolidation, however, this lack of effect is consistent with other studies showing a failure of anisomycin to impair extinction (Lattal & Abel, 2001; Lattal et al., 2006). There was a reliable main effect of drug group during the test, with the anisomycin groups showing less freezing behavior overall compared to the vehicle groups ($F(1,43) = 10.96$, $p= .002$). The anisomycin-treated mice froze less than did the vehicle-treated mice in the Acquisition group ($t(21)= 3.6$ $p =.002$), but there was no simple effect of drug within the Retrieval group. This indicates that while the 12 min non-reinforced exposure on reversal was successful in matching freezing in the Retrieval and Acquisition groups, the retrieval trial also resulted in significant extinction, which may have masked any reconsolidation deficit that might have existed. Thus, in the next experiments, the retrieval trial was shortened in an attempt to prevent significant extinction in the vehicle-treated reconsolidation group.

Experiment 2: Short Non-reinforced Exposure (systemic). To engage memory reactivation while minimizing behavioral extinction, the Day 2 CTX- retrieval trial was

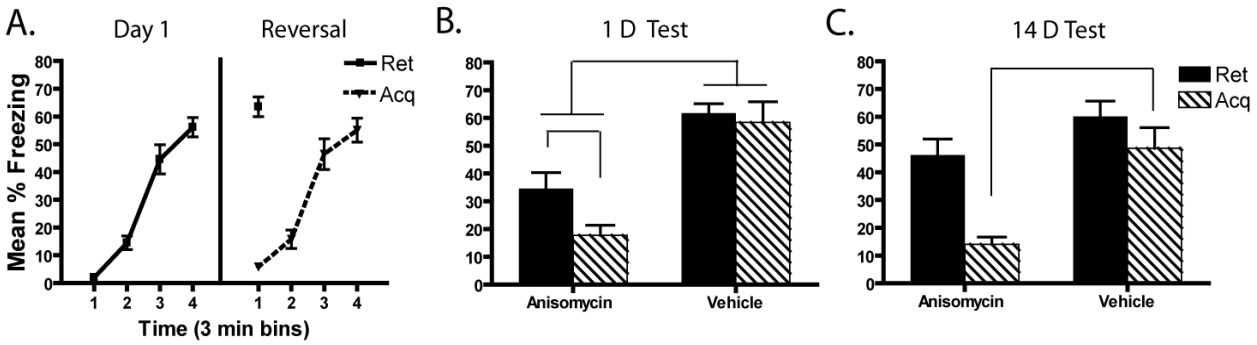


Figure 7. Systemic anisomycin-induced consolidation deficits are initially larger in magnitude and more persistent (to at least 14 days) compared to reconsolidation deficits. A) During the last 3 min of the reversal fear conditioning session (CTX+), the Acquisition group showed similar levels of freezing to the 3-min retrieval session in the Retrieval group. Immediately and 2 h after this reversal day mice received either anisomycin or vehicle treatment. B) When tested 1 d later, the vehicle-treated mice from the Retrieval and Acquisition groups showed equivalent levels of freezing. Mice treated with anisomycin after initial memory acquisition or retrieval showed freezing deficits compared to their vehicle controls. This deficit was larger in those mice treated after acquistion than it was in the mice treated after retrieval. C) The anisomycin-induced deficit only persisted in the Acquisition group; freezing recovered to vehicle levels in the Retrieval group during the 14 d test.

shortened to 3 min. To equate the non-reinforced context experiences in the Acquisition group, their Day 1 CTX- pre-exposure also was shortened to 3 min. Figure 7A shows that these treatments resulted in fear acquisition throughout the Day 1 session in the Retrieval group (main effect of Time Block; $F(1.5, 35.1) = 95.3$ $p < .001$) with little freezing in the pre-exposed Acquisition group. During Reversal (Day 2) freezing in the Acquisition group increased throughout the CTX+ session ($F(1.9, 45.5) = 1.5$ $p < .001$) to the same level of performance during the 3-min CTX- memory retrieval session (no difference between the last 3-min block of freezing in the Acquisition group relative to the Retrieval group's 3-min retrieval session; $p > .05$). On both Days 1 and 2, the mice to be treated with anisomycin or vehicle did not differ in freezing levels within each Context Treatment (all ps >.05). This procedure was therefore successful in matching the asymptotic behavioral performance, context exposure, and shock exposure of the Retrieval and Acquisition groups prior to amnesic treatment and testing.

During the 1-D test (Figure 7B), freezing was lower in the anisomycin-treated mice compared to vehicle-treated mice in both the Acquisition and Retrieval groups. This deficit appeared larger in the Acquisition group compared to the Retrieval group. A significant overall effect of Drug group indicated that anisomycin-treated animals froze less than did the vehicle-treated animals during the 1-D test ($F(1,47) = 39.2$, $p < .002$). Further analysis of this effect revealed that the anisomycin-treated mice froze less than did vehicle-treated mice in both the Acquisition ($t(24) = 4.9$ $p < .001$) and Retrieval groups ($t(23) = 3.9$, $p = .001$). There was no reliable interaction between Drug and Context Treatment ($F(1,47) = 1.6$, $p = .21$). Despite a lack of significant interactions, the *a priori* hypothesis that the effects of anisomycin on post-acquisition and -retrieval processes was tested. This revealed that while the vehicle-treated mice did not differ, the anisomycin-treated Retrieval group froze significantly more than did the anisomycin-treated Acquisition group ($t(24) = 2.5$ $p = .02$). Together, these results indicate that the consolidation deficit was larger than was the reconsolidation deficit during the 1-D test.

During the 14-D test (Figure 7C), freezing was lower in the anisomycin-treated mice compared to vehicle-treated mice in the Acquistion group, but not in the Retrieval group. There was a reliable main effect of Drug ($F(1,47) = 18.7$ $p < .001$) and Context Treatment ($F(1,47) = 15.0$, $p < .001$) during the 14-d test, with no reliable interaction ($F(1,47) = 3.4$, $p = .07$). The anisomycin- and vehicle-treated mice in the Retrieval group did not differ ($p = .1$), but the anisomycin-treated Acquisition group continued to show less freezing than their vehicle controls ($t(24) = 4.5$ $p < .001$). The 14-Day test therefore suggests that the consolidation deficit persisted to 14 days, but the reconsolidation deficit did not.

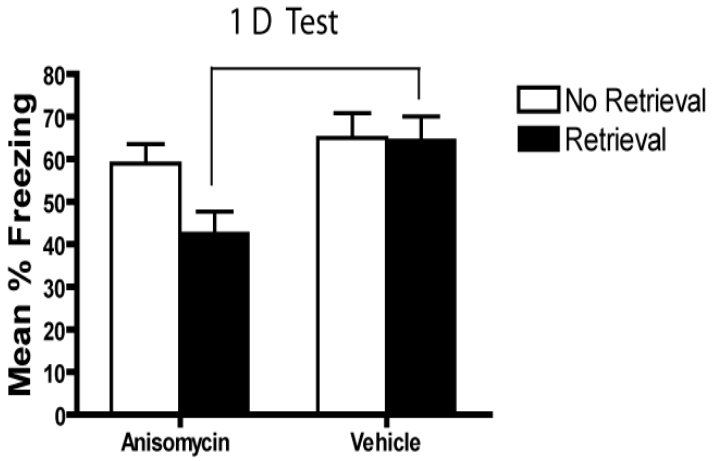


Figure 8. Anisomycin treatment has no effect when it does not follow a memory retrieval trial. One day after contextual fear conditioning mice either received a 3 min re-exposure to the conditioning CTX (Retrieval) or were moved to the experimental procedure room and handled (No Retrieval). Mice were then injected with either anisomycin or vehicle immediately and 2 hours later. When tested 1 d later, mice treated with anisomycin following memory retrieval had a significant impairment while those that did not experience a retrieval trial showed no such deficit.

Experiment 3. No Retrieval (systemic). To evaluate whether the reconsolidation deficit observed in Experiment 2 (Figure 7) was actually due to disruption in memory processes during retrieval and not some non-specific action of anisomycin, the effects of anisomycin were compared in groups that did or did not receive the retrieval trial on Day 2. Visual inspection of Figure 8 suggests that the animals who received anisomycin after retrieval showed a significant freezing impairment when tested 1 D later. In contrast, the group that received anisomycin treatment without memory reactivation showed no deficit. A main effect of Drug ($F(1, 33)= 7.5$, $p = .01$) in the absence of a significant Context Treatment X Drug interaction ($F(1,33) = 1.5$ $p = .24$) confirmed that there was less freezing in the anisomycin-treated animals compared to controls. This drug effect was due to the freezing deficit in the Retrieval Group ($t(17) = 2.7$, $p = .014$) as the No Retrieval Group showed no such deficits ($p =.29$).

Experiment 4a. Short Non-reinforced Exposure (intrahippocampal) with 1-d and 14-d tests. Figure 9 shows cannula tip placement for all mice and a representative brain slice from a cannulated mouse. Mice that received conditioning on Day 1

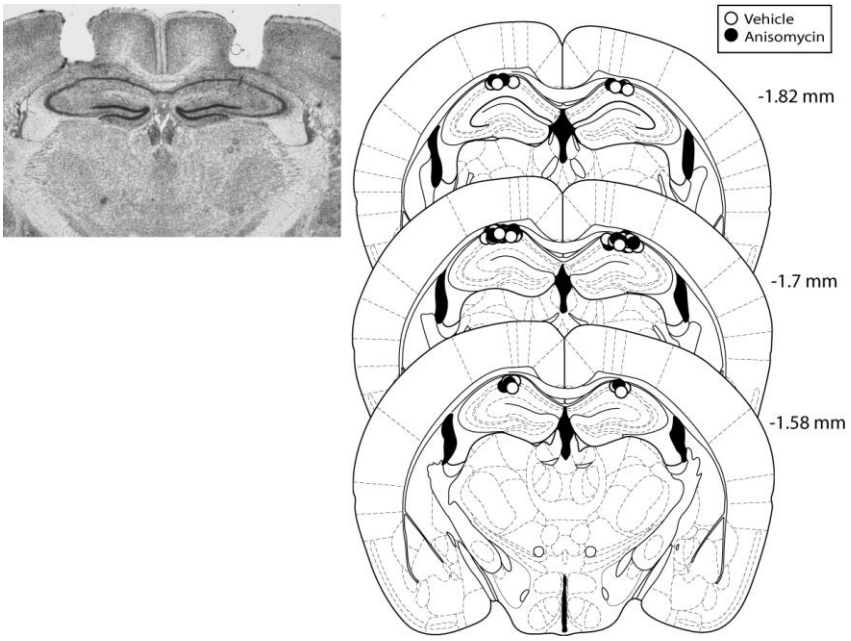


Figure 9. Hippocampal cannula placements. Photomicrograph depicting a representative sample of an accurate bilateral dorsal hippocampal injector placement. The coronal slice was stained with cresyl violet and was taken from -1.7mm bregma. Actual cannula tract placements in each animal used in Experiment 4are in the left panel.

(Retrieval groups) increased freezing over the course of the session ($F(3,68.9) = 83.5$ $p<.001$), whereas mice that received pre-exposure showed very low levels of freezing (Figure 10A). On Day 2 (Reversal), the Acquisition groups increased freezing during the course of the session ($F(3,56.2) = 91$, $p < .001$). Freezing during the last 3 min of the conditioning session in the Acquisition group was not statistically different from freezing during the 3-min retrieval session in the Retrieval group ($p=0.054$). On both Days 1 and 2, there was no effect of post-reversal drug treatment on freezing (all ps >.05). These results are consistent with those reported in Experiment 2; the Acquisition and Retrieval groups were brought to similar levels of freezing prior to amnestic treatment and testing.

Figure 10B shows that when anisomycin was injected directly into the hippocampus, anisomycin-treated mice in both the Acquisition and Retrieval groups showed similar levels of freezing that were lower than vehicle-treated mice during the Test 1 d following reversal. During the 1-day test, anisomycin-treated mice froze less than did vehicle-treated mice, as revealed by a significant main effect of Drug ($F(1, 30)$

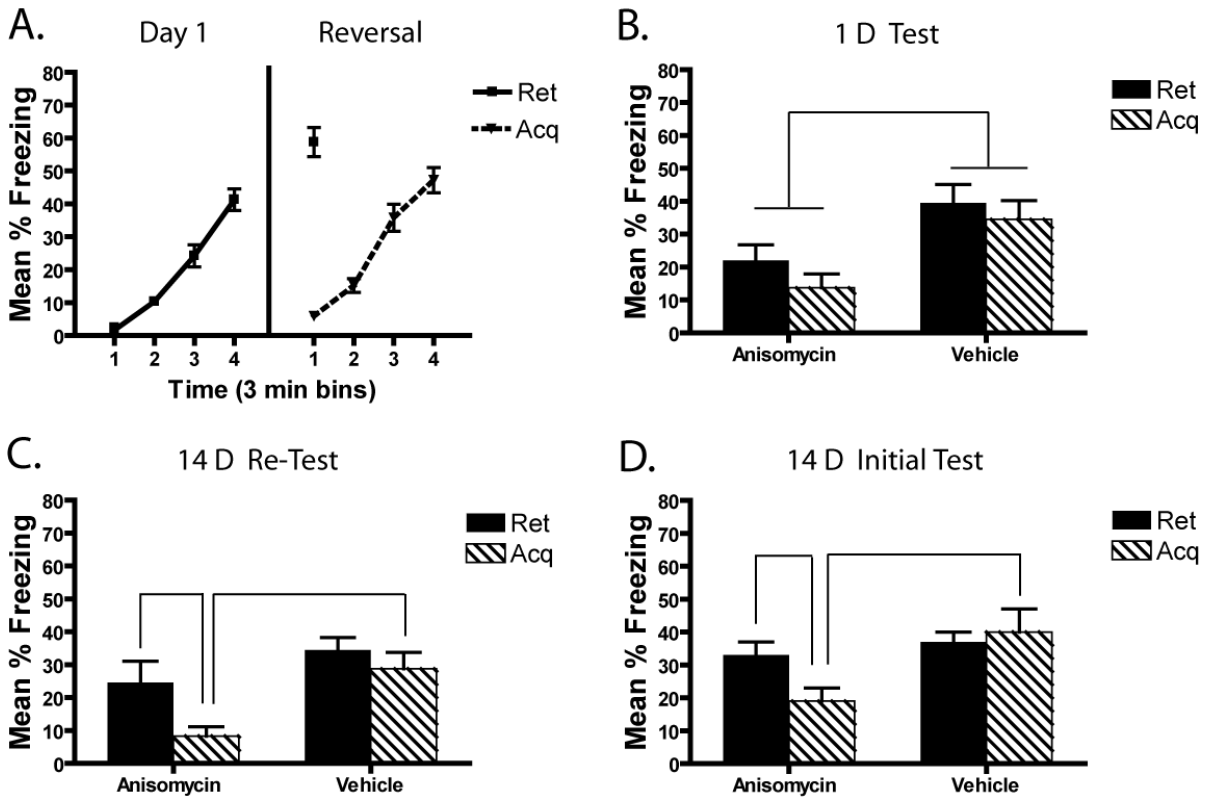


Figure 10. Intrahippocampal anisomycin treatment matches the initial magnitude of consolidation and reconsolidation deficits, but only the consolidation deficit persists. A) During the last 3 min of reversal fear conditioning (CTX+), the Acquisition group freezing level did not differ from the Retrieval group during their 3 min retrieval session. Immediately after this Reversal day mice received bilateral hippocampal infusions of either anisomycin infusions or vehicle. B) When tested 1 d later, the vehicle-treated mice from the Retrieval and Acquisition groups showed equivalent levels of freezing. The anisomycin-treated mice in the Retrieval and Acquisition groups showed significant freezing deficits that were equal in magnitude. C) When re-tested 14 days later only the Acquisition group continued to show a deficit. D) When the 14 day test was the first test following the Reversal Day, the freezing deficit also was persistent only in the Acquisition group.

= 14.8, p =.001) and no reliable Drug X Context Treatment interaction (F(1, 30) = .11 p =.75). This effect was driven by less freezing in the anisomycin-treated relative to the vehicle-treated mice in both the Acquistion (t(15) = 3.0, p= .009) and Retrieval groups (t(15) = 2.5 p = .026), suggesting the presence of consolidation and reconsolidation deficits. The two anisomycin-treated groups did not differ (p=0.19) during the first test.

The second test 14 days later (Figure 10C) continued to reveal an anisomycin-induced consolidation deficit, but not a reconsolidation deficit. Statistical analysis of the

14-day test revealed a reliable main effect of both Context Treatment ($F(1,30) = 12.2$ $p = .002$) and Drug ($F(1,20)=6.2$, $p = .019$), but no reliable Drug X Context Treatment interaction ($F(1,30) = 1.4$, $p = .25$). There was less freezing in the anisomycin Acquistion group relative to their vehicle ($t(15) = 3.6$ $p = .002$) and relative to the anisomycin-treated Retrieval mice ($t(15)=2.6$ $p = .019$), but the Retrieval drug groups showed no differences in freezing levels ($p = 0.15$). The intrahippocampal results therefore demonstrate that even when the magnitude of the initial deficit is matched, consolidation deficits persist longer than do reconsolidation deficits.

Experiment 4b. Short Non-reinforced Exposure (intrahippocampal) with only 14-d test. To investigate whether the initial 1 D test influences the recovery or persistence of memory deficits on the 14 Day test, we tested a subset of animals only at the 14-d retention interval. As in Experiment 4a, the Acquisition group continued to show a freezing deficit, whereas the reconsolidation deficit was not present (see Figure 10D). During the 14 d Test, there was a reliable main effect of Drug Group ($F(1,29) = 7$, $p = .011$) and no reliable Context Treatment X Drug group interaction ($F(1,29) = 3.4$, $p = .08$). Further analyses confirmed that anisomycin-treated mice in the Acquisition group froze less than did their vehicle controls ($t(13) = 2.6$, $p = .02$), but there was no reliable simple effect of drug within the Retrieval group ($p = 0.46$).

Summary of Major Findings. Studies of recovery sometimes examine the change in performance from one test (e.g., 1 d) to a later test (e.g., 14 d), but this change in behavior across time is not an ideal comparison because performance fluctuates and the first test itself will influence performance on the second (Estes, 1997; Kamin, 1957). Instead, a more direct and appropriate comparison for spontaneous

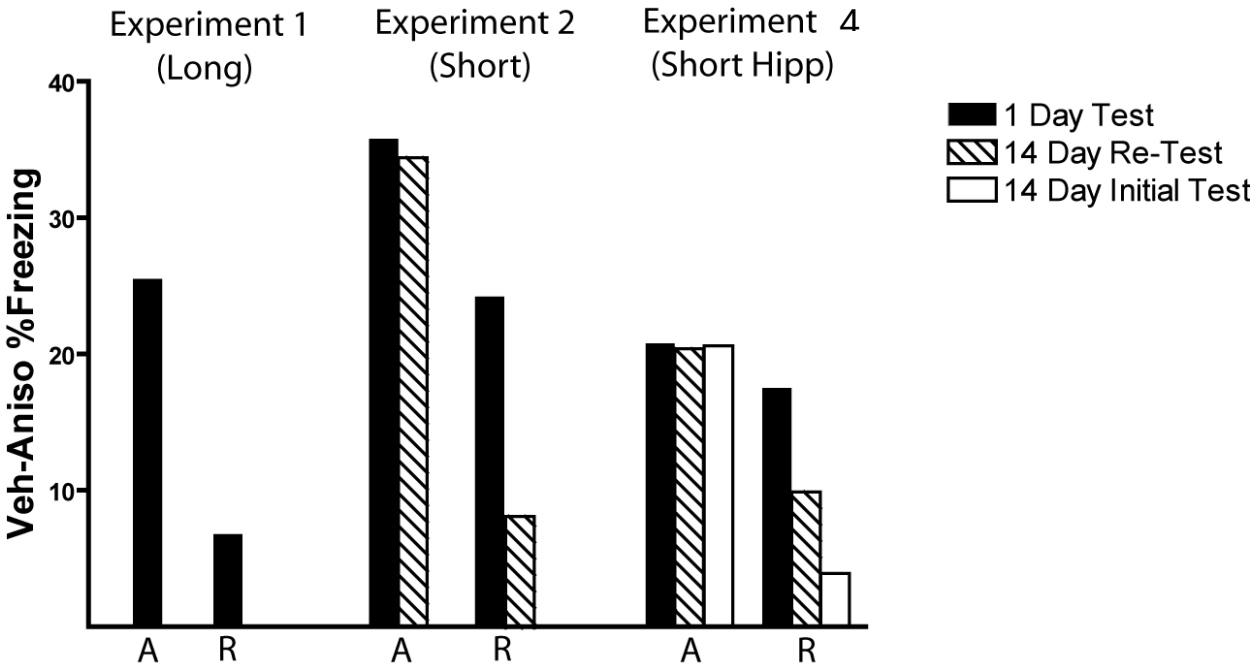


Figure 11. Summary of major experimental findings with memory deficits on test days expressed as the difference between vehicle and anisomycin treated animals within Retrieval (R) and Acquisition (A) groups. In Experiment 1, a robust consolidation deficit was seen whereas no reconsolidation deficit was observed. When the memory retrieval and pre-exposure sessions were shortened to 3 min, a reliable consolidation and reconsolidation deficit was produced by systemic anisomycin treatment (Experiment 2). The Consolidation deficit was initially larger than the reconsolidation deficit (1 d test) and persisted to 14 days while the reconsolidation deficit did not (14 d test). Following intrahippocampal injections in Experiment 5, the size of the reconsolidation and consolidation deficits was matched statistically (1 d test). Only the reconsolidation deficit persisted to the 14 d test. Removing the confound of repeated testing yielded an even less persistent reconsolidation deficit (open bars). In Experiments 2 and 5, the consolidation deficit remains flat from the first to the second tests, whereas the reconsolidation deficit decreases in size across tests.

recovery is to examine differences between two groups at a common test point [see (R. A. Rescorla, 1988, 2004) for further discussion]. As such, Figure 11 summarizes the findings from these experiments as a difference score between mean freezing levels in vehicle- and anisomycin-treated mice during the 1-d and 14-d tests. While the statistical analyses of these data are discussed above, visualizing the data in this way facilitates general comparisons of the size and persistence of memory deficits as well as the influence of repeated testing on the persistence of such memory deficits. In Experiments 1 and 2, the size of the consolidation deficit was larger compared to the

reconsolidation deficit during the first 1-d test, but in Experiment 4 the size of this deficit was matched. What is striking from this figure is that the consolidation impairment (defined as the difference between the means of anisomycin- and vehicle-treated groups) is flat across tests; there was no evidence that this deficit decreased with time. However, in both Experiments 2 and 4, the reconsolidation deficit got smaller during the 14-d test, even when the initial deficit was statistically identical in magnitude to the consolidation deficit. This recovery effect in the Retrieval group was particularly pronounced when the 14-d test was not confounded by the 1-d test (open bars in Figure 11). This meta-analysis revealed that indeed anisomycin had a large and more persistent effect post-acquisition than post-retrieval and that repeated testing was capable of masking the recovery of behavior following anisomycin treatment.

Discussion

The main finding from these experiments is that when overall experience and levels of performance prior to injections were matched, anisomycin-induced post-acquisition (e.g., consolidation) deficits were larger and more persistent than were post-retrieval (e.g., reconsolidation) deficits. All mice received the same amount of reinforced and nonreinforced context exposure with anisomycin injections delivered immediately after the second exposure. This treatment also resulted in vehicle-treated mice in both acquisition and retrieval groups showing identical levels of freezing during the test sessions, which makes direct comparisons more meaningful between anisomycin-treated groups. This comparison revealed larger behavioral deficits in freezing when systemic anisomycin followed a reinforced session (acquisition) compared to when it followed a nonreinforced session (retrieval). The size of the

deficits was matched statistically with intrahippocampal injections, but only the acquistion deficit persisted during a retention test.

One reason that has been offered for the differences in persistence between consolidation and reconsolidation deficits is that these deficits may differ in initial size (e.g., Duvarci & Nader, 2004). If reconsolidation deficits are smaller compared to consolidation deficits, the size of the deficit may contribute to the persistence, particularly when repeated testing is used. As can be seen in Figure 11, which summarizes the results of our experiments, there was no evidence for recovery in the Acquisition groups in any experiment – performance in anisomycin-treated groups relative to vehicle-treated groups did not change from the 1-d to the 14-d test. In the Retrieval groups, however, the difference between anisomycin and vehicle groups decreased from the 1-d to the 14-d test, even when the 1-d difference was statistically identical to that of the consolidation group (Experiment 4). This recovery effect was more pronounced when the 14-d test was not confounded by the 1-d test. Together, these findings demonstrate that a major factor influencing whether behavior recovered with time was whether the initial impairment followed the formation of context-shock memories or the retrieval of those memories, independent of the size of the deficit.

Clearly, there are different theoretical interpretations for results like ours. The important points are that multiple processes need to be considered when analyzing differences in recovery and that learning processes can be effectively enhanced through depressions in different memory systems. This is especially true with drugs such as anisomycin that have a variety of biological effects not limited to protein synthesis inhibition (Canal, Chang, & Gold, 2007). The value in the experience matching

approach used here is that it allows for direct comparisons to be made between different memory processes at behavioral and molecular levels that are not confounded by overall experience with the stimulus or levels of performance before or after the memory deficit. Several recent experiments have demonstrated important cellular and molecular differences between post-acquisition and post-retrieval processes (J. L. Lee et al., 2004; Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006; von Hertzen & Giese, 2005). Applying this behavioral experience matching approach to both molecular and theoretical investigations would therefore facilitate direct comparisons between the molecular and behavioral consequences of these memory processes.

