THE EFFECTS OF OVEREXPRESSING CREB IN THE LATERAL AMYGDALA ON THE CONSOLIDATION OF AUDITORY FEAR EXTINCTION MEMORY

by

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ABSTRACT

Fear extinction refers to the ability of organisms to adapt as circumstances change by learning to inhibit a previously learned fear. This process is characterized by a progressive reduction in the capacity of the CS to elicit fear following repeated presentations of the CS in the absence of an aversive US. Although the process by which fear memories are acquired and consolidated has been extensively studied, little is known about the neural mechanisms underlying fear extinction. One important advance made towards understanding fear extinction derives from studies showing that extinction represents a form of new learning that actively inhibits, rather than erases, the original CS-US association. As such, it is thought that fear extinction usurps similar mechanisms as fear acquisition. Here, we show that mice receiving CREB infusions in the LA exhibit less conditioned fear than GFP controls during an extinction test given 24 hours following training for extinction. In contrast, both groups of mice displayed a similar reduction in conditioned fear in a training session. Collectively, these results support the notion that overexpressing CREB in the LA facilitates the consolidation of extinction learning.

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INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1. Learning and Memory

The ability to record and store information about stimuli in the environment is fundamental for survival. Learning and memory allows organisms to use past experiences to guide future actions and behaviours. However, due to changing environmental circumstances, it is also important that organisms be able to modify their responses to stimuli. For instance, a child may be reluctant to get on a bike after falling off and hurting himself the first time. This is because the child has formed an association between the bike and getting hurt. If, however, the child got on a bike and rode it without falling off, his fear of bikes will diminish and he would be less reluctant the next time. This reduction of fear caused by repeated exposure to a fearful stimulus without aversive consequences is known as fear extinction. In the laboratory, fear acquisition and extinction have been modeled using classical fear conditioning, especially auditory fear conditioning (Figure 1). Although experiments using this paradigm have produced a wealth of literature on the acquisition of fear memory, much less is known about the neural basis of the extinction of a fear memory. This thesis will explore the role of the transcription factor CREB (cAMP response element binding protein) in the lateral amygdala (LA) on the extinction of a fear memory.

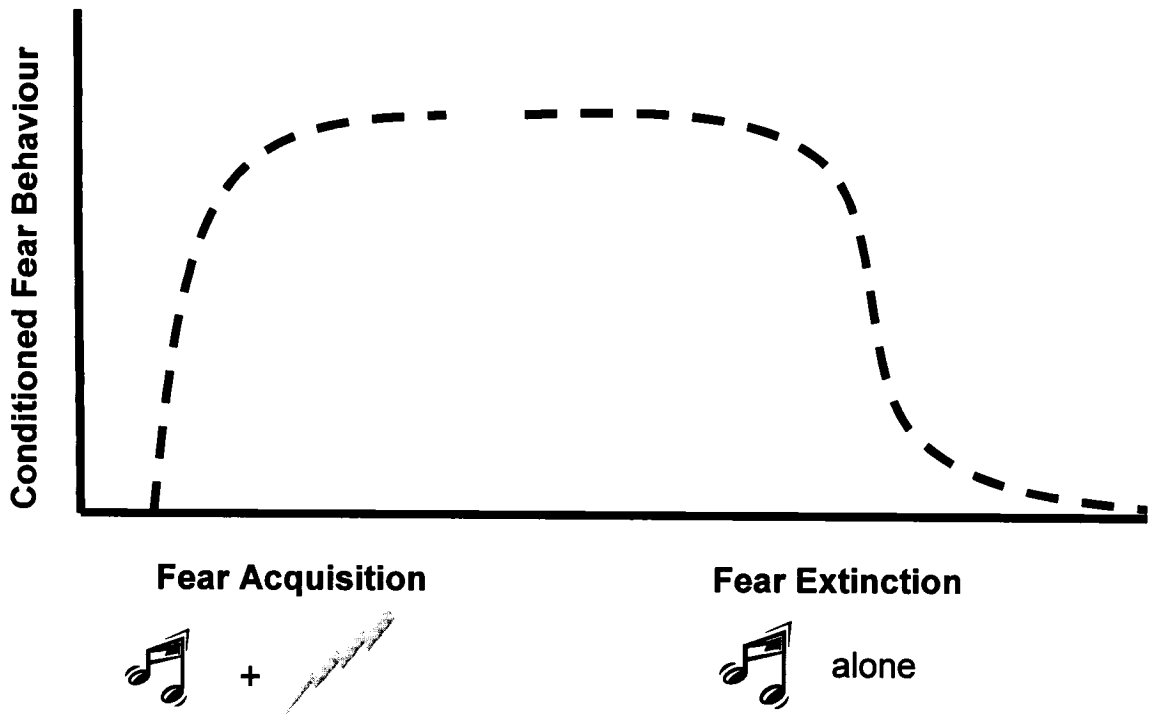


Figure 1. Diagram showing conditioned fear behaviour of fear acquisition (where a previously neutral tone is paired with a footshock) and extinction (where a tone is presented in the absence of a footshock.

1.1.2. Short-Term Memory and Long-Term Memory

Memories come in a variety of forms; some memories are transient while others last a lifetime. In general, these memory phases are classified as short-term memory (STM) and long-term memory (LTM). These types of memories are mediated by distinct molecular mechanisms.

As its name suggests, STM lasts for a short period of time, persisting for minutes to hours. STM is thought to be mediated by covalent modification of pre-existing synaptic proteins (i.e., phosphorlyation of ion channels), producing a short-lived alteration of synaptic function (Stork and Welzl, 1999).

In contrast, LTM lasts for days or longer and is thought to be mediated by the growth and restructuring of synapses, and the formation of new synaptic connections (Bailey and Chen, 1989). Moreover, it is well-established that LTM, but not STM, requires RNA and protein synthesis (Davis and Squire, 1984; Goelet et al., 1986; Kandel, 2001; Kandel et al., 1986; Matthies, 1989).

1.1.3. Molecular Basis of Short-Term Memory

STM is thought to be triggered by calcium influx through N-Methyl-D-Aspartate receptors (NMDARs) (Parada-Turska, J. and Turski, W. A., 1990; Sharma, A. C. and Kulkarni, S. K., 1991). Once inside the cell, calcium activates downstream effectors, such as alpha calcium/calmodulin-dependent protein kinase II (α-CaMKII) (Silva et al., 1992a, 1992b). Activated α-CaMKII undergoes autophosphorylation and proceeds to phosphorylate a second glutamate receptor, alpha-amino-3-hydroxy-5-methyl-4-isoxaolepropionic acid receptor (AMPAR). Phosporylation of AMPARs enhances their

conductance and promotes their insertion into synapses (Barria et al., 1997; Silva et al., 1992a, 1992b). Studies show that glutamate acting on metabotropic glutamate receptors (mGluRs), in particular, mGluR5, contribute to STM (Fendt and Schmid, 2002; Rodrigues et al., 2002). It is believed that they do so by stimulating calcium- and phospholipid-dependent protein kinase C (PKC) via their activation of second messenger cascades and stimulation of calcium release from intracellular stores (Hermans and Challiss, 2001). PKC in turn, enhances NMDAR conductance via phosphorylation of the NR2B subunit (Anwyl, 1999; de Blasi et al., 2001; Liao et al., 2001; see Figure. 2). These mechanisms lead to a short-lived facilitation of synaptic function that mediates STM.

1.1.4. Molecular Basis of Long-Term Memory

Additional signaling pathways are recruited to transform the short-lasting STM into the stable and persistent changes in synaptic function underlying LTM. This process is referred to as memory consolidation. Whereas calcium entry through NMDARs is sufficient to produce STM, calcium influx through both NMDARs and L-type voltage-gated calcium channel (L-VGCCs) may be required to sustain the molecular events involved in LTM for many behaviours, including conditioned fear (Bauer et al., 2002). The rise in intracellular calcium during memory acquisition through NMDARs and L-VGCCs leads to the activation of protein kinase second messenger pathways culminating in the activation of transcription factors (Bauer et al., 2002). For instance, calcium activates a number of protein kinases, including cAMP-dependent protein kinase A (PKA), α-CaMKII, and PKC, which in turn, phosphorylate mitogen-activated protein

kinase (MAPK) (Adams and Sweatt, 2002; Wang et al., 2004; Lin et al., 2001). In its active state, MAPK translocates to the nucleus, where it phosphorylates, and thereby activates, gene transcription factors, such as CREB (Alberini et al., 1995; Silva et al., 1998; Milner et al., 1998). Phosphorylated CREB increases the transcription of a number of target genes that may be important for the structural changes underlying LTM. Protein synthesis is believed to lead to changes in cell morphology that stabilizes the memory, presumably by altering the actin cytoskeleton (Bailey and Kandel, 1993; Woolf, 1998; van Rossum and Hanisch, 1999; Matus, 2000; Rampon and Tsien, 2000; Kasai et al., 2003; Sweatt, 2004). In conclusion, the consolidation of many forms of memory appears to involve the CREB signaling pathway (PKA/PKC→ MAPK → CREB → RNA and protein synthesis → structural changes) (Figure 2).

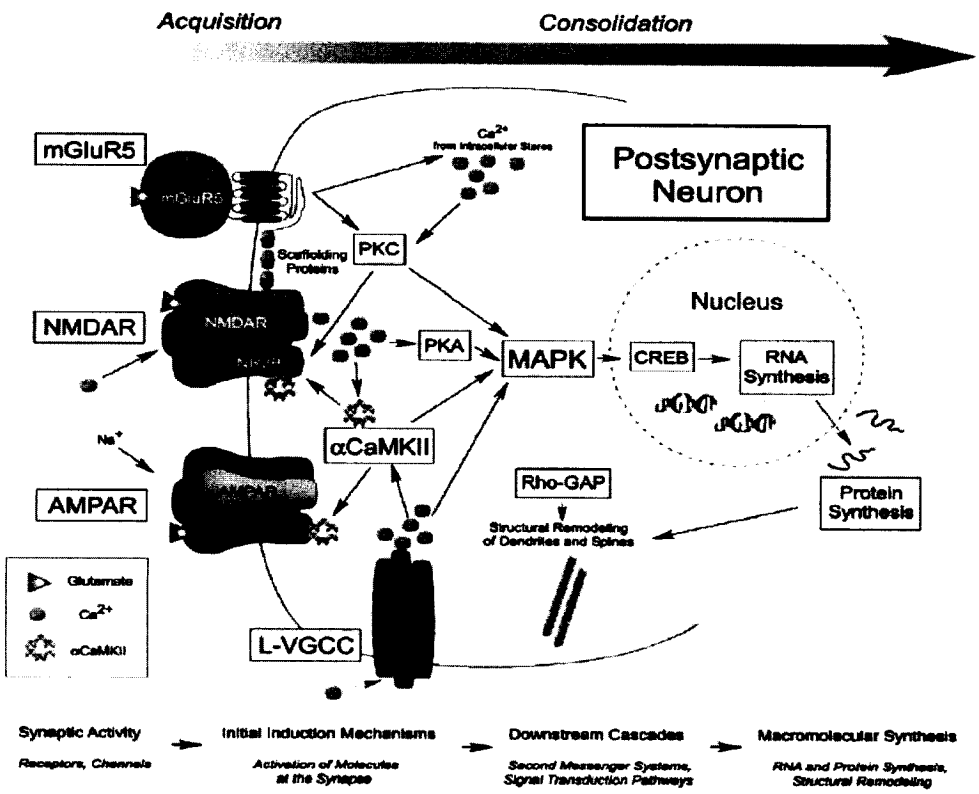


Figure 2. Molecular mechanisms underlying the acquisition and consolidation of fear memory (Adapted from Rodrigues et al., 2004).

1.2. CREB (cyclic adenosine 3,5-monophosphate response element binding protein)

The importance of CREB function in LTM was first shown in studies using invertebrate species (Kaang et al., 1993; Mohamed et al., 2005). CREB is a transcription factor that regulates the expression of genes containing the cAMP response element (CRE) sequence. Many second messenger pathways including, α-CaMKII, PKA, and MAPK (Rodrigues et al., 2004) activate CREB via phosphorylation at a key Ser133 residue (Silva et al., 1998). CREB regulates gene transcription by binding as a dimer to CRE sites within the promoter region of target genes. Upon phosphorylation at Ser133, CREB recruits the transcriptional co-activator CREB-binding protein (CBP) and together, they initiate gene transcription (Mayr and Montminy, 2001).

Converging evidence indicates that LTM in mammals also critically depends on CREB function. First, LTM for conditioned taste aversion was impaired in mice with either a chronic ($CREB^{\alpha\delta-/-}$) or a temporally induced ($CREB^{IR}$) disruption of CREB function (Josselyn et al., 2004). Second, disrupting CREB function by using either an antisense (Lamprecht, Hazvi, and Dudai, 1997) or knock-out approach (Balschun et al., 2003) attenuated LTM measured in conditioned taste aversion and Morris water maze tasks. Third, correlational studies show that robust CREB activation, as measured by CREB phosphorylation, is positively correlated with the formation of LTM in both conditioned taste aversion (Swank, 2000) and contextual fear conditioning (Stanciu et al., 2001). Finally, Josselyn et al. (2001) showed that overexpressing CREB in the lateral amygdala (LA) via viral vector-mediated gene transfer enhanced the consolidation of fear conditioning, as measured by fear-potentiated startle. In summary, this reciprocal

demonstration of loss- and gain-of-function by manipulating CREB levels highlights the importance of CREB function in LTM formation. Based on the well-established role of CREB in memory consolidation, we elected to investigate the effects of increasing CREB levels on the consolidation of memory for fear extinction.

1.3. Pavlovian Fear Conditioning

One way to study fear memory in the laboratory is by using Pavlovian fear conditioning. In this paradigm, a previously neutral stimulus, (the conditioned stimulus, CS) such as a light or tone, acquires the ability to elicit conditioned fear responses after being paired with an aversive stimulus, (the unconditioned stimulus, US) such as a footshock (Figure 3). Two commonly used indices of conditioned fear are behavioural freezing (defined as the cessation of movement except for respiration; Blanchard and Blanchard, 1972; Fendt and Fanselow, 1999) and conditioned suppression (defined as the reduction of bar pressing for a food reward; Kamin, Brimer, and Black, 1963; Bouton and Bolles, 1980). In a typical conditioned suppression experiment, the subject is first trained to press a lever for a food reward. After reaching a steady-state pattern of responding, the Pavlovian conditioning phase is initiated, in which the CS (i.e., tone) is paired with an aversive US (i.e., foot shock). In a subsequent test, subjects receive presentations of the CS in the absence of the US. A high level of conditioned fear is inferred if the animal decreases its ongoing bar pressing during the CS. The observation that animals resume food-rewarded lever pressing soon after the CS is terminated, indicates that the suppression (and fear) is specific to the CS. Although freezing and suppression are treated as separate measures of conditioned fear, they may also overlap in the sense that

suppression of lever pressing occurs because the animal freezes (Bolles, 1970). In other words, subjects cannot press a lever when they are freezing.

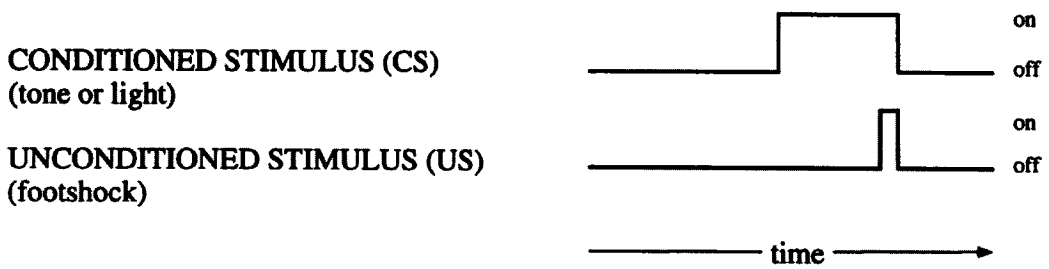


Figure 3. Classical fear conditioning. During fear training, the CS precedes and co-terminates with the US (LeDoux and Muller, 1997).

1.3.1. Auditory Fear Conditioning

The neural circuitry essential for the acquisition of auditory fear conditioning has been extensively studied.

1.3.2. Amygdala

A large body of evidence points to the amygdala, especially the lateral nucleus of the amygdala (LA), as the key neural system subserving auditory fear conditioning. The LA is ideally positioned for this role, as auditory (CS) and nociceptive (US) inputs converge on single neurons in the LA (Romanski et al., 1993; Blair et al., 2001).

