Compensatory Neural Circuits for Learning Fear Without the Basolateral Amygdala

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in The University of Michigan
2010

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Dedication

This dissertation is dedicated to

my amazing wife Britt

for her endless support.

Acknowledgements

I would like to thank my advisor, Dr. Stephen Maren, for his support throughout my graduate career. His insight and leadership have helped me to become a successful scientist and provided me with the skills necessary succeed in my future career. I would additionally like to thank Dr. Geoff Murphy for supporting and teaching me, both as a member of my committee and throughout my rotation in his lab. I would also like to thank the other members of my committee, Dr. Kent Berridge, Dr. Gina Poe, and Dr. Michael Sutton, for their continued support and insight as I complete my dissertation.

I would sincerely like to thank all of my past and present laboratory colleagues, Kevin Corcoran, Jen Hobin, Steve Merino, Jinzhao Ji, John Perkowski, Brandon McKinney, Christine Rabinak, and Caitlin Orsini. These individuals have taught me the skills necessary to succeed in science and provided the support necessary to persevere, even in cases of complete experimental failure. I would also like to thank all of the friends, of whom there are too many to name, that have supported me throughout my graduate career.

Last, but certainly not least, I must thank my family, my wife Britt for celebrating with me on the good days and consoling me on the bad, my parents Pamela and Joel, my brother Kyle, and all my grandparents for always supporting me and teaching me that I can achieve anything I put my mind to.

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CHAPTER 1

INTRODUCTION

The ability to express an appropriate fear response to dangerous situations is evolutionarily advantageous for all species and likely evolved to allow animals to escape from dangerous situations. However, expressing extreme fear in the absence of danger can be extremely debilitating. Such is the case with many psychological disorders including panic, anxiety, and posttraumatic stress disorders (PTSD).

Anxiety disorders such as PTSD have interfered with human lives for centuries. While the term posttraumatic stress disorder wasn't coined until the publication of DSM-III in 1980 by the American Psychiatric Association (APA), references to the symptoms associated with PTSD can be found in classic literature as far back as Homer's *Iliad* written in approximately 720 B.C. (Ray, 2008) and can be traced through time to our current understanding of the disorder.

Around the time of the Bohr war and American Civil War Myers (1870) wrote about "soldiers' heart" to describe a condition amongst soldiers that included extreme fatigue, dyspnea, and sweating. At this point in time, the symptoms of the disorder were considered to be of the heart, not the mind. Following World War I, doctors began to realize that many of the symptoms

being diagnosed were psychiatric in nature and were in direct response to the psychological trauma associated with war; a syndrome being diagnosed as “shell shock” (Myers, 1915).

It was not until after World War II, as reports about survivors of Nazi concentration camps became more readily available, that researchers began to link the environmental and psychological stressors with physiological responses; realizing that the symptoms of “combat fatigue” were not unique to combat, but could be generalized to emotionally and physically stressful situations including rape and natural disasters (Ray, 2008). Currently the most widely accepted diagnostic criteria for PTSD comes from the current version of the DSM, the 4th edition (1994), requiring that an individual must have experienced, witnessed, or been confronted with an event or events that involved actual or threatened death or serious injury or a threat to the physical integrity of self or others, and responded to the event with intense fear, helplessness, or horror. As the history has demonstrated, PTSD is extremely prevalent among war veterans, with lifetime prevalence rates ranging from 6-31% (Richardson et al., 2010). Through a better understanding of the biology of PTSD we can better target treatments for the disorder and help the 6.8% of the US adult population currently suffering from PTSD (National Center for Posttraumatic Stress Disorder, 2007).

Pavlovian Conditioning

While the history of PTSD has helped us to describe the symptoms and refine the diagnoses of the disorder, it provides little insight into the biological

basis. To gain a better understanding of the biological etiology of PTSD we turn to animal models of fear learning and anxiety disorders.

In the early 1900s, while collecting saliva samples for his research on gastric function in dogs, Ivan Pavlov stumbled upon the discovery of conditioned reflexes (now known as classical or Pavlovian conditioning) (Pavlov, 1927). He noticed that dogs began to salivate before they received food in response to food-associated cues. Through further research, Pavlov discovered that through the pairing of a biologically neutral conditioned stimulus (CS - such as the sound of a metronome) with a biologically relevant unconditioned stimulus (US – such as food) the dogs began to display a conditioned response (CR – such as salivation), even if the CS was presented in the absence of the US.