Are acquisition and retrieval processes equally susceptible to pharmacological *enhancements*? (Experiments 5-7)

NOTE: *This study was previously published (Stafford et al., 2012). See acknowledgements for contributions of each author.*

In the following experiments, we investigate the ability of the HDAC inhibitor sodium butyrate (NaB) to produce lasting enhancements in memory following initial learning or retrieval under different conditioning (strong or weak), retrieval, and administration protocols (pre-session systemic and post-session systemic and intra-hippocampal). Because of the critical importance of matching learning experiences when comparing drug effects on fear conditioning and extinction (Lattal & Stafford, 2008; R. A. Rescorla, 2002), different groups received equal total exposure to the context and shocks surrounding NaB administration.

Methods

Subjects

A total of 328 male C57BL/6 mice (Jackson Laboratory; Bar Harbor, ME (eight-twelve weeks) were housed under identical conditions to Experimental Series 1.

Cannulations

The bilateral hippocampal cannulation technique followed Experimental Series 1, Experiment 4.

Injections

Systemic. Sodium butyrate (Millipore, Billerica, MA) was delivered at 1.2 g/kg in1X phosphate buffered saline as vehicle.

Intracranial. Mice received bilateral intrahippocampal injections or unilateral NaB (55 mM) or vehicle (sterile saline) under identical conditions used Experimental Series 1, Experiment 4.

Procedure

Fear Conditioning. Mice received 0.35 mA footshocks in a chamber equipped with behavioral monitoring equipment (Context, CTX; described in Experiment 1).

Habituation. Mice were habituated to handling and injection procedures as in Experiments 1-4.

Matching Approach. To compare NaB effects on conditioning and extinction, groups were matched for total exposure to the context and shocks surrounding NaB administration (Experiments 5, 6, and 7A), using a matching approach identical to that reported above (See Figure 5).

Experiment 5: Pre-session systemic injections with a strong conditioning protocol . The habituation, apparatus, drug injection and general methods used in this experiment are described above. Fifteen min prior to the Day 2 reversal session, mice

were injected with either 1.2 g/kg NaB or vehicle to maximally increase acetylation during the critical memory formation time period (15 min to 1 hour post-learning, Bourtchouladze et al., 1998; Schroeder, Lin, Crusio, & Akbarian, 2007). Mice were assigned to groups that matched levels of Day 1 freezing. Mice were tested 1D and re-tested 14 D following Day 2. A separate group of mice was tested 14 D after Day 2 in the absence of the 1 D test (14 D Initial test).

Experiment 6: Post-session systemic injections

A) Strong conditioning. Methods were identical to Experiment 5, except injections occurred immediately after the Day 2 reversal session to avoid effects of NaB on freezing during that session while isolating effects of NaB on memory consolidation.

B) Weak conditioning. Methods were identical to those used in Experiment 6A except a single 0.35 mA shock was used during conditioning to evaluate whether NaB would enhance consolidation of a weaker contextual fear memory.

Experiment 7. Post-session intrahippocampal injections.

A) Strong Conditioning. Acquisition and Retrieval treatments were identical to those used in Experiment 6. NaB and vehicle infusions were made directly into the hippocampus to evaluate the involvement of the hippocampus in driving NaB mediated memory enhancements. Testing was conducted as above except only the Retrieval group was run in the 14 Day Initial test group as the only persistent effect was seen in the Retrieval in all prior experiments.

B) *Weak conditioning*. Methods were identical to those used in Experiment 7A except a single .35 mA shock was used during conditioning to evaluate whether NaB would enhance consolidation of a weaker contextual fear memory.

C) *Delayed microinfusions*. To ensure that the behavioral effects of NaB on extinction were due to its effects on extinction memory consolidation and not a non-specific effect, intrahippocampal injections were administered 4 hours following a 3 min retrieval session (Abel & Kandel).

Data Analysis

Freezing was measured and evaluated, with appropriate statistics in an identical fashion to the anisomycin studies above (Experiments 1-4).

Results

Experiment 5: Pre-session systemic injections with a strong conditioning protocol. In this and all subsequent experiments, there was very little freezing (<5%) during Day 1, before shocks were delivered (data not shown). During Reversal, the Retrieval groups showed high levels of freezing independent of drug treatment, whereas in the Acquisition groups, NaB-treated mice froze more than vehicle-treated mice (Figure 12). This was confirmed by a lack of significant Drug X Conditioning Order interaction ($p>.05$) between and significant and a main effect of Drug [$F(1,38)=6.39$, $p=0.016$]. The Drug effect was largely driven by higher freezing in the NaB Acquisition group [$t(19)=2.95$, $p=0.008$] and lack of significant difference between the Retrieval groups [$p>0.3$]. This was not due to NaB having non-specific effects on locomotion, response to the shock (Appendix 1) or anxiogenic effects of NaB (Kumar et al., 2005; Peters, Dieppa-Perea, Melendez, & Quirk, 2011). Thus, the increased freezing may be a non-specific action of the drug during conditioning or a pre-existing difference in baseline levels of freezing between NaB and Veh treated mice.

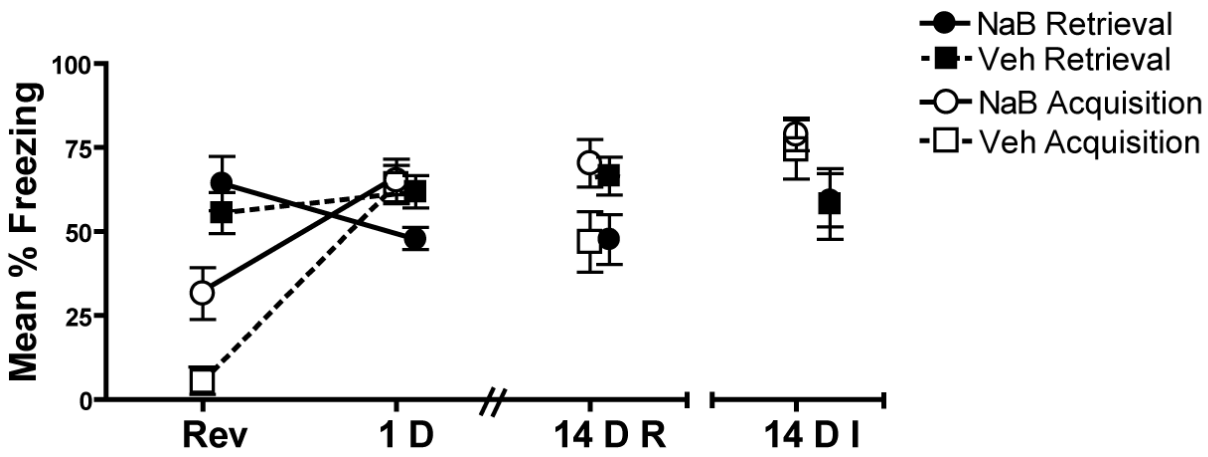


Figure 12. Pre-Retrieval NaB injections induced persistent extinction enhancements to 14 D in the presence of repeated testing. Mice in the Acquisition group who received NaB injections prior to the 2 context-shock pairings froze significantly more than Veh on Reversal. When injections preceded memory retrieval, Veh and NaB treated mice did not differ in performance on Reversal. When tested 1D later, NaB treated mice in the Retrieval group showed a significant decrement in freezing relative to vehicle treated controls indicative of enhanced extinction. This effect persisted when mice were re-tested 14D later (14DR) but not when the 14 D test was the initial test (14 DI). No reliable difference between NaB and Veh treated mice was seen on any test in the Acquisition Group.

NaB delivered prior to retrieval decreased freezing during the 1D and 14D re-test and, when delivered prior to the conditioning session, increased freezing during the 14D re-test. There was no interaction between or main effect of Drug or Conditioning Order (all ps>.05). During Test 1, NaB treated mice in the Retrieval group froze significantly less than vehicle-treated mice [$t(19)=2.35$, $p=0.03$], but there was no drug effect in the Acquisition group.

During the 14D re-test, mice in the Acquisition group that received NaB displayed greater freezing. NaB generated a persistent decrease in freezing within the Retrieval groups (Figure 12, 14DR). A significant Drug Treatment X Conditioning Order interaction confirmed this effect [$F(1,39)=6.78$, $p=0.013$]. This persistent extinction enhancement was not observed in mice that received the 14D test as their first test after NaB treatment [Figure 12, 14DI; ps >0 .1]. Thus, long- term enhancements were

revealed by repeated testing, but were not present when the 14D test was not preceded

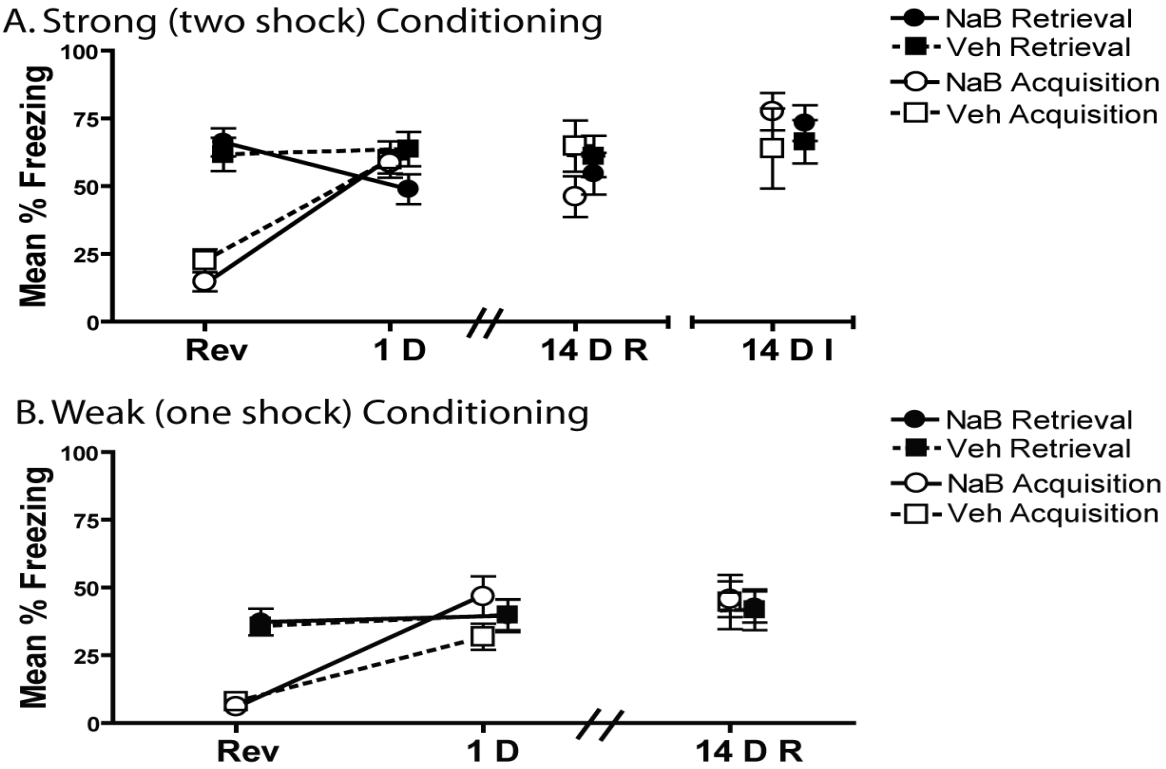


Figure 13. Post-Extinction Systemic NaB injections cause an initial extinction enhancement. A) Mice received two shocks on CTX+ days. During Reversal, NaB and Veh treated mice did not differ within Acquisition or Retrieval groups. Mice injected with NaB immediately after retrieval showed an extinction enhancement relative to vehicles when tested 1D later (1D). This effect was not persistent to 14D when the mice were re-tested (14DR) or when the 14 D test was the initial test (14DI). B) To examine whether NaB would enhance a weak CTX-shock memory all mice received only one shock on CTX+ days. Freezing levels were identical within Acquisition and Retrieval Drug groups. Mice injected with NaB immediately following weak conditioning (1 CTX-shock pairing) did not differ from Veh treated mice when tested 1D later. No difference between Drug groups was observed when mice were tested again 14D (14DR) later or when the 14 d test was the first test (14DI). There was no difference between NaB and Veh treated mice on any test in the Extinction group.

by a 1D test.

Experiment 6. Post-session systemic injections

A) Strong conditioning protocol. During the first test, the NaB treated mice showed a significant extinction enhancement (Figure 13A). There was no interaction or main effect of Conditioning Order or Drug Treatment [ps >0.1], or significant difference between drug and vehicle treated Retrieval mice. However, a difference score between

Reversal and the 1D test revealed that NaB treated extinction mice showed a significantly greater decrease in freezing from Reversal to Test than did the vehicle treated mice [t(22)=2.53 p=0.019]. No difference between groups was observed on the 14 D test when this test was either a retest or initial test [all ps >0.1].

B) *Weak conditioning protocol.* During Test 1, only the NaB treated Acquistion group showed an increase in freezing from Reversal day (Figure 13B), but this was not reliably different from vehicle treated mice [p=0.09]. Examination of the first minute of the 1D test showed that the NaB Acquisition mice [M=60.1, SE = 7.8] froze significantly more than the Veh treated Acquistion mice [M=38.8, SE=5.9]. This finding was confirmed by a significant Drug X Conditioning Order interaction (F(1,50)=4.2), p=.05) and difference between NaB and Veh treated Acquisition mice (p=.04). Together these results suggesting that NaB caused a modest conditioning memory enhancement under very sensitive temporal parameters.

During the first 3 min of either the 14 D Re-Test or 14 D Initial Test, no differences were observed between any groups [all ps>0.1]. Although the weaker conditioning protocol produced lower levels of freezing compared to the stronger, 2-shock protocol, NaB still had no significant effect on a newly formed fear memory suggesting that these null effects were not due to a behavioral ceiling.

Experiment 7. Post-session intrahippocampal infusions

A) Strong conditioning protocol. Intrahippocampal injection of NaB induced a persistent extinction enhancement (Figure 14A). A Conditioning Order X Drug interaction [F(1,34)=4.75, p=0.04] during the 1 D test confirmed the initial extinction enhancement as the NaB treated Retrieval mice froze significantly less than vehicle-

treated mice [t(15) = 4.1, p=0.001] while there was no difference between Drug groups

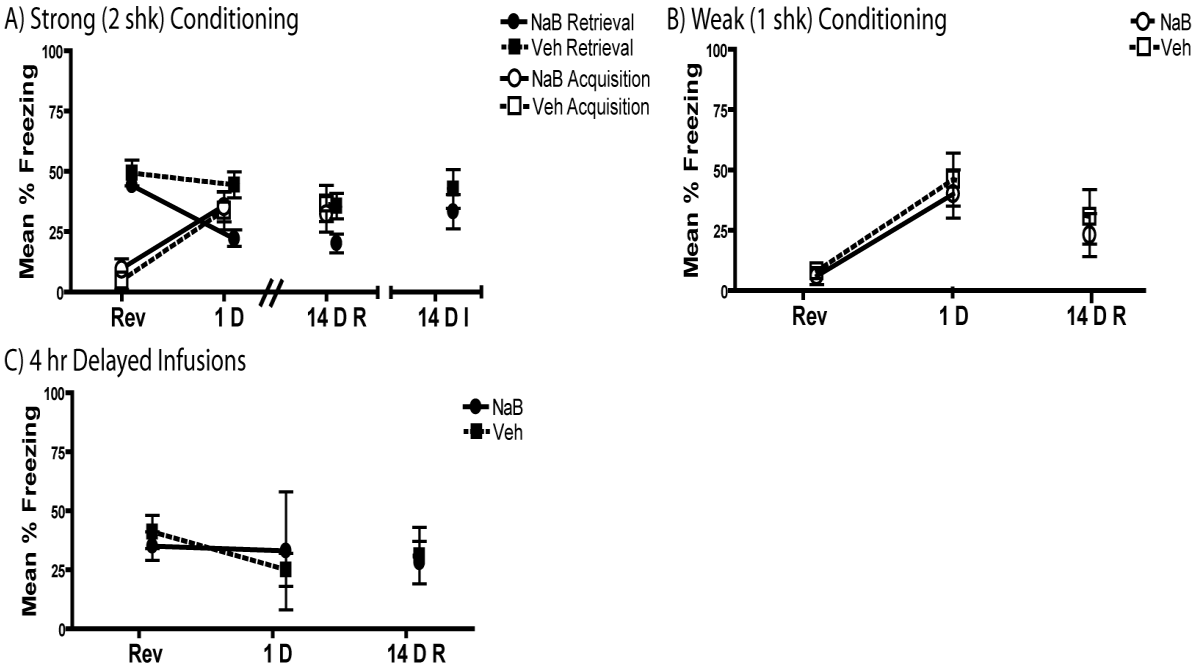


Figure 14. Intra-hippocampal NaB injections selectively cause persistent extinction enhancements. A) During Reversal, mice receiving either NaB or Veh following conditioning (NaB and Veh groups) or retrieval did not differ. Mice receiving post-extinction NaB injections showed a significant extinction enhancement relative to controls when test 1D later. This extinction enhancement persisted to 14 D only when mice were re-tested (14DR). No conditioning enhancement was seen on any test. B) NaB infused into the hippocampus immediately after weak (1 shk) conditioning did not result in a significant difference in freezing from Veh when test 1 and 14 D later. C) Infusion of NaB into the hippocampus 4 hr following 3 min extinction had no significant effect on freezing relative to Veh when tested 1D later mice when tested 1D later. No difference between Drug groups was observed when mice were tested again 14D (14DR) later or when the 14 d test was the first test (14DI). There was no difference between NaB and Veh treated mice on any test in the Extinction group.

in the Acquisition Group [p>0.05].

When re-tested 14 days later, this effect persisted with NaB treated mice in the Retrieval group freezing less than vehicle treated controls. While there was no interaction or main effect of Conditioning Order and Drug Group [all p>0.1] there was significantly less freezing in the NaB treated Retrieval mice [t(15)=2.65, p=0.018]. In contrast, when the 14 D test was the initial test, this effect was not present [p=0.69]. No

differences were observed in the Acquisition group on any of the 14D retention tests [p>0.05]. Effects of hippocampal NaB are consistent with the results of Experiment 5 which showed a persistent extinction enhancement only when mice were repeatedly tested.

B) Weak conditioning protocol. Post-session intra-hippocampal NaB did not enhance a newly formed weak contextual fear memory when tested either 1D or 14D following acquisition [Figure 14B; ps >0.6].

C) Delayed intrahippocampal injections. When injections were administered 4 h after retrieval, there was no difference between NaB and vehicle treated animals [Figure 14D, t(10)=.37 p=0.72] indicating that these effects were due to NaB's effects on extinction and not some nonspecific drug effect.

Summary of Behavioral Findings

Table 1 shows the p-values for NaB induced enhancement in expression of the conditioning or extinction memory relative to Veh. NaB was able to induce persistent

	Initial Test (1 D)		Persistence (14 D†)	
	Conditioning	Extinction	Conditioning	Extinction
Pre-session systemic (Exp 1)	p>.05	***p =.03***	***.013****	***.013***
Post-session systemic (Exp 2)				
Strong Conditioning	p>.05	***p=.019***	p>.05	p>.05
Weak Conditioning	p>.05	p>.05	p>.05	p>.05
Post-Session Intrahippocampal (Exp 3)				
Strong Conditioning	p>.05	***p=.001***	p>.05	***p=.018***
Weak Conditioning	p>.05	NT	p>.05	NT

Table 1. Summary of effects of NaB following acquisition and retrieval. P-values for differences between NaB and vehicle during the 1 and 14D tests. NaB induced persistent extinction enhancements under a range of conditioning (pre-session systemic injections, post-session intracranial infusions), whereas enhancements in the conditioning memory were more restricted across preparations. †All persistent extinction enhancements were only found if the mice were repeatedly tested (14D Re-Test) and not if the 14 D test was the initial test (14 D Initial Test). *The NaB induced freezing enhancement at 14 D is confounded by the pre-conditioning NaB injections which resulted in freezing greater than Veh at baseline. NT signifies "not tested" as certain tests were not required in all conditions.

extinction enhancements under a range of conditions (pre-session systemic injections, post-session intracranial infusions) while enhancements of the acquisition memory were much more restricted across procedures.

Discussion

The key finding from these experiments was that the HDAC inhibitor sodium butyrate promoted long-term behavioral extinction when administered either systemically or directly into the hippocampus. The other important finding was that HDAC inhibitor-induced extinction enhancements occurred under a wider range of conditions (pre- or post-session systemic injections, post-session intra-hippocampal injections) compared to the initial conditioning effects. Our findings suggest that NaB can enhance memories that form during initial learning and extinction, but the long-term effects of this drug are sensitive to several behavioral parameters, including conditioning/extinction strength and testing conditions. These findings add to other recent demonstrations of the limitations of HDAC inhibitor-induced memory enhancements (Bredy & Barad, 2008; Miller, Campbell, & Sweatt, 2008; Reolon et al., 2011).

From a theoretical perspective it is possible that the learning that occurs during extinction is simply more vulnerable to pharmacological manipulations compared to initial conditioning. Some studies have demonstrated that the rate of extinction may be slower compared to the rate of initial acquisition (R. A. Rescorla, 2002). A slower rate of learning during extinction would theoretically leave more room for enhancements than would the relatively fast rate of learning associated with initial acquisition. In turn,

this would translate into smaller drug-induced enhancements in initial consolidation. Indeed, recent studies indicate that the memory enhancing effects of NaB are critically dependent on the strength of learning and the subsequent memory. For example, NaB transforms a weak or impaired memory into a robust long-lasting memory (Fischer, Sananbenesi, Wang, Dobbin, & Tsai, 2007; Stefanko, Barrett, Ly, Reolon, & Wood, 2009). These studies are also consistent with our finding that the ability of NaB to enhance an extinction memory depends on the strength of the extinction memory.

From a preclinical perspective, our findings suggests that HDAC inhibitors like NaB may be more likely to enhance fear memory extinction than exacerbate future fear expression when paired with exposure-based therapies. Together, our findings demonstrate promise for the future clinical application of HDAC inhibitors, like NaB to exposure-based therapies.

Do *behavioral manipulations* of post-acquisition and retrieval processes produce similar behavioral effects? (Experiments 8-10)

NOTE: *This study is currently being submitted for publication. See acknowledgements for author contributions.*

The first two experimental series compare pharmacological manipulations of acquisition and retrieval. However this Experimental series takes a behavioral approach to ask whether extinction during post-acquisition and retrieval periods of lability re-writes the original memory, as a safe, extinction memory (Chan, Leung, Westbrook, & McNally, 2010; Monfils, Cowansage, Klann, & Ledoux, 2009; Schiller, Levy, Niv, LeDoux, & Phelps, 2008).

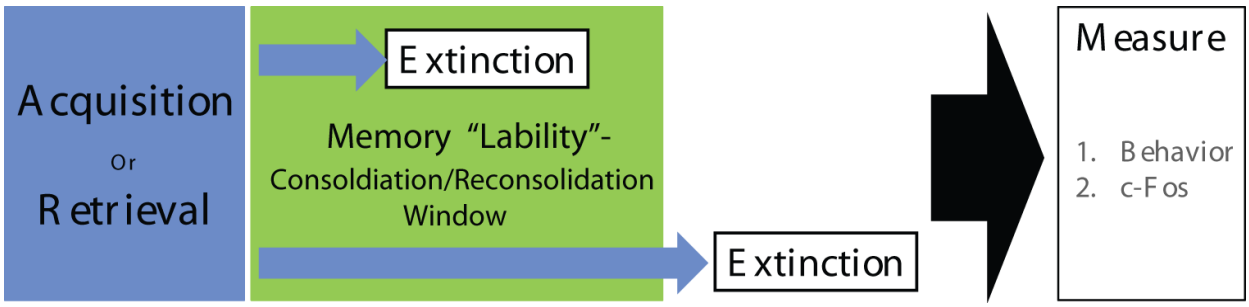


Figure 15. General Experimental Design. Contextual fear extinction occurred inside or outside of previously demonstrated post-acquisition or post-retrieval periods of memory vulnerability. Behavior was tested and c-Fos immunohistochemistry was examined following extinction.

Here, I explicitly test this hypothesis by placing extinction directly within post-acquisition and post-retrieval windows that have previously shown to be shown to be within periods of memory lability (Bourtchouladze et al., 1998; Monfils et al., 2009; Stafford & Lattal, 2009). I compared the effects of immediate extinction to extinction placed outside this period of memory lability (Figure 15). Second, I sought to understand some of the brain regions that may be responsible for differences in immediate and delayed extinction by examining changes in the product of the immediate early gene, c-Fos induced by these treatments. I found that across a variety of behavioral conditions, extinction soon after acquisition or retrieval prevents the retention of extinction during subsequent test sessions. These effects correlated with differential responses in the prelimbic cortex and in the subpopulations of the amygdala (e.g., ITC, BA, CeA).

Materials and Methods

Subjects. A total of 282 male 8-12 week old C57Bl/6J were cared for as in Experiments 1-7.

General Procedure. General habituation, and fear conditioning procedures were identical to those used in Experiment 2. To ensure the reliability of our findings, each experiment was conducted in at least two replications.