1.3.2.1. Anatomical Organization

The amygdala is an almond-shaped limbic structure buried deep within the temporal lobe (Davis, 1997; Fendt and Fanselow, 1999; Lavond et al., 1993; LeDoux, 1996). Information about the CS and US enters the amygdala through the LA (LeDoux, 1996; Aggleton, 2000), where the CS-US association is believed to take place. The LA projects to the central nucleus of the amygdala (Ce) (Pare et al., 1995, 2003; Pitkanen et al., 1997; Savander et al., 1997), which is thought to be the main amygdala output structure sending projections to various autonomic and somatomotor centers that regulate the expression of conditioned fear responses. The LA communicates with the Ce both directly and indirectly via connections to the intercalated cell mass (ITC) and basal nucleus (B) of the amygdala (Sotres-Bayon et al., 2004; Figure 4).

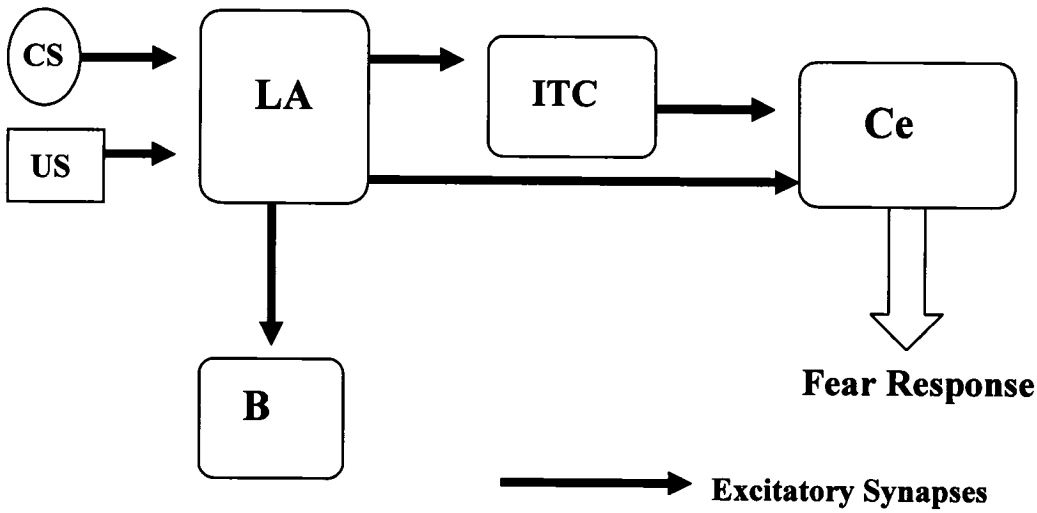


Figure 4. Anatomy of fear conditioning. CS and US inputs converge in the LA, where they are integrated and subsequently transmitted either directly to the central nucleus (Ce) of the amygdala or indirectly via projections to the intercalated cell mass (ITC). The LA also projects to the basal nucleus (B) of the amygdala.

The LA and B are composed of glutamate-containing spiny multipolar cells (pyramidal-like cells) and aspiny GABAergic interneurons (Pitkanen, 2001; Rainnie, 2003). The GABAergic interneurons constitute the main source of local inhibition, whereas the excitatory pyramidal-like cells give rise to connections with the Ce (McDonald, 1985; McDonald and Augustine, 1993; Smith and Pare, 1994; Pare et al., 1995). ITC cells, in contrast, are small, interconnected, densely packed spiny inhibitory GABAergic neurons that receive afferents from the LA and B and project to the Ce (McDonald and Augustine, 1993; Pare and Smith, 1993, 1998; Ghashghaei and Barbas, 2002; Royer and Pare, 2002). Thus, the connections from the LA and B to the CE and ITC are excitatory, whereas the connections from the ITC to the Ce are inhibitory.

1.3.2.2. Neuroanatomical Pathways Underlying Auditory Fear Conditioning

Auditory CS information from the ears is transmitted to the auditory thalamus, specifically the medial division of the medial geniculate nucleus (mMGN) and adjacent posterior intralaminar nucleus (PIN) (LeDoux et al., 1985, 1987; 1990; Doron and LeDoux, 1999). From there, the auditory signal is carried to the LA either directly from the mMGN and PIN (the thalamo-amygdala pathway) or indirectly via the auditory cortex (the thalamo-cortico-amygdala pathway) (LeDoux et al., 1990; Romanski and LeDoux, 1993; Doron and LeDoux, 1999; Pitkanen et al., 1997; McDonald, 1998).

Although each pathway is fully capable of supporting auditory fear conditioning (Romanski and LeDoux, 1992), lesion studies show that they participate to different degrees. For example, pre-training lesions of the direct pathway, but not the indirect pathway, impairs fear conditioning to a tone CS (Romanski and LeDoux, 1992; DiCara et

al., 1970; Romanski and LeDoux, 1992b; Teich et al., 1988). Furthermore, Morris et al. (1999) observed that amgydala activity measured during fear conditioning was more strongly correlated with activity in subcortical (thalamic and collicular), rather than cortical, areas (Morris et al., 1999). These findings suggest that the direct pathway is sufficient to mediate fear conditioning and that the auditory cortex may not be critical for this task.

While the CS pathway to the amygdala is well characterized, the US pathway to the amygdala is poorly understood. However, several studies indicate that multiple parallel pathways originating in the cortex, thalamus, and spinal cord may be involved in relaying nociceptive (US) information to the LA during fear conditioning (Figure 5; LeDoux et al., 1987; Shi and Davis, 1999; Brunzell and Kim, 2001; Lanuza et al., 2004).

Once inputs from the direct and indirect pathways are processed in the amygdala, the signal is thought to be sent to the Ce, which in turn, innervates brainstem regions that control the expression of conditioned fear responses (Pare et al., 1995; Pitkanen et al., 1997).

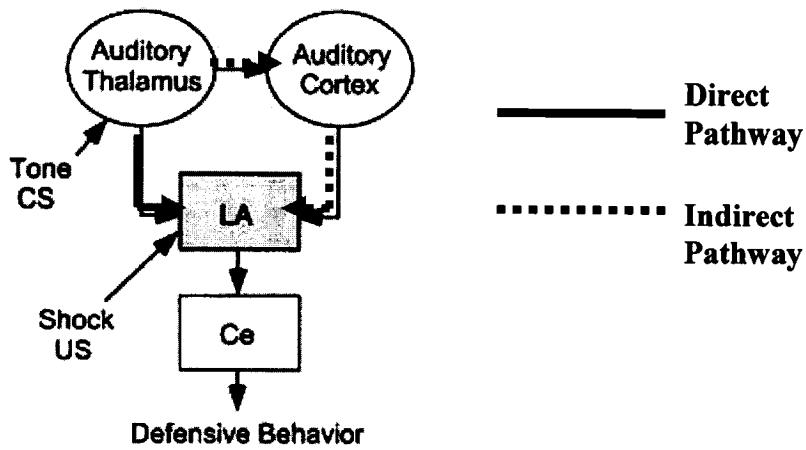


Figure 5. CS and US pathways converge in the LA. During auditory fear conditioning, the tone CS input reaches the LA via a direct thalamic pathway and an indirect cortical pathway (Blair et al., 2001). Information about the US is also directed to the LA by pathways that are poorly characterized. The LA, in turn, projects the integrated signal to the Ce, which drives defensive and autonomic responses to the CS.

1.3.2.3. The LA is Critical for Auditory Fear Conditioning

Results derived from lesion, electrophysiological, behavioural and pharmacological studies indicate that the LA is critical for the acquisition of fear conditioning.

1.3.2.3.1. Ablation of the LA Disrupts the Acquisition of Conditioned Fear

Electrolytic and excitotoxic lesions of the LA impaired the acquisition of Pavlovian fear conditioning to a tone or light CS (LeDoux et al., 1990; Amorapanth et al., 2000; Goosens and Maren, 2001; Nader et al., 2001; Anglada-Figueroa and Quirk, 2005). Furthermore, functional inactivation of the LA with the GABA agonist muscimol during fear conditioning prevented the acquisition of auditory fear conditioning (Muller et al., 1997).

Importantly, electrolytic lesions of the LA reduced freezing and blood pressure responses elicited by the CS to levels seen in a non-associative control group that received the same number of CS-US presentations, but in an unpaired fashion (LeDoux et al., 1990). This suggests that LA lesions do not interfere with the processing of the CS and US, but instead interfere with the ability to form the CS-US association.

Although these findings suggest that neural activity in the LA is important for the acquisition of auditory fear conditioning, there is considerable evidence indicating that an intact LA is also necessary for the expression of unconditioned fear responses (Walker and Davis, 1997; Davis, 1997). Therefore, it is difficult to dissociate the effects of lesions on learning from the effects on performance (Fanselow and LeDoux, 1999; Cahill

et al., 1999, 2001). As a result, lesion studies alone cannot fully substantiate the notion that the LA is critically involved in the acquisition of conditioned fear.

1.3.2.3.2. LTP-like Mechanisms in the Amygdala: A Cellular Model of Fear Conditioning?

Long-term potentiation (LTP) is thought to be a cellular model of learning (Malenka and Nicoll, 1993, 1999; Milner et al., 1998). Electrophysiological recording studies showed the existence of LTP in sensory input synapses to the LA. For example, several studies using *in vitro* brain slices demonstrated that LTP can be induced in CS pathways (thalamo-LA and cortico-LA) to the LA (Clugnet and LeDoux, 1990; Maren and Fanselow, 1995; Rogan and LeDoux, 1995; Huang and Kandel, 1998; Weisskopf and LeDoux, 1999). Furthermore, *in vivo*, pairings of the CS and US during fear conditioning lead to changes in the electrophysiological responses of cells in the LA that resemble LTP. For example, the response of cells in the LA to a tone CS or to electrical stimulation of CS pathways to the LA is enhanced following fear conditioning (Quirk et al., 1995, 1997; McKernan and Shinncik-Gallagher, 1997; Rogan et al., 1997; Weisskopf et al., 1999; Collins and Pare, 2000; Maren, 1999, 2000; Repa et al., 2001). Therefore, neural activity in the LA is altered during auditory fear conditioning in a manner that is similar to artificial LTP induction. Together, these findings strongly suggest that LTP in the LA may be a mechanism underlying fear conditioning.

1.3.2.3.3. Pharmacological Studies

Pharmacologically blocking NMDARs in the LA has been shown to disrupt the acquisition of fear conditioning. For example, systemic administration of the competitive NMDAR antagonist CPP given prior to auditory fear conditioning abolished the acquisition of conditional freezing and conditional single-unit activity in the LA in rats (Goosens and Maren, 2004). Similarly, pre-training infusions of the NMDAR antagonist APV directly into the LA blocked the acquisition of contextual (Fanselow and Kim, 1994) and auditory fear conditioning measured using fear-potentiated startle (Miserendino et al., 1990; Campeau et al., 1992; Gewirtz and Davis, 1997; Walker and Davis, 2000) and freezing to a visual CS (Lee and Kim, 1998). However, it is not clear whether APV specifically blocks acquisition, rather than performance, as similar infusions of APV before testing have also been shown to block the expression of conditioned fear as measured by freezing (Fendt, 2001; Lee et al., 2001; Maren et al., 1996; Lee and Kim, 1998), but not fear-potentiated startle (Gewirtz and Davis, 1997). To address this issue, Rodrigues et al. (2001) showed that pre-training infusion of ifenprodil, a selective blocker of NR2B-containing NMDARs, into the LA impaired acquisition of auditory fear conditioning, but did not affect the expression of a previously learned fear response (Rodrigues et al., 2001). Together, these findings suggest that NMDARs, in particular the NR2B subunit, are essential for the acquisition, but not expression, of fear memory.

L-VGCCs have also been implicated in the consolidation of LTM of auditory fear conditioning. Pre-training infusion of the L-VGCC blocker verapamil into the LA dose-

dependently impaired conditioned freezing to a tone CS when tested 24, but not 1, 3, and 6 hours, after training (Bauer et al., 2002).

Collectively, these data suggest that calcium entry through NMDARs and L-VGCCs is necessary for fear memory formation in the LA, however, NMDARs appear to be more important for fear acquisition, whereas L-VGCCs are selectively required for the long-term storage of fear memory.

During fear learning, the increase in intracellular calcium through both NMDARs and L-VGCCs leads to activation of a number of protein kinases. There is evidence suggesting that α-CaMKII is involved in fear conditioning. Specifically, intra-LA infusions of the α-CaMKII antagonist KN-62 dose-dependently impaired the acquisition, but not expression, of auditory and contextual fear conditioning measured 1 and 24 hours after conditioning in rats (Rodrigues et al., 2004). Moreover, the behavioural training used to induce fear memory was associated with an increase of the autophosphorylated (active) form of α-CaMKII in the LA. Finally, using the tetracycline transactivator system, Mayford et al. (1996) showed that high levels of expression of a mutant form of α-CaMKII in the LA and striatum during training caused a deficit in both contextual and cued fear conditioning. Interestingly, this effect was reversed when transgene expression was suppressed with doxycycline. This suggests that calcium-dependent activation of α-CaMKII and its subsequent autophosphorylation in the LA may be an important mechanism underlying the formation of fear memory.

As with other forms of LTM, previous studies have shown that LTM for auditory fear conditioning also depends on PKA and MAPK. For example, intra-amygdala infusions of the PKA inhibitor Rp-cAMPS immediately after conditioning spared STM, but dose-dependently impaired the formation of LTM for auditory fear conditioning (Schafe and LeDoux, 2000). Furthermore, transgenic mice that overexpress R(AB), an inhibitory isoform of PKA, in forebrain regions, including the LA and hippocampus, showed deficits in LTM, but not STM for contextual fear conditioning (Abel et al., 1997). Considerable evidence indicates that the MAPK signaling pathway is required for memory consolidation (Adams and Sweatt, 2002; Chen et al., 1998; Wang et al., 2004; Lin et al., 2001). For example, disrupting MAPK activity by intra-LA infusions of U0126 dose-dependently interfered with LTM for auditory fear conditioning (Schafe et al., 2000). Likewise, rats injected intraventricularly with PD098059, a MAPK inhibitor, prior to conditioning exhibited a selective impairment in LTM for both auditory and contextual fear conditioning (Schafe et al., 1999).

PKA and MAPK signals converge on the transcription factor CREB (Alberini et al., 1995; Silva et al., 1998; Milner et al., 1998). A number of studies in rodents implicate CREB in LTM for fear conditioning. First, genetic disruption of the alpha and delta isoforms of CREB ($CREB^{\alpha\delta-/-}$) selectively impaired LTM for fear conditioning (Bourtchuladze et al., 1994; Kogan et al., 1996; Falls et al., 2000). Second, overexpression of CREB in the LA using viral transfection methods facilitates long-term fear memories in both auditory fear conditioning (Wallace et al., 2004) and fear-

potentiated startle (Josselyn et al., 2001). Third, an increase in the phosphorylated form of CREB was observed in the LA after contextual fear conditioning (Stanciu et al., 2001).

It is well-established that RNA and protein synthesis are required for LTM. Consistent with this view, rats receiving microinjections of the RNA synthesis inhibitor actinomycin-D into the LA, before or after training, showed a selective impairment of LTM for auditory and contextual fear conditioning (Bailey et al., 1999; Duvarci et al., 2003). Furthermore, intra-LA infusion of the protein synthesis inhibitor anisomycin immediately following fear training disrupts LTM, but not STM, for auditory fear conditioning (Bailey et al., 1999; Schafe et al., 1999, 2000; Nader et al., 2000; Schafe and LeDoux, 2000; Maren et al., 2003).

In summary, these findings support the notion that calcium influx through NMDARs and L-VGCCs in the LA during fear learning activates a number of protein kinases, including α-CaMKII, PKA and MAPK, which in turn, activate CREB via phosphorylation. Phosphorylated CREB then promotes the consolidation of fear memories by initiating the production of new RNA and proteins. Although CREB has been the best studied transcription factor with regard to memory consolidation, it is probably not the only one involved in this process.

1.4. Fear Extinction

Once acquired, conditioned fear may last a lifetime (Gale et al., 2004). However, the expression of conditioned fear acquired under one set of circumstances may be inappropriate or even disadvantageous under a different set of circumstances. For example, reacting to a CS that no longer predicts a realistic threat may cost the organism valuable time foraging for food. Therefore, it is also important to understand the neural mechanisms underlying the process of fear inhibition or reduction. From a clinical perspective, findings from studies of fear inhibition may provide insights into the clinical states associated with dysfunctions in this process, including anxiety disorders.

The predominant model system for studying fear inhibition in the laboratory is extinction. Extinction of learned fear represents the ability to adapt to changing circumstances in the environment. It is characterized by a progressive reduction in the capacity of the CS to elicit fear following presentations of the CS in the absence of an aversive US.