Following the work of Ivan Pavlov, John B. Watson with the help of his assistant Rosalie Rayner set out to demonstrate classical conditioning in humans using a 9-month old child known as Little Albert for a subject (Watson and Rayner, 1920). After first demonstrating that Little Albert was unafraid of a white rat (CS), Watson and Rayner proceeded to hit a steel beam with a hammer creating a loud noise (US) causing Little Albert to cry (unconditioned response [UR] and CR) every time he reached out to touch the rat. Following the pairing of the CS with the US, whenever Little Albert was presented with the rat (CS) he began to cry (CR). Furthermore, Little Albert generalized the CS (stimulus generalization) to other furry objects. This experiment, for the first time, demonstrated Pavlovian fear conditioning in humans. Furthermore it

demonstrated the phenomenon of stimulus generalization, a hallmark trigger of the symptoms associated with PTSD.

Pavlovian fear conditioning is a variation of classical conditioning that has proven to be very insightful into the neurobiological mechanisms of fear learning and anxiety disorders (Davis, 1992; Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001a, 2005). Pavlovian fear conditioning consists of a neutral conditioned stimulus (CS), such as a tone or the conditioning context, paired with an aversive unconditioned stimulus (US), such as a mild footshock that elicits an unconditioned response (UR). After conditioning, the CS alone triggers a variety of conditioned fear responses (CRs), including increases in blood pressure (Romanski and LeDoux, 1992), potentiated acoustic startle (Davis, 2001), and freezing behavior (Fanselow and Bolles, 1979; Fanselow, 1980; Fendt and Fanselow, 1999). The behavior displayed during the process of fear conditioning requires a number of active processes. First, the animal must learn, or acquire, the CS/US association. Second, they must store the learned memory, a process known as consolidation. Finally, the animal must recall the CS/US association to express the fear when presented with the CS alone. Memory for the CS/US association can be obtained in as little as a single pairing (Blanchard and Blanchard, 1972; Davis et al., 1989; Maren, 2001b). Moreover, the memory obtained following the acquisition of conditioned fear has been shown to last at least one year (Gale et al., 2004).

Through lesions, temporary inactivation, and/or other pharmacological techniques applied at various points in the fear conditioning process we can

examine the underlying neurobiology and molecular processes required for Pavlovian fear conditioning.

The Neurobiology of Conditioned Fear

In recent years, understanding the neurobiology of conditioned fear has become of particular interest. As a result it is now widely agreed that the amygdala is an essential structure for the acquisition, consolidation, and expression of conditioned fear (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001a). The amygdala lies deep in the medial temporal lobe and is composed of several discrete nuclei (Krettek and Price, 1978): the basolateral complex (BLA), consisting of the lateral (LA) and basal (BA) (including basolateral (BL), and basomedial (BM) nuclei) nuclei of the amygdala and the central nucleus of the amygdala (CEA), composed of lateral (CEl) and medial (CEm) subdivisions. Separating the BLA and CEA is a cluster of inhibitory GABAergic neurons know as the intercalated cell mass (ITC).

Information flow within the amygdala is primarily unidirectional beginning in the LA and terminating in the CEm (Figure 1.1) (Pitkanen, 2000). Neurons in the LA project to the BA, ITC, and CEA. The BA in turn projects to the ITC and CEA. Importantly, Royer and colleagues have demonstrated lateral inhibition between inhibitory clusters within the ITC that allow neurons in the BLA to indirectly excite neurons in the CEA (Royer et al., 1999, 2000).

The BLA is widely regarded as the sensory interface of the amygdala, the first point of convergence for sensory information. Information regarding the CS is processed by, and sent to the BLA from the medial geniculate nucleus of the thalamus (MGN) (Iwata et al., 1986; Doron and Ledoux, 2000) and the primary auditory cortex (Romanski and LeDoux, 1992). Simultaneously, US information is provided to the BLA from the posterior intralaminar nucleus of the thalamus (PIN) (Doron and Ledoux, 2000; Linke et al., 2000), while the hippocampus (HIP) provides contextual information to the BLA about the time and place of the stimuli (O'Reilly and Rudy, 2001; Sanders et al., 2003).