Exp 8. Effect of post-acquisition delay on extinction of fear expression and sensitivity to extinction. A) Effect of acquisition to extinction interval. Mice were removed from the chambers and received extinction (12 min context exposure) either immediately 0 hr, 1 hr, 4hr or 24 hrs following acquisition. Previous research has found that pharmacological manipulations can affect memory consolidation at one or more of these intervals (Bourtchouladze et al., 1998; Monfils et al., 2009; Stafford & Lattal, 2009). Each group was returned to the home cage and transported into a small procedure room between acquisition and extinction (the 0 hr group was in the home cage for ~30-60s; enough time to clean the chambers and reset the computer program). The first test (12 min context exposure) was conducted immediately after extinction (EXT-Imm) and the next test was conducted 1D later. Immediately following the 1 D test mice were again tested for 12 min (1 D-Imm). *B) Sensitivity of immediate or delayed extinction to extinction strength.* To examine the effect of sensitivity of extinction strength, mice received either 3 min or 24 min extinction immediately (0 h) or 24 h (delayed) following acquisition. Testing occurred 1 D later.

Exp 9. Effect of post-retrieval delay on extinction on fear expression and sensitivity to extinction. A) Effect of retrieval to extinction interval. All subjects received memory retrieval (3 min context exposure) 1 D following acquisition. Previous studies from our lab indicate that this retrieval duration followed by a protein synthesis inhibitor causes impairments in performance (Stafford & Lattal, 2009). Following retrieval, mice

were removed from the chambers, placed back into their homecage and brought into an adjoining procedure room. They then received extinction (12 min context exposure) either immediately 0 hr, 1 hr, 4hr or 24 hr following retrieval. Testing (12 min context exposure) occurred 1 D and 14 D following extinction. *B) Sensitivity of immediate or delayed extinction to extinction strength.* Mice received either 3 min or 24 min extinction immediately (0 hr) or 24 h following retrieval and were tested at 1 and 14D following extinction.

C) Immediate extinction deficit depends on memory retrieval. One day following acquisition, one group of mice received retrieval immediately followed by 24 min extinction (0 hr), a second group received 24 min extinction in the absence of retrieval (0hr-No Ret), a third group received retrieval followed 24 h later by 24 min extinction (24 hr) and a fourth group received 24 min extinction 24 hr following the retrieval day in the absence of retrieval (0hr-No Ret). Testing (12 min context exposure) occurred 1 D and 14 D following extinction.

Exp 10. Effect of Extinction Recency on c-Fos expression . Behavioral procedures were similar to Exp 8B and Exp 9B. Briefly, mice were separated into groups that received 24 min extinction either immediately or 24hr following either fear acquisition or retrieval. A separate group underwent acquisition with no extinction immediately on the same day the other groups received extinction to control for the presence of shock prior to sacrifice. Immunohistochemistry (IHC) for the immediate early gene c-Fos in select brain regions was examined in each group. Briefly, mice were sacrificed 30 min following extinction with brains subsequently fixed in formaldehyde and cryoprotected in sucrose. The No Ext group was sacrificed 1hr and

24 min following acquisition to equate the interval between behavior and sacrifice in the other groups. After sectioning 30 um slices on a cryostat, IHC was performed on representative slices standardized to the same bregma level across brain regions (Stafford et al., 2012). Briefly, .3% hydrogen peroxide was used to inhibit endogenous peroxide activity with blocking performed in 3% goat serum. Slices were later incubated with antibody recognizing c-Fos (1: 2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The Vecstatin ABC kit (Vector Laboratory, Burlingame, CA) and metal enhanced DAB kit (Pierce, Rockford, IL) was used for immunoreaction detection. Three slices per brain region were analyzed in all experiments with data (cell counts; see below) averaged per animal across slices.

Distinction of ITC Subpopulations. We distinguished c-Fos^{+} neurons in the main nucleus (In) vs. the medial paracapsular (ImP) using methods identical to those used by Busti et al., 2011 to evaluate Zif268 expression in these nuclei as well as other studies of ITC populations (Palomares-Castillo et al., 2012; Whittle et al., 2010). Briefly, these nuclei were distinguished from other amygdalar nucluei as their activation patterns cluster together and the background staining produced by our IHC technique allows for distinction from other nuclei (Appendix 4).

In/ImP proportion analysis. We calculated the proportion of c-Fos+ In neurons to ImP neurons as; 1) Shock prior to c-Fos quantification led to significant activation of each of the individual ITC populations and obscured the main effect of extinction recency on these populations. To overcome this activation that masked these effects, we calculated a pr oportion score which normalized these substantially elevated activation patterns, and 2) Reciprocal connections between the Imp and In clusters

functionally inhibit one another. Indeed, it is the relative output of these nuclei that influences excitability within the CeL and CeM ultimately impacting fear expression (i.e., ImP active during fear states and IN active exclusively during low fear/extinction). Current theoretical accounts require that the relative effects of these distinct ITC masses are considered when evaluating their functional output. We therefore calculated the proportion of active IN to ImP neurons to account for their combined role in mitigating the effect of extinction recency in a way consistent with current amygdala functional findings (Busti et al., 2011; Manko et al., 2011; Palomares-Castillo et al., 2012; Pare & Duvarci, 2012; Whittle et al., 2010).

Data Analysis. Fear was evaluated and analyzed as in Experiment 1. Quantification of c-Fos was performed by counting c-Fos positive nuclei in each brain region by an experimenter blinded to experimental conditions. Group differences were analyzed with and ANOVA. Between subjects ANOVA with Extinction Recency (time between extinction/retrieval and extinction) and other factors such as Pre-extinction Conditions (acquisition vs. retrieval) Extinction Duration, Retrieval Conditions were performed where appropriate. Simple planned post-hoc comparisons were tested using a Fisher's LSD. For all statistical tests the α was set ≤ .05.

Results

Exp 8. Effect of post-acquisition delay on extinction of fear expression and sensitivity to extinction. A) Effect of acquisition to extinction interval. Both groups receiving a short delay between acquisition and extinction (0 hr and 1 hr) showed significantly more spontaneous recovery than those receiving a long delay between acquisition and extinction (4 and 24 hr) during the first 3 min of the 1 D Test (effect of

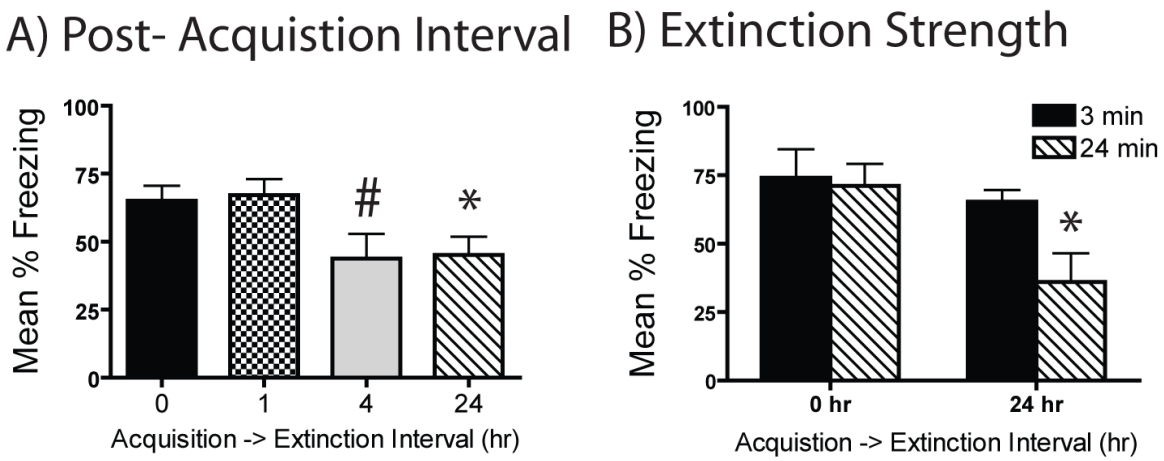


Figure 16. Extinction immediately following acquisition impairs extinction and decreases sensitivity to extinction strength. (A) Extinction at short post-acquisition delays caused less robust response loss than did longer delays (24 hr) when tested 1 D after extinction. **(B)** Mice receiving extinction immediately following acquisition were insensitive to extinction strength (3 vs. 24 min) whereas mice receiving extinction at a 24 hour delay showed more robust response loss in the presence of strong extinction. * $P < 0.05$, # $P < 0.05$.

Extinction Recency; Fig. 16A). A main effect of Extinction Recency [$F(3,48)=2.94$, $p=.048$] followed by post-hoc confirmed this as both the 24 hr and 4 hr groups froze less than either the 1 hr or 0 hr (all $p<.05$). Importantly, mice were brought to similar levels of performance prior to the 1D test and following the 1 D Test (Ext-Imm and 1 D-Imm, all $p > .1$; Appendix 2A). A follow-up experiment showed that even a very remote (50D) interval between acquisition and extinction produced more robust behavioral extinction than extinction immediately following acquisition (Appendix 2C). This study shows that long delays between acquisition and extinction, particularly those outside the hypothesized "consolidation window" attenuate spontaneous recovery more strongly than do short acquisition-extinction intervals. *B) Sensitivity of immediate or delayed extinction to extinction strength.* The robust attenuation of spontaneous recovery with a 24 hr acquisition-extinction delay extended to mice receiving strong extinction (24 min) but not weak extinction (3 min) 24 hr following acquisition (Fig. 16B). Prior to test mice

were brought to common levels of performance by the end of the extinction sessions (Appendix 2B; all ps >.1). There was no Extinction Recency X Extinction Strength interaction (p>.05). However, a main effect of Extinction Recency during the 3 min of Test 1[(F(1,32) = 5.7, p =.024] followed by post hoc indicated that the mice receiving strong extinction (24 min) at a 24hr delay froze significantly less than those receiving extinction immediately following acquisition (p=.02). This further replicated the basic finding that immediate extinction produces more spontaneous recovery and revealed that mice were insensitive to the duration of the extinction session when extinction occurred immediately after acquisition or retrieval.

Exp 9. Effect of post-retrieval delay on extinction on fear expression and sensitivity to extinction. A) Effect of retrieval to extinction interval. A 24 hr delay between fear memory retrieval and extinction produces more robust and persistent extinction (1 and 14 D test) than do shorter delays (Fig. 17A). A significant main effect of Extinction Recency [F(3,28)=4.1, p=.016] followed by post-hoc analysis revealed that indeed that the 24hr groups indeed froze less than the 0 hr group on the 1D Test [p=.003]. When tested 14D later, a 24hr post-retrieval interval induced significantly less freezing than a 0 hr interval (p=.044). Differences on test were not due to differences in performance prior to testing as all groups showed similar levels of freezing during both retrieval and the last block of extinction (Appendix 3A; all p>.1). *B) Sensitivity of immediate or delayed extinction to extinction strength.* Strong extinction 24 hrs following retrieval produced more robust extinction than a short retrieval-extinction delay (Figure 17B). There was no Extinction Recency X Extinction Strength Interaction (p>.05). However, both Extinction Strength (F(1,28)=5.8, p=.02) and Extinction Recency

(F(1,28)=7.9, p=.01) significant effects on freezing during the first test. This was driven by the 24 hr 24 min group freezing significantly less than groups receiving a 0 hr post

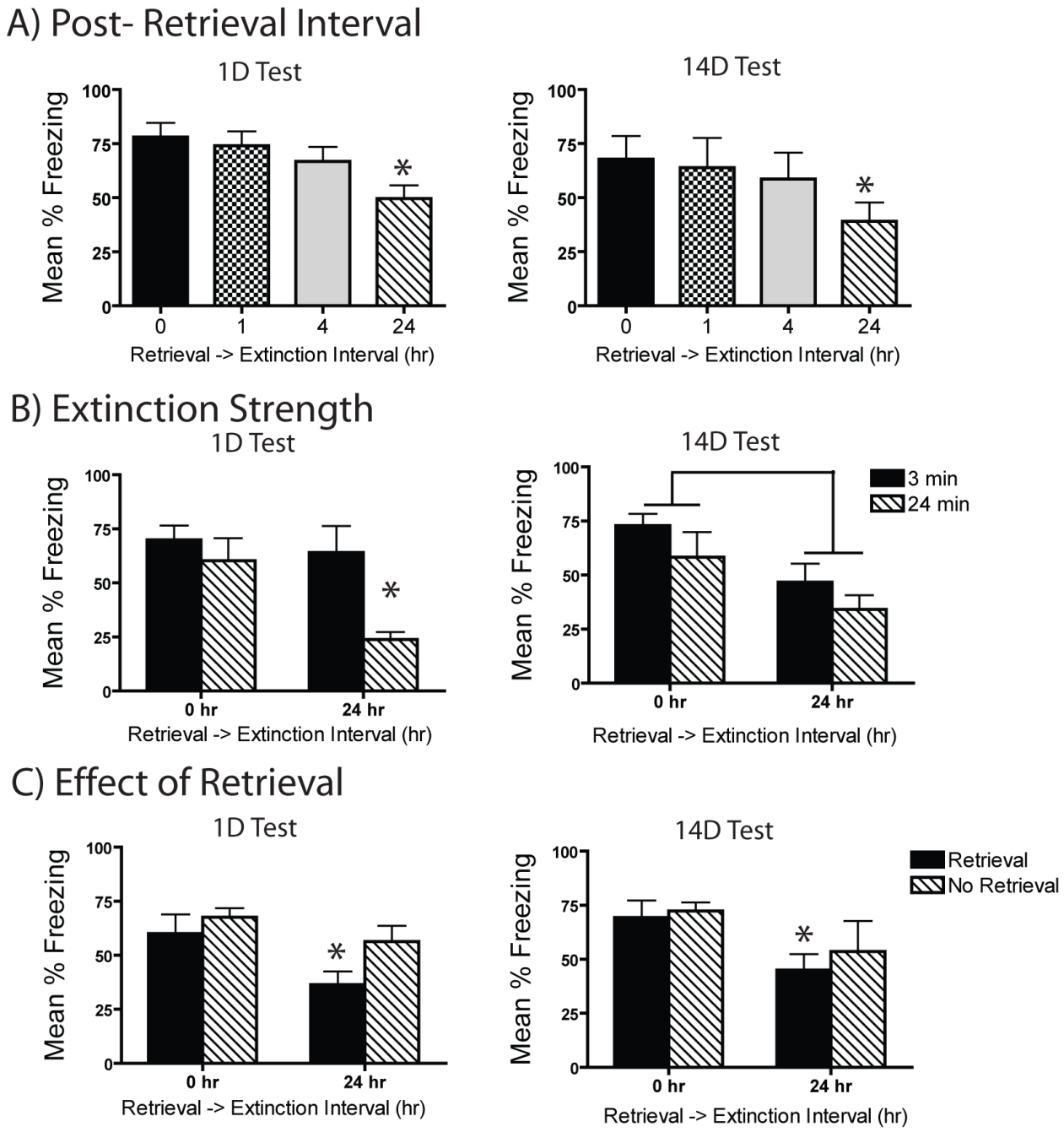


Figure 17. A short retrieval to extinction interval produces lasting impairments in extinction and decreases sensitivity to extinction strength. (A) Extinction 0 hrs post-retrieval caused less robust response loss than did longer delays (24 hr) when tested 1 and 14D after extinction. **(B)** Mice receiving extinction immediately following acquisition were insensitive to extinction strength (3 vs. 24 min) whereas mice receiving extinction at a 24 hour delay showed more robust response loss in the presence of strong extinction. * $P < 0.05$.

extinction delay regardless of extinction duration (all p<.01) or the group receiving 3 min extinction at a 24hr interval (p=.003). A similar effect was seen at the 14D test with a main effect of Recency [F(3,28=3.9, p=.02] and the 24hr 24min extinction group freezing significantly less than the 0hr 3 min group (p=.03) and the 0hr 24min group (p=.05). Like Exp 1 and 2, these results indicate that a short retrieval-extinction interval led to greater spontaneous recovery and decreased sensitivity to extinction strength. *C) Effect of Retrieval* Attenuated spontaneous recovery was only seen at 1 and 14 D tests if retrieval preceded delayed extinction (24 hr) but not if retrieval was absent or if extinction occurred imme diately following extinction (Fig. 17C). On both the 1 D test, there was no Retrieval X Recency interaction (p>.05). There was, however, a main effect of Recency [F(1,22)=6.1, p=.02]. Evaluation of the hypothesis that retrieval was required for the effect of extinction recency showed that extinction 24 hr following retrieval resulted in less freezing than any group that did not receive retrieval prior to extinction (all p<.05) or the group that received extinction immediately following retrieval (p<.03). A similar effect was found at the 14D test with the main effect of Recency Retrieval Conditions [F(3,22)=2.3, p=.014] being driven by extinction 24 hr following retrieval resulting in significantly less freezing than the group receiving extinction in the absence of retrieval (p=.031). A baseline difference at the end of extinction (Appendix 3C; p<.02) may contribute to differences observed during tests. However, the main effect of the interval between retrieval and extinction has been repeatedly replicated without this baseline difference (Exp 9A and 9B) making it unlikely that this difference significantly confounds the effects of this experiment.

Together, these studies provide critical evidence for short delays between retrieval and extinction impair extinction and highlight the importance of retrieval prior to extinction in mitigating these effects.

Exp 10. Effect of Extinction Recency on c-Fos Expression.

mPFC. Immediate extinction following both acquisition and retrieval strongly activates the prelimbic (PrL) cortex (Figure 18; Main effect of Extinction Recency; $F(1,27)=9.7$, $p=.004$). Mice receiving fear conditioning in the absence of extinction did not display elevated PrL c-Fos indicating that this effect was contingent on extinction delay rather than simply activation post-acquisition.

No consistent effect of extinction delay on c-Fos in the IL cortex was seen. A significant interaction between Extinction Recency X Pre-extinction Conditions [$F(1,27) = 6.9$, $p<.014$] followed with post-hoc revealed that the only simple main effect was with the immediate acquisition group showing greater c-Fos expression than the delayed acquisition group ($p=.014$).

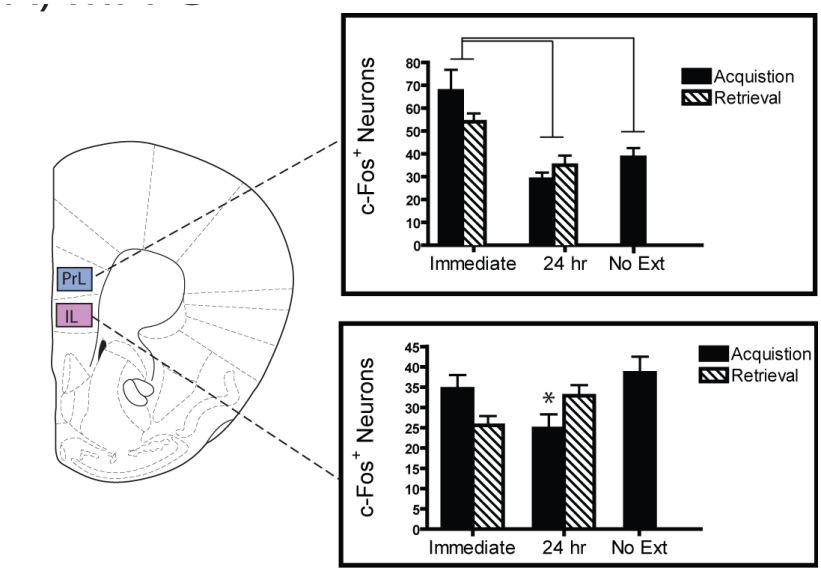


Figure 18. Differential activation of mPFC is associated with the immediate extinction deficit. Immediate (0 hr) extinction induced strong c-Fos expression in the prelimbic (PrL) cortex compared to delayed extinction (24 hr). Activation within the infralimbic (IL) cortex showed variations in sensitivity to extinction recency. Lines between bars denote P < .05 *Acquisition Delay < Acquisition Immediate P<.05.

Amygdala. Activation within amygdala subregions varied across immediate and delayed extinction treatments, with some regions showing greater activation following immediate extinction (e.g., BA, CeA) while others showed the opposite with patterns that depended relative activation in related nuclei e.g., ITC; Figure 19). *Basal Amygdala.* Within the BA, a Extinction Recency X Pre-extinction Conditions interaction [$F(1,26)=5.28$, $p=.03$] with planned comparison follow-up indicates that Immediate Extinction induced greater c-Fos than delayed extinction (24 hr group ; all $p \leq .05$). The interaction was driven by the immediate acquisition group displaying greater activation than the immediate retrieval group ($p=.03$). The No-Ext group differed only from the delayed groups (all $ps<.01$). *Central Nucleus.* Within the central lateral nucleus, a main effect of Extinction Recency [$F(1,27)=11.1$, $p=.003$] revealed that immediate extinction induced greater c-Fos expression than delayed extinction. Interestingly, there was no difference between the No Ext group and the delayed extinction groups, indicating that this effect was not generally due to shock prior to c-Fos quantification. In contrast, a main effect of Recency ($F(1,27)=8.1$, $p=.01$) and Pre-Extinction Conditions ($F(1,27)=6.0$, $p=.02$) within the central medial nucleus (CeM) indicates that while immediate extinction produced greater c-Fos activation, so did being shocked prior to extinction. The main effect of pre-extinction conditions (e.g., fear acquisition vs. retrieval) was likely due to shock immediately prior to extinction as the No Ext group also showed Fos levels above the delayed group ($p<.001$).

Intercalated Cells. Detailed analysis of the intercalated cells of the amygdala revealed that the proportion of main nucleus (In) c-Fos^{+} neurons relative to medial paracapsular (ImP) neurons was greater in mice receiving extinction 24 hr following

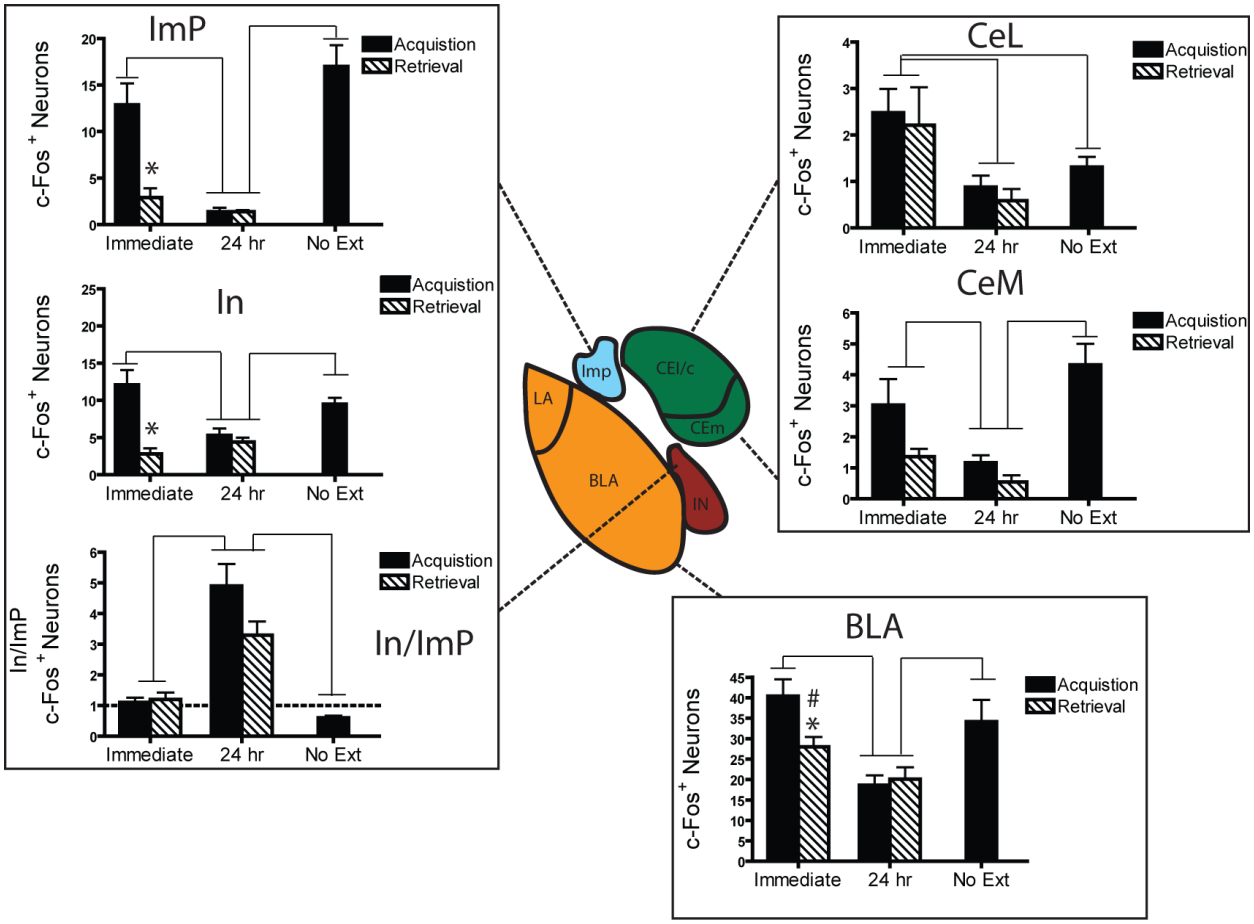


Figure 19. Differential activation of amygdala is associated with the immediate extinction deficit. A 24 hr delay between acquisition or retrieval and extinction induced less c-Fos expression in the central (CeL/CeM) and basolateral amygdala (BLA) than did immediate or No –Extinction. In contrast, delayed extinction induced strong In:ImP activation, the immediate extinction group showed neutral In:ImP activation and the No Extinction group showed an inverse In:ImP ratio. Lines between bars denote P < .05. *Acquisition Delay < Acquisition Immediate P<.05.

acquisition/retrieval than in mice receiving immediate extinction (Figure 19). This was confirmed by a main effect of Extinction Recency ($F(1,26)=40$), $p<.001$ and no significant Extinction Recency X Pre-extinction Conditions interaction. Furthermore, the No Ext groups significantly differed from both the Immediate and Delayed Extinction groups ($p=.024$ and $p<.001$, respectively). When evaluating the subpopulations alone, the consistent finding is that shock strongly activates both intercalated cell masses, with greater c-Fos expression in the immediate acquisition and No EXT groups (all $p<.05$) Combined with the behavioral data, the IHC suggests that the activity of the In relative to ImP may serve to signal contingencies that result in strong extinction.