A large body of literature indicates that, contrary to conventional wisdom, extinction is a form of new learning that actively inhibits, rather than erases, the original CS-US association (Bouton 2002; Davis and Myers 2002). In other words, during extinction, animals learn that the CS no longer predicts the US and this newly generated CS-noUS contingency competes with the original fear memory to inhibit conditioned fear responses (Figure 6). Support for this notion comes from studies showing that extinguished fear can re-emerge with the passage of time (spontaneous recovery) (Pavlov 1927; Robbins 1990), following unsignaled exposure to the US (reinstatement) (Rescorla and Heth 1975; Bouton and Bolles 1979a), or when tested in a context different from the

one in which extinction training occurred (renewal) (Bouton and Bolles 1979b; Bouton and Swartzentruber 1986). This indicates that extinction forms a new memory (CS-noUS) that actively suppresses, rather than erases, the original fear memory.

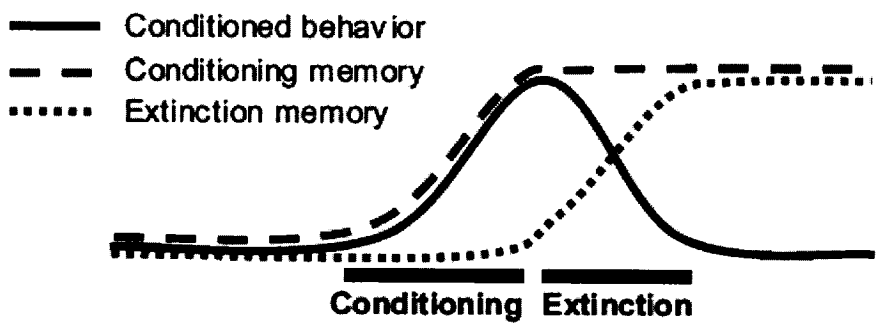


Figure 6. Schematic diagram showing the competition between an established conditioned memory (long dashed curve) and a newly formed extinction memory (short dashed curve) for control over conditioned fear behaviour (Quirk et al., 2006).

Extinction of fear can be divided into 2 temporally distinct phases. Within-session extinction refers to the attenuation of fear that occurs over the course of CS presentations in a single session. On the other hand, between-session extinction or the consolidation of extinction refers to the reduction of fear evoked by the first CS presentation on each day (Figure 7). Within-session extinction and between-session extinction are analogous to STM and LTM, respectively. This thesis will specifically examine the effects of CREB overexpression on the consolidation of extinction.

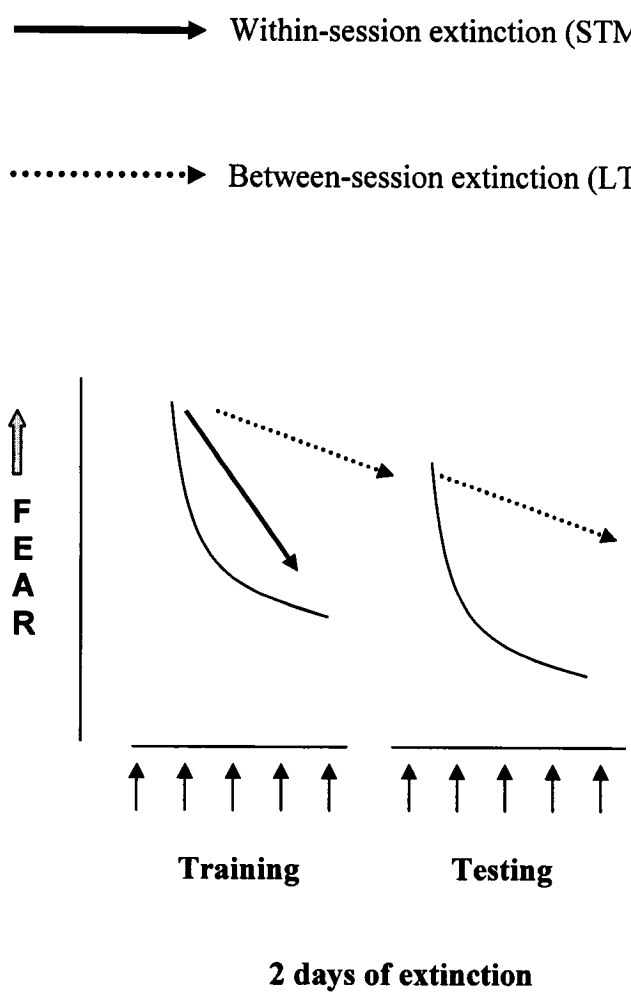


Figure 7. Schematic illustration of within- and between-session extinction of a fear memory. Each arrowhead on the x-axis represents that presentation of a CS alone. During a session, the CS elicits a fear response which diminishes over the course of the five presentations (within-session extinction or STM). The fear evoked by the first CS presentation decreases across days (between-session extinction or LTM).

1.4.1. Neuronal Substrates of Fear Extinction

In contrast to the acquisition of fear conditioning, research on the neural basis of fear extinction is still in its infancy. However, findings from recent studies implicate two brain regions in fear extinction; the medial prefrontal cortex (mPFC) and the LA.

1.4.1.1. Medial Prefrontal Cortex (mPFC) – Infralimbic Cortex (IL)

Several lines of evidence suggest that the medial prefrontal cortex (mPFC), especially the infralimbic (IL) subregion of the mPFC, is important for extinction of conditioned fear.

1.4.1.1.1. Disrupting IL Function Prevented the Consolidation of Extinction Memory

Electrolytic lesions of the IL made prior to fear conditioning and extinction training disrupt the consolidation of extinction learning, as measured by freezing and suppression of bar pressing, 24 hours after extinction training (Morgan et al., 1993; Quirk et al., 2000). Importantly, lesions of the IL had no effect on the acquisition of conditioned freezing or extinction that occurred within a session. Furthermore, rats given bilateral infusions of the protein synthesis inhibitor anisomycin into the mPFC prior to extinction training extinguished normally within an extinction training session, but did not show evidence of extinction the next day (Santini et al., 2004). As such, extinction and acquisition of fear appear to be similar, to the extent that in both, LTM, but not STM, is disrupted by protein synthesis inhibition

1.4.1.1.2. Neural Activity in the mPFC Is Correlated With and Important for Fear Extinction

Electrophysiological data also support a role for the mPFC in extinction. For instance, neurons in the IL (but not in adjacent prelimbic or medial orbital cortex) fire to a tone CS only when rats were tested for extinction 24 hours after extinction training, but not during fear conditioning or extinction training (Milad and Quirk, 2002). Moreover, rats that froze the least during an extinction test (24 hours after extinction training) showed the greatest IL tone responses. Interestingly, conditioned tones paired with electrical stimulation of the IL produced low freezing on a 24 hour test in rats that had not been extinguished, suggesting a causal relationship between IL activity and extinction memory. Also, Herry and Garcia (2002) observed that induction of plasticity by applying LTP-inducing high-frequency stimulation of thalamic inputs to the mPFC before extinction training had no effect on within-session extinction, but enhanced extinction of freezing to a conditioned tone measured 1 week after extinction training (Herry and Garcia, 2002). These data suggest that activity in the IL is important for the consolidation of extinction memory.

Barrett et al. (2003) mapped metabolic neural activity after extinction using a radiolabeled glucose analog and showed that mPFC activity is elevated during a retrieval test 24hours after extinction training (Barrett et al., 2003). Consonant with animal research, PET and fMRI imaging studies have shown that areas of the mPFC in humans were engaged during recall of extinction learning (Hugdahl et al., 1995; Phelps et al., 2004) and subjects with posttraumatic stress disorder have reduced mPFC activity when recalling a traumatic event compared to non-traumatized healthy subjects (Bremner et al., 1999).

Collectively, the above findings suggest that the mPFC may be a critical storage site for extinction memory. However, several studies have questioned this. For example, Gewirtz et al. (1997) reported normal extinction of freezing and fear-potentiated startle following electrolytic lesions of the mPFC in rats. Likewise, a recent study showed that electrolytic lesions of the mPFC made either 1 week before conditioning or 1 day after extinction training did not disrupt STM or LTM for extinction of auditory fear conditioning in rats (Garcia et al., 2006). In addition, Farinelli et al., (2006) has shown that under certain circumstances, fear extinction can be recalled without mPFC potentiation. Therefore, the precise role of the mPFC in the consolidation of fear extinction memory is debatable.

1.4.2. Neural Circuitry – mPFC-Amygdala Interactions

One theory posits that extinction involves a process by which neural activity in the mPFC comes to regulate the amygdala-mediated expression of conditioned fear responses (Milad et al., 2004; Sotre-Bayon et al., 2004). Currently, there are two competing neural models for mPFC regulation of amygdala output during extinction of conditioned fear. According to the first model (Figure 8A), activation of the IL and PL excite inhibitory interneurons within the LA and B, respectively, with a consequent inhibition of LA and B activation of CE neurons (Grace and Rosenkranz 2002). The second model (Figure 8B) argues that IL stimulation excites inhibitory projection neurons in the ITC, which in turn, inhibit CE output (Quirk et al., 2003). In both cases, however, mPFC-induced suppression of amygdala output is thought to be one mechanism

underlying the reduction of conditioned fear that occurs during extinction (Royer and Pare 2002; Rosenkranz et al., 2003).

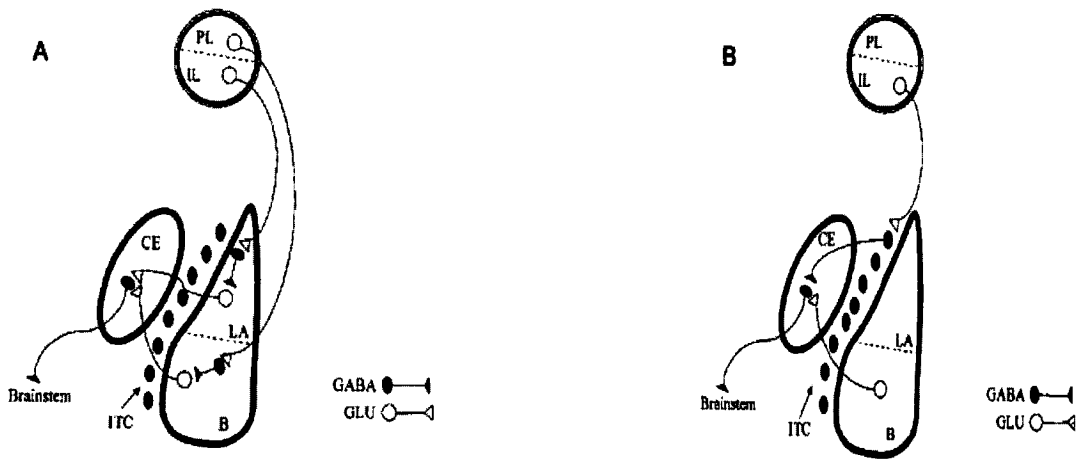


Figure 8. Neural models for mPFC regulation of amygdala-mediated conditioned fear responses during extinction (Sotres-Bayon et al., 2004). (A) Activation of the IL and PL excite inhibitory interneurons within the LA and B, respectively, with a consequent inhibition of LA and B activation of CE (called Ce in Figure 4) neurons. (B) IL stimulation excites inhibitory projection neurons in the ITC, which in turn, inhibit CE output.

1.4.3. Lateral Amygdala (LA)

Accumulating evidence from pharmacological studies suggests that the LA may be critically involved in the extinction of fear memory. For example, intra-amygdala infusions of APV in rats immediately before extinction training dose-dependently blocked the consolidation of extinction of conditioned fear, as assessed with fear-potentiated startle measured 24hours following extinction training. In contrast, infusions of APV into the interpositus nucleus of the cerebellum failed to block extinction consolidation (Falls et al., 1992; Lee and Kim 1998; Lin et al., 2003), indicating that the effect of APV on extinction is brain-region specific. Conversely, infusions of D-cycloserine, a partial agonist at the strychnine-insensitive glycine-recognition site on the NMDAR, into the LA 15 minutes prior to extinction training facilitated extinction of conditioned fear measured 24 hours later. Furthermore, this effect was offset by HA-966, an antagonist at the glycine-recongnition site. Importantly, in a separate group of rats that did not receive extinction training, it was found that neither DCS nor HA-966 altered fear-potentiated startle responses when injected either 30 or 40 minutes before testing. Thus, it is unlikely that these drugs influence extinction by increasing fear or by disrupting CS processing (Walker et al., 2002; Ledgerwood et al., 2003). Taken together, these findings suggest that NMDARs in the LA are involved in the consolidation of fear extinction.

Similar to the consolidation of fear conditioning, the consolidation of fear extinction seems to depend on MAPK and BDNF signaling in the LA. For example, inhibiting MAPK activity in the LA with PD98059 blocked extinction of conditioned fear assessed 24 hours after extinction training (Lu et al., 2001). Furthermore, because fear

extinction is a form of new learning, it might be expected to depend on protein synthesis (Davis and Squire, 1984). Indeed, infusions of anisomycin into the LA following extinction training impaired extinction measured 20 minutes after extinction training (Lin et al., 2003). However, whether this treatment also blocked the consolidation of extinction, measured 24 hours following training, was not examined.

Recent evidence shows that brain-derived neurotrophic factor (BDNF) acting through tyrosine kinase B receptors (TrkB) within the LA is also important for the consolidation of extinction. Previously, it has been shown that disrupting BDNF function in the LA blocks the consolidation of fear memory (Barbacid, 1995; Rattiner et al., 2004). Moreover, it was shown that extinction training induced expression of BDNF mRNA in the LA 2 hours, but not 30 minutes or 4 hours, after training (Chhatwal et al., 2006). Consistent with this, disrupting BDNF function blocked the consolidation of fear extinction. Specifically, expressing a dominant-negative truncated tyrosine kinase B receptor in the LA using lentiviral vectors blocked the consolidation of extinction of conditioned fear assessed 2 days after training, but spared within-session extinction. Therefore, BDNF activation of TrkB receptors in the LA is important for the consolidation of fear acquisition and extinction. Interestingly, BDNF is intimately involved with CREB. For instance, BDNF is a target gene of CREB activation (West et al., 2001) and BDNF signaling through TrkB receptors activate MAPK and CREB (Patterson et al., 2001).

Together, these findings emphasize the possibility that the LA is crucial for the consolidation of extinction memory and that similar signal transduction cascades may be involved in both the consolidation of fear memory and extinction of fear memory.

Based on these results and the similarities between the molecular mechanisms underlying fear acquisition and extinction, the present thesis will investigate whether increasing CREB function in the LA enhances the consolidation fear extinction as it does in fear acquisition. In order to assess the effect of increasing CREB function on extinction of fear memory, it was crucial that all mice acquired fear conditioning equally. To this end, mice were fear conditioned in a drug-free state and then CREB levels were increased specifically in the LA before extinction training.

1.5. Herpes Simplex Virus Type 1 (HSV-1)

To increase CREB function in a temporally and spatially specific manner, we used a viral vector, specifically, the replication-defective herpes simplex virus (HSV) type 1 (the common cold sore virus). Increasing CREB function could not be accomplished by injecting drugs into the brain because there are no pharmacological agents that specifically increase CREB function. Additionally, transgenic mice that overexpress CREB could not be used because the technology available today lacks the required spatial specificity.

1.5.1. HSV-1 Structure and Infection

HSV-1 is a 150kb double-stranded enveloped DNA virus that carries over 75 genes (Neve et al., 2005). Unlike other viruses, the capsid is enclosed in a lipid bilayer membrane bearing glycoproteins that play a role in cellular infection (Figure 9). HSV-1 is capable of infecting most adult mammalian differentiated cell types, including neurons (Neve et al., 2005). HSV-1 infection is initiated when the virus attaches to the host membrane by non-specific charge interactions between viral envelope glycoproteins and proteoglycans on the cell surface (Spear, 1993; Neve et al., 2005). The virus enters the host cell by a process called receptor-mediated endocytosis. Within the cell, the virus is transported along microtubules from the site of entry to the nucleus of the neuron, into which viral DNA is released. From here, the virus has the option of replicating or entering a latent (dormant) state (Neve et al., 2005).

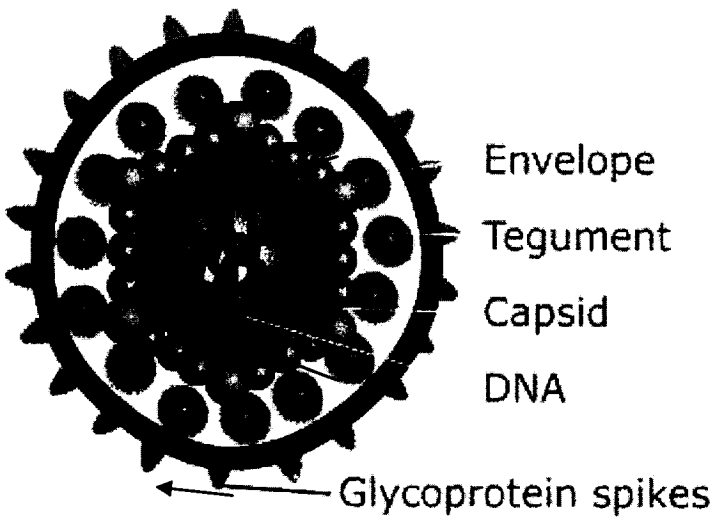


Figure 9. Cartoon depicting HSV-1 structure (Josselyn).

1.5.2. Advantages of Using HSV-1

I elected to use HSV for several reasons. First, neurons are the natural target of HSV and HSV vectors infect adult neurons with high efficiency. In addition, the DNA from HSV vectors remain episomal, thus avoiding potentially confounding effects associated with integration into the host DNA. Following infusion, we observe strong localized transgene expression produced by HSV vectors. Importantly, infusion of HSV vectors produces minimal cytotoxic damage (similar to that produced by vehicle injection) (Carlezon et al., 1997; Bursztajn et al., 1998; Coopersmith and Neve, 1999).

1.5.3. Methods Used to Generate HSV-1 Vectors

There are two methods of using HSV to produce replication-defective viral vectors. The first involves cloning the gene of interest into the viral genome itself. These are known as genomic vectors (Roizman and Jenkins, 1985; Neve et al., 2005). The second involves creating a plasmid carrying minimal sequences from the wild-type HSV genome that allows it to be packaged into viral particles with the aide of a helper virus (also replication-defective). These are referred to as amplicon vectors and they are the ones that we have chosen to use. The amplicon contains a packaging "a" site and an origin of replication site (Ori_S), which enables them to replicate and be packaged into virus particles in the presence of replication-defective helper virus (Neve et al., 2005). The amplicon also includes an HSV immediate early (IE) 4/5 promoter that drives the expression of the inserted cDNA (in our case, CREB-GFP or GFP) and a simian virus 40 (SV40) poly-adenlylation site which is important for RNA processing (Spaete and Frenkel, 1982, 1985; Stowe and McMonagle, 1982; Neve et al., 2005; Figure 10). To

visualize infected neurons, we will fuse a green fluorescent protein (GFP) tag to the 3' end of the CREB cDNA. As a control for viral infusion, we will infuse HSV-GFP.

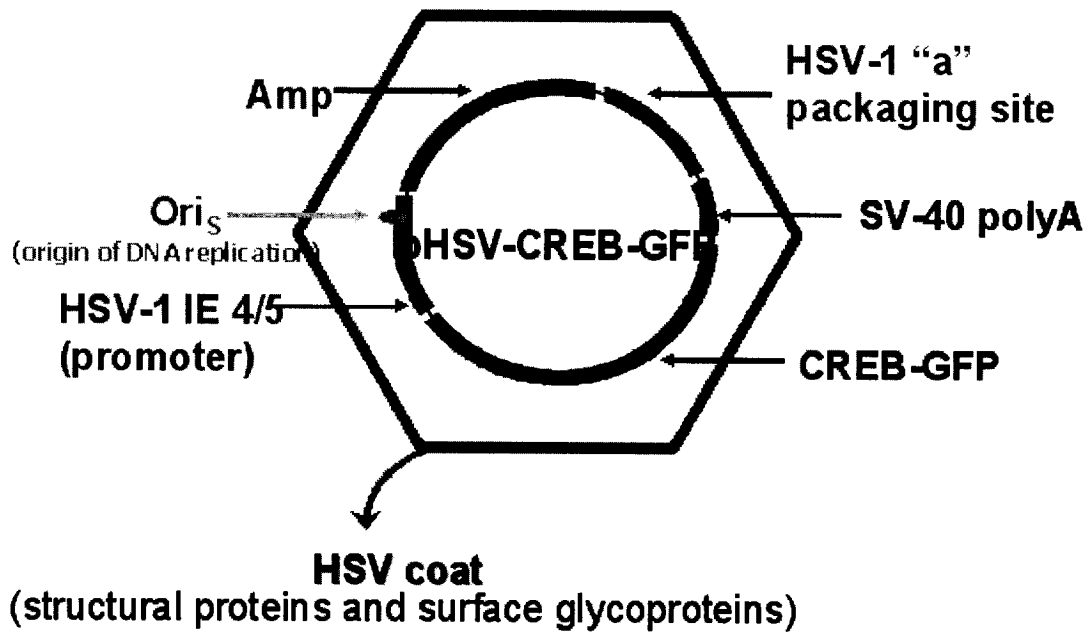


Figure 10. Cartoon depicting the HSV amplicon vector pHSV-CREB-GFP that will express CREB-GFP (Josselyn).

1.6. Hypothesis

We predict that overexpressing CREB in the LA will enhance the consolidation of extinction memory. Specifically, we expect that mice injected with HSV-CREB-GFP into the LA will show greater levels of extinction, measured 24 hours after extinction training, than mice injected with only HSV-GFP.

METHODS

CHAPTER 2: MATERIALS AND METHODS

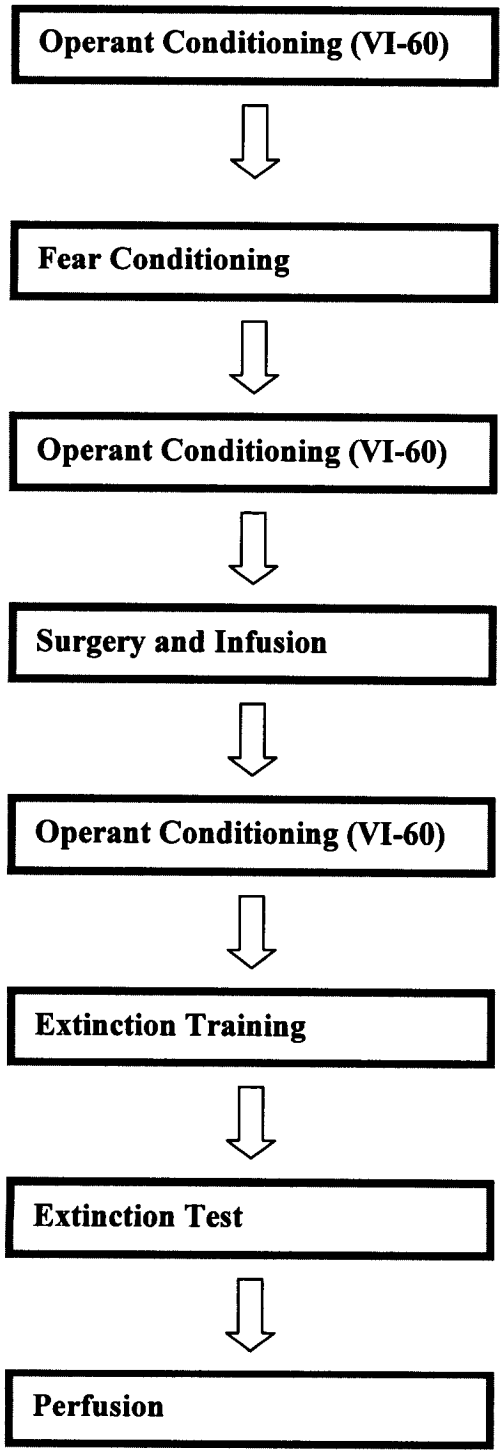
2.1. Subjects

Female F1 hybrid C57Bl/6 x 129Svev mice bred in our colony and aged at least 2 months were used for the experiments. Mice were housed in groups of 4-5 in transparent polyethylene cages and maintained on a 12 hour light/dark schedule with free access to water. Before starting the experiments, the mice were deprived of food overnight to achieve a weight of 90% of their free-feed weight. Thereafter, they were kept on a food-restricted diet to maintain them at 90% of their free-feed body weight. The mouse-housing facilities have been certified by the Canadian Council of Animal Care. All experimental procedures were conducted in accordance with the Hospital for Sick Children Animal Care and Use Committee Guidelines.

2.2. Generation of Recombinant HSV Viral Vectors

cDNAs for GFP and CREB-GFP were inserted into the herpes simplex virus (HSV) amplicon HSV-PrpUC as previously described (Lim et al., 1996; Carlezon Jr. et al., 1997; Neve et al., 1997). The resulting amplicon was packaged with the aid of a helper virus containing a deletion in the *5dl*1.2 gene (Neve et al., 1997). The replication-defective virus was subsequently purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose. The average titer of the recombinant virus stocks was $4.0x10^7$ infectious U/ml and was similar for HSV-CREB-GFP and HSV-GFP. Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene IE 4/5. Using this promoter, transgene expression peaks 3-4 days post-infusion and is absent 10 days post-infusion (Carlezon et al., 1998).

2.3. Experimental Design



6 Weeks - 3 Months
mice trained to bar-press for sucrose pellets.
Reinforcement schedules gradually increased to VI-60

Day 0
mice received 6 tone-shock pairings

Day 1
mice re-trained on VI-60 schedule

Day2
mice received either HSV-CREB-GFP or HSV-GFP

Days 3-4
mice re-trained on VI-60 schedule

Day 5
mice received 6-tone alone presentations

Days 6
mice received 20-tone alone presentations

Day 7
brains processed for histology to confirm placement of viral infections

2.3.1. Bar-Press Training

The overall training protocol for extinction training and testing was adapted from Quirk et al. (2000; see above). Mice were trained to press a bar for sucrose pellets in a standard operant chamber (18 x 25 x 21cm) with plexiglas walls and a steel bar floor. The chamber included retractable response levers and a magazine hopper on one wall. The chamber was housed in a sound-attenuating box (Med Associates). Pellet delivery was controlled by a computer running commercial behavioural software (MED-PC). Before training, mice were given novel sucrose pellets (10 mg; BioServ) used for bar-press training (with free access to water) in separate cages. On the first day of training, mice were placed in the operant chambers with the levers retracted and received a sucrose pellet delivered to the hopper every 60 seconds for 30 minutes (magazine training). This magazine training continued until mice consumed at least 15 pellets. Following this, bar-press training was initiated. First, mice received one sucrose pellet for each bar press (continuous reinforcement schedule). Once mice earned 15 pellets under this schedule, the reinforcement ratio was gradually reduced in the following order; variable interval 5 (VI-5) → VI-10 → VI-30 → VI-45 → VI-60. Mice had to acquire a criterion of 30 pellets (on the VI-5, VI-10, and VI-30 schedules) or 60 pellets (on the VI-45 schedule) within the 60-minute session to progress to the next reinforcement schedule. Training continued until mice obtained a criterion of 91 pellets within 60 minutes under a variable interval 60 (VI-60) schedule of reinforcement. By the end of training, the average number of bar presses in the 60-minute session was 5300, which corresponded to a maximum total of 91 pellets. This high level of bar pressing was needed in order for the effects of conditioned suppression to be investigated. Bar-press training lasted 6 weeks

to 3 months, after which mice were randomly assigned to experimental (HSV-CREB-GFP) and control (HSV-GFP) groups. Throughout training, mice were given a piece of standard mouse chow approximately 4 hours after training to maintain the desired weight.

2.3.1.1. A Note on VI Reinforcement Schedules

VI schedules have previously been shown to induce high levels of bar pressing over long periods of time (Catania and Reynolds, 1968). On a VI reinforcement schedule, the first response after the passage of a specified period of time has elapsed, since the last reinforcement, is rewarded. However, the required amount of time to have passed from one reinforcement to the next varies in an unpredictable manner (Commons and Nevin, 1991). This schedule is expressed as VI-x, where x is the specific period of time between reinforcements. For example, if you have a boss who checks your work periodically you have to be working hard at all times in order to be ready since you do not know when the next 'check-up' might come.

2.3.2. Auditory Fear Conditioning

Twenty-four hours after mice reached the criterion performance in bar press responding, they were trained for auditory fear conditioning. Mice received 6 tone-shock pairings in a novel fear chamber (20 x 25 x 31cm) that differed from the operant chamber in which bar-press training took place in that the fear chamber had been cleaned with an ethanol solution before training, was not enclosed in a sound-attenuated chamber, and did not contain bars or food pellets. Three minutes following placement in the chamber, the conditioned stimulus (CS, 2.8kHz pure tone lasting 30 seconds with an intensity of 75

dB) was presented. The tone co-terminated with the unconditioned stimulus (US), a scrambled footshock delivered to the floor bars with an intensity of 0.4 mA and duration of 2 seconds. The intertrial interval between the tone-shock pairings varied from 60 to 240 seconds. The percentage of time mice spent freezing to the tones was collected using an automated system (Freeze Frame). The following day (Day 1), mice were re-trained under a VI-60 schedule in the operant chambers to determine whether fear conditioning affected their bar-pressing behaviour. Surgery was then performed on the mice.

2.3.3. Surgery

After pretreatment with atropine (0.15ml, 0.08mg/ml, i.p.), the mice were anesthetized with chloral hydrate (400mg/kg, i.p.) and placed into a stereotaxic apparatus. After exposing the cranium and marking bregma, holes were made bilaterally over the lateral amygdala with a dental drill. The coordinates were AP: -1.4 mm, ML: ± 3.5mm, DV: - 5.0 mm relative to bregma (Paxinos and Franklin, 2001). Viral vectors were administered bilaterally in a volume of 1.5 µl per side at a rate of 0.1µl/min for 15 min using sharp glass micropipettes attached to a 10 µl Hamilton syringe. After infusion, the pipette was left in place for 5 min to allow for diffusion away from the pipette tip.

2.3.4. Extinction Training and Testing

Twenty-four hours after surgery, mice received 2 days of additional bar-press training sessions under a VI-60 reinforcement schedule in the operant chambers (Days 3-4). This additional training was performed to maintain high levels of bar pressing. On experimental day 5, mice were given auditory fear extinction training in the operant

chambers. Prior to training, baseline levels of responding for a sucrose reward on a VI-60 schedule was assessed for 4 minutes, where no tones or shocks were given. This was followed immediately by 6 extinction trials during which the tone CS was presented without the footshock (tone alone trials). As with fear conditioning, the tones were 30 seconds in duration and spaced 60 seconds apart. During training, the number of bar presses both before and during the tones was recorded to examine within-session extinction of the fear conditioning memory (or short-term memory for extinction). Twenty-four hours later (Day 6), mice were tested for long-term memory of extinction training for auditory fear conditioning. In this memory test, mice were presented with 20 CS tones without the shocks following a 4 minute baseline period. During extinction training and testing, food reward was continuously available on a VI-60 schedule. A computer controlled the delivery of tones and sucrose pellets, and recorded the number of bar presses. Acquisition and extinction of auditory fear conditioning were conducted in distinct chambers to eliminate contextual factors that may confound the results. That is, we wanted fear responses measured during extinction to be specific to the tone.

Conditioned fear responses to the tone were measured using suppression of bar pressing during the tone. The suppression ratio was calculated using the formula (Bouton and Bolles, 1980; Armony et al., 1997):

$$\text{Suppression Ratio} = \frac{(\text{Pretone} + \text{Posttone}) - \text{Tone}}{(\text{Pretone} + \text{Posttone}) + \text{Tone}}$$

In this equation, the pretone variable refers to the number of bar presses recorded 30 seconds prior to tone onset. The tone variable refers to the number of bar presses recorded over the duration of the 30 second tone. One advantage of using suppression ratio is that it takes into account variability in baseline bar pressing rates for each mouse. A value of 1 indicates complete suppression of bar pressing during the tone (an indication of strong fear to the tone CS), whereas a value of 0 indicates no suppression (an indication of either weak or no fear to the tone CS).

2.3.5. Histology

After the completion of all extinction tests, the mice were given an overdose of chloral hydrate (400mg/kg, i.p.) and perfused transcardially with 80ml of 1x PBS at 10ml/min, followed by 80ml of 4% paraformaldehyde (PFA) at 10ml/min. Brains were extracted and stored in 4% PFA for 3 hours then transferred to 30% sucrose-PBS solution for storage in 4°C. Subsequently, 50μm brain sections were generated with a cryostat and mounted on slides using Vectashield mounting medium with DAPI. Sections were analyzed under an epifluorescent microscope to confirm infusion placements.