Together these results indicate that hyperactivity in the PrL and select amygdalar subregions (CeA and BA) may underlie a deficit in extinction while the proportion of In to ImP active neurons signals contingencies are associated with robust extinction.

Discussion

The critical finding from these studies was that under a variety of conditions, an extinction session conducted soon after acquisition or retrieval produced poor behavioral extinction, relative to longer delays. These effects were replicated both within and between experiments with consistent results across experiments. Importantly, control experiments showed that poor extinction produced by immediate extinction critically depended on the interval between behavioral manipulations under common testing conditions. Immunohistochemistry for the IEG c-Fos revealed that hyperactivity in the prelimbic cortex and the relative activity of In to ImP regions, and other amygdala subregions are correlated with the deficits in extinction after short delays.

These results provide new insight into a growing body of literature on the effects of time before extinction following acquisition or retrieval. These findings are consistent with those indicating that extinction shortly after fear learning or fear cue exposure impairs extinction and exacerbates spontaneous recovery, renewal and reinstatement (Chan et al., 2010; Costanzi, Cannas, Saraulli, Rossi-Arnaud, & Cestari; Ishii et al., 2012; R. W. Morris, T. M. Furlong, & R. F. Westbrook, 2005). Other studies have found opposing results – that extinction soon after acquisition (Myers et al., 2006) or retrieval (Monfils et al., 2009) promotes the retention of extinction. Some of these differences may be attributed to how behavior is assessed (e.g., change in behavior from extinction to test as in (Monfils et al., 2009) vs. common test performance as in (Chan et al., 2010), the type of preparation used (e.g., fear-potentiated startle in (Myers et al., 2006) or cued fear conditioning in (Maren & Chang, 2006), species used, or the particular intervals used in the different experiments. However it is important to note that even when species and experimental paradigms are nearly identical there is still discordance between results (e.g, (Chan et al., 2010; Monfils et al., 2009)). Furthermore, other studies have shown similar effects to Monfils, 2009 using contextual fear preparations in mice (Rao-Ruiz et al., 2011) while others, like us fail to replicate these findings in contextual paradigms (Ishii et al., 2012). While important to study, it is unlikely that the discrepant findings in the literature are simply due to cued vs. contextual, immediate vs. 10 min post-retrieval intervals or species differences. Regardless of slight parametric differences all of these studies, including ours, are based on decades of work showing that the immediate post-acquisition and post-retrieval intervals chosen are those that are the most vulnerable to pharmacological and electrophysiological disruption.

Therefore, there is a bigger issue of theoretical importance that overshadows these parametric distinctions—that is, whether extinction during a period of memory vulnerability can effectively re-write or erase the pervious memory.

One of the most striking findings from the IHC data was that high Fos expression within the PrL and certain amygdala subregions (CeA and BLA) was found in mice receiving immediate extinction, suggesting that hyperactivity in these brain regions is associated with poor extinction retention. This is consistent with data suggesting that heightened activity within these brain regions results in increased fear retention and expression (Xue et al., 2012), but see (Kim, Jo, Kim, Kim, & Choi, 2010).

Detailed analysis of the ITCs showed that the proportion of main nucleus (In) c-Fos^{+} neurons relative to medial paracapsular (ImP) neurons was greater in mice receiving extinction 24 hr following acquisition or retrieval than mice receiving immediate extinction (Figure 19). This finding is consistent with studies indicating that activation of these two neuronal populations compete during learning, with the paracapsular cluster (ImP) more active during both acquisition and extinction and the main nucleus (IN) activated preferentially active during fear extinction (Busti et al., 2011; Whittle, et al., 2011; Manko et al., 2011).

These ITC data fit well with our other amygdala data and recent amygdala connectivity studies (reviewed in Palomares-Castillo et al., 2012; Pare & Duvarci, 2012). For example, it has been postulated that the dorsal ImP group drive fear expression states by functionally disinhibiting the CeM via inhibition of CeL “off” neurons while inhibiting the IN. Conversely, when not under inhibitory influence from the ImP, the IN is thought to drive extinction via direct inhibition of the CeM. Therefore, when there is

more ImP activation relative to the IN, the CeM should be more active. In contrast, more relative IN activation should result in less CeM activity. This is the pattern seen in our data, with the relatively greater ImP/greater CeM activation in the group showing the greatest fear (Immediate extinction, No EXT) while relatively greater IN/ less CeM activity was associated with stronger extinction (delayed extinction; Figure 5). Combined with the hyperactivity seen in the PL and BA when extinction was poor (i.e., immediate), this pattern of data suggests a complex network that extends from distinct mPFC populations to these very specific amygdala populations in mitigating the effect of extinction recency. However, more work is needed to determine the specificity of these microcircuits as the cell-type (PKCδ+/-; glutamate/GABAergic receptor sub-types) and the precise afferents/efferents of these amygdala subregions are critically important in regulating fear expression vs. extinction (Ciocchi et al., 2010; Dobi et al., 2012; Haubensak et al., 2010).

There is great promise for therapeutic strategies that place extinction-related therapy at the optimal temporal window following psychological trauma or trauma retrieval. However, the extension of our findings and findings like these into the clinic is premature. While some studies in humans suggest that immediate behavioral intervention (e.g., extinction) may dampen fear and drug-seeking behavior (Schiller et al., 2008; Xue et al., 2012), there are human studies, which indicate that this approach may have little effect or actually enhance memory expression on (Kindt & Soeter, 2011; Potts & Shanks, 2012; Soeter & Kindt, 2011; Wichert, Wolf, & Schwabe, 2011). Particularly problematic for clinical applications are rodent and preclinical studies like ours suggesting that across a variety of conditions, immediate extinction may actually

strengthen the fearful response and underlying neural circuitry (Chan et al., 2010; Kim et al., 2010; R.W. Morris, T.M. Furlong, & R. F. Westbrook, 2005). The idea that memories can be re-written or erased by extinction is exciting from a theoretical and clinical standpoint, but as mentioned, the literature is mixed and suggests that the effect of extinction recency are better explained by associative and non-associative learning accounts. Moreover, from an ethical standpoint, approaches that improve inhibitory learning rather than erase memories may be more beneficial to patients as they leave their memories and experiences intact while giving them the ability to learn from and cope with these powerful life events (Glannon, 2006; Henry, Fishman, & Youngner, 2007). Thus, advancing these basic findings into the clinic requires more work to determine the conditions and neural circuits that strengthen the inhibitory learning that occurs during extinction without necessarily appealing to erasure mechanisms.

General Discussion

A unifying result from these experimental series is that identical pharmacological manipulations produce different behavioral results post-acquisition and post-retrieval. Generally, those manipulations which produce behavioral deficits (e.g., anisomycin) produce larger and more persistent deficits when administered following acquisition that when administered following retrieval (Experiments 1-4). Furthermore, retrieval processes were more vulnerable to HDAC inhibition than were acquisition processes (Experiments 5-7). In contrast to the different effects of pharmacological manipulation of acquisition and retrieval processes, extinction following acquisition or extinction appeared to produce similar behavioral results—that is, heightened fear compared to

delayed extinction. However, even in Experiments 8-10, there were differences in the brain activation patterns induced by acquisition and retrieval (e.g., heightened amygdala activity post-acquisition) and some slight behavioral differences with a 4 hr acquisition to extinction interval producing behavioral deficits while no such difference was observed with a 4 hr retrieval-extinction interval.

It is important to note that Experiments 8-10 differs slightly from the approach taken in Experiments 1-7 in that we did not use a matching approach (e.g., no context pre-exposure in the acquisition group). We did however, ensure that the experimental timeline allowed for testing under common post-acquisition and retrieval conditions. As a result of some of these procedural differences we do not make direct comparisons between post-acquisition and post-retrieval extinction manipulations. We only observe that relative to appropriate within experiment controls, the effects of extinction recency are similar post- acquisition and retrieval. Regardless of any procedural differences between these experiments, there are some important unifying conclusions with broad theoretical implications that stem from this work:

Pharmacological Manipulations

When considering the pharmacological manipulations alone, it is important to note that we use an approach that allows us to examine different memory processes in common assessments that match history and conditions for performance. This approach has been used in several recent experiments examining the amount and persistence of learning during initial acquisition and extinction (e.g., Bradfield & McNally, 2008; Leung & Westbrook, 2008; McNally & Westbrook, 2006; Rescorla, 2002, 2005). Although our procedures are slightly different, the logic of our approach is the same:

match the overall history of the organisms as closely as possible and assess their learning against a common testing baseline. This allows for a much more direct comparison of consolidation and reconsolidation processes because differences in levels of performance and overall experiences are less likely to influence the organism's ability to express the memory on test day (cf. Biedenkapp & Rudy, 2004, 2007; Estes, 1997). It is important to note, however, that although our approach matches the overall experience that the organisms have with the context as well as the level of freezing prior to pharmacological manipulation, it does not match all factors that may influence subsequent performance. Evaluating these factors—such as timing of the injection relative to shock and the quality of the experience during reinforced and nonreinforced exposures—will be important for future studies, especially those using a within-subjects approach to consider.

One consistent finding from Experiments 1-4 and 8-10 is that the persistence of the pharmacological effect appeared to depend on whether the long-term test was the first or second test. Indeed there are a number of experimental factors that may influence the persistence of any effect on memory. One long-appreciated factor is the test-retest problem; the first memory test soon after learning is itself a learning experience that will influence performance on subsequent tests (Estes, 1997; Rescorla, 1988). In an extensive review, Estes (1997) argues that the use of repeated testing in studies of memory has severely limited the conclusions that can be made about memory processes because of the clear effects that initial tests have on subsequent tests. This is especially true in studies with animals that use extinction testing; during the first test, the animal learns that the previously conditioned stimulus is no longer

associated with the unconditioned stimulus. If animals express a memory deficit during this test, they will freeze less compared to their control group, which may result in a greater association between the cues on that test with extinction and the absence of conditioned responding (Estes, 1955; 1997). Thus, when testing occurs again sometime later, any recovery from the initial behavioral deficit may be masked by the new extinction learning that occurred during that first test.

Many studies examining post-acquisition and post-retrieval processes use repeated testing, often including short-term (immediate), long-term (24 hr), and longer term (3+ days) tests in the same subjects. Thus, by the time the longer term test occurs, animals have had multiple extinction tests that are likely to impact the performance on the final test and make any behavioral deficit appear more persistent than it may actually be in the absence of repeated extinction testing. Although studies of memory consolidation have demonstrated that repeated testing may not contribute to the persistence of the memory deficit (e.g., Luttges & McGaugh, 1967), many studies have demonstrated that spontaneous recovery after extinction is weakened by repeated testing (e.g., Pavlov, 1927). Our findings are consistent with this, where in Experimental Series 1, post-acquisition deficits were unaffected by repeated testing, but post-retrieval deficits appeared more persistent when repeated testing was used (see also Lattal & Abel, 2004). In Series 2, we also show that a persistent behavioral deficit, interpreted as an extinction enhancement was only seen under repeated testing conditions.

Behavioral manipulations

The critical theoretical implication of Experimental Series 3 is that extinction during periods when the original fearful memory has been shown to be most labile

according to consolidation and reconsolidation accounts, does not erase or prevent a memory from forming. One would therefore need an account with mechanisms that prevent a labile memory from being updated with the new information that occurs during the extinction trial. Many such theories exist, although they do not necessarily make assumptions about memory lability after conditioning or retrieval. For example, according to sometimes-opponent process (SOP) theory, more of the contextual memory will be in a secondary state of activity during extinction soon after acquisition or retrieval. This will result in less of that contextual representation being retrieved to a primary state of activity during extinction and consequently impair the development of inhibitory learning (see (Brandon et al., 2003; R.W. Morris et al., 2005)). Similar accounts would predict that effects of extinction recency may also be attributable to proactive interference where the first active memory trace (i.e., new fear acquisition or fear retrieval) proactively interferes with the second learning event (i.e., extinction; (Gleitman & Jung, 1963)).

An account that brings together aspects of each of the aforementioned theories is one alluded to by Chan et al., 2010. This account suggests that animals are sensitive to the differences between the fearful or acquisition “state” and the extinction “state” (Capaldi, 1966; Redish, Jensen, Johnson, & Kurth-Nelson, 2007). In the context of these experiments, when extinction closely follows acquisition or retrieval (which strongly engages the original fearful CS-US memory), the subjects have trouble distinguishing between whether the non-reinforced context/CS exposure during extinction still predicts the original fear contingency. This creates an ambiguous state where the mice are forced to maintain the original fear memory as the most salient

association. Therefore, when tested on subsequent days the mice retrieve the original fear memory and express fear at the cost of extinction because of the “ambiguity” of the immediate extinction contingency (fear memory most salient). This means that despite subsequent extinction on test days, they continue to retrieve the fear state on future tests (resulting in spontaneous recovery) as the context/CS configuration best predicts the fear contingencies. This is akin to other theories such as partial reinforcement and related theories which make explicit predictions about behavior when animals aren’t sure if extinction contingencies are in effect.

Remarkably, the ITC data support these theoretical mechanisms as their relative activation represented whether the context was a good, poor, or ambiguous predictor of shock. In the delayed groups, a test ambiguity model suggests that the long delay between acquisition or retrieval and extinction makes the extinction context no longer a good predictor of shock because there is strong temporal discrimination between the “fearful” state engaged by the acquisition/retrieval context and the non-reinforced extinction context. Indeed, the delayed group showed the strongest extinction and the most In:ImP activation. This suggests that when extinction contingencies are in effect, the In cluster (selectively associated with extinction contingencies) is strongly active relative to the more ambiguous ImP cluster. When the contingencies are ambiguous such as in the immediate extinction groups, where the fearful acquisition and retrieval states cannot be temporally discriminated from the extinction state (i.e., the proportion of In:ImP active neurons is 1:1), suggesting a more ambiguous activity state leading to default fear expression. However, when the context is a good predictor of shock (i.e., No Extinction condition), a significantly lower In:ImP ratio is seen. Together, these data

suggest that a high ratio of In:ImP neurons is indicative of contingencies associated with strong extinction retention, a neutral In:Imp ratio is indicative of ambiguous contingencies and a high Imp:In ratio indicates that the context is a reliable predictor of shock. This fits well with our other amygdala data (CeL, CeM, BLA) and mPFC data (discussed above) as well as recent physiological accounts suggesting that that very specific neuronal groups are involved in predictive fear learning and expression (Palomares-Castillo et al., 2012; Pare & Duvarci, 2012).

Conclusion and Implications

During all of these Experimental Series we found little evidence for retrieval inducing a period of memory lability (e.g., re-consolidation) that leaves a memory vulnerable to permanent disruption similar to that induce by initial memory formation (e.g., consolidation). As noted in these reviews and elsewhere, all-or-none reconsolidation accounts make for good theories, in that they make explicit predictions about whether behavioral deficits should reverse with time, but they also likely fail to capture the complexities in the system. Further, the many documented differences between effects following initial learning and retrieval suggest that the label "reconsolidation," which implies a very specific theoretical process, may not accurately characterize the nature of the plasticity that follows these different experiences (e.g, Amaral, et al 2008; Biedenkapp & Rudy, 2004; McGaugh, 2004; Miller & Matzel, 2006).

Our results, and reconsolidation-like results in general, also are consistent with other ways of talking about performance that do not appeal to reconsolidation processes. Although the label "reconsolidation" has become synonymous with "post-

test performance impairment," it is a theoretical term that describes only one of a number of theoretical possibilities. For example, many modern theories of reconsolidation-like effects are variants of stimulus sampling theory (see e.g., Amaral et al. 2008; Riccio, Millin, & Bogart, 2006), which contains memory storage and memory retrieval mechanisms that can account for differential effects of spontaneous recovery (e.g., Bower, 1994; Estes 1955). Theories based in the logic of stimulus sampling need only assume that the components of a stimulus representation that are active during an amnestic treatment will be most vulnerable to the effects of that treatment. After an initial learning experience, a large proportion of the stimulus representation will be active, whereas after retrieval of a learning experience, only a portion of that representation will be active (see Riccio, et al. 2006). Thus, post-retrieval manipulations should affect a more vs. fewer elements of the stimulus, and, as time passes, sampling of the intact stimulus representation should increase. This reasoning was used by Estes to account for extinction, spontaneous recovery, and memory erasure (e.g., Estes, 1955).

Any time a manipulation is administered after a nonreinforced retrieval trial, actions on extinction processes also must be considered. Extinction and reconsolidation are often pitted against each other as distinct processes at the molecular, neural systems and behavioral level (Duvarci & Nader, 2004; Mamiya et al., 2009; Riccio, et al. 2006). Although extinction is often described as new memory formation, many theories of extinction have long appealed to depressions and other modifications to some aspect of the original memory without appealing to reconsolidation mechanisms (reviewed in Delamater, 2004; Lattal, Radulovic, &

Lukowiak, 2006; Lattal, 2007; Myers & Davis, 2007). Thus, an alteration in aspects of the original memory is a perfectly plausible mechanism for extinction to be enhanced. Further, the absence of spontaneous recovery, renewal, and reinstatement have often been used as evidence that extinction processes are not facilitated in studies of reconsolidation, but it is certainly true that any manipulation that should enhance extinction should also weaken spontaneous recovery and associated phenomena (see Davis, Ressler, Rothbaum, & Richardson, 2006). Studies of reconsolidation-like processes in humans is consistent with these extinction accounts, because behavior in these experiments is often eliminated without affecting the subjects' knowledge of the original contingencies (e.g., Kindt, Soeter, & Vervliet, 2009; Norrholm, et al. 2006, 2008). Indeed, this distinction between observed behavior and knowledge of the original association forms the cornerstone of modern thinking about extinction in animals (Rescorla, 2001).

The main implication from this work is that the label "reconsolidation," which implies very specific post-retrieval periods of memory lability does not accurately capture the processes induced by retrieval. This conclusion stems from the consistent finding that across a variety of manipulations and conditions, post-retrieval processes are different than those processes engaged by acquisition. This is indeed problematic for reconsolidation theories which suggest that it describes a process akin to consolidation using similar evidence that was used to form modern consolidation theory. The remaining chapters of my dissertation move away from comparing post-acquisition and retrieval processes to study specific process engaged by retrieval (e.g., extinction) and how those might inform theoretical accounts of retrieval.

Chapter 3: How does retrieval duration affect the molecular and behavioral expression of memory?

Introduction

The act of retrieving a memory has potential to change a memory at a variety of levels including its behavioral expression, molecular signature and neurobiological substrates. As reviewed in the Introduction, retrieval can cause three major behavioral outcomes on subsequent tests; 1) an increase in performance, 2) a decrease in performance or 3) no change in performance. The underlying cause of these different behavioral outcomes is of great theoretical importance and is the subject of some debate in the literature (e.g., extinction and reconsolidation accounts). However, there is a general consensus that when retrieval conditions provide an organism sufficient time to learn that the CS no longer predicts the US, CS evoked responding decreases or extinguishes. In contrast, retrieval conditions that do not allow sufficient time to learn these extinction contingencies may result in little change in behavior or exacerbate CS-induced responding through a variety of processes. The purpose of Experiments 11-13 was to systematically determine the retrieval conditions (i.e., context exposure duration) that lead to increases, decreases and no change in context evoked freezing behavior relative to appropriate controls. My hypothesis was that long retrieval durations would lead to less freezing, short durations would increase freezing and intermediate durations would not change freezing on long-term tests.

The unifying goal of these experiments is to understand the conditions that lead to retrieval-induced behavioral enhancements and decrements.

General Methods

Mice were cared for as in Chapter 2 except that they were divided into cages of 2 to decrease within cage variability in behavior and facilitate experiments requiring short retrieval durations.

Habituation. Mice were carted into the procedure room and remained there for 1h before being handled for 30s. Habituation occurred for 3 days prior to fear conditioning.

Fear Conditioning. Examining the effect of retrieval trial on subsequent behavior required a conditioning procedure that would allow me to detect increases and decreases in behavior following retrieval. Therefore, I sought a conditioning procedure that brought behavior to an intermediate level on test day. Based on a meta-analysis of the different conditioning procedures used in this dissertation, I used a 3 min context exposure combined with 2-.35 mA foot shocks as in Experiment 5.

Behavioral Testing. Testing occurred either 1D or 14D following the retrieval day (Day 2) and consisted of a 12 min non-reinforced CTX exposure.

Data Analysis. Behavior was measured as in Chapter 2 with infrared monitors and scored as % freezing during the session. ANOVAs were used where appropriate and followed by simple planned comparisons (Fishers LSD or t-tests; see text). Alpha was set at .05.

Experiment 11. Effect of Retrieval Duration on Behavior

Experiment 11 evaluates the effect of a variety of retrieval durations (re-exposure to a previously shock paired context) on behavior. These experiments were designed so that groups receiving different retrieval durations could be compared to a control group that did not receive explicit retrieval (e.g., no CTX exposure). One potential confound for

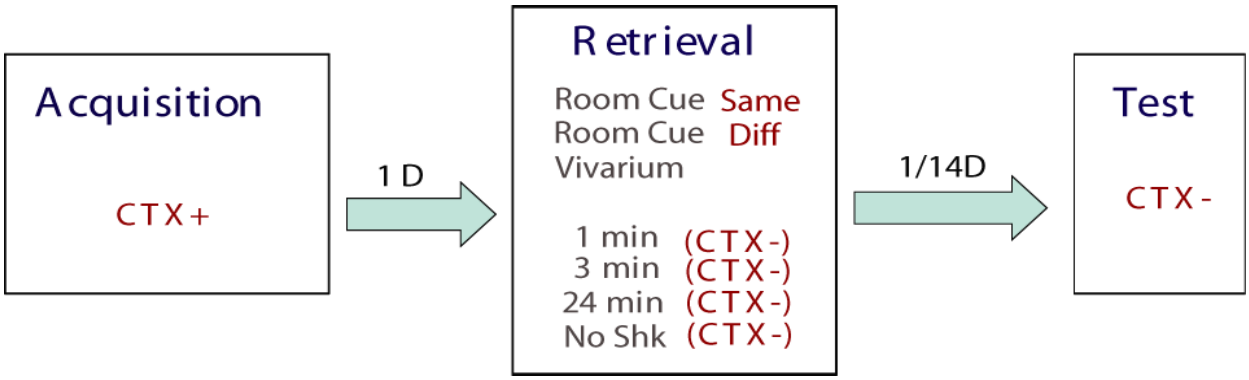


Figure 20. General Experimental Design. Mice were fear conditioned on Day 1 (Acquisition). On Day 3 (Retrieval), were divided into different groups that received various retrieval conditions. All mice were tested 1 and 14D following the Retrieval day.

interpreting effects of retrieval duration relative to a no-retrieval control is that numerous factors such as handling, transport cues and environmental cues may induce retrieval of the original fear memory. Currently, little is known about how control conditions impact performance, thus different no-retrieval control groups were evaluated.

Experimental Design

Mice were habituated and fear conditioned (CTX+) as described above. The No Shock Group received a 3 min nonreinforced context exposure (CTX-) while the other mice were being fear conditioned.

On the Retrieval Day (Day 2) mice were divided into 3 Control Groups and 4 Experimental groups to evaluate the effect of retrieval duration on subsequent behavioral expression (Figure 20 and description below):

1. Control Groups
    a. *Same Room Cue:* These mice were brought back into the outer procedure room using the cart used during conditioning but were never brought into the fear conditioning chambers themselves. They remained in their home cages in the procedure room for 1-2 h before

being handled for 10-20s. Timing of handling was counterbalanced to match the Experimental groups.

b. *Different Room Cue:* These mice were carried from the vivarium in paper bags to a novel room on a different floor. After 1-2 h of being in this novel room they were handled by a separate experimenter than was used for conditioning. These variables were changed to give different transport, room and handling cues on Day 2.

c. *Vivarium:* Mice were left undisturbed in the vivarium during Day 2.

2. Experimental Groups

a. *1 min*: These mice were brought to the outer procedure room using the cart and remained in their home cages in the procedure room for 1-2 h. After this period they were placed in the fear context for 1 min and then placed back into the home cage.

b. *3 min*: These mice were treated identically to the 1 min group except they were placed in the fear context for 3 min and then placed back into the home cage.

c. *24 min*: These mice were treated identically to the 1 min group except they were placed in the fear context for 24 min and then placed back into the home cage.

d. *No shk*: These are the mice that were placed in the conditioning chambers on Day 1 but were not shocked. On Day 2, they were treated identically to the 24 min group.

Mice were tested 1 and 14D after retrieval as described above.