RESULTS

CHAPTER 3: RESULTS

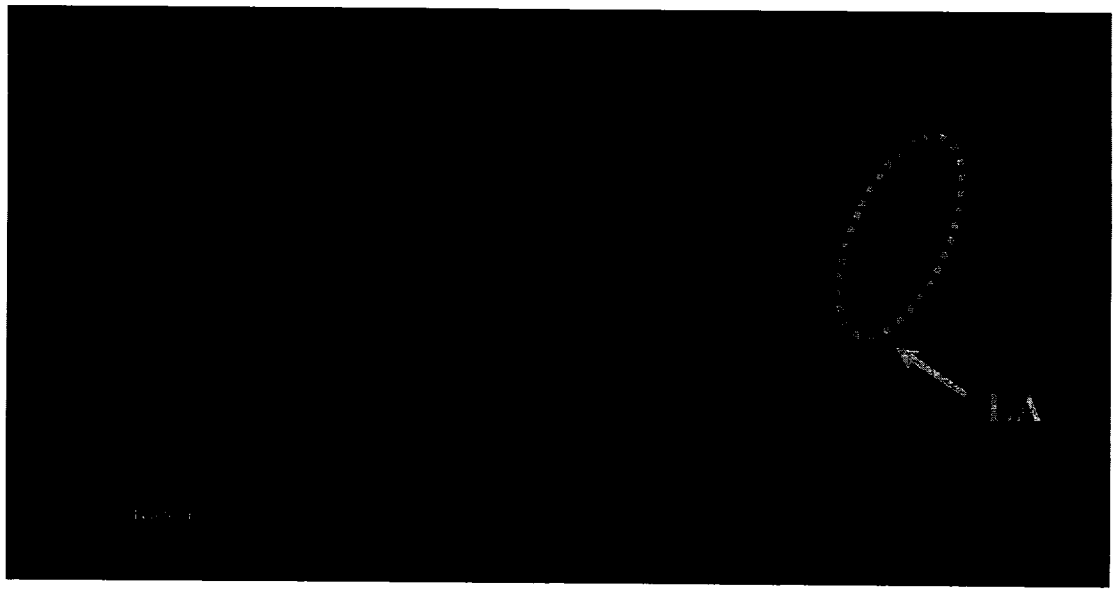
3.1. Histology – Infusion of HSV Vectors Produces High Transgene Expression in the LA

Previous studies show that the LA is important for the consolidation of fear extinction memory (Lu et al., 2001; Chhatwal et al., 2006). Therefore, we targeted this region with HSV vectors expressing either CREB-GFP or GFP alone. Figure 11 depicts a typical brain section with GFP expression in the LA following microinjection of HSV-GFP. Infusions of both HSV-CREB-GFP and HSV-GFP into the LA produced strong, localized transgene expression throughout the LA (approximately 1000 infected LA cells; see Appendix). In contrast, little to no expression was detected in regions outside the LA (less than 500 infected cells; see Appendix).

Typically, our viral infusions cause minimal damage (similar to that produced by saline infusion) (Figure 12A). Occasionally, however, infusion of HSV vectors causes extensive cytotoxic damage (Carlezon et al., 2000). Because the amygdala has previously been shown to be critical for both the acquisition and expression of fear memory (LeDoux et al., 1990; Amorapanth et al., 2000; Goosens and Maren, 2001; Nader et al., 2001; Anglada-Figueroa and Quirk, 2005), two mice that sustained LA damage (Figure 12B) were excluded from subsequent data analysis. Only mice with strong bilateral GFP expression in the LA were included in further statistical analysis. Of the 27 mice that underwent surgery, 8 died, 4 showed unilateral GFP expression, and 3 showed no GFP expression in the LA (a miss). Therefore, a total of 9 mice showed

robust bilateral GFP expression in the LA. Of these, 6 received HSV-CREB (experimental group) and 3 received HSV-GFP (control group) (Figure 13).

4X



10X

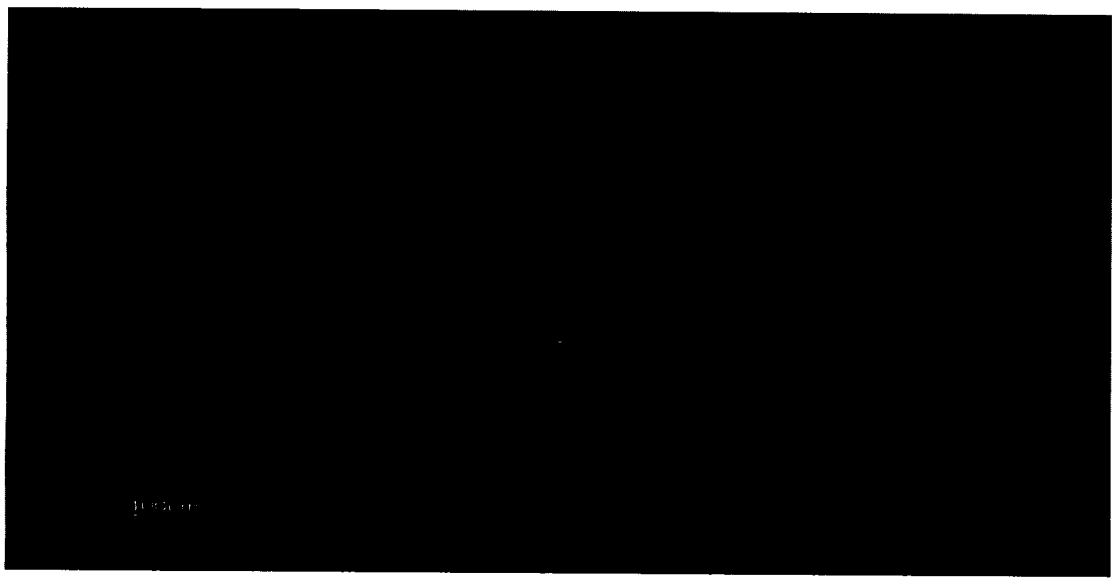
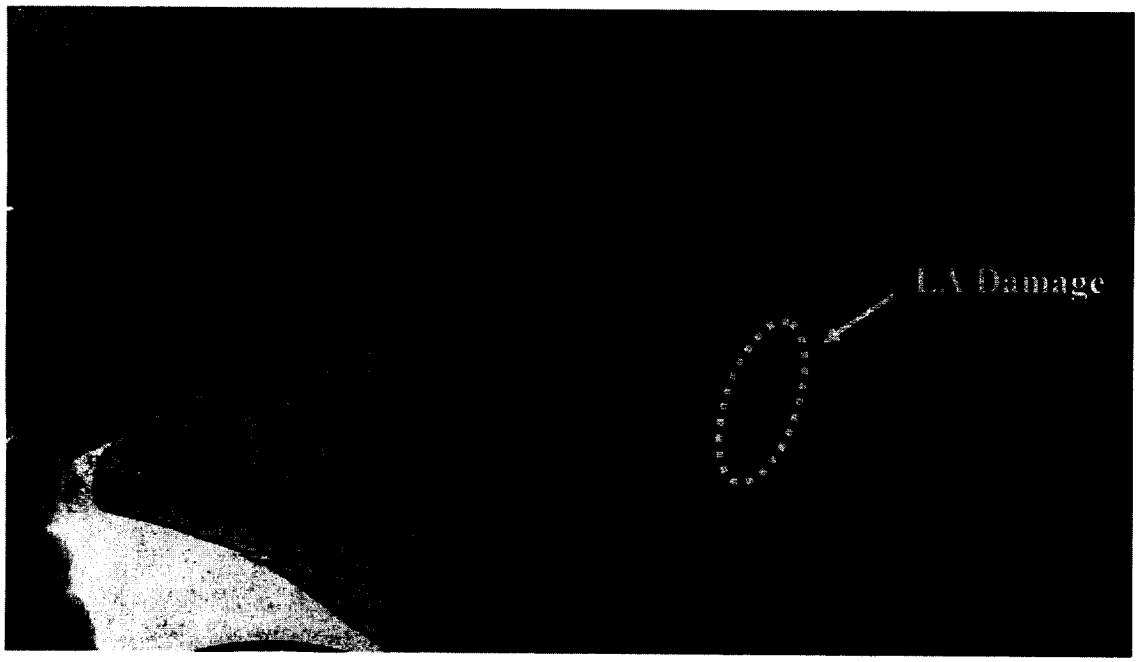


Figure 11. Representative images taken from an epifluorescent microscope showing strong, localized HSV-mediated GFP expression within the lateral amygdala (LA) 5 days post-injection. Images were acquired at 4X (to show the general location of HSV infection) and 10X (to show individual GFP-labeled cells) magnifications.

A Minimal LA Damage



B Extensive LA Damage

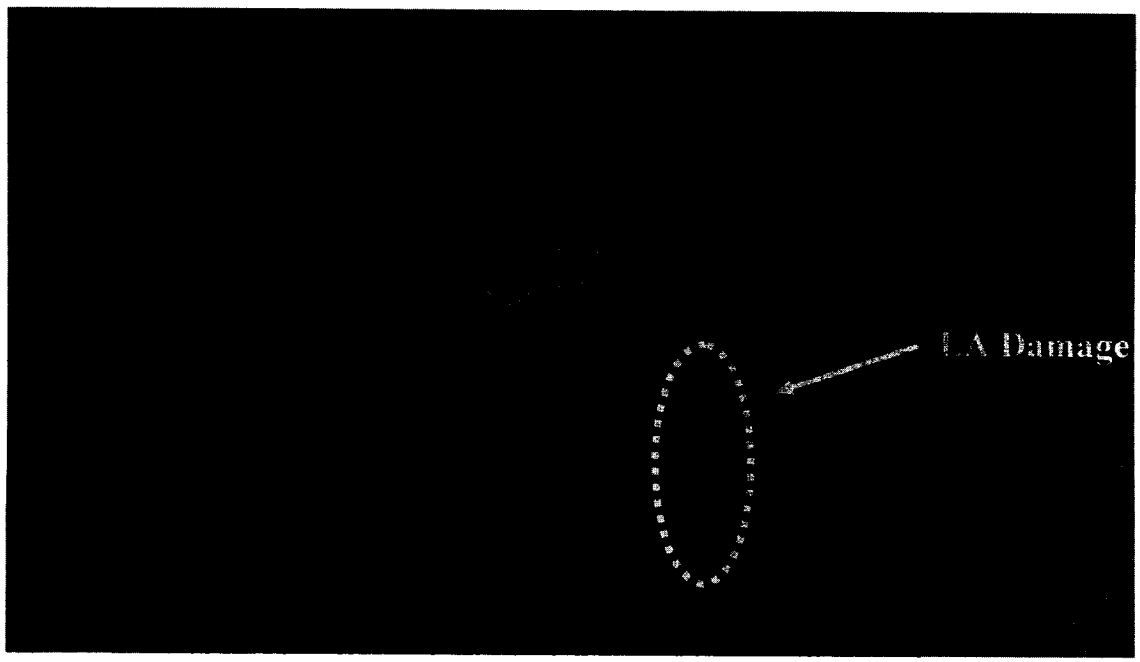


Figure 12. Light microscopic images depicting limited damage (associated with viral infusions and similar to that produced by infusion of saline) (A) and extensive damage (B) in the LA.

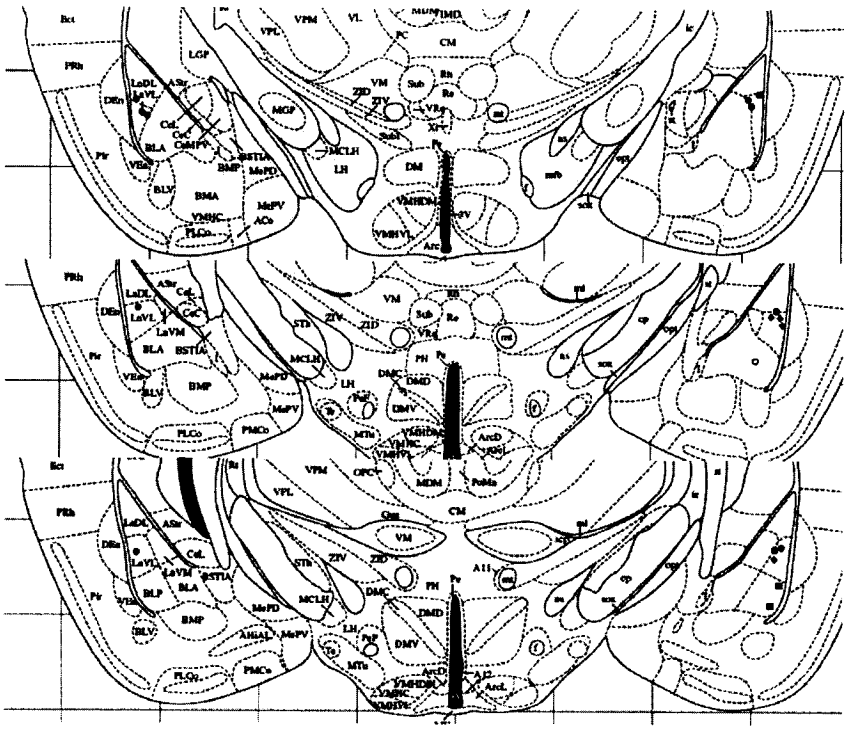


Figure 13. Diagram showing the location of maximum GFP expression at four different rostrocaudal planes. All coordinates are relative to bregma and adapted from the mouse brain atlas (Paxinos and Franklin, 2001). ● CREB hit ♦ GFP hit ○ CREB miss ■ GFP miss.

3.2. Fear Acquisition

All mice were trained for auditory tone conditioning prior to surgery (Day0). In this training session, mice received 6 tones (30 sec, 85 db, 2.8 kHz) that co-terminated with a footshock (intensity of 0.4 mA). Figure 14 shows the percentage of time mice spent freezing to each of the 6 conditioning tones. As can be seen from this graph, the freezing levels increased over successive tones (indicating fear acquisition) and there did not appear to be a difference between the freezing levels in mice that later received HSV-CREB or HSV-GFP. An Analysis of Variance (ANOVA) with between-group factor Virus (HSV-CREB, HSV-GFP) and within-group factor Time (6 tones) showed a significant effect of Time [$F(5,35) = 14.4$, $p < .001$], but no significant effect of Virus [$F(1,7)= 0.48$, $p >.05$] or interaction of Time by Virus [$F(5,35) = 1.33$, $p > .05$]. Therefore, both groups of mice acquired auditory fear conditioning to a similar degree.

Day 0

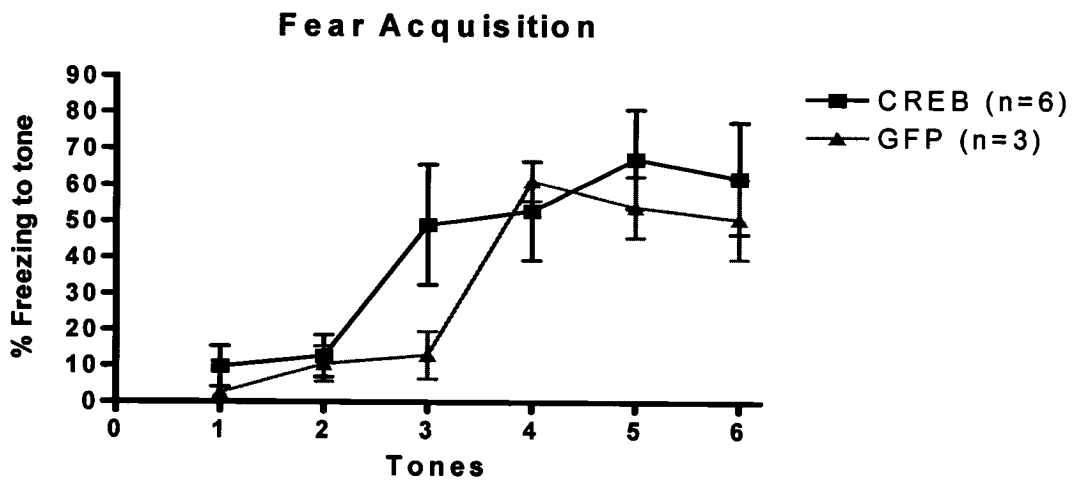


Figure 14. Mean (+/- SEM) freezing levels during the acquisition of auditory fear conditioning. Training consisted of pairing 6 tones with a footshock. Freezing is expressed as the percentage of time spent motionless during each of the 30 second tones. Mice that were subsequently microinjected with HSV-CREB and HSV-GFP exhibited increased freezing over the course of the 6 tones. Importantly, no significant difference in freezing levels was found between HSV-CREB and HSV-GFP groups.

3.3. Within-Session Fear Extinction (STM)

Two days after surgery (Day5), at a time when transgene expression is thought to be maximal (Carlezon et al., 1998), previously fear conditioned mice were given extinction training, during which 6 tones were presented without the footshock.

3.3.1. CREB Overexpression Did Not Affect Baseline Levels of Bar Pressing

To measure changes in conditioned fear during this session, suppression of bar pressing was used. As such, it was first important to determine whether microinjection of HSV vectors produced effects on baseline bar press responding. To this end, we examined the number of bar presses during a 4 minute baseline period that preceded tone onset, where mice were placed in operant chambers and allowed to bar press for sucrose pellets under a VI-60 schedule. As seen in Figure 15, both HSV-CREB and HSV-GFP mice exhibited similarly high levels of bar pressing in this 4 minute period. An ANOVA with between factor Virus showed no difference in the number of bar presses in this 4 minute period [$F(1,7) = 1.39$, $p > 0.05$]. Therefore, both groups of mice began extinction training with similar high levels of bar pressing.