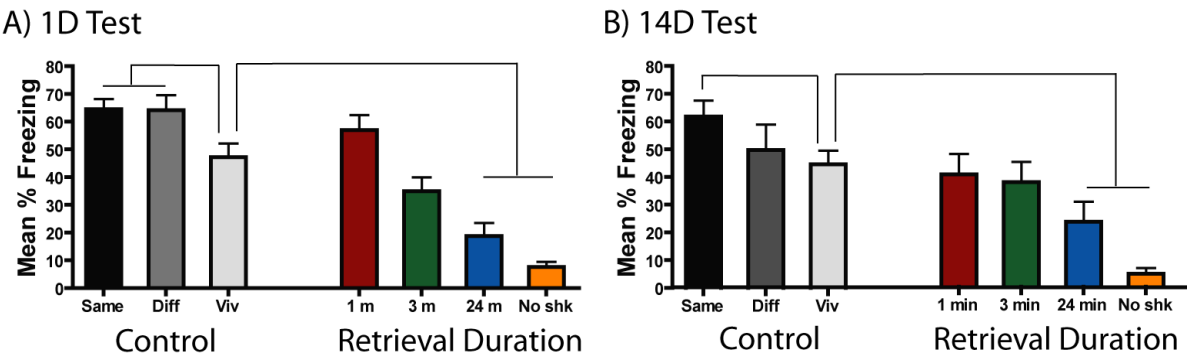


Figure 21. Retrieval conditions and duration modify the long term expression of a fear memory. A) Mice that received a 24 min retrieval trial or were not shocked on acquisition day froze significantly less than the Vivarium Group (Viv) when tested 1D later. Mice in Room Cue Same (Same) and Room Cue Different groups (Diff) froze significantly more than the Vivarium group. **B)** All of the effects observed on Test 1 persisted to 14D except for the difference between the Room Cue Same and Vivarium groups. Bars indicate significant differences.

Results and Discussion

There was no difference between any groups on the conditioning day (data not shown). On Retrieval Day, all groups were matched during the first 1 min block of time, except the No Shk group which displayed freezing lower than any group. The 24 min group decreased freezing to levels of the No Shock group by the end of the retrieval session (data not shown).

When tested 1D after retrieval, retrieval conditions had a significant effect on behavior with longer durations producing the greatest response loss while other conditions (e.g., Room Cue) caused enhanced responding relative to Vivarium controls (Figure 21A). These effects were confirmed by a main effect of Retrieval Condition ($F(6,91)=16.5$, $p<.01$). Planned comparisons were between the Vivarium group and the other retrieval groups as the Vivarium group represent a control that receives no retrieval via handling, room cues or explicit fear CTX re-exposure. The 1 and 3 min groups were not significantly different than the Vivarium group whereas both the 24 min and No Shock Groups showed greatly attenuated freezing relative to Vivarium ($p<.01$

and p<.01, respectively). In contrast both the Room Cue Same and Room Cue Different Groups showed significantly elevated freezing relative to Vivarium (p<.01 and p=.04, respectively).

The 14D retention test (Figure 21B) showed a similar main effect of Retrieval Condition ($F(6,91)= 4.1$), p<.01). Similarly, the 24 and No Shock groups both froze significantly less than Vivarium (p=.02, p<.01). Similarly, the 1 and 3 min groups still showed no difference relative to Vivarium, However, only the Same Room Cue group maintained elevated fear relative to Vivarium (p=.03).

The hypothesis that relatively long retrieval durations would result in persistent response loss (e.g. extinction) was well supported by the 24 min group. In addition, certain retrieval durations (1 and 3 min) indeed produced little change in behavior. However, there was no significant evidence to suggest that relatively short durations would produce behavioral enhancements although the directional effect was present in the 1 min group (i.e., froze more than Viv controls). Surprisingly, mice in both the Room Cue Same and Room Cue Different groups showed significantly elevated freezing when tested 1D later.

The results of the Room Cue groups are interesting as they suggest that direct exposure to the actual context where fear conditioning occurs is not required to change behavior. It is curious that even when procedure room cues, transport cues and handling cues were changed (Room Cue Different), heightened fear was still observed. However, Room Cue Different mice were still handled and had some environmental cues that may have been associated with the previous day's fear conditioning. Any number of these variables may have contributed to the heightened fear. It is important

to note that processes engaged in the Room Cue Same group made the behavioral enhancement more resistant to extinction and/or decay as the Room Cue Different group did not show persistent increases in behavior on the 14D test.

Together, these results show that certain retrieval conditions do lead to response loss (long durations), response gain (room cue conditions) and no change in behavior (intermediate durations). While some of these retrieval-effects came from unexpected sources, they allow for the study of how retrieval can change the behavioral and molecular expression of contextual fear.

Experiment 12. Effect of Retrieval Day Handling on Behavior

Experiment 11 showed that it is possible enhance behavioral performance under certain retrieval conditions. The exact cause of this enhancement is unclear as numerous variables such as environmental cues and handling may have changed behavior through any number of associative (e.g., second-order conditioning) or non-associative processes (fear sensitization). A common variable in both of the groups (Room Cue Same and Different) that displayed elevated freezing was that they were both handled 1D following fear conditioning. Therefore, Experiment 12 examines whether handling 1D following conditioning may be responsible for this freezing enhancement. To examine this possibility, 3 groups that received handling under different conditions on Day 2 including one that replicated the Room Cue Same Group, were compared to a group that was not handled (Vivarium).

Experimental Design

Habituation and Fear Conditioning was identical to Experiment 11. On the Retrieval Day, mice were divided into 3 groups:

1) *Vivarium:* Treated the same as in Experiment 11.
2) *Vivarium Handled:* Treated the same as the Vivarium group except they were handled on the Retrieval day (Day 2) and were immediately returned to the housing rack.
3) *Room Cue Short*: Treated the same as the Room Cue Same group except they remained in the procedure room for only 5 min before handling.
4) *Room Cue Long*: Identical to Room Cue Same group in Experiment 11.

Testing (1 and 14D) occurred as in Experiment 11. Data analysis was performed using planned comparisons as in Experiment 11.

Results and Discussion

The procedural similarities between the Room Cue Short and Room Cue Long groups resulted in nearly identical levels of freezing between these groups on both Test 1 (Long; M=74.8, STErr=3.0, Short; M=69.2, STErr=3.4) and Test 2 (Long; M=60.7, STErr=6.6, Short; M=64.1, STErr=4.3). Therefore, The Room Cue Short and Long groups were pooled on all analyses.

During the first test (1 D) there was a general effect of handling with those mice that were handled (Vivarium Handled and Room Cue) freezing significantly more than all those mice that were not handled on Day 2 (Vivarium; Figure 22A). This was confirmed by a main effect of Retrieval Conditions ($F(1,35)=5.7$, $p=.02$). Planned comparisons showed only a trend towards elevated freezing in the Vivarium Handled ($p=.08$) mice while there was a significant effect in the Room Cue treatment ($p=.05$).

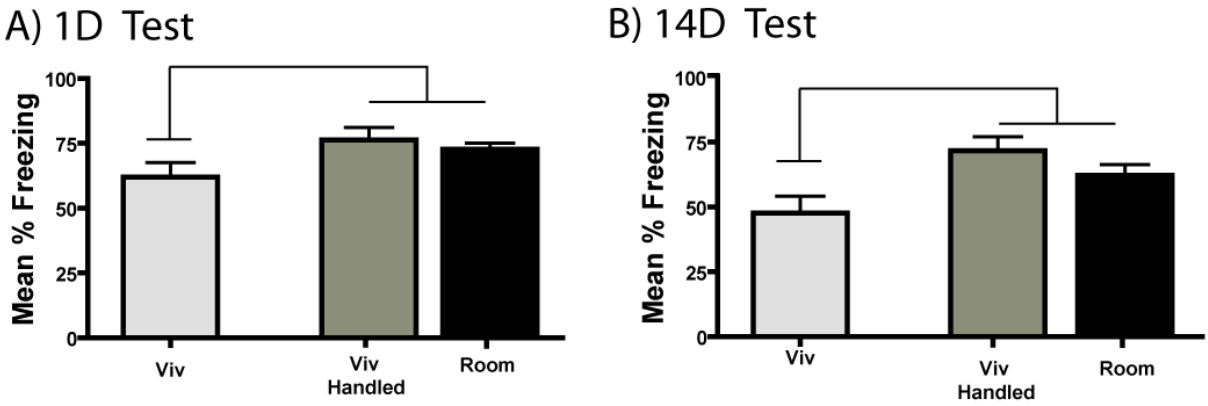


Figure 22. Handling 1D following Acquisition leads to persistent increases in freeing. A and B) Mice that were handled on the retrieval day (both Vivarium Handled and Room Cue groups froze more than Vivarium when tested 1 and 14D later. Bars indicate significant differences.

Test 2 (14 D-Figure 22B), yielded a similar main effect of Retrieval Conditions ($F(1,35)=7.0$, $p=.012$). On this test both the Vivarium Handled and Room Cue groups froze significantly more than Vivarium ($p=.013$ and $p=.05$, respectively).

The critical finding from this experiment was that handling 1D following fear conditioning is capable of enhancing freezing on subsequent tests. The exact mechanism by which handling enhances freezing is unclear, however it is possible that handling functions as a retrieval cue, an aversive unconditioned stimulus (US) or both. For example, handling as a US would mean that the handling US gets paired with whatever retrieval cues are present, thus adding new excitatory value to the other retrieval cues (Hui, Hui, Roozendaal, McGaugh, & Weinberger, 2006). Even though the Vivarium Handled group does not receive any explicit retrieval cues, handling itself may function as a retrieval cue that can potentiate fear responding. Another possibility is that handling enhances fear behavior through some non-associative process such as sensitizing the fear response the next day.

It is important to note that while significant, the effect of handling is not particularly strong and thus may not be solely responsible any behavioral enhancement seen. More studies are required to better understand the mechanisms by which retrieval associated-handling induces behavior change.

Experiment 13. Effect of Short Retrieval Durations on Behavior.

My original hypothesis was that short durations would lead to enhanced fear the next day. While there was evidence that certain conditions (e.g., handling in the absence of fear CTX exposure) can produce these enhancements, there was no significant effect of the short 1 min durations. However, an ordinal difference was present with the 1 min group freezing more than the Vivarium group (Experiment 11). This leaves the possibility that shortening the retrieval trial even more may lead to enhanced fear. Experiment 13 evaluates whether short , 10s and 30s retrieval durations are capable of enhancing freezing behavior on subsequent tests.

Experimental Design

Habituation and Fear Conditioning was identical to Experiment 11. On the Retrieval Day, mice were divided into 3 groups:

1) *Vivarium:* Treated the same as Experiment 11.
2) *10s:* These mice were brought to the outer procedure room using a cart and remained in their home cages in the procedure room for 1h. After this period they were placed in the fear context for 10s and then placed back into the home cage.
3) *30s:* Mice were brought to the outer procedure room using a cart and remained in their home cages in the procedure room for 1h. After this

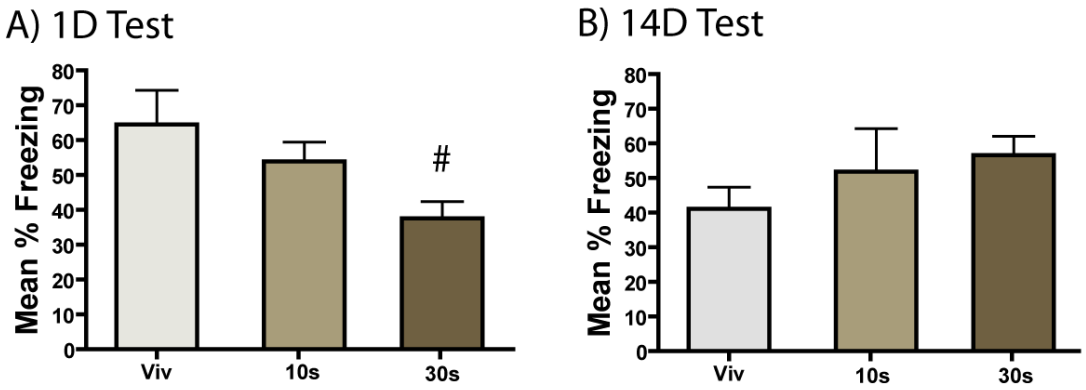


Figure 23. A 30s retrieval duration reduces freezing. A) Following a 30s retrieval trial, mice froze less than the Vivarium group. This effect did not persist to 14 D(**B**).

period they were placed in the fear context for 30s and then placed back into the home cage.

Testing (1 and 14D) occurred as in Experiment 11. Data analysis was performed using planned comparisons as in Experiment 11.

Results

Unexpectedly, we saw that the 30s context re-exposure produced less freezing when tested 1D later (Figure 23). A significant Retrieval Condition ANOVA ($F(2,23)=3.7$, $p=.04$) followed by planned comparisons confirmed that the 30s group indeed froze less than the Vivarium group ($p=.03$) while there was no effect in the 10s group.

The 14D retention test suggests that the effect seen on the 1D test was reversed with the 10s and 30s groups showing more fear than the Vivarium group. However, there was no significant effect of Retrieval Conditions indicating that whatever effect was present in the 30 s group on the 1D test did not survive repeated testing or long-term retention intervals.

Though unexpected, the reduced freezing in the 30 s group is interesting as it is the opposite result of studies suggesting that short durations may powerfully reactivate a memory and potentiate its expression through a variety of processes (Rohrbaugh & Riccio, 1972; Inda et al., 2011). In fact, this empirical result is counter to the predicted result of my original hypothesis. This result is especially curious given that a 1 min trial slightly elevates freezing and a 24 min trial produces robust response loss (Exp. 11). It is important to note that the decreased fear in the 30 s group is not as robust or persistent as the decrement produced by the 24 min group. This likely means that whatever processes produced some response loss in these groups were not the same.

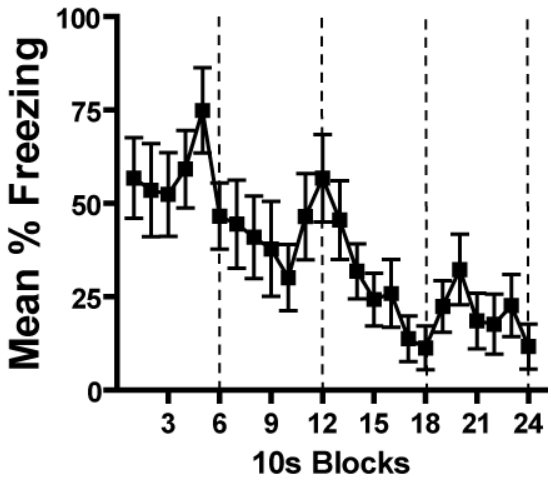


Figure 24. Freezing behavior at 10s resolution during the first 4 min (240s) of retrieval.

Looking at the freezing behavior of the 24 min group from Experiment 11 at a tight, 10 s temporal resolution, yielded some clues as to what may be occurring in the 30 s group (Figure 24). The basic trend is that early in the session (10-30 s) freezing behavior is high but less than it is by about 50 s. After that first minute, freezing shows a gradual decrease, with an increase in freezing at 2 min (where the shock normally occurred during conditioning) and declining to consistent low level during minutes 3 to 4. An explanation of the current results may therefore be that during the first 30 s of the retrieval trial, the mice are exploring the fear context and are removed prior to fully expressing the freezing response. The exploratory behavior may then become

reinforced as they learn during the 30 s retrieval that the shock does not come if they are moving. The same might be expected in the 10 s group, however these mice are put into the chamber and taken out so quickly that they may not have any time to process the fear context thus not being subject to the same contingencies as the 30 s group. Those mice that receive a 1 min retrieval trial express robust freezing behavior by 50 s right before they are removed from the conditioning context and thus have not extinguished and are not subject to the same movement-reinforcing effects of the 30s group. In contrast, the 24 min group had already fully extinguished f breezingy 4 min. This extinction contingency is therefore expressed the next day and over subsequent tests. Other fear retrieval studies have also suggested that a behavior may become reinforced during retrieval as the behavior is predictive of the absence of shock (Rohrbaugh & Riccio, 1972).

General Discussion

The key finding from these experiments was that retrieval is capable of modifying the expression of a memory in a number of ways that is not solely dependent on the duration of the trial itself. For example, we found little evidence that short retrieval durations were capable of producing robust increases in fear. We did, however, find that conditions associated with handling on retrieval day enhanced fear. Our results confirmed a large literature showing that long non-reinforced exposure to a stimulus produces robust and persistent response loss. However, it was not simply that the longer retrieval duration itself was responsible for the response loss as extremely short durations were also able to produce some response loss.

These data add to a growing body of literature suggesting that retrieval is a dynamic process that modifies memory expression through a variety of processes that depend on retrieval parameters, the behavior of the organism during the trial as well the state of the organism at the time of retrieval (Rescorla, 1998; Brandon & Wagner, 2003). The exact underlying mechanisms that produce these changes are not entirely clear, however a few interesting possibilities emerge. With regard to the behavioral enhancements, handling and/or other associated cues may function to retrieve a memory and strengthen its expression and/or representation. A plausible mechanism for this to occur may be that handling confers new excitatory value to the original memory through second order conditioning or may somehow sensitize the fear response the next day. The behavioral decrements seen in the long, 24 min retrieval condition are likely due to extinction as we saw robust response loss during the retrieval session that led to attenuated responding and decreases spontaneous recovery on long-term (14D) tests.

Importantly, I found little evidence for the contemporary view that retrieval induces opposing processes (e.g., extinction vs. reconsolidation) as a direct result of duration (Eisenberg & Dudai, 2004; Mamiya et al., 2009). Instead, it appears that multiple processes are engaged during retrieval and some of these processes do not depend on explicit re-exposure to the original fear context itself. In fact, the duration of exposure to the original fear context itself doesn't necessarily predict the outcome of retrieval as both short and long durations are capable of producing response loss. Together, these finding suggest that an organism relies on many factors during retrieval to modify its behavior in the face of changing contingencies and these factors are not

simply mutually exclusive, opposing processes. In Chapter 4, I follow the behavioral study of retrieval with brain region specific manipulations of HA and transcription to examine whether we can modify the outcome of memory retrieval by engaging specific neural substrates.

Chapter 4: How do pharmacological manipulations of brain-region specific histone acetylation change the outcome of memory retrieval?

NOTE: *Portions of this study are previously published (Stafford et al., 2012). Contributions of each author are defined in the acknowledgements.*

Introduction

A rich literature as well as experiments conducted in Chapter 3 show that memory retrieval induces behavioral change under a variety of conditions. There are also retrieval conditions such as intermediate durations that produce little change in behavior. However, as reviewed in the Introduction, multiple retrieval processes may be engaged even though behavioral change is not changed as a result of retrieval. These different processes such as extinction or reactivation likely rely on different neural substrates (e.g., infralimbic vs. prelimibic cortex; reviewed in Quirk & Mueller, 2008) as well as the engagement of different epigenetic processes (Stafford & Lattal, 2011). Therefore, I hypothesize that manipulating brain regions that drive excitatory or inhibitory processes following retrieval will produce behavioral enhancements and decrements, respectively.

To test this hypothesis, I used the intermediate, 3 min retrieval duration from Chapter 3 as this duration produced no significant change relative to control. Following the 3 min retrieval duration I injected the HDAC inhibitor sodium butyrate (NaB) to enhance acetylation and transcriptional processes in discrete brain regions that may play opposing roles in retrieval processes. My focus was on the hippocampus, amygdala as well as subregions of the medial prefrontal cortex (mPFC) as these brain

regions are known to be important in mitigating retrieval processes. The underlying

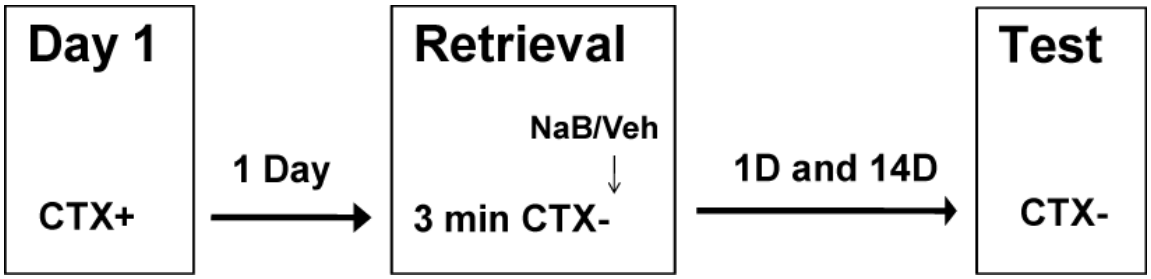


Figure 25. Experimental Design. Mice were fear conditioned on Day 1. Mice received an identical retrieval trial followed by infusion of the HDAC inhibitor, NaB or vehicle into either the hippocampus, infralimbic cortex, prelimbic cortex or amygdala. The effects of brain regions-specific NaB infusions were evaluated 1 and 14 D following retrieval.

hypothesis was to demonstrate that multiple, competing processes are simultaneously active during retrieval by selectively manipulating their neural substrates following an identical retrieval trial.

General Methods

General Experimental Design. Mice were housed, habituated and fear conditioned identically to those in Chapter 3. One day following acquisition mice received a 3 min re-exposure to the fear conditioning CTX in the absence of shock (except Experiment 14 and the No Retrieval controls, see below). Mice were tested 1 and 14D following retrieval (Figure 25).

Data Analysis. Data analysis was performed using planned comparisons as in Chapter 3.

Experiment 14. Effect of Post-retrieval Hippocampal NaB on Behavioral Expression and mPFC Transcriptional Markers.

Chromatin modifications and transcriptional changes within the hippocampus have been shown to be important in mediating extinction and other retrieval mediated

processes (reviewed in Stafford & Lattal, 2011). In Chapter 2, I demonstrated that NaB following an intermediate retrieval duration leads to robust and persistent extinction enhancements. A remaining challenge for the field is to understand the molecular processes that mediate enhanced extinction effects induced by HDAC inhibition (Stafford & Lattal, 2011). There is increasing evidence that transcriptional changes in the hippocampus and medial prefrontal cortex (mPFC) as well as signaling from the hippocampus to the mPFC are critical for extinction memory formation and modulation (e.g., Marek et al., 2011; Peters et al., 2011; e.g., Quirk & Mueller, 2008). However, it is unknown whether manipulating chromatin modifications such as HA in the hippocampus during retrieval modulates transcription in specific subregions of the mPFC. Furthermore, we know little about whether enhancing extinction actually brings the behavioral and molecular expression of that memory to levels commensurate with strong extinction produced by long retrieval durations.

In this study I evaluate whether enhanced extinction induced by hippocampal NaB (Chapter 2) generates behavioral expression and molecular changes in the extinction memory in the mPFC consistent with a strong, extinction experience.

Methods

Method. Methods were consistent with those used in Experiment 7except that in addition to the 3 min retrieval group, a separate group received a 24 min context exposure. Immediately following retrieval mice received hippocampal NaB or Vehicle infusions. These mice were then tested for behavior 1 and 14D later or were sacrificed 30 min after infusion for IHC analysis (see below)

Intracranial Infusions. Mice were cannulated and received bilateral intrahippocampal injections of either NaB (55 mM) or vehicle (sterile saline) as in Experiment 7.

Immunohistochemistry (IHC). Immunohistochemistry for histone acetylation (HA) and the immediate early gene c-Fos (1: 2,000 dilution; Santa Cruz Biotechnology) in the mPFC and hippocampus was performed and analyzed as in Experiment 10.

Results

Behavior.

Intrahippocampal injection of NaB induced a persistent extinction enhancement only when infused following an intermediate, 3 min extinction duration (Figure 26A) but not when infused following a long, 24 min retrieval duration (Figure 26B). The results of Figure 26A are described in detail in Experiment 7 as these data are reproduced from that experiment. Importantly, there was no interaction between Extinction and Drug Treatment ($p>.05$). However, the long (24 min) retrieval produced robust extinction as

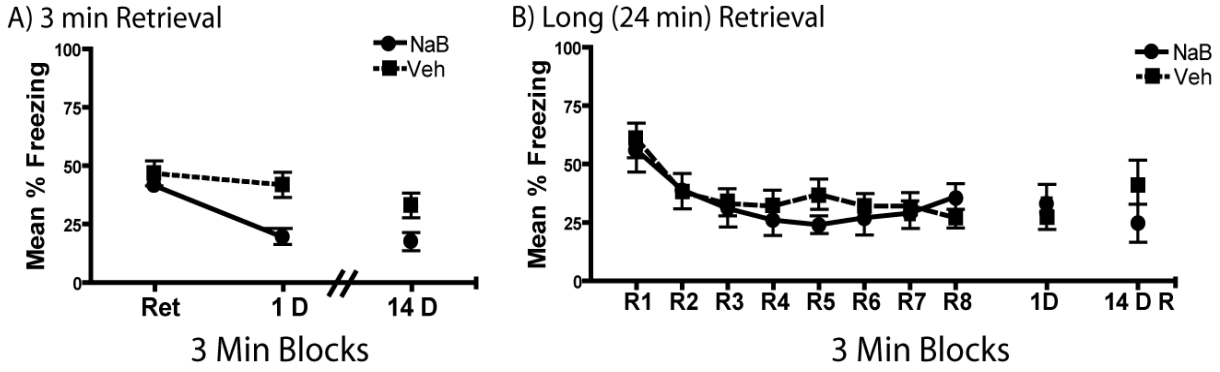


Figure 26. Intra-hippocampal NaB injections selectively cause persistent extinction enhancements only in the presence of weak extinction. A) During Retrieval, mice receiving either NaB or Veh following conditioning or retrieval did not differ. Mice receiving post-extinction NaB injections showed a significant extinction enhancement relative to controls when test 1D later *these data are reproduced from Chapter 2. B) Long (24 min) retrieval led to persistent decreases in freezing 1 and 14D later. Post-retrieval NaB hippocampal infusions was not able to induce any change in freezing relative to Veh.

revealed by a within session main effect of Extinction ($F(3.9, 78.1)=10.8$, $p<.01$). The groups did not differ during either the 1D or 14D test (e.g., no interaction or main effects; ps>0. 25).

Immunohistochemistry

Hippocampus. NaB targeted dorsal hippocampal CA1 (Paxinos & Franklin, 2007) enhanced acetylation and c-Fos in CA1 (right panel Figure 27A; left panel shows injector placements). A significant main effect of Drug Treatment confirmed greater acetylation stain density [$F(1,23)=10$, $p=0.005$] and c-Fos^{+} nuclei [$F(1,15)=17.02$, $p=0.002$] in NaB treated mice across extinction durations. There was no interaction between Drug Treatment and Retrieval Duration or main effect of Retrieval Duration on acetylation or c-Fos [all ps >0.1; Figures 27].

Infralimbic Cortex. Long retrieval (24 min) resulted in more histone acetylation as well as c-Fos^{+} nuclei in the infralimbic cortex (Paxinos & Franklin, 2007) than did short retrieval (3 min). Furthermore, intra-hippocampal NaB increased acetylation and c-Fos following 3 min retrieval but not following 24 min retrieval (Figure 28A left panels).

A significant Retrieval Duration X Drug interaction [$F(1,25) = 5.88$, $p =0.024$] combined with a main effect of Retrieval Duration ($F(1,25)=4.94$, $p=0.037$) and Drug [$F(1,25)=7.81$, $p=0.037$] confirmed the differences in histone acetylation intensity. Simple main effects revealed that indeed the Veh treated 3-min retrieval group had significantly lower levels of infralimbic acetylation than NaB or Veh treated 24 min groups or the 3-min NaB treated group [all ps ≤0.01].

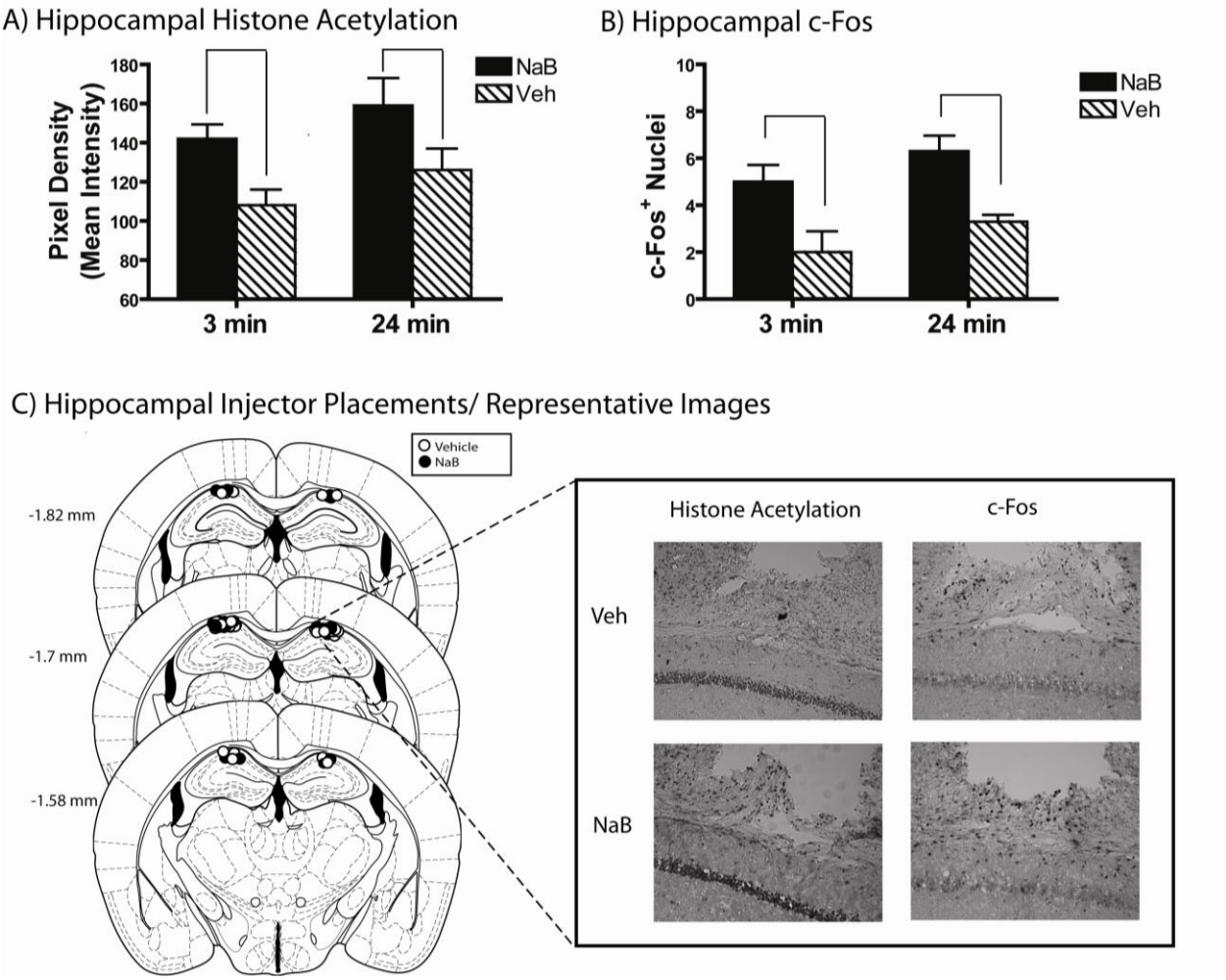


Figure 27. Injecting NaB Into the Dorsal Hippocampus Increases CA1 Histone Acetylation. A) NaB injected into the dorsal hippocampus enhanced acetylation (A) and c-Fos (B) in the dorsal hippocampus relative to vehicle (n=6,7) regardless of retrieval duration. C) Individual injector placements are shown in the left panel. Representative immunohistochemistry demonstrating that dorsal hippocampal infusions of NaB increases H3 Lys14 acetylation and c-Fos in the dorsal hippocampus are shown in the inset right panel. As no difference was observed between Retrieval Duration only representative images from NaB and Veh .

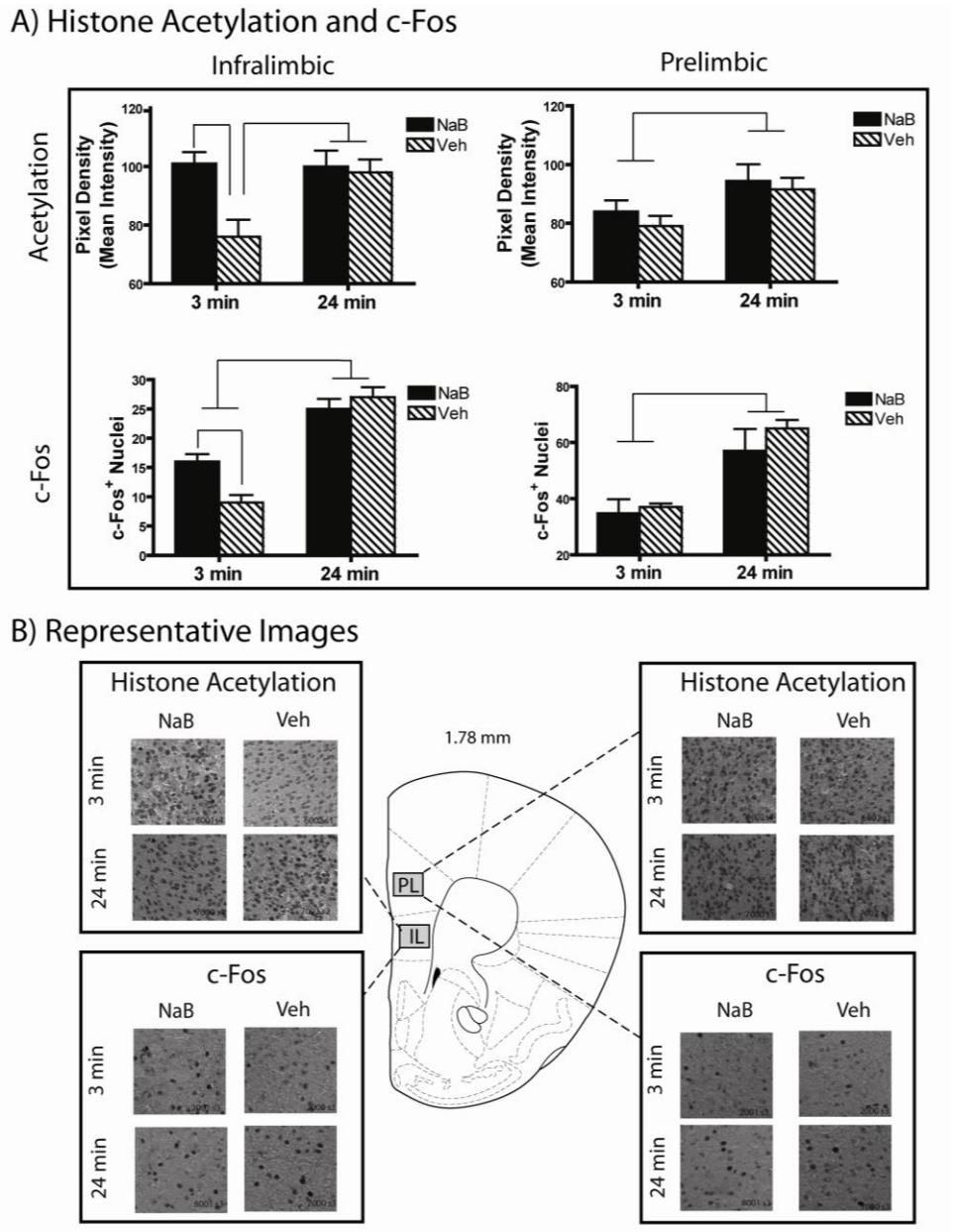


Figure 28. Intra-hippocampampal NaB Enhanced Histone Acetylation and c-Fos Expression Following a 3 min Retrieval in the Infralimbic Cortex but not Prelimbic Cortex. A) Mice receiving long, 24 min retrieval showed greater H3 Lys14 acetylation in both the infralimbic cortex and prelimbic cortex compared to short retrieval (3 min; top panels). Intra-hippocampal NaB enhanced histone acetylation in the infralimbic cortex following 3-min retrieval above Veh levels bringing them to levels commensurate with long retrieval (top left panel). In contrast to the infralimbic effects, intrahippocampal NaB infusion had no effect on prelimbic acetylation following either weak or strong extinction relative to vehicle (top right panel).

The 24 min group showed greater c-Fos expression in both the infralimbic and prelimbic cortices than did the 3 min group (top panels). Similar to the acetylation findings, intra-hippocampal NaB enhanced c-Fos following 3 min retrieval above Veh levels with no effect following 24 min retrieval (bottom left panel). No effect of hippocampal NaB on c-Fos in the prelimbic cortex was seen (bottom right panel).

C) Representative histone acetylation and c-fos immunohistochemistry images from of the infralimbic and prelimbic cortices. A unilateral sample is presented here for illustration, however the IHC was quantified in the entire (bilateral) infralimbic and prelimbic cortices. Stereotaxic image reproduced with permission from (Paxinos & Franklin, 2007)

Veh treated 24 min retrieval groups showed significantly more c-Fos positive neurons than both the NaB and Veh treated 3-min retrieval groups [all ps<0.01]. The 3-min NaB group showed a greater number of infralimbic c-Fos positive neurons than the 3-min vehicle treated mice [p=0.01].

Prelimbic Cortex. In contrast to the infralimbic IHC, no effect of intra-hippocampal NaB was seen in the prelimbic cortex. Only elevated acetylation and c-Fos was found following 24-min vs. 3-min retrieval (Figure 28A right panels). A main effect of Retrieval Duration on both acetylation [$F(1,15)= 25.6$, $p<0.001$] and c-Fos [$F(1,25) =6.5$ $p=0.018$] confirmed this with no interaction or effect of drug in any group [all ps>0.3].

Representative images of infralimbic and prelimbic IHC are shown in Figure 28B, respectively. Together, these results suggest that transcriptional modulations in the hippocampus drive infralimbic transcription supporting extinction.

Discussion

The key finding from these experiments was that the HDAC inhibitor sodium butyrate promoted long-term extinction, as revealed through behavioral and molecular measures. When a brief extinction session, that on its own had little impact on behavior, was followed by intra-hippocampal NaB administration, the behavioral and molecular consequences of that session were similar to those induced by a long extinction session. Indeed, NaB infusion into the hippocampus drove increases in histone acetylation and c-Fos expression consistent with strong extinction in the infralimbic, but not prelimbic cortex.

We showed that modulating acetylation and c-Fos expression in the hippocampus is sufficient to drive transcriptional changes in the infralimbic cortex and

that these changes are associated with strong extinction. A remaining question is whether these hippocampal driven changes in the mPFC are necessary to promote strong extinction. Our basic finding is consistent with studies showing the hippocampus and infralimbic cortex interact to promote fear extinction (Hoover & Vertes, 2007; Peters et al., 2011). Within this network, we observed changes in acetylation at L14 of H3 as well as c-Fos expression, which are generally associated with permissive, transcriptionally active chromatin states. These chromatin states are associated with downstream increases in the expression of genes critical for excitatory and inhibitory memory formation (e.g., BDNF, Nr4a1; Barrett et al., 2011; Cheung et al., 2000; Lubin et al., 2008; Vecsey et al., 2007). Interestingly, a recent study indicates that inhibiting enzymes that remove acetyl groups (e.g., p300) in the mPFC enhances extinction memory (Marek et al., 2011) demonstrating the need for future studies characterizing the global chromatin state required for extinction memory formation.

The specificity of this effect to the infralimbic but not prelimbic cortex is consistent with growing evidence that enhanced extinction is driven by transcriptional events in the infralimbic but not prelimbic cortex (Marek et al., 2011; Whittle et al., 2010) as well as the involvement of the hippocampus in mediating such changes specifically in the infralimbic cortex (Peters et al., 2011). Furthermore, anatomical studies in rats show the dorsal hippocampus (CA1) has more projections to the infralimbic than the prelimbic cortex, which may explain why the molecular effects of CA1 NaB infusion were present in the infralimbic and not the prelimbic cortex (Hoover & Vertes, 2007).

These results also shed light into recent studies which indicate that the memory enhancing effects of NaB are critically dependent on the strength of learning and the

subsequent memory. For example, NaB transforms a weak or impaired memory into a robust long-lasting memory (Fischer et al., 2007; Stefanko et al., 2009). Sensitivity of memories to the enhancing effects of NaB has also been shown at the molecular level—NaB transforms relatively low levels of histone acetylation following weak training into robust levels of acetylation commensurate with strong training and memory expression (Federman, Fustinana, & Romano, 2009). These studies are also consistent with our finding that the ability of NaB to enhance an extinction memory at both behavioral and molecular levels depends on the strength of the extinction memory; if the learning during extinction is strong, increases in histone acetylation in the hippocampus may not have further downstream effects on changes in the infra-limbic cortex. In light of our current results this suggests that the strength of the memory may be a critical determinant in the ability of HDAC inhibitors to enhance memory.

A major implication of this work is that increases in acetylation and associated transcriptional processes in the infralimbic cortex but not the prelimbic cortex drive extinction. Experiment 15 explicitly tests this hypothesis.

Experiment 15. Effect of Dorsal and Ventral mPFC NaB Infusions Following an Intermediate Memory Retrieval Duration.

Studies of fear memory retrieval over the last 25 years implicate a critical role for the mPFC in mediating persistent response loss (Morgan, Romanski, & LeDoux, 1993; Quirk & Mueller, 2008). As reviewed in the Introduction and evidenced in Experiment 14, different subregions of the mPFC may play very different roles in memory retrieval. Specifically, activation in infralimbic cortex (IL) tends to be associated with behavioral inhibition while prelimbic (PL) activation is associated with fear expression (Quirk &

Muller, 2008). However we know little about how directly manipulating HA in these brain regions will affect the outcome of memory retrieval. Therefore, based on the previous experiment the driving hypothesis behind this experiment was that infusions of the HDAC inhibitor, NaB directly into the infralimbic cortex would enhance extinction while infusion into the prelimbic cortex would have little effect on behavior.

Methods

General Experimental Design. In these experiments we used identical housing, habituation and fear conditioning protocols described in Experiment 14. The 3 min retrieval duration was again used in this experiment as it was previously shown to be sensitive to extinction enhancements. Infusions of NaB or Veh into the mPFC occurred immediately following retrieval or after handling in the No Retrieval control group (Experiment 15B). Testing occurred 1 and 14D later. Two independent iterations of this experiment were performed:

1. Experiment 15A: In this experiment injectors were guided to the IL. I therefore relied on IL misses as the PL infusions (see below for coordinates).
2. Experiment 15B: Here, I repeated the IL NaB and Veh infusions. However, I also included another group which had injectors directed at the dorsal portion of the mPFC encompassing the PL and anterior cingulate cortices with the intention of more robustly and specifically targeting those brain regions (see below for coordinates). This experiment also included a No retrieval control group that received infusions into these brain regions 1D following conditioning in the absence of retrieval.

Cannulations and Infusions. To direct injections into the mPFC, an angled, unilateral injection procedure was used to avoid damaging the dorsal portions of the mPFC when injecting into the IL.

For the unilateral, angled IL cannulations, mice were mounted on a stereotax, skulls leveled and rotated 30°. A hole was then drilled (1.7 AP, 1.67 ML) with a 7.0 mm, 26 ga guide cannula lowered –1.43 DV and glued to the skull with ketac dental cement (3M).

PL cannulations were conducted as above except the drilling coordinates were 1.7 AP, 1.42 ML and the cannula was lowered -1.1 mm.

Infusion into these brain regions was performed by lowering an injector that extended 1.0 mm below the cannula and infusion .25µL of NaB (55mM) or vehicle.

Results and Discussion

Experiment 15A. Infusion of NaB into the infralimbic but not prelimbic cortex immediately following retrieval induced a persistent extinction enhancement (Figure 29A). A significant of effect Drug Infusion Placement indicated a difference at both 1 and 14 D tests [$F(2,19)=3.63$, $p=0.049$ and $F(2,19)= 5.14$, $p=0.019$]. Further analysis indicated that at both 1D and 14 D tests mice receiving infralimbic NaB froze significantly less than vehicle [$p=0.04$ and $p=0.03$]. No effect of prelimbic infusion was seen on any test day [all $ps>0.2$].

Mice were identified as receiving infralimbic or prelimbic NaB infusions depending on injector tip placement (Figure 29B; Paxinos & Franklin, 2007). Mice receiving vehicle infusions into prelimbic and infralimbic did not differ on any day and were thus combined into a single vehicle group.

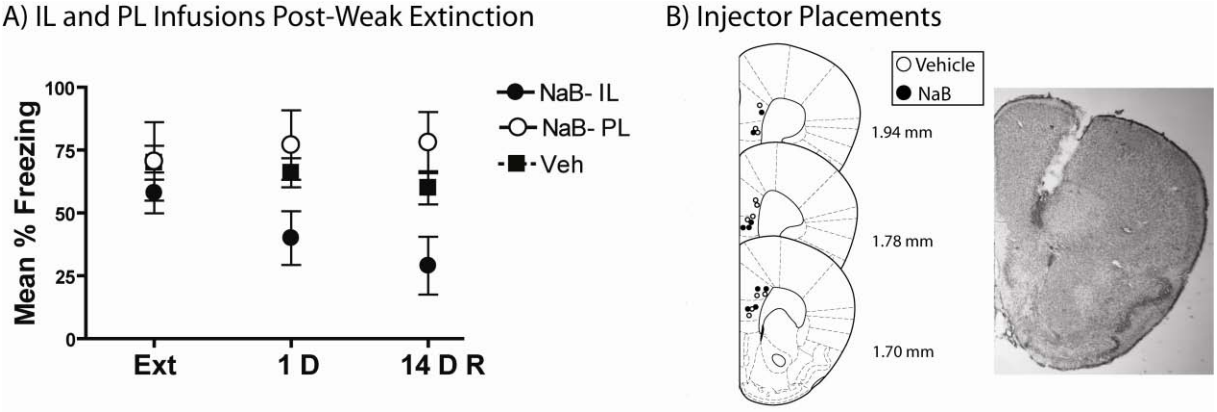


Figure 29. Infralimbic but not Prelimbic NaB Infusions Caused Persistent Extinction Enhancements. A) During retrieval mice receiving NaB into the infralimbic or prelimbic cortex did not differ from vehicle. Only the mice injected with NaB following retrieval froze significantly less than vehicle on the 1 and 14D tests. B) Cannula placements with a representative angled placement in the IL and PL.

Experiment 15B. Infusions of NaB into the IL replicated the extinction enhancement seen in Exp. 15A. In contrast, NaB infusions into the dorsal mPFC (PL) resulted in enhanced freezing on the 1D test (Figure 30). This was confirmed by a main effect of Drug Infusion Placement ($F(2,20)=9.2$, $p<.01$) and planned comparisons showing that indeed the NaB-IL group froze less than Veh ($p=.02$), and the NaB-PL group froze more than Veh ($p=.04$).

The 14D test suggests that the behavioral enhancements and decrements seen on the 1D test did not persist as no significant effect was observed (all ps>.05).

Importantly, NaB infused into either the IL or PL in the absence of retrieval resulted in no effect on behavior when tested 1 and 14D later (Figure 30B; all ps >.05).

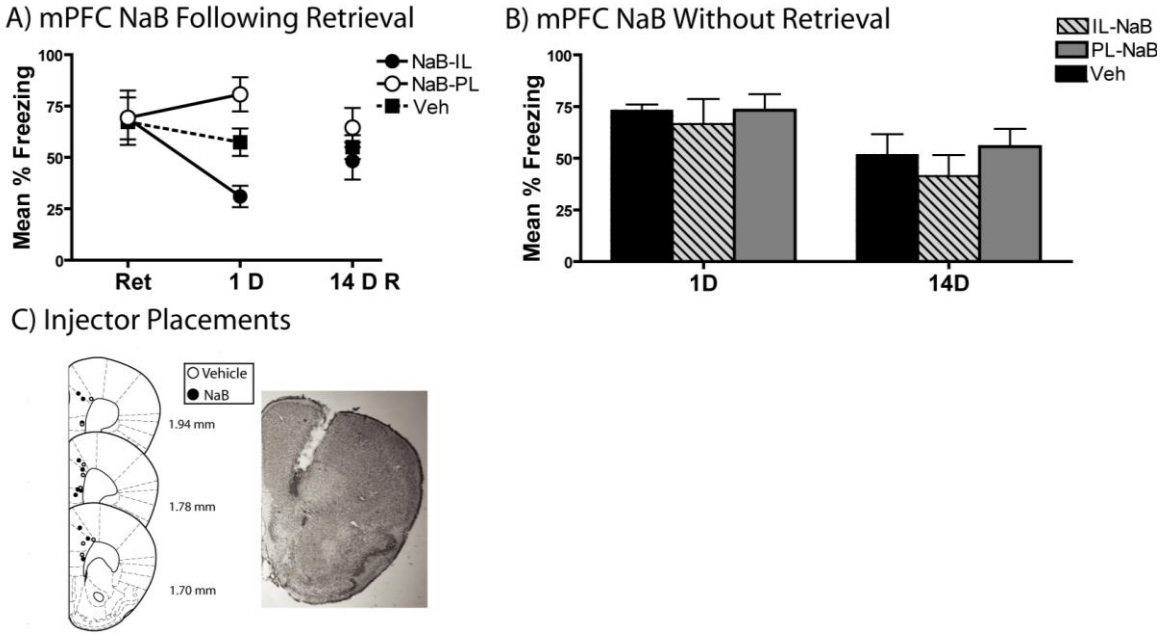


Figure 30. Infralimbic NaB Infusions Caused Extinction Enhancements while Dorsally Guided PL Infusions Resulted in Behavioral Enhancements. A) During retrieval mice receiving NaB into the infralimbic or prelimbic cortex did not differ from mice receiving vehicle. Only the mice injected with NaB into the IL following retrieval froze significantly less than mice receiving vehicle on the 1D tests. NaB infusions into the PL following retrieval induced behavioral enhancements. B) Infusions of NaB into the mPFC in the absence of retrieval had no effect on behavior. C) Cannula placements with a representative angled placement in the IL and PL.

The critical finding from these two experiments is that even when the retrieval trial is identical, NaB produces opposite effects on behavior depending on the brain region it is infused. Generally, post-retrieval IL infusions resulted in enhanced extinction while infusions directed specifically at the dorsal portion of the mPFC (e.g., PL) generate enhanced fear.

The results of Experiment 15A and 15B differed slightly. One difference was that NaB infusions into the IL caused a persistent extinction enhancement to 14 D in Exp. 15A but the NaB-induced extinction enhancement only persisted to 1 D in Exp. 15B. The reason for this is unclear, however there are often some discrepancies between replications of the same experiment. It is also possible that the injection sites differed enough between experiments that different results resulted.

In addition, post-retrieval infusions of NaB into the PL in Exp 15A resulted in no significant increase in freezing while PL infusions in Exp 15B were able to produce reliable freezing enhancements. The most likely explanation is that in Exp 15A I was not successful in selectively targeting the PL with NaB diffusing into the IL. In Exp 15B I was able to create a large distance between the PL and IL injection sites thus dissociating their behavioral effects. In addition, these more dorsally-directed infusions (Figure 30C) may have engaged the anterior cingulate, a brain region recently implicated in reconsolidation-like processes (Einarsson & Nader, 2012).

Experiment 16. Effect of Amygdala NaB Infusions Following an Intermediate Memory Retrieval Duration.