Day 5

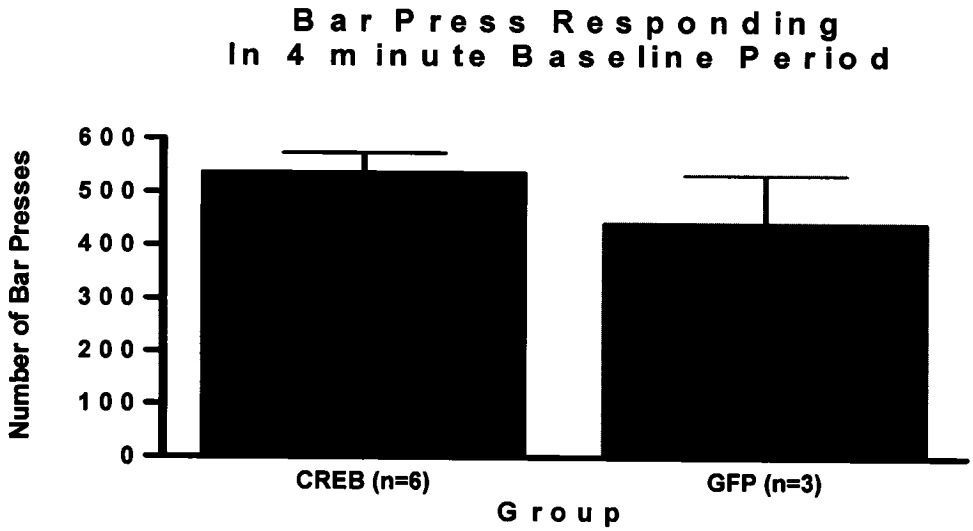


Figure 15. Mean (+/- SEM) baseline number of bar presses was unaffected by viral infusions. Mice that received HSV-CREB and HSV-GFP exhibited similar high levels of bar pressing in the 4 minute period preceding tone presentations. Therefore, baseline levels of bar press responding were unaffected by viral infusions.

3.3.2. Surgery and Viral Infusions Did Not Affect Baseline Levels of Bar Pressing

As a further control, we examined whether surgery and viral infusion of the HSV vectors changed the rate of bar pressing. In other words, whether there was any difference between the rates of bar pressing before and after surgery. Figure 16 shows the bar pressing rates before (A) and after (B) surgery for mice that received HSV-GFP and HSV-CREB. As seen from this graph, both groups of mice showed similar rates of bar pressing before and after surgery. An ANOVA with within factor Time (before vs. after surgery) and between factor Virus (HSV-GFP vs. HSV-CREB) showed no effect of Time [$F(1,7) = 1.94$, $p>0.05$], Virus [$F(1,7) = 1.71$, $p>0.05$] or Time by Virus interaction [$F(1,7) = 0.02$, $p>0.05$]. Therefore, surgery and viral infusion did not interfere with the ability of the mice to bar press.

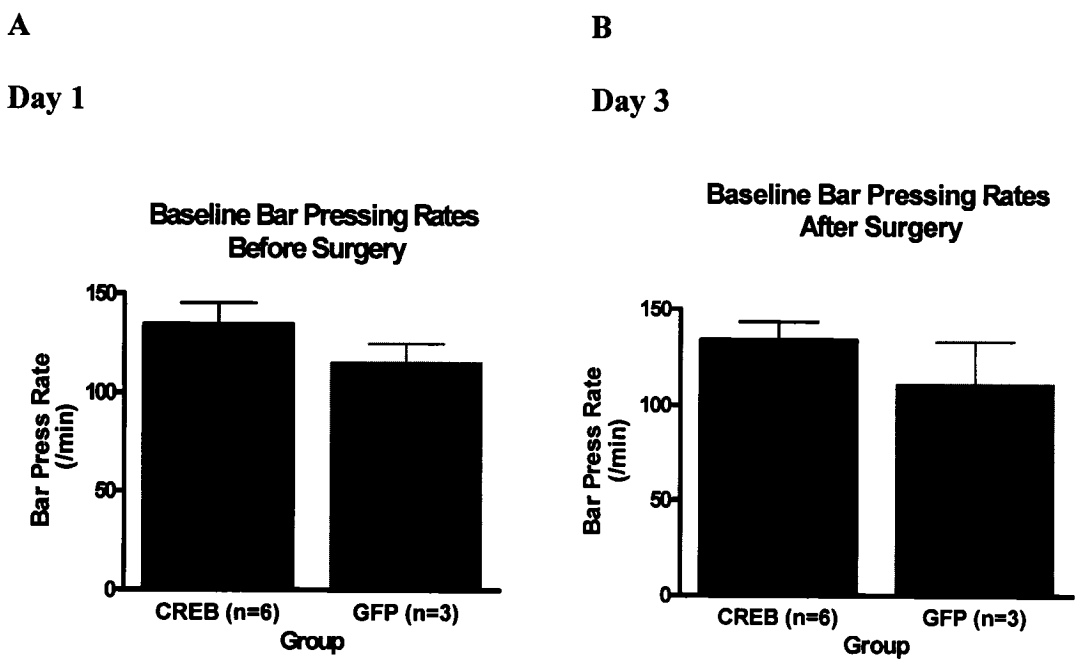


Figure 16. Mean (+/- SEM) rate of bar pressing (number of bar presses per minute) before and after surgery. Surgery had no effect on the rates of bar pressing. Mice that received HSV-GFP and HSV-CREB showed similar rates of bar pressing before (A) and after (B) surgery.

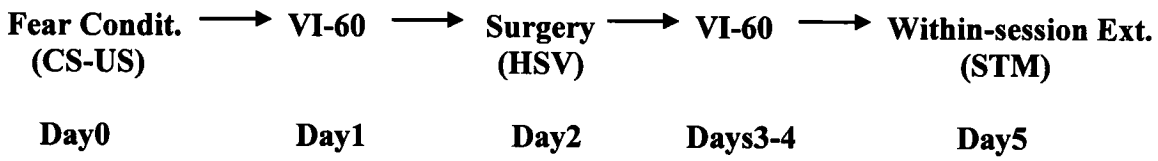
3.3.3. Within-Session Fear Extinction (STM) is Unaffected by CREB Overexpression in the LA

Immediately following the 4-minute baseline period, mice were given 6 tone-alone presentations (extinction trials) and the level of extinction within a session was assessed. To measure the degree of fear (and hence, the amount of fear extinction) we used several measures. The first is a modified suppression ratio based on previous work by Quirk et al. (2000). This ratio was calculated by dividing the difference between the average number of bar presses in the 30 seconds before and after the tone, and during the 30 second tone by the sum of the 2 values:

$$\text{Quirk Suppression Ratio: } \frac{(\text{Pretone} + \text{Posttone}) - \text{Tone}}{(\text{Pretone}+\text{Postttone}) + \text{Tone}}$$

Using this index of conditioned fear, a high ratio (up to a maximum of 1) indicates high levels of fear to the tone CS whereas, a low ratio indicates low or no fear to the tone CS. As shown in Figure 17, both groups of mice exhibited a decrease in suppression ratio (or fear) over the course of the 6 tones, indicating within-session extinction. Moreover, this within-session extinction did not appear to differ between the two groups. In support of this observation, an ANOVA comparing Virus (HSV-CREB vs. HSV-GFP) and within-group factor Tone (6 tones) revealed a significant effect of Time [$F(5,35) = 3.51$, $p<0.05$], but no significant effect of Virus [$F(1,7) = 0.77$, $p>0.05$] or interaction between Time and Virus [$F(5,35) = 0.53$, $p>0.05$]. These data indicate that both groups of mice displayed similar within-session extinction and hence, extinguished their fear responses to a similar degree.

A



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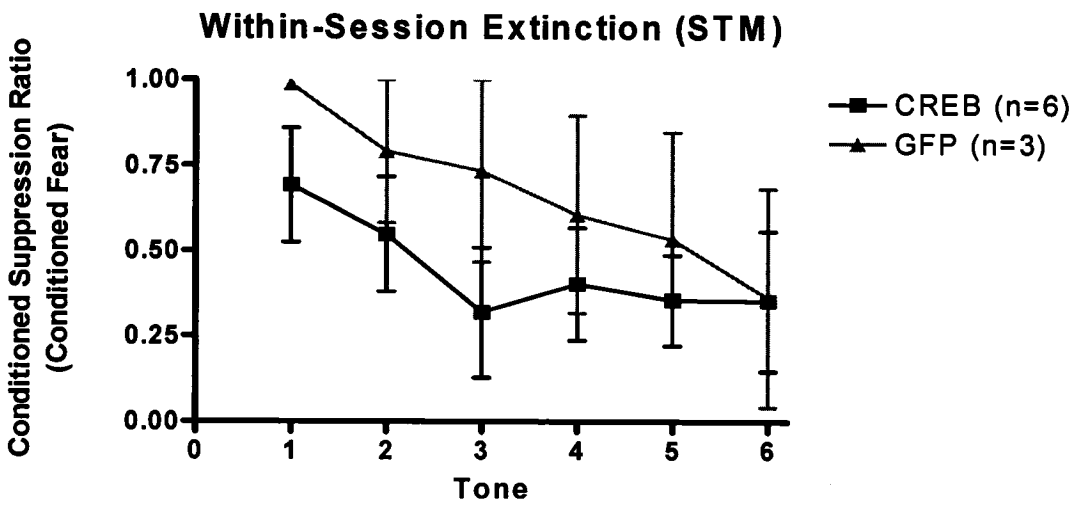


Figure 17. (A) Behavioural procedure used. (B) Changes in conditioned fear responses over the course of 6 non-reinforced tones. There was no significant difference in within-session extinction (or STM for extinction) between mice receiving HSV-GFP and HSV-CREB as measured by conditioned suppression of bar pressing. Both groups of mice showed similar decreases in conditioned suppression ratios and hence, conditioned fear, to 6 successive non-reinforced tone presentations within the training session. Points are mean conditioned suppression ratios (+/- SEM).

3.3.4. No Difference Between HSV-CREB and HSV-GFP Mice in Bar Press Numbers During the 6 Training Tones

A limitation of using suppression ratios as a measure of conditioned fear is that they can sometimes obscure subtle effects in the data. For example, it is possible that changes in bar press activity caused by effects of the treatment on non-associative factors (e.g., motor function and motivation processes that heavily influence baseline bar pressing levels), rather than a genuine learning phenomenon, may account for the observed results. To address this issue, we also analyzed the raw number of bar presses to detect differences in dynamic changes of bar pressing over repeated non-reinforced tone presentations among and between the 2 groups. First, we compared the levels of bar pressing during the 6 tones, where high levels of bar pressing during this period indicate less fear (or extinction of fear). As shown in Figure 18, both groups of mice showed a similar increase in bar pressing levels over the course of the 6 tones. An ANOVA revealed an effect of Time [$F(5,25) = 3.32$, $p<0.05$], but no significant effect of Virus (HSV-CREB vs. HSV-GFP) [$F(1,5) = 2.92$, $p>0.05$] or interaction of Time by Virus [$F(5,25) = 0.50$, $p>0.05$].

Day 5

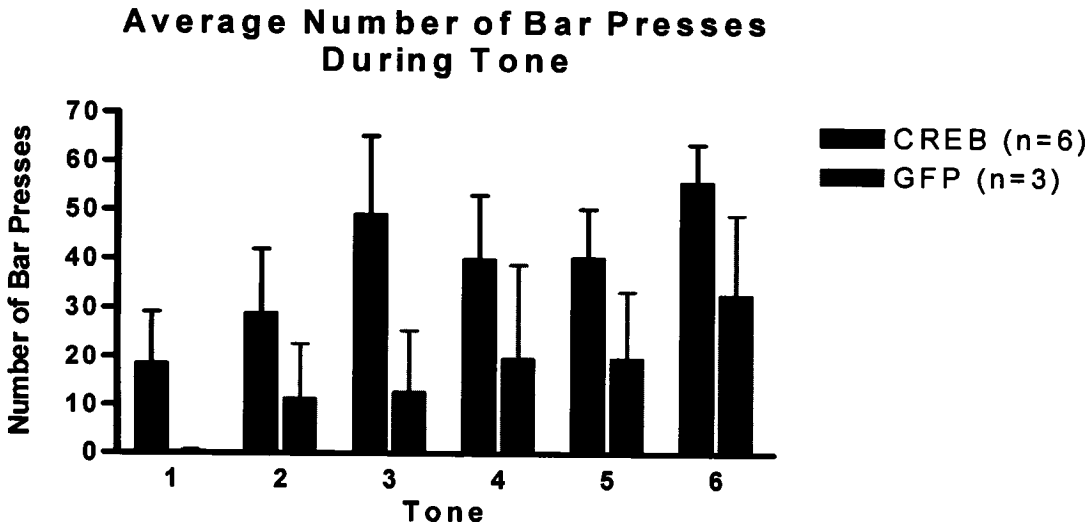


Figure 18. Mean (+/- SEM) number of bar presses over the course of 6 non-reinforced tones during extinction training (within-session extinction). Both groups displayed a similar increase in bar pressing levels over the 6 tones, indicating a reduction in conditioned fear to the tone. Furthermore, the two groups of mice did not differ in their number of bar presses during the tone.

3.3.5. HSV-CREB Mice Exhibited Slightly Higher Bar Press Responding Between Training Tones than HSV-GFP Controls

Second, we compared the average number of bar presses before and after the tone between the two groups to determine if the observed suppression of bar pressing was specific to the tone. As shown in Figure 19, the number of bar presses in this period did not vary across the 6 tones for either HSV-CREB or HSV-GFP mice, indicating consistent levels of bar pressing in the absence of a tone. Overall, however, the number of bar presses between tones appeared to be slightly higher in mice that were injected with HSV-CREB. An ANOVA with between-group factor Virus and within-group factor Time (6 tones) showed a significant effect of Virus [$F(1,7) = 6.00$, $p<0.05$], but no effect of Time [$F(5,35) = 2.46$, $p>0.05$] or Time by Virus interaction [$F(3,35) = 0.28$, $p>0.05$].

Day 5

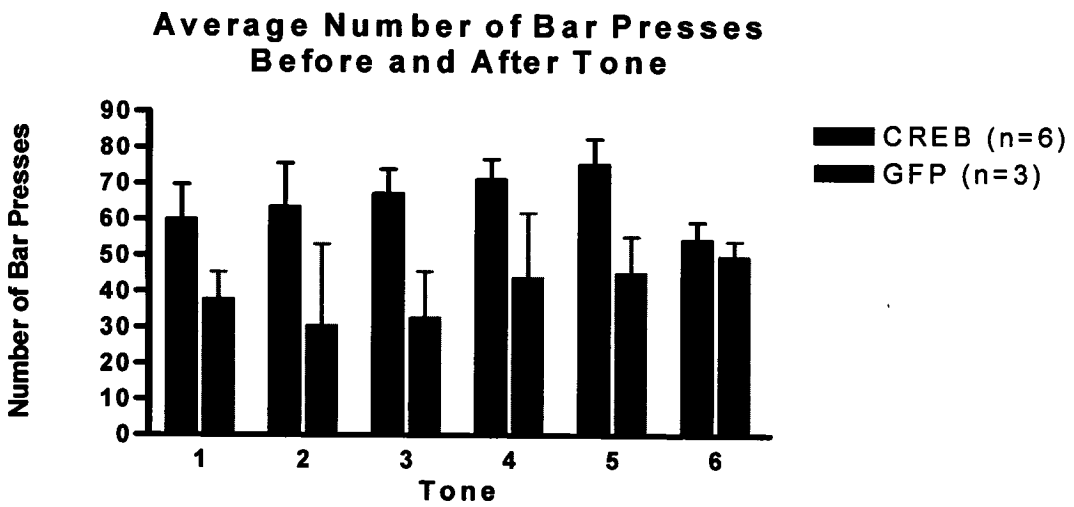


Figure 19. Mean (+/- SEM) number of bar press responses in the 30 seconds before and after the tones during extinction training (within-session extinction). Mice that received HSV-CREB and HSV-GFP did not show differences in the average number of bar presses across the 6 tones. However, HSV-CREB mice exhibited significantly higher levels of bar pressing between tones.

Together, the results presented in Figures 18 and 19 preclude definitive conclusions as to whether viral infusions produced behavioural effects that are not related

to learning. This is because while the two groups of mice exhibited similar increases in bar press levels over the course of the tones (indicating fear extinction; Figure 18), they differed in bar pressing levels between tones. Despite this, the finding that both groups exhibited similar bar pressing levels prior to the tones seems to suggest that viral infusions did not cause deficits in motor function or motivation. Alternatively, the observed differences may be a direct consequence of the low of number of mice in each group.

3.4. Between-Session Extinction (LTM)

Twenty-four hours after extinction training (Day6), mice were tested for extinction consolidation, in which 20 tones were presented without the footshock.