Studies of HDAC inhibition following memory retrieval have revealed that HA in the hippocampus and mPFC are important in mediating response loss and enhancements (Bahari-Javan et al., 2012; Lattal, Barrett, & Wood, 2007; Stafford et al., 2012). However, we know relatively little about the role of HDAC inhibition in the amygdala following retrieval. What is known is that post-retrieval infusion of memory enhancing agents such as HDAC inhibitors and PKA activators into the amygdala can potentiate fear the next day (Maddox & Schafe, 2011; Tronson, Wiseman, Olausson, & Taylor, 2006). My hypothesis for this experiment was therefore that infusions of NaB into the amygdala following retrieval would enhance responding on subsequent tests.

Methods

General Experimental Approach. In these experiments, we used identical housing, habituation and fear conditioning protocols described above. The 3 min retrieval duration was again used in this experiment as it was previously shown to be

sensitive to pharmacologically-induced enhancements and decrements in performance . Infusions of NaB or Veh into the amygdala occurred immediately following retrieval or after handling in the No Retrieval control group. Testing occurred 1 and 14D later.

Cannulations and Infusions. Mice were mounted on a stereotax with skulls prepared as described above. Two holes were then drilled (-1.46 AP, ±3.1ML) with two 7.0 mm, 26 ga guide cannula lowered –1.8 DV and glued to the skull with ketac dental cement (3M). Only bilateral amygdala hits were included in the analysis (Figure 31C)

Infusion into the amygdala was performed by lowering an injector that extended 3.0 mm below the cannula and infusion .25µL of NaB (55mM) or vehicle.

A sham infusion group was also included that were cannulated like the other amygdala mice, however, instead of placing an injector into the brain, mice were handled on the retrieval day.

Results and Discussion

When tested 1D following retrieval, vehicle mice froze less than either the NaB or Sham treated animals (Figure 31A). An Infusion Conditions ANOVA ($F(2,20)=6.8$, $p=.006$) followed by planned comparison confirmed this effect as the vehicle treated mice froze less than either the NaB or Sham mice (both $ps<.01$).

No significant effect was seen on the 14D test or in the No Retrieval Groups (Figure 31A and 31B, respectively; all $ps >.05$)

The most striking result from this study was that vehicle infusions into the amygdala following retrieval caused a deficit in freezing relative to Nab and sham

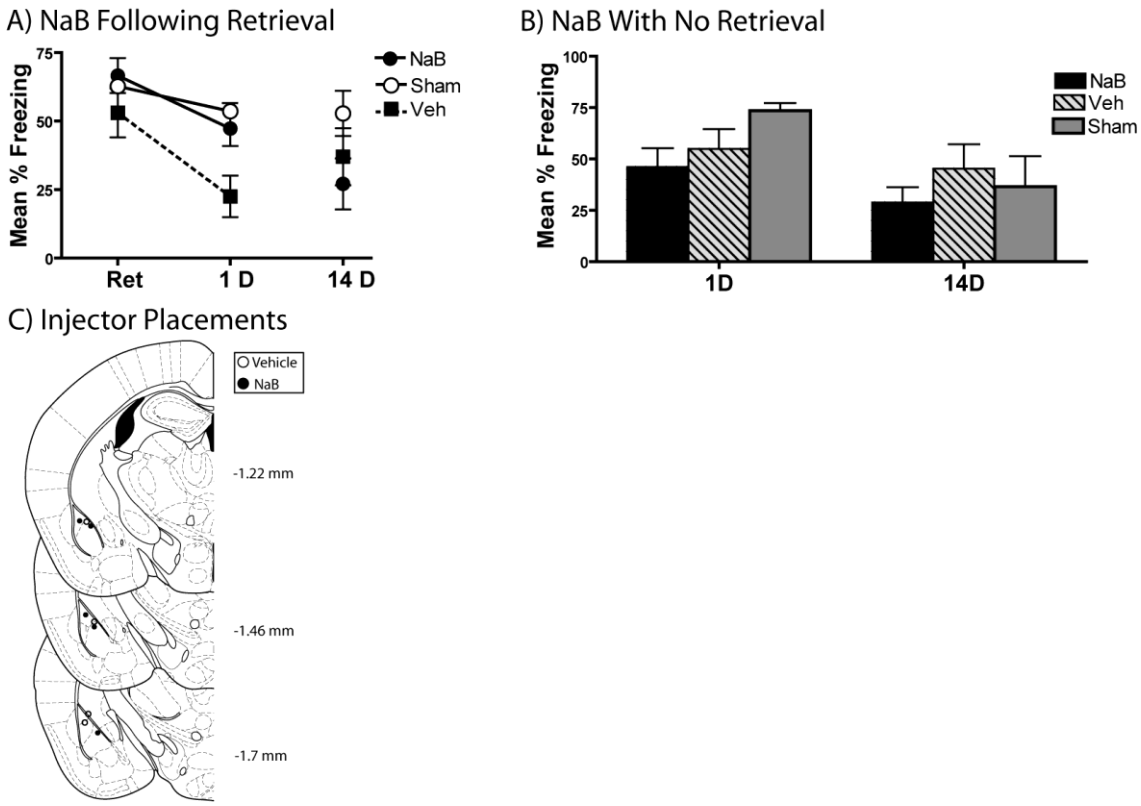


Figure 31. NaB into the Amygdala Prevents Vehicle-Induced Deficits. A) Vehicle treated mice froze less than Sham and NaB –treated mice 1D following retrieval. B) Infusions conditions produce no effect when retrieval was omitted. C) Amygdala placements from one hemisphere of the brain.

treated mice. These results would be exactly in line with the original hypothesis (post-retrieval amygdala Nab enhances responding) in the absence of the sham group. However, after running a pilot experiment, I recognized that the vehicle group was displaying a deficit in freezing relative to vehicle treated mice in other experiments such as the mPFC experiments. Therefore, the sham group was added and revealed that indeed post-retrieval vehicle infusion produces a behavioral deficit.

Behavioral deficits induced by vehicle infusion into the amygdala are not surprising as the brain may be damaged following infusion especially since the injectors descend deep into the brain through multiple structures including the striatum. In this case, it is likely that NaB confers some protective effect as HDAC inhibitors have been

shown to decrease inflammation surrounding neurological insult and improve stroke prognosis (Chuang, Leng, Marinova, Kim, & Chiu, 2009).

As mentioned, other studies of HDAC inhibition show that HDAC inhibitor into the amygdala following retrieval can bolster response relative to vehicle. However, a close inspection of some of this data suggests that their experiments may also suffer from a similar vehicle-induce decrement that is recovered by HDAC inhibition (Maddox & Schafe, 2011). Combined with our results, this seems to indicate that HDAC inhibition in the amygdala may not enhance reconsolidation or associated processes *per se.* HDAC inhibition is more likely protecting against some vehicle induced deficit. Whether this infusion-induced deficit and deficits like these are effects on memory storage, a retrieval effect on test or some other process is unclear (Riccio, Millin, & Bogart, 2006). However it is clear that when considering intracranial microinfusions, especially those that are deep, the effect of the vehicle on behavior needs to be considered before making claims about specific processes that are affected by the drug manipulation.

General Discussion

Together, these experiments suggest that even when following an identical, intermediate retrieval trial, the HDAC inhibitor NaB can have very different effects on behavior that are dependent on the brain region into which it is infused. Specifically, post-retrieval infusion into the hippocampus enhanced extinction and drives increases in HA selectively in the infralimbic cortex. Similarly, infralimbic cortex NaB enhances fear extinction while infusion into the prelimbic cortex enhances fear expression. Like the PL infusions, NaB infusion into the amygdala produces enhancements relative to vehicle, but no difference from sham treated animals.

At a broad level, these data indicate that selectively targeting the neural substrates of retrieval-induced excitatory and inhibitory processes can shift the outcome of memory retrieval to generate fear enhancements and decrements (respectively). Because these bidirectional behavioral effects follow from an identical retrieval trial, it suggests that multiple processes are simultaneously engaged by retrieval (see Discussion below for explanation and theoretical interpretation).

Discussion and Conclusions

The overarching goal of this dissertation was to understand how memory retrieval changes behavioral memory expression by manipulating behavioral conditions, neural substrates and molecular mechanisms underlying these retrieval processes.

Summary of major findings

In Chapter 2, I asked whether the learning and memory processes that occur following retrieval are similar to those that occur during acquisition. I found that identical pharmacological manipulations produce different behavioral results post-acquisition and post-retrieval. Generally, those manipulations which produce behavioral deficits (e.g., anisomycin) produce larger and more persistent deficits when administered following acquisition than when administered following retrieval (Experiments 1-4). Furthermore, retrieval processes were more vulnerable to HDAC inhibition than were acquisition processes (Experiments 5-7). In contrast to the different effects of pharmacological manipulation of acquisition and retrieval processes, extinction following acquisition or retrieval appeared to produce similar behavioral results—that is, heightened fear compared to delayed extinction. However, even in Experiments 8-10, there were differences in the brain activation patterns induced by post acquisition and retrieval extinction (e.g., heightened amygdala activity post-acquisition).

In Chapter 3, I asked how retrieval conditions affect the behavioral expression of memory. The key finding from these experiments was that retrieval is capable of modifying the expression of a memory in a number of ways that do not solely depend on the duration of the trial itself. For example, we found little evidence that short retrieval

durations were capable of producing robust increases in fear. We did, however, find that conditions associated with handling on retrieval day were capable of enhancing fear. We confirmed a large literature showing that long non-reinforced exposure to a stimulus produces robust and persistent response loss. However, it was not simply that the longer retrieval duration was responsible for the response loss as extremely short durations were also able to produce some response loss.

Chapter 4 examined whether pharmacological manipulations of brain-region specific histone acetylation change the outcome of memory retrieval. These experiments suggest that even when following an identical, intermediate retrieval trial, the HDAC inhibitor NaB can have very different effects on behavior that are dependent on the brain region into which it is infused. Specifically, post-retrieval infusion into the hippocampus enhances extinction and drives increases in HA selectively in the IL. Similarly, IL NaB enhances fear extinction while infusion into the PL enhances fear expression. Like the PL infusions, NaB infusion into the amygdala produces enhancements relative to vehicle, but no difference from sham treated animals.

Implications for Memory Retrieval Processes

A few general implications for theoretical accounts of retrieval emerge from these studies. The first is that while sharing some similarities with acquisition, the learning and memory consolidation processes induced by retrieval are different than those engaged by new learning and consolidation (Chapter 2). Then second is that, multiple processes are engaged by retrieval and the dominance of any one of these processes critically depends on the conditions of retrieval (e.g., duration), the state of the animal and the neural substrates engaged (Chapters 2-4).

Retrieval-induced Learning

In contrast to my original hypothesis, I found that retrieval duration alone does not determine the outcome of memory retrieval. For example, I found that despite differences in the magnitude of response loss, both short and long retrieval durations are capable of producing response loss. In addition, even retrieval manipulations that do not explicitly re-expose the animal to the original fear cue (e.g., context) are still capable of producing behavioral changes. Together, these studies suggest that behavior change induced by retrieval depends on a number of factors that include the physiological and affective state of the organism, the availability of salient information at the time of retrieval, as well as the behavior of the organism during retrieval itself (Rohrbaugh & Riccio, 1972; Rescorla, 1998; Brandon & Wagner, 2003).

Together, these results suggest multiple processes that are dynamically and sometimes simultaneously engaged by retrieval (summarized in Figure 32). In the context of Experiment 13, early during context-induced retrieval of a fearful memory, a mouse may orient itself to the contextual cues present and this orientation requires some movement. Early movement may become reinforced because the mouse associates this movement with the absence of shock (movement-associated reinforcement). As the retrieval trial progresses, orientation decreases while the mouse retrieves the fear memory (context-shock) by virtue of the contextual cues. Freezing behavior is elicited and may become reinforced as the mouse associates freezing with the lack of shock (freezing reinforcement). Importantly, other studies also strongly implicate a role for instrumental process governing the reinforcement of those behaviors

that predict the absence of an aversive outcome (Graf & Bitterman, 1963; Rohrbaugh & Riccio, 1970; Rohrbaugh et al., 1972).

Second order conditioning is another processes that is likely to be engaged early in the retrieval trial as the mouse is in a heightened state of activation allowing the association of additional contextual cues (those weakly or not associated with the original fear memory) with the original fear memory (Helmstetter, 1989; R.A. Rescorla, 1984). While not explicitly tested here, a strong case can also be made for handling (Experiment 12) conferring some second-order excitatory information to the original context-shock association in the absence of explicit extinction (Hui et al., 2006).

Later in the retrieval trial, as non-reinforced exposure to the context increases, the mouse learns that the contextual cues originally associated with the shock are poor predictors of the shock (Bouton, 2004; Delamater, 2004). Extinction learning results and the freezing response is suppressed which may lead to additional reinforcement of movement as the mouse associates this movement with the absence of shock (Experiment 11).

An exciting possibility emerges which suggests that during retrieval; any number of these learning processes may be simultaneously engaged by retrieval. Evidence for the concurrent development of excitatory and inhibitory value to stimuli comes from studies of stimulus pre-exposure, backwards conditioning and the transition from second-order conditioning to conditioned inhibition (Pavlov, 1927 pp71-73; Kiernan & Westbrook, 1993; Tait, 1986; Yin et al., 1994). In the studies of retrieval presented in this dissertation, evidence for concurrent excitatory and inhibitory processes comes indirectly from the intermediate retrieval durations which produce little effect on

behavior. However, direct evidence comes from the site-specific studies showing that by selectively targeting certain neural substrates we can shift the outcome of a retrieval trial towards either decrements (e.g., IL engagement) or enhancements (e.g., PL engagement) in performance.

Consolidation of Retrieval-induced Learning

Each of the aforementioned retrieval-induced learning processes may induce a period of time-dependent memory consolidation where the most salient learning processes are stabilized into long-term memory (Figure 32). This conclusion draws largely from those studies which show that post-retrieval processes are sensitive to both systemic and brain region-specific pharmacological manipulation (Experiments 1-7, 14-15). For example, the consolidation of extinction processes was enhanced by systemic, hippocampal and IL infusion of NaB. A growing body of evidence also points towards the expression of extinction learning requiring a time-dependent consolidation process (Lattal et al., 2006; Quirk & Mueller, 2008). Evidence for the consolidation of other excitatory processes comes from the finding that post-retrieval infusions of NaB into the PL enhance fear responding on subsequent tests as well as a budding literature showing that excitatory retrieval processes such as second-order conditioning are sensitive to manipulation of their neural substrates (Parkes & Westbrook, 2010, 2011).

In summary, these studies of retrieval learning and consolidation suggest that the dominant learning process induced by retrieval undergoes consolidation and is later expressed on tests of memory.

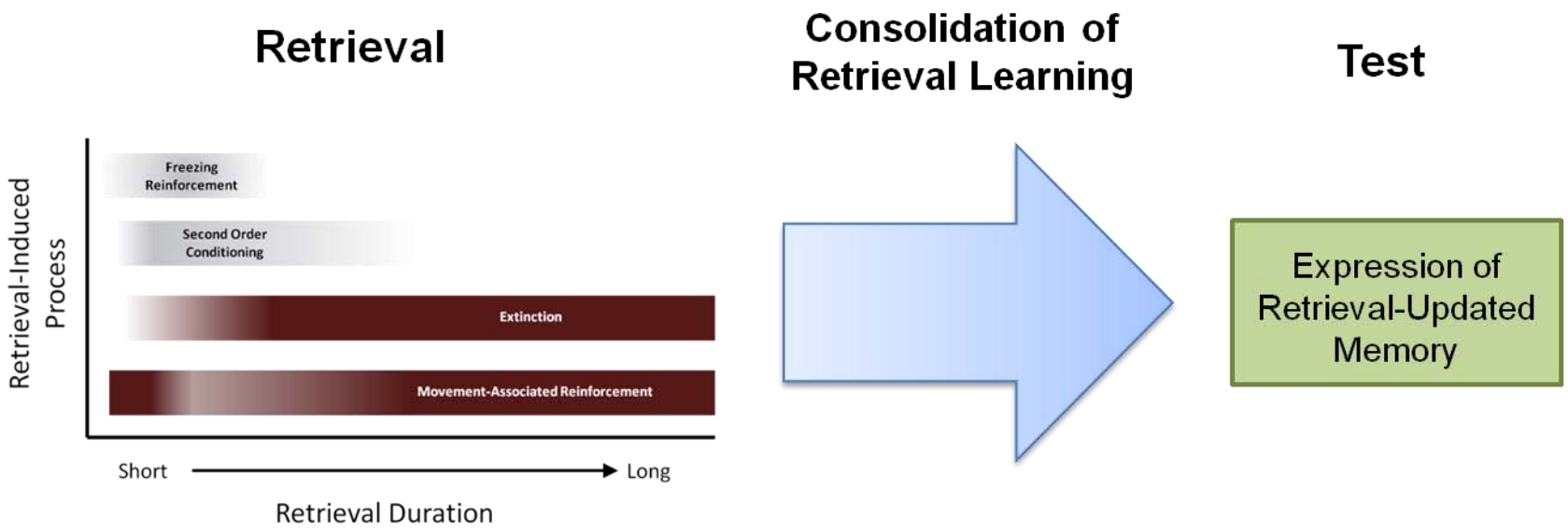


Figure 32. Learning processes engaged by retrieval critically determine subsequent memory consolidation and expression. *Left Panel*-Early during context-induced retrieval of a fearful memory, a mouse orients itself to the contextual cues present resulting in some movement. This early movement may be reinforced as the mouse associates its movement with the absence of shock (movement-associated reinforcement). As the retrieval trial progresses, orientation decreases while the mouse retrieves the fear memory (context-shock) by virtue of the contextual cues. Freezing behavior is elicited and may become reinforced as mouse associates freezing with the lack of shock (freezing reinforcement). Second order conditioning may concurrently occur if the mouse begins to associate additional contextual cues (those weakly or not associated with the original fear memory) with the original fear memory. As non-reinforced exposure to the context increases, the mouse learns that the contextual cues originally associated with the shock are poor predictors of the shock. Extinction learning results and the freezing response is suppressed which may lead to additional reinforcement of movement as the mouse associates this movement with the absence of shock. The exact responses and processes engaged are critically dependent on the state of the animal (e.g., high fear). *Middle Panel*-Each of these speculated retrieval-induced learning processes can induce a period of consolidation where the most salient learning processes are stabilized into long-term memory. *Right Panel*-Those dominant retrieval processes are then expressed on subsequent tests.

Implications for Neurobiology

One of the most exciting results of my dissertation work is the demonstration that excitatory and inhibitory retrieval processes have distinct neural substrates (summarized in Figure 33).

Excitatory Processes

When context-induced fear retrieval results in excitatory processes that serve to strengthen or maintain the original fear memory while impairing extinction, the central medial amygdala (CeM) fear output circuits become strongly engaged by virtue of converging input from amygdalar, prefrontal and hippocampal sources.

At a systems circuit level, it is well established that the hippocampus provides input to the central lateral (CeL), basal (BLA) and lateral (LA) amygdala as well as the PL and dorsal anterior cingulated (dACC) to modulate their activity based on contextual information (Cenquizca & Swanson, 2007; P. Gabbott, Headlam, & Busby, 2002; Gross & Canteras, 2012; Hoover & Vertes, 2007). The PL and dACC project to amygdala regions that serve to excite and generally disinhibit the CeM [e.g., the LA, BLA and paracapsular island of intercalated cells; ImP (P. L. Gabbott, Warner, Jays, Salway, & Busby, 2005; Gutman et al., 2012; Vertes, 2004)]. My site-specific drug infusions are consistent with engagement of the PL and dACC facilitating subsequent fear responding (Experiment 15A) while the c-Fos data in Experiment 10 suggests that when the PL is strongly engaged, there is a concurrent enhancement in those amygdalar circuits that lead to increased fear output. In addition, local inhibitory interneurons in the PL also may serve to inactivate the IL (Miller & Marshall, 2004).

Within the amygdala, the lateral and basal amygdala directly activates CeM fear output neurons or indirectly activates those CeM neurons through activation of “On” neurons (PKCδ-) in the CeL . The ImP also plays a role in CeM activation by inhibiting GABAergic input to the CeM from the main intercalated cell mass (IN) as well as inhibiting “Off” neurons (PKCδ+) in the CeL (reviewed in Palomares-Castillo et al., 2012).

Inhibitory processes

When extinction contingencies or those processes that serve to dampen fear responding are strongly engaged, the fear output of the CeM is decreased through a shift in the balance of PFC and amygdala input. The hippocampus provides input to the amygdala based on contextual stimuli, while recruiting the IL. In addition, the IL may inhibit the PL through local GABAergic interneurons (Miller & Marshall, 2004). This claim is strongly supported by data from Experiment 14 showing that directly engaging the hippocampus with NaB leads to strong extinction and a concurrent recruitment of the IL but not the PL. Direct evidence for the IL enhancing extinction comes from Experiment 15 where IL NaB infusion enhanced extinction. Under these strong extinction contingencies, projections of the IL may then dampen CeM fear output through inhibitory networks with the BLA or IN (Amir, Amano, & Pare, 2011; Gutman et al., 2012; Quirk & Mueller, 2008). In turn, the IN may inhibit CeM fear output directly or indirectly through inactivation of the ImP. For example, in the absence of inhibitory input from the ImP, the CeL “Off” neurons inhibits CeM output (Palomares-Castillo et al., 2012). Experiment 10 provides some correlational c-Fos evidence for this possibility as

those extinction contingencies leading to strong extinction are associated with increased c-Fos expression in the IN as well as decreased expression in the BLA, CeA and ImP.

Combined, the site-directed brain region manipulations and the c-fos data, while largely correlational, suggest and intricate balance in diffuse neural systems that regulate fear expression and inhibition.

Histone Acetylation and Gene Expression

These data provide valuable information to a growing literature on the involvement of HA in mediating transcriptional events underlying excitatory and inhibitory retrieval processes. Generally, I find that increased H3K14acetylation is associated with increased c-Fos expression and increasing these events in the IL and hippocampus enhances fear extinction. Conversely, HDAC inhibition in the PL following retrieval impairs extinction and increases fear expression. Together these studies are consistent with those suggesting that an increase in HA drives transcriptional processes underlying learning and memory (Barrett & Wood, 2008).

A layer of complexity is added to these interpretations by the suggestion that so-called HDAC inhibitors may derive some of their effects by driving acetylation of non-histone targets (Choudhary et al., 2009). However, the behavioral effects of NaB in these studies are linked to increased HA and c-Fos expression. In addition, other studies using similar HDAC inhibitors have directly linked HA driven transcriptional changes to memory processes (Vecsey et al., 2007). This suggests that the effects of NaB on behavior seen here are likely the results of its activity on HDACs and transcriptional processes underlying memory formation.

However, the exact HDAC isozyme target of NaB is difficult to determine as it is largely a non-selective Class I HDAC inhibitor (Kilgore et al., 2010). Other pharmacological and transgenic approaches have specifically revealed a role for HDAC2 and HDAC3 in memory formation, making these isozymes the most likely targets of NaBs' effects (Guan et al., 2009; McQuown et al., 2011). Another strong possibility is that NaB exerts its effects on HA and transcription through HDAC inhibition and the subsequent recruitment of HATs such as CBP (Vecsey et al., 2007). Future studies will be required to determine how HA- and HDAC inhibition-induced modifications of the overall chromatin landscape affect post-retrieval memory formation.