3.4.1. CREB Overexpression Did Not Affect Baseline Levels of Bar Pressing

Again, because a suppression ratio was used as an index of fear, it was important to assess whether baseline bar press responding was different between the two groups. Before testing, baseline bar press responding was measured for 4 minutes prior to tone onset. As seen in Figure 20, both groups of mice exhibited similar high levels of bar pressing during this 4 minute period. An ANOVA with between factor Virus showed no difference in bar press responding in this 4 minute period [$F(1,7) = 1.08$, $p>0.05$]. Therefore, both groups of mice began extinction testing with similar high levels of bar pressing.

Day 6

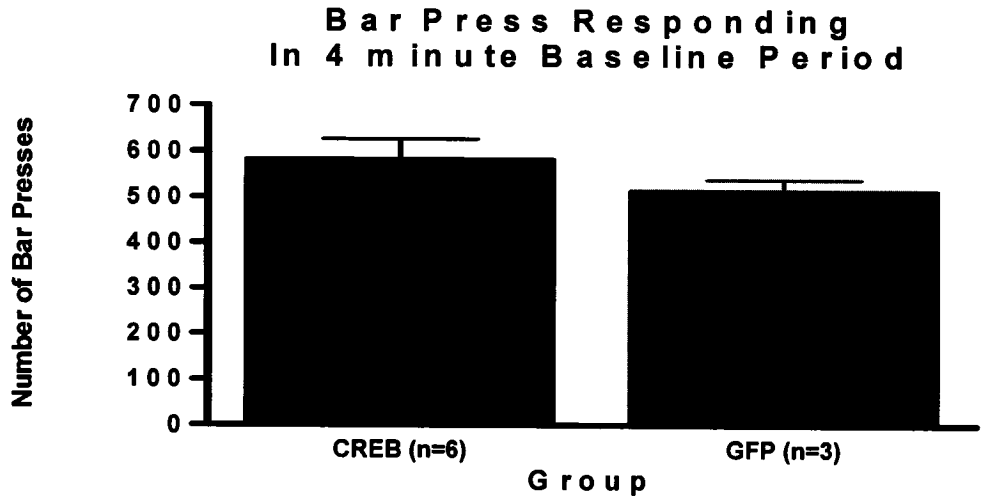
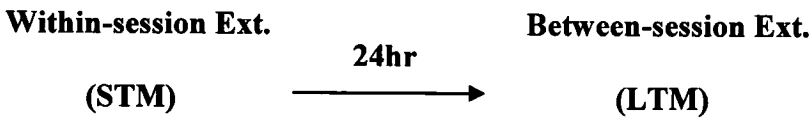


Figure 20. Mean (+/- SEM) number of bar presses during a 4-minute baseline period was unaffected by viral infusions. Mice receiving HSV-CREB and HSV-GFP exhibited similar high levels of bar pressing in the 4 minute period preceding tone presentations.

3.4.2. Overexpression of CREB in the LA Enhances Fear Extinction Consolidation (LTM)

Immediately following the 4-minute baseline period, mice were given 20 tone-alone presentations to assess between-session extinction (extinction consolidation). Notably, only the data for the first 4 tones was plotted, as mice did not show any further signs of fear to the tone thereafter. That is, all mice virtually reached complete extinction after the first 4 tones. As seen in Figure 21, both groups of mice showed a reduction in suppression scores (indicating decreasing fear) over the course of the tones. By tone 3, both groups of mice had a suppression ratio of near 0 (an indication of little to no fear). However, HSV-CREB infused mice showed a lower suppression ratio (less fear) to the first test tone than did GFP controls. This suggests that HSV-CREB mice showed weaker fear memory (or enhanced fear extinction) on the first tone. In support of this observation, an ANOVA with within-group factor Tone (4 tones) revealed a significant effect of Time [$F(3,21) = 9.46$, $p<0.05$] and interaction of Time by Virus [$F(3,21) = 3.9$, $p<0.05$], but no significant effect of Virus [$F(1,7) = 1.41$, $p>0.05$]. Planned comparisons of the significant interaction revealed that the difference between groups is attributed to HSV-CREB mice exhibiting lower conditioned suppression on the first tone [$F(1,7) = 7.47$, $p<0.05$]. By contrast, the suppression ratios on the other tones did not differ significantly between the 2 groups [tone2: $F(1,7) = 0.14$, $p>0.05$, tone3: $F(1,7) = 0.19$, $p>0.05$, tone4: $F(1,7) = 0.43$, $p>0.05$]. The finding, that mice infused with HSV-CREB exhibited lower levels of fear on the first tone of the extinction test given 24 hours following extinction training, supports the conclusion that increasing CREB levels in the LA facilitated the consolidation of extinction learning.

A



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Day 6

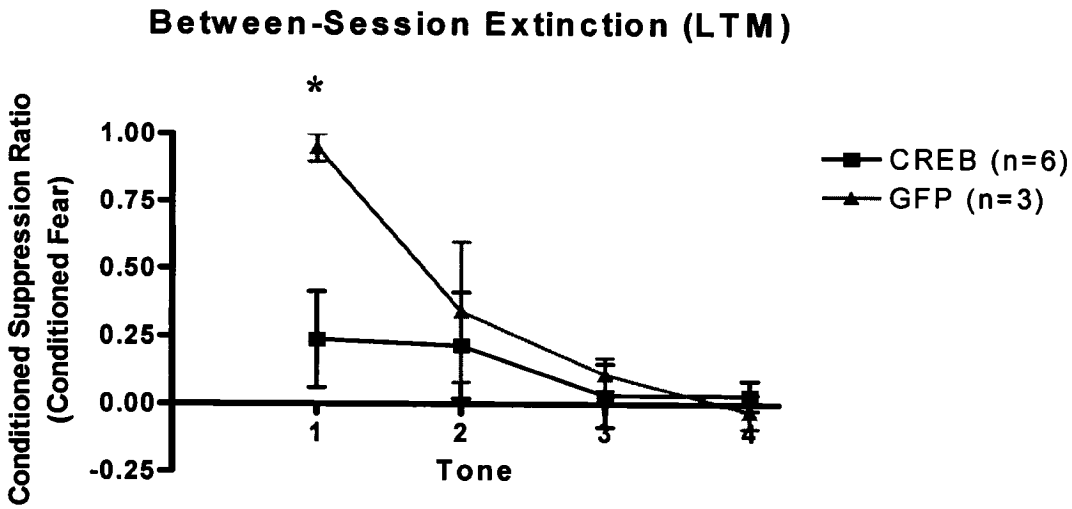

Figure 21. (A) Behavioural procedure used. (B) Changes in conditioned fear responses over the course of 4 non-reinforced tones. Increasing CREB in the LA facilitated extinction consolidation. Mice that received HSV-CREB showed enhanced extinction of conditioned fear (lower suppression ratio) to the first test tone. However, there was no significant difference in suppression ratios on 3 subsequent tones. Points are mean conditioned suppression ratios (+/- SEM).

3.4.3. No Overall Difference Between HSV-CREB and HSV-GFP Mice in Bar Press Numbers During the 4 Testing Tones

As with extinction training, we examined the number of bar presses during and between the tones to determine whether the results can be attributed to non-specific changes in bar pressing. Figure 22 shows the average number of bar presses during the first 4 tones. From this graph, it appears that HSV-GFP mice show an increase in the number of bar presses over the course of the 4 tones (indicating a decrease in conditioned fear to the tone or fear extinction). On the other hand, HSV-CREB mice maintained relatively constant high levels of bar pressing across the tones. In addition, these mice showed a higher level of bar pressing (less fear) on the first test tone compared to GFP controls. By contrast, bar pressing levels were similar between the two groups on the 3 tones that followed. An ANOVA with within-group factor Time (4 tones) and between-group factor Virus showed a significant effect of Time [$F(3,21) = 5.51$, $p<0.05$], but no significant effect of Virus [$F(1,7) = 2.47$, $p>0.05$] or interaction of Time by Virus [$F(3,21) = 2.26$, $p>0.05$]. Planned comparisons of the significant effect of Time revealed a significant effect of Virus on tone 1 [$F(1,7) = 6.19$, $p<0.05$], but not tone 2 [$F(1,7) = 0.80$, $p>0.05$], tone 3 [$F(1,7) = 0.95$, $p>0.05$], or tone 4 [$F(1,7) = 0.31$, $p>0.05$].

Day 6

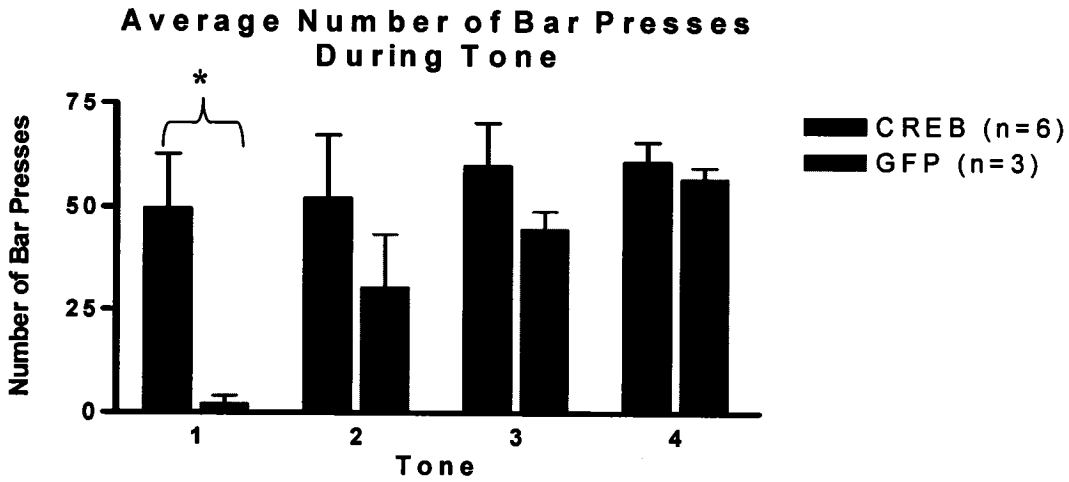

Figure 22. Mean (+/-SEM) number of bar presses over the course of 4 non-reinforced tones during the extinction test. Both groups of mice exhibited similar levels of bar pressing on tones 2-4, but HSV-CREB mice showed a higher level of bar pressing on the first tone than GFP controls.

3.4.4. No Difference Between HSV-CREB and HSV-GFP Mice in Bar Press Numbers Between Testing Tones

Next, we compared the average number of bar presses before and after the tone between the two groups. As seen in Figure 23, both groups of mice exhibited similar levels of bar pressing between tones. Furthermore, bar pressing levels for either group did not vary over the course of the 4 tones. This observation is supported by an ANOVA with between-group factor Virus and within-group factor Time showing no significant effect of Virus [$F(1,7) = 0.82$, $p>0.05$], Time [$F(3,21) = 0.05$, $p>0.05$] or interaction of Time by Virus [$F(3,21) = 0.18$, $p>0.05$].

Based on these findings, the calculated conditioned suppression ratios cannot simply be explained by non-associative factors that might affect bar pressing levels.

Day 6

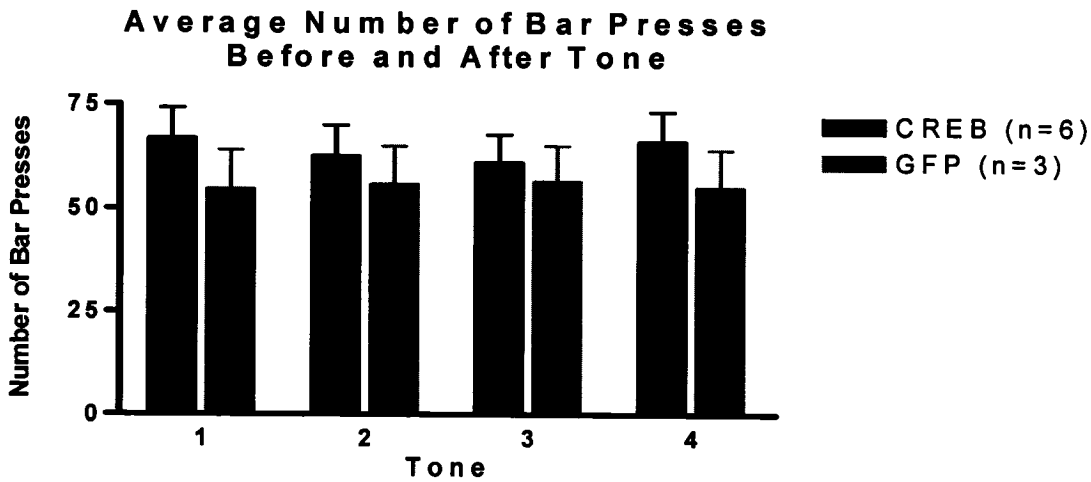

Figure 23. Mean (+/- SEM) number of bar presses 30 seconds before and after the tone during the extinction test. Both groups of mice showed similar levels of bar pressing between tones.

DISCUSSION

CHAPTER 4: DISCUSSION

4.1. Summary of Results

In the present study, we examined the effects of increasing CREB expression in the LA on the acquisition and consolidation of extinction memory. We found that overexpression of CREB in the LA prior to extinction training did not have a significant effect on the acquisition of fear extinction. That is, mice receiving either HSV-CREB or HSV-GFP displayed a similar within-session reduction in fear over the course of 6 non-reinforced tones. In contrast, mice infused with HSV-CREB showed enhanced consolidation of fear extinction measured 24 hours following extinction training. Specifically, HSV-CREB infused mice exhibited a lower suppression ratio (or less fear) to the first tone of the consolidation test. However, the suppression ratios on subsequent tones did not differ between the two groups. Moreover, by the fourth tone, both groups showed virtually complete extinction. In other words, the mice did not seem to show further signs of fear to the tone. This finding suggests that CREB in the LA enhances the consolidation of fear extinction memory; an effect that cannot be attributed to factors such as, HSV-induced changes in baseline bar pressing rates or different levels of fear acquisition and within-session extinction. However, due to the low numbers of mice in each group, additional studies are required to strengthen our conclusions.

4.2. Methodology

4.2.1. Fear Acquisition

Previous studies showed that overexpressing CREB in the LA via HSV-mediated gene transfer facilitated LTM for fear conditioning (Josselyn et al., 2001; Wallace et al., 2004). Therefore, to examine the effects of increasing CREB levels on long-term fear extinction, we performed our CREB manipulation after fear conditioning. Importantly, both groups of mice acquired auditory fear conditioning to a similar degree.

4.2.2. Extinction Training and Testing

4.2.2.1. Measuring Conditioned Fear

In accordance with studies by Quirk and colleagues (e.g., Quirk et al., 2000), we planned to use the suppression ratio and percent freezing to measure conditioned fear during extinction training and testing. However, due to poor lighting conditions in the experimental room and problems with the cameras (factors that strongly affect the freezing data), freezing could not be reliably used to assess extinction. Nevertheless, it can be argued that the suppression ratio is a more sensitive measure of conditioned fear than freezing (Quirk et al., 2000). In particular, freezing is a 'low cost' behaviour, in that no cost is incurred on an animal that freezes to a CS that no longer predicts a US. As a result, an animal may freeze during the time between CS presentations or freezing may not reflect a state of fear. By contrast, conditioned suppression of bar pressing is a relatively 'high cost' behaviour because when a food-deprived animal ceases bar pressing to a non-reinforced CS, it misses an opportunity to obtain food. Therefore, using a food-motivated bar pressing task ensured that animals would be active rather than stationary in

the inter-trial period and thus, establish a more instructive relationship between behaviour and the state of fear.

4.2.2.2. Contextual Effects

It is well established that contextual cues influence the expression of conditioned fear responses (Dexter and Merrill, 1969) and extinction performance (Rescorla and Heth 1975; Bouton and Bolles 1979; Bouton and King, 1983). Therefore, in addition to learning the tone-footshock contingency, mice may have also formed an association between various contextual cues present during fear training (e.g., odours, background noise, experimental room) and the footshock. Accordingly, all cues (tones and context) have to be present during retrieval (or extinction training) to elicit maximal fear (Morgan, Schulkin, and LeDoux, 2003). Since mice received fear and extinction training in distinct rooms, it is possible that they were unable to completely transfer the learning (CS-US association) that occurred in the fear room to the extinction room. As a result, the fear memory may not be fully expressed in the extinction room, thus, tipping the balance in favour of extinction. This may explain the high rate of within-session extinction in our study. For instance, in the present experiment, only 3 tones were required to produce the same level of within-session extinction that Quirk et al. (2000) achieved with 7 tones. However, other factors, including the footshock intensity, number of conditioning trials, and species (rats versus mice), may also account for this discrepancy. One way to address this issue would be to compare freezing levels during fear acquisition (prior to CS-US pairings) with those on a retention test given the next day (without tones). Although the issue of contextual influences on extinction performance is important, it

may not be relevant in the interpretation of our results because both HSV-GFP and HSV-CREB mice underwent similar treatments (e.g., both groups were exposed to the same stimuli during training and testing).