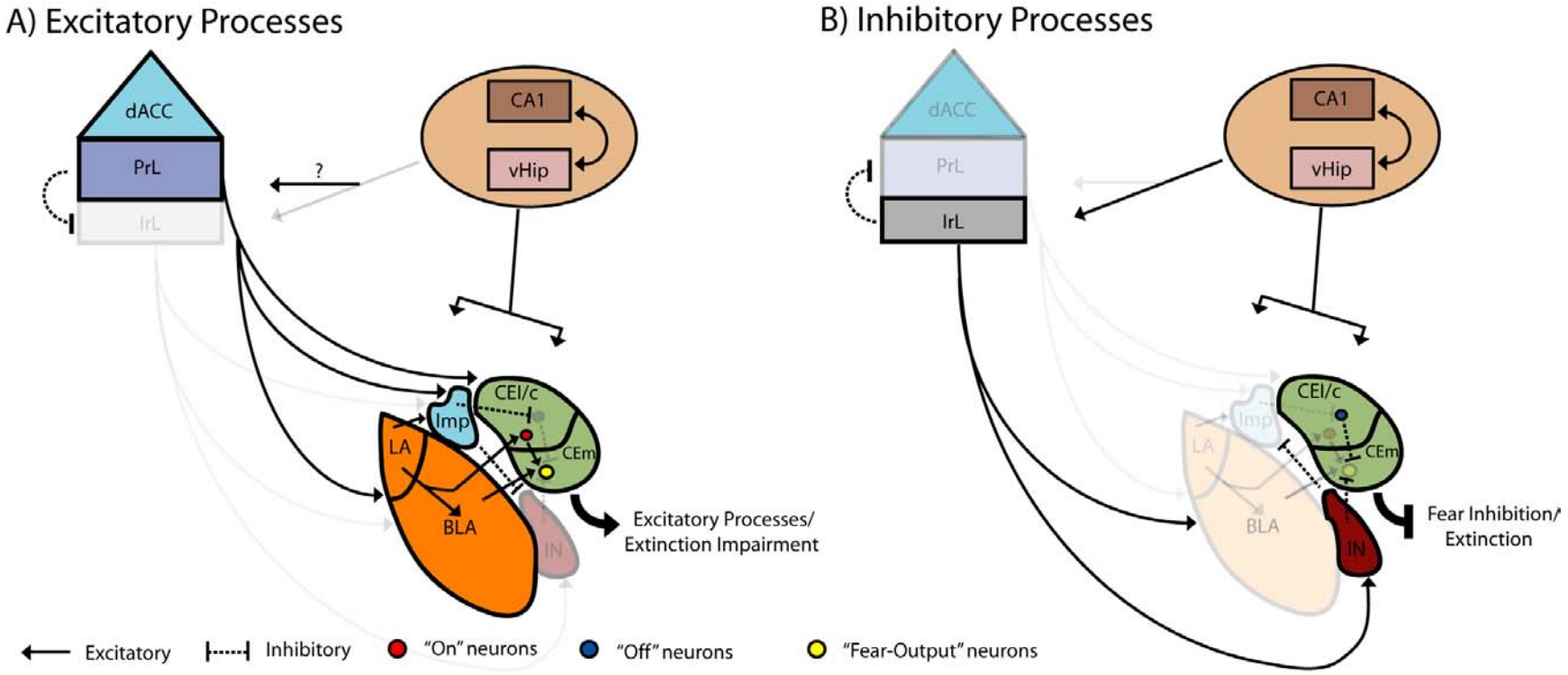


Figure 33. Differential Circuits Engaged by Contextual Fear Memory Retrieval. Those circuits strongly engaged are shown at full opacity while those inhibited or exhibiting decreased activity are made transparent. **A)** When context-induced fear retrieval results in excitatory processes that serve to strengthen or maintain the original fear memory while impairing extinction, the central medial amygdala (CeM) fear output circuits become strongly engaged by virtue of converging input from amygdalar, prefrontal and hippocampal sources. Generally, the hippocampus provides input to the central lateral (CeL), basal (BLA) and lateral (LA) amygdala as well as the prelimbic (PrL) and dorsal anterior cingulated (dACC) to modulate their activity based on contextual information. The PrL and dACC project to amygdala regions that serve to excite and generally disinhibit the CeM (e.g., the LA, BLA and paracapsular island of intercalated cells; ImP). Local inhibitory interneurons in the PrL also serve to inactivate the infralimbic cortex (IrL). Within the amygdala, the lateral and basal amygdala directly activate CeM fear output neurons or indirectly activate those CeM neurons through activation of "On" neurons (PKCδ-) in the CeL. The ImP also plays a role in CeM activation by inhibiting GABAergic input to the CeM from the main intercalated cell mass (IN) as well as inhibiting "Off" neurons (PKCδ+) in the CeL. **B)** In contrast, when extinction contingencies are strongly engaged, the fear output of the CeM is decreased through a shift in the balance of PFC and amygdala input. The hippocampus remains in a similar role, providing input to the amygdala based on contextual stimuli, however under extinction, the hippocampus recruits the IrL. The IrL in turn inhibits the PrL through local GABAergic interneurons and activation of the IN. The IN inhibits CeM fear output directly and indirectly through inactivation of the ImP. In the absence of inhibitory input from the ImP, the CeL "Off" neurons inhibit CeM output.

Implications for Reconsolidation and Retrieval-induced Memory Erasure

A critical implication of this dissertation is that the pervading reconsolidation view does not accurately capture retrieval learning or mnemonic process. The reconsolidation view suggests that a retrieval trial which strongly reactivates a memory (e.g., short duration) induces a period of plasticity where the original memory is vulnerable to disruption [e.g., a period of memory reconsolidation (Eisenberg & Dudai, 2004; Nader & Hardt, 2009)]. As reviewed in the Introduction, the major prediction of this account is that disrupting reconsolidation leads to behavioral decrements and these behavioral decrements are not the same as those inhibitory processes engaged by extinction. In essence, the reconsolidation view predicts that extinction and reconsolidation are mutually exclusive retrieval processes with disruptions in reconsolidation producing permanent disruptions as the original memory must be consolidated anew following retrieval. Chapter 2 explicitly tested this possibility and showed that disruptions in post-retrieval mnemonic processes are smaller and less persistent than disruptions in post-retrieval processes. As reviewed above, the results of Chapters 2-4 suggest an alternative to the reconsolidation view where retrieval induces new learning which adds information to the original intact memory. Depending on the learning induced by retrieval, both behavioral enhancements and decrements can result. Thus our studies, in large part, make a case for the inaccuracies of erasure and reconsolidation accounts.

However, it is still tempting to employ erasure accounts in those studies which show that retrieval associated manipulations reverse molecular or cellular events induced by new memory formation. For example, manipulations that impair memory

also impair synaptic plasticity, as revealed through effects on long-term potentiation (LTP) and long-term depression (e.g., Massey & Bashir, 2007). Recent studies have found that under some circumstances, extinction causes depotentiation, reversing the potentiated neuronal firing observed during LTP. During depotentiation many of the molecular mechanisms underlying LTP are reversed (e.g., AMPA receptors are internalized; protein kinases such as Akt, MAPK, CaMKII are dephosphorylated; see Zhou & Poo, 2004). Mao et al. (2006) showed that learning induced expression of certain AMPA receptor subtypes (GluR1) within the amygdala was abolished by extinction 1 hr but not 24 hr after auditory fear acquisition. This finding is consistent with extinction soon (1 hr) but not long (24 hr) after acquisition depotentiating the molecular substrates for LTP. In contrast, Kim et al. (2007) found that extinction 24 hr after the acquisition of fear conditioning decreased AMPA receptor expression in amygdala synaptosomes. Although these different findings may be attributed to procedural differences (e.g., extinction strength, molecular preparation) it is still difficult to say when or if extinction reverses certain molecular markers of LTP. Even if we knew with certainty when this occurs, we are still faced with the challenge of demonstrating that this specific process leads to memory erasure.

The question ultimately is a behavioral one: what are the long-term behavioral consequences of decreased plasticity and are there ways to think about this other than memory erasure? Synaptic plasticity in vivo is controlled through a variety of signaling (e.g., GABA, dopamine, K channels, transporters) and molecular mechanisms (e.g., histone acetylation, actin rearrangement; for review see Kim and Linden, 2007). Although many of these cellular and molecular mechanisms are involved in memory

consolidation, we still know very little about how these processes translate into long-term storage of memories (e.g., Kim & Jang, 2006; Shors & Matzel, 1997). Thus, changes among receptors or signaling molecules in one brain region may not be sufficient evidence for the existence (or nonexistence) of a memory. Further, seemingly opposing processes at the molecular level (e.g., phosphorylation and dephosphorylation, protein synthesis and proteolysis, histone acetylation and deacetylation) are likely all involved in initial memory formation and extinction. These factors highlight the fact that interpretations of response loss following retrieval are subject to multiple explanations.

A clear example of a case in which multiple explanations are available for similar effects on behavior comes from studies examining the role of NF-kB in retrieval and extinction. A 2007 paper by Lubin et al. in mice shows that inhibiting the NF-kB pathway following fear memory retrieval leads to deficits in fear memory expression the next day as well as decreases in histone marks typically associated with memory formation. The authors therefore concluded that they had blocked the reconsolidation of the memory as its behavioral and molecular expression were both reduced by blocking NF-kB. Similarly, a later study by Merlo & Romano, 2008 also showed that NF-kB inhibition was associated with loss of fear memory expression. *However, Merlo & Romano interpreted their results to mean that NF-kB inhibition was required for memory extinction rather than blockade of reconsolidation*. There are many other examples in the literature of this issue, in which different studies show similar behavioral effects resulting from similar molecular manipulations, but interpret these results in terms of reconsolidation impairments or extinction enhancements (Bernardi, Ryabinin,

Berger, & Lattal, 2009; Isiegas, Park, Kandel, Abel, & Lattal, 2006; Tronson et al., 2006). Until a clear picture is developed of the behavioral and molecular consequences of epigenetic manipulations, there will always be multiple possible interpretations for any behavioral result.

This problem of proving the absence of a memory becomes particularly problematic at a neural systems level as the engram migrates from one structure to another (Kim and Fanselow, 1992), may be stored in a sensory modality specific manner (Shema et al., 2007), and is likely distributed among different structures (e.g., Gold, 2004). The question for a molecular approach to retrieval thus becomes not only when do we look for memory erasure, but also where do we look? This is difficult to answer because activation of a memory stored in one brain region may be inhibited by another brain region during extinction. Using a tool such as functional magnetic resonance imaging (fMRI) during acquisition and extinction permits analysis of a memory's brain-wide functional signature during extinction. In this regard, a systems level analysis may provide insight into erasure and inhibition during extinction. Along, these lines, Anderson and Green (2001) found that subjects who were asked to actively suppress memories showed greatly attenuated recall when tested, even when offered monetary incentive for correct responses. The degree of memory suppression correlated with higher fMRI activity in the dorsolateral prefrontal cortex and less activity in the hippocampus (Anderson et al., 2004). Although instruction-based memory suppression may differ from extinction, both processes rely on the PFC to modulate brain regions associated with memory formation, retrieval, and expression (e.g., amygdala and hippocampus; Milad, et al., 2007; Phelps, Delgado, Nearing, & LeDoux,

2004). The PFC sends inhibitory signals to those regions (e.g., Das, et al 2005; Hariri et al 2003), although these outputs are not always inhibitory in nature (see Quirk & Beer, 2006). It is therefore plausible that decreases in the molecular/systems expression of a memory in the amygdala do not reflect erasure as the mPFC increases in activation to drive this amygdala suppression. This explanation fits well with our data where we show that strong extinction memories strongly engage transcriptional processes in the IL and decreases in transcriptional events in the BLA and CeA.

In summary, an engram can take many different forms at a molecular and neural systems level and these signatures are not always expressed in behavior. Together, these findings caution against those accounts that rely on erasure to explain their data. I would instead argue in favor of more conservative accounts that acknowledge multiple interpretations for response loss such as those theories suggesting that the absence of expression is due to an inhibitory form of that memory.

Limitations and Future Directions

It is important to note that the conditions used induce memory retrieval are determined by the experimenter in these studies. The exact cues and information the subject (the mouse) uses to retrieve and modify the memory may or may not reflect the intentions of the experimenter. Therefore, despite trying to explicitly control features such as handling and retrieval duration, other variables may indeed be responsible for some of the retrieval-induced effects we see on memory expression.

At a molecular level, these studies focus on single molecular mechanism at a time in a brain region using immunohistochemistry (IHC). This approach has the benefit of being able to look at molecular changes in very small, specific brain regions.

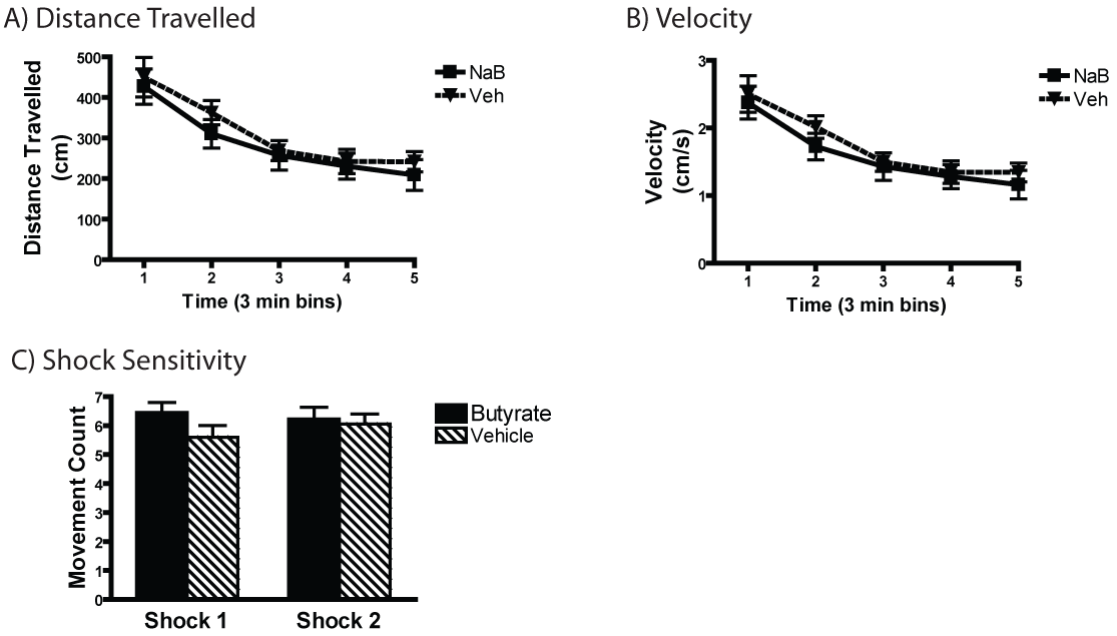
However, aside from being semi-quantitative, IHC for HA has the limitation of looking at bulk histone modifications and therefore may miss subtle changes in acetylation at specific promoters. Furthermore, we are only looking at the expression of the product of a gene whose transcription is known to be coupled to HA (e.g., c-fos) making it difficult to directly draw any causal conclusions about HA.

Despite some of these limitations, these experiments still provide important information that will ultimately set the stage for more brain region specific and molecularly detailed investigations of retrieval. Future directions will capitalize on techniques that allow for dissection of specific neuronal populations (e.g., laser-capture microdissection, flow –assisted cell sorting) for downstream evaluation of chromatin modifications regulating gene expression (e.g., chromatin immunoprecipitation and RNA sequencing genome wide).

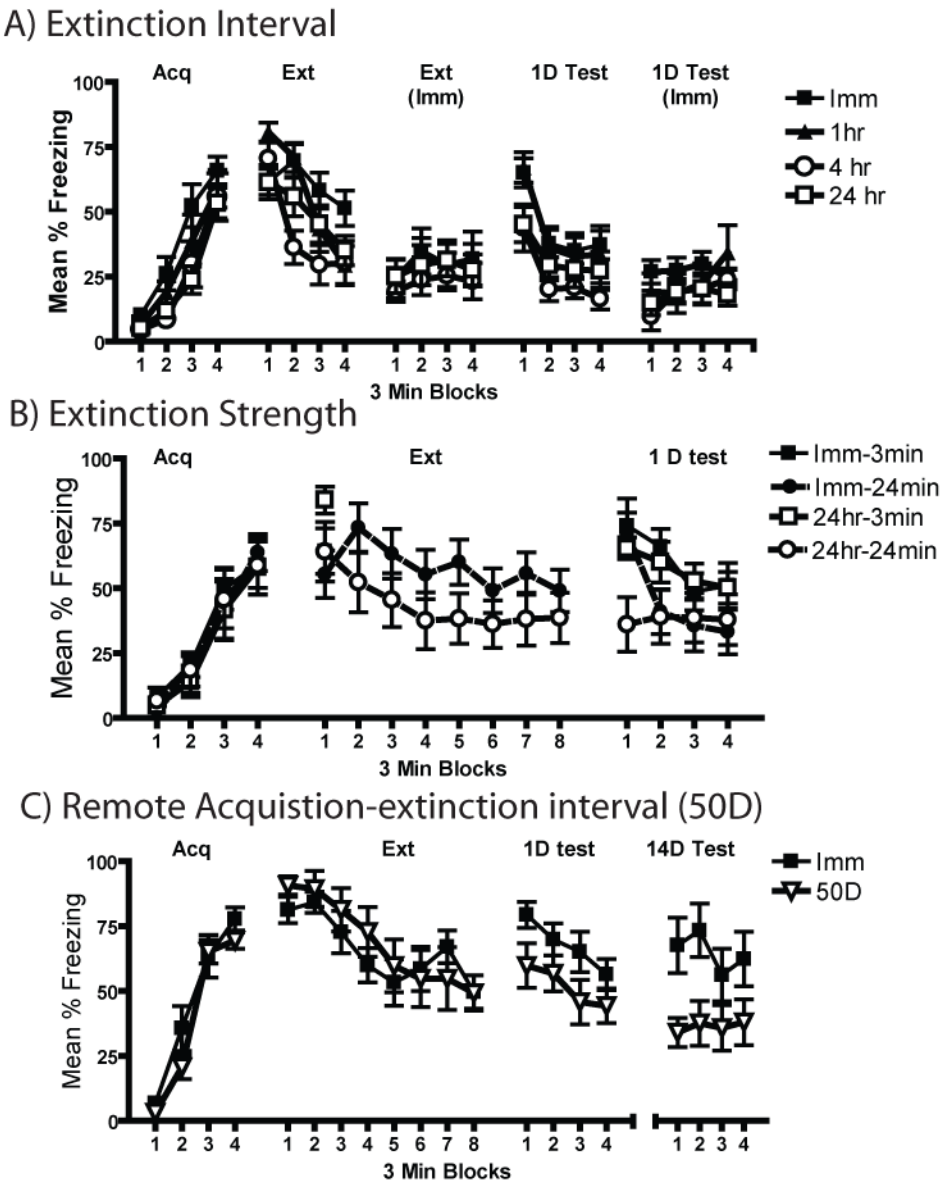
Summary

The critical finding from this dissertation is that memory retrieval can modify a memory through a number of mechanisms with very specific neural substrates. In addition, the idea that retrieval induces reconsolidation or allows for memory erasure is not well-supported by my findings. By revealing some of the conditions and neural substrates that lead to behavioral response loss and enhancements, my hope is that this knowledge can be applied to preclinical and clinical studies seeking to improve those diseases characterized by pathological memories (e.g., PTSD, drug addiction).

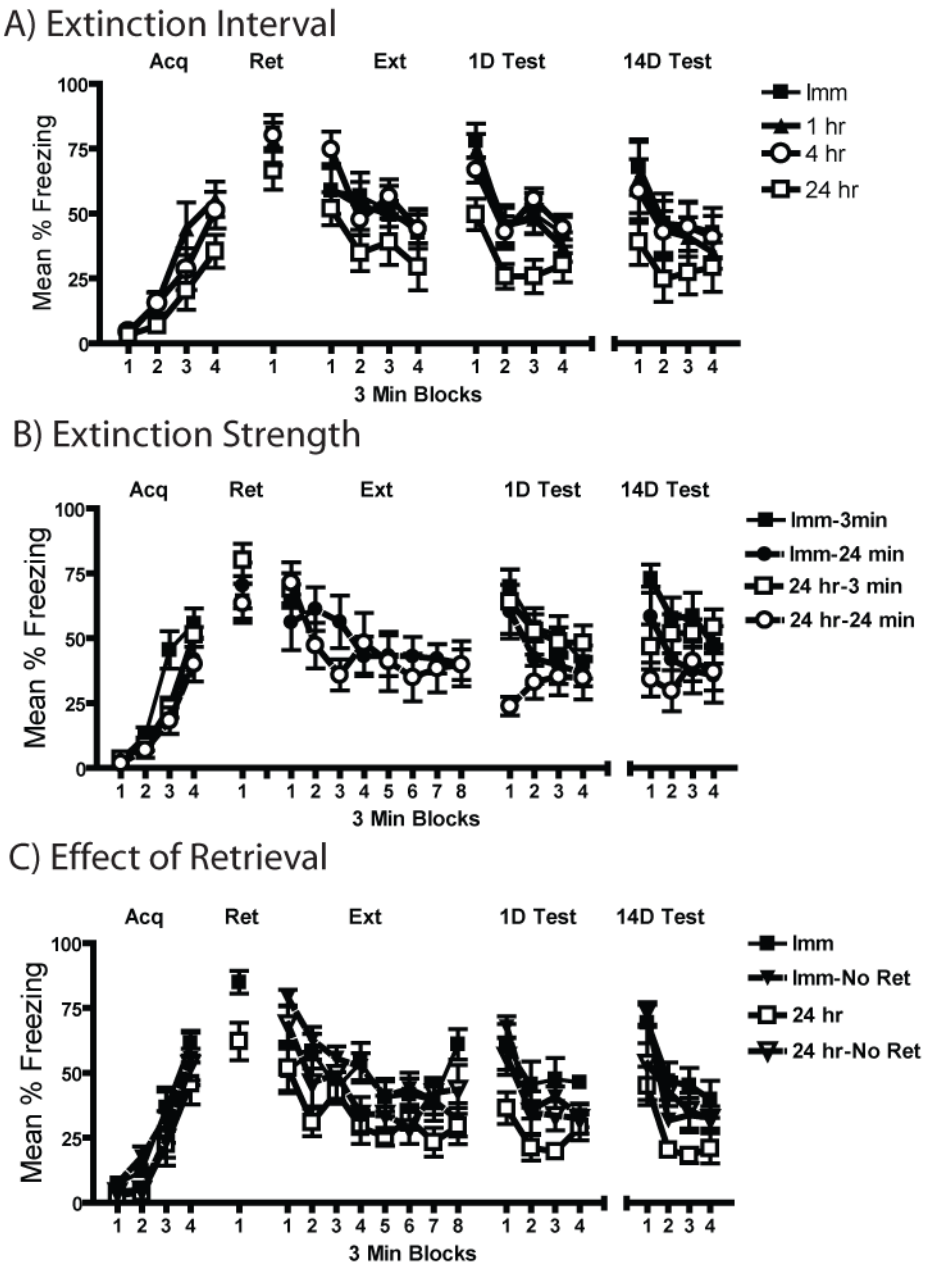
Appendix



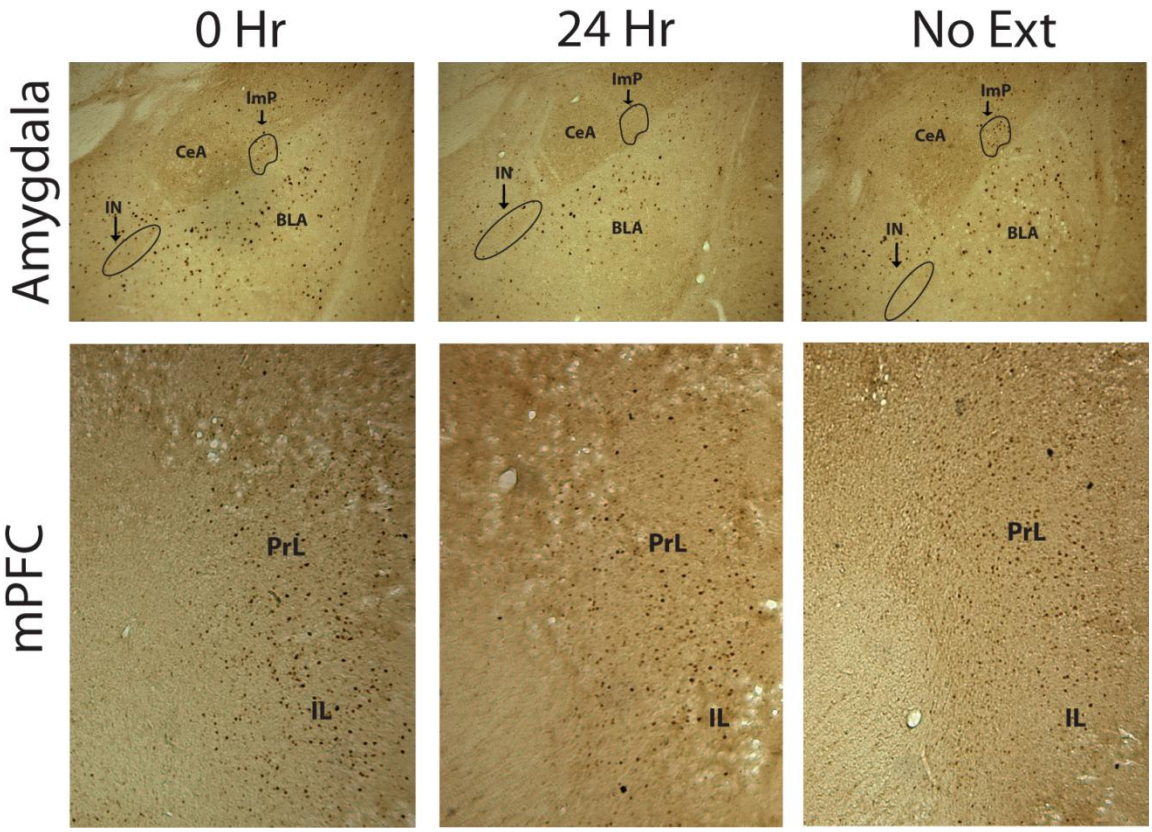
Appendix 1. Sodium butyrate (NaB) effects on locomotor behavior and shock response. **(A, B)** Injection of NaB 15 min prior to a 15 min locomotor test does not alter distance travelled or velocity relative to vehicle treated mice. **(C)** NaB injected 15 min prior to two 2 s footshocks does not alter response to shock during the shock relative to vehicle treated mice.



Appendix 2. A) Both groups receiving a short delay between acquisition and extinction (Imm and 1 hr) showed significantly more spontaneous recovery than those receiving a long delay between acquisition and extinction (4 and 24 hr) during the first 3 min of the 1 D Test (effect of Extinction Recency). Importantly, mice were brought to similar levels of performance levels prior to the 1D test and following the 1 D Test. **B)** Prior to test mice were brought to common levels of performance by the end of the extinction. Mice receiving strong extinction (24 min) at a 24hr delay froze significantly less than those receiving strong extinction immediately following acquisition. **C)** Extinction 50 D following acquisition produces more robust extinction than does immediate extinction, suggesting that even significantly delayed extinction produces more robust response loss than short intervals.



Appendix 3. A) Extinction 24hr after retrieval resulted in less freezing and spontaneous recovery even after mice were brought to common levels of performance. **B)** Only those mice receiving extinction 24hrs after retrieval showed sensitivity to extinction strength.



Appendix 4. Representative c-Fos IHC from recency study

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