4.2.2.3. Number of Trials

We chose to use 6 tones for extinction training for several reasons. First, we wanted to prevent complete extinction so that a potential facilitatory effect of CREB overexpression in the LA on extinction consolidation could be revealed. Quirk et al. (2000) used 15 tones during extinction training and found this number of tones produced near complete extinction (suppression ratio near 0). In our experiment, the conditioned suppression ratio declined to a value of approximately 0.3 for both groups after the 6 training tones. Second, we wanted to ensure that our CREB manipulation acted specifically on extinction as opposed to reconsolidation. Some scientists currently believe that when memories are retrieved, they return to a labile (unstable) state that is susceptible to disruptions (Sara, 2000). Previous studies have shown that like memory consolidation, reconsolidation depends on protein synthesis (Nader et al., 2000; Kida et al., 2002) and CREB activation (Hall et al., 2001; Kida et al., 2002). A recent study reported that memory retrieval initiates the opposing processes of extinction and reconsolidation (Suzuki et al., 2004). Moreover, memory processing following retrieval is in part, determined by the duration of re-exposure to the CS, such that brief re-exposures lead to reconsolidation whereas, longer re-exposures result in extinction (Debiec et al., 2002; Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004). Therefore, by re-exposing the mice 6 times to the CS without the US (a high

number of exposures) our manipulation more likely affected extinction, rather than reconsolidation.

4.2.3. HSV Methodology

Our results suggest that increasing CREB function in LA neurons enhanced extinction consolidation. To increase CREB function in the LA we used HSV vectors, however, we did not investigate which neurons in the LA were affected. This could yield important information because the LA consists of excitatory pyramidal cells and inhibitory interneurons (Pitkanen, 2001; Rainnie, 2003). As excitatory cells project to and activate Ce neurons that generate fear responses, this pathway may be more important for fear conditioning and elevating CREB levels in these neurons would be expected to facilitate LTM for fear conditioning. By contrast, inhibitory interneurons in the LA have been shown to play a role in fear extinction by inhibiting LA and B activation of Ce neurons (Grace and Rosenkranz, 2002). Therefore, increasing CREB levels in these neurons may be expected to facilitate LTM for fear extinction. However, this view is complicated by the finding that excitatory LA neurons also project to GABAergic intercalated cells (located between the LA and Ce), which have also been implicated in fear extinction (Quirk et al., 2003).

To determine the type of neuron (excitatory or inhibitory) that was infected by our viral vector, immunohistochemical experiments could be performed. For example, co-localization of GFP signals (indicating an infected neuron) with immunohistochemical markers of excitatory (e.g., CaMKII) or inhibitory (e.g., GAD67) neurons would indicate whether HSV preferentially infects excitatory neurons, inhibitory neurons, or both. The

viral vector that we used contained a HSV IE 4/5 promoter (a promoter for HSV immediate early genes) that would not be specific for excitatory or inhibitory neurons. The use of different promoters would allow selective transgene expression in different cell types.

4.2.4. Effects of CREB Overexpression Are Not on Extinction Memory Retrieval

In our study, we examined the effects of increasing CREB function in the LA on fear extinction consolidation by measuring conditioned fear 24 hours after extinction training. It is interesting to note that CREB enhanced extinction performance only on the first test tone. Therefore, an alternative interpretation of the results could be that the CREB effects were on memory retrieval, rather than consolidation. This is not likely, given that within-session extinction was the same between the 2 groups of mice. Moreover, previous studies have shown that CREB is not important for the retrieval of conditioned fear memory (Kida et al., 2002) and that CREB overexpression in the LA does not affect retrieval processes (Josselyn et al., 2001).

4.3. Mechanism of CREB Action in the LA Underlying Fear Extinction

Several lines of evidence from different species indicate that CREB function is important for the formation of LTM in a variety of behavioural paradigms. For example, previous studies showed that CREB overexpression in the LA enhances the consolidation of fear memory without affecting fear acquisition (Josselyn et al., 2001; Wallace et al., 2004). By contrast, very few studies have examined CREB function in the extinction of fear memories. In one of a few studies that have examined this question, Lin et al. (2003)

showed that extinction training reduced CREB phosphorylation (pCREB) in the LA and BLA measured 20 minutes after training (fear-potentiated startle). At first glance, this may seem to contradict our results. However, this study did not measure pCREB levels at a later time point (e.g., 24 hours) after training (a time point that would correspond to extinction consolidation). Furthermore, they did not examine the effects of manipulating CREB function on extinction.

There is extensive evidence from a variety of model systems showing that protein synthesis is required for the consolidation of various memory tasks (Davis and Squire, 1984; Matthies, 1989; Abel et al., 1997; Schafe et al., 1999). Many studies have shown that CREB is important for initiating the protein synthesis necessary for LTM (Bailey, C. H., Bartsch, D., and Kandel, E. R., 1996; Frankland et al., 2004). However, the few studies that have examined the effects of protein synthesis inhibitors on extinction have produced widely varying results that may depend on the region and/or the behavioural paradigm used. Berman and Dudai, (2001), for example, reported that pre-extinction training infusions of anisomycin into the insular cortex impaired extinction of conditioned taste aversion. By contrast, similar infusions into the Ce had no effect (Berman et al., 2001). Furthermore, Lin et al. (2003) found that pre-training blockade of protein synthesis in the LA with anisomycin prevented extinction measured 10 minutes after fear training. This latter finding is surprising given that protein synthesis inhibitors typically block LTM, but not STM. However, whether this treatment also blocks the consolidation of extinction learning measured 24 hours following training was not tested.

Recently, BDNF in the LA has been implicated in fear extinction. Disruption of BDNF signaling in the LA via a lentivirus encoding a dominant negative TrkB blocked

the consolidation of fear extinction as measured 2 days after extinction training (Chhatwal et al., 2006). Interestingly, BDNF is closely associated with CREB. For example, BDNF is a target gene of CREB activation (Shieh et al., 1998; Tao et al., 1998; West et al., 2001) and BDNF signaling through the TrkB receptor activates CREB (Patterson et al., 2001). In conjunction with the findings of the present study, these results suggest that both CREB and BDNF in the LA are important for the consolidation of fear extinction and that their activation may result in protein synthesis.

4.4. Parallels Between Fear Acquisition and Extinction

Our results, along with others, suggest that fear acquisition and extinction share common molecular mechanisms. Both, processes for example, require glutamate-activation of NMDA receptors in the LA. The consolidation of fear acquisition and extinction also involves many of the same intracellular signaling pathways, such as PKA, MAPK, α-CaMKII, and CREB. These cascades may in turn, initiate gene transcription and protein synthesis. Together, these findings suggest that fear extinction represents another form of learning that actively inhibits the original fear memory.

4.5. Other Issues

It could be argued that CREB overexpression did not lead to an increase in CREB function. One way to verify that our manipulation resulted in an increase in CREB function would be to examine whether injecting a mutant form of CREB that lacks the S133 phosphorylation site also produces a behavioural effect. Alternatively, if CREB function was indeed increased, it would be expected that pharmacological blockade of

S133 phosphorylation prior to extinction training would abolish the facilitatory effect of CREB overexpression. Finally, another method would be to compare levels of CREB-dependent immediate early genes (e.x. c-fos) in mice that received CREB with GFP controls using immunohistochemistry. A higher level of immediate early genes in CREB-treated mice would indicate that the infused CREB was functional.

Another issue concerns the amount of CREB overexpression achieved with the viral vectors in a single cell. This can be approximated using double-labeling techniques whereby, cells with co-labeling of GFP signals with an antibody for CREB are compared with cells in which only a CREB signal from the antibody is detected. As different cells are infected to different degrees, this method would only yield a range infection intensities rather than an absolute value.

One question that naturally arises from our CREB manipulation is whether CREB overexpression affected endogenous levels of different CREB isoforms. Although this may be the case, the net result would always be an increase in CREB-mediated transcription in neurons. The mechanisms by which this occurs is of interest but is not that relevant to the project at hand.

It was observed that mice receiving HSV-CREB exhibited less fear (lower suppression ratio) on the first tone during extinction training than GFP mice. This could be interpreted as an effect of CREB overexpression on retrieval processes. However, this is unlikely, as previous studies have shown that CREB overexpression does not affect

retrieval processes (Josselyn et al., 2001). Alternatively, the presence of CREB during the period between surgery and extinction training, in which mice were re-trained under a VI-60 schedule, may enhance learning of safety mechanisms and therefore, reduce the impact of the tone on conditioned responding. To test whether safety learning is occurring, one could include a group that does not receive re-training during the recovery period, but rather left in the home cage and therefore, would not be exposed to any safety cues.

4.6. Future Studies

Here, we have shown data that is consistent with the conclusion that CREB function in the LA is important for fear extinction consolidation. However, this does not preclude the involvement of other brain structures. Previous data on the role of the IL in extinction is mixed. Some groups report that lesions of the IL and protein synthesis inhibition in the IL impair extinction measured 24 hours after extinction training (Morgan et al., 1993; Quirk et al., 2000; Santini et al., 2004). However, other studies have shown that the IL is not important for the consolidation of fear extinction (Gewirtz et al., 1997; Garcia et al., 2006). All of these studies attempt to infer the function of the IL by disrupting the function of the IL. Another way to approach this question is to use a similar gain-of-function approach to the one employed here. For instance, one could test whether overexpressing CREB in the IL enhances fear extinction consolidation.

Although the present data are consistent with the notion that CREB in the LA is important for the consolidation of fear extinction, it may be that CREB is not essential for this process. To establish an essential role for CREB in fear extinction consolidation, one

should demonstrate that the disruption of CREB disrupts extinction memory consolidation. One way to do this would be to similarly infuse a dominant negative form of CREB into the LA and determine whether this disrupted consolidation of extinction.

Given that the present data support the notion that CREB is involved in extinction consolidation, it will become important for future studies to identify targets of CREB activation during the consolidation of extinction memories. As mentioned earlier, BDNF is a likely candidate target of CREB activation.

Our study examined the effects of CREB overexpression on extinction consolidation at a behavioural level. More information can be gleaned by examining the effects of our manipulation at an electrophysiological level. This can be accomplished by measuring the electrical properties of input synapses to the LA before and after increasing CREB levels. As the LA consists of a heterogeneous population of neurons, selective potentiation of synapses in this region would yield insight into which neurons and pathways are engaged during fear extinction consolidation. Based on our results and the notion that memory for fear extinction is inhibitory, it may be expected that increasing CREB levels in the LA will specifically potentiate input synapses onto inhibitory interneurons, leading to an increase in inhibition of Ce output neurons that generate conditioned fear responses.

To test whether increasing CREB levels in the LA produces long-term effects on extinction memory, one can examine whether this manipulation affects the processes of spontaneous recovery and reinstatement of extinguished conditioned fear. To investigate spontaneous recovery, one approach would be to compare conditioned fear levels in mice receiving CREB or GFP 24 hours after training and at a later time point (e.g., 1 month

post-training). To investigate reinstatement, one could compare conditioned fear produced by an unsignaled footshock, in mice infused with CREB and GFP, 1 month following extinction training. For both spontaneous recovery and reinstatement, if the effects of CREB overexpression persists for at least 1 month after training, CREB-infused mice would be expected to exhibit lower levels of conditioned fear (better extinction retention) than control mice on a test given 1 month following extinction training. It is important to note that transgene expression using our viral vectors declines to near zero levels 7 days following infusion. Therefore, any behavioural effect measured past this point would be attributed to high levels of CREB at the time of extinction training.

Finally, a recent study has found that extinction of fear and drug use may involve similar mechanisms within the LA (Schroeder and Packard, 2004). This raises the question of whether increasing CREB levels in the LA may also facilitate the extinction of drug use as it does for conditioned fear.

4.7. Concluding Remarks

The results from the present study support the conclusion that CREB in the LA is important for the consolidation, but not acquisition, of fear extinction. However, it does not rule out the possibility that other transcription factors and brain regions also contribute to this process. Our findings help to provide insight into the molecular mechanisms that govern extinction. A better understanding of normal extinction processes holds great promise for the development of improved therapeutic strategies for the treatment of anxiety disorders, which are believed to arise from a dysfunction in normal extinction processes.

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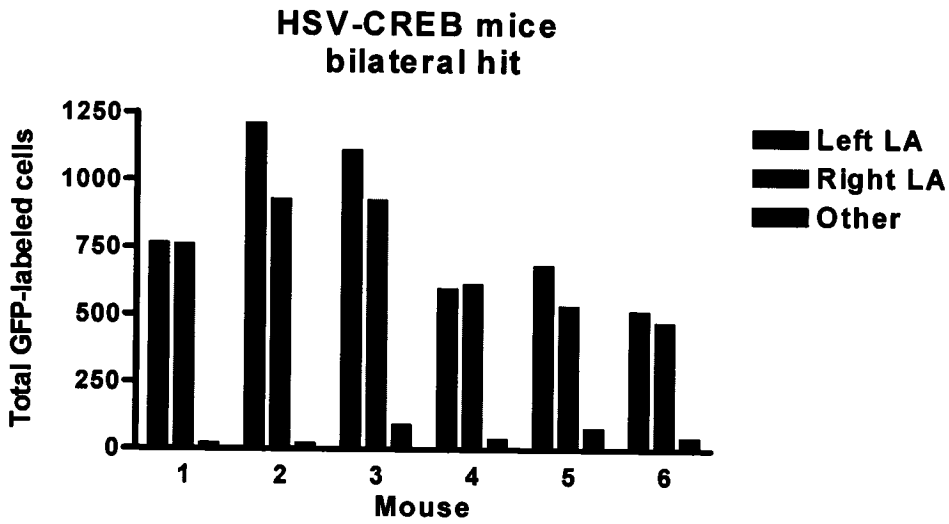
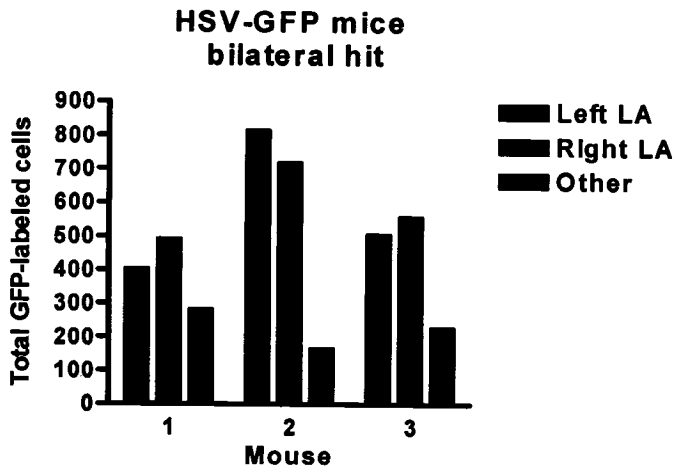
APPENDIX

Cell Counting Data

A



B



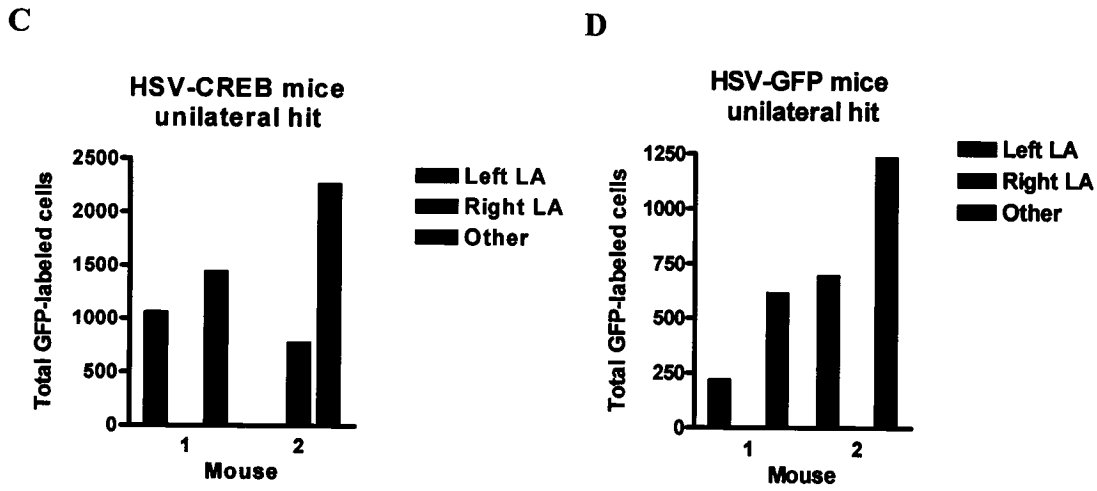


Figure 23. Number of GFP-positive cells. Comparison of the number of GFP-labeled cells in the left LA, right LA, and regions outside of the LA for mice with GFP expression on both sides (A) and (B) or on only one side (C) and (D).