Associative plasticity within the BLA is believed to underlie the formation of fear memory. The best-studied model for associative plasticity is long-term potentiation (LTP), a process by which brief repetitive stimulation of a synaptic pathway results in a long-term enhancement of the synaptic efficacy of the connections within that pathway (Bliss and Lomo, 1973). While originally described in hippocampal pathways by Bliss and Lomo (1973), similar protocols have been effectively utilized in the thalamo-amygdala pathway believed to underlie conditioned fear (Sah et al., 2008).

Projections from the thalamus to the amygdala are primarily glutamatergic in nature (Mahanty and Sah, 1999). The release of glutamate from thalamic inputs into the amygdala activates two types of glutamatergic receptors, AMPA and NMDA. AMPA receptors mediate fast excitatory potentials through the influx of sodium and potassium, opening at the resting membrane potential when glutamate is bound (Sah et al., 2008). AMPA receptors are therefore believed to

be primarily involved in basal excitatory transmission (Sah et al., 2008). NMDA receptors are often co-expressed in the post-synaptic density with AMPA receptors (Bekkers and Stevens, 1989; Mahanty and Sah, 1999). Like AMPA receptors, NMDA receptors require glutamate to open. Unlike AMPA receptors, NMDA receptors act as coincidence detectors with the additional requirement that the cellular membrane be sufficiently depolarized to remove the Mg2+ ions that bind to the open channel and obstruct the flow of current (Nowak et al., 1984). Once activated, NMDA receptors are permeable to sodium, potassium, and calcium. Therefore, NMDA receptors are believed to underlie some forms LTP. When the cell is sufficiently depolarized in the presence of glutamate, the influx of calcium through the NMDA receptor is believed to activate second messenger cascades altering protein expression and ultimately strengthening the synapse (Sah et al., 2008). One such mechanism that has been demonstrated at amygdala synapses is the trafficking of additional AMPA receptors to the post-synaptic density, resulting in a potentiation of synaptic transmission (Malinow and Malenka, 2002; Malenka, 2003). As such, the blockade of NMDA receptors blocks the induction of LTP (Bauer et al., 2002; Humeau et al., 2003; Humeau et al., 2005).

While a direct link between the molecular mechanisms required for LTP induction and fear learning has never been demonstrated, many pharmacological studies suggest such a link exists. Infusions of AMPA receptor antagonists into the BLA block the expression of conditioned fear (Falls et al., 1992; Kim et al., 1993; Walker et al., 2005), supporting a role for AMPA receptors in basal

synaptic transmission. Just as NMDA receptor antagonism blocks the induction of LTP, it similarly blocks the acquisition of conditioned fear when infused into the BLA (Miserendino et al., 1990; Campeau et al., 1992; Cox and Westbrook, 1994; Fanselow and Kim, 1994; Maren et al., 1996b; Lee and Kim, 1998; Rodrigues et al., 2001; Lin et al., 2003; Goosens and Maren, 2004; Maren and Quirk, 2004; Walker et al., 2005).

While the BLA is widely regarded as the input of the amygdala, the CEA is functionally and anatomically positioned to serve as the output. Believed to primarily receive sensory information from the BLA, the CEA sends this information to the downstream nuclei responsible for the behavioral and physiological responses associated with fear (Ledoux et al., 1988; Davis, 1992; Maren and Fanselow, 1996; Maren, 2005). These nuclei include the periaqueductal grey (PG) for the production of freezing responses, the lateral hypothalamus (LH) controlling changes heart rate, the parabrachial nucleus (PB) responsible for the startle response, and the nucleus reticularis pontis caudalis (NR) required for changes in respiration (Figure 1.1). Importantly, the CEA also receives projections directly from the thalamus carrying information regarding the CS and US (Linke et al., 2000; Pare et al., 2004).

Based upon the findings discussed above, the most common and long-held model of intra-amygdaloid processing is a serial model whereby CS/US information converges in the BLA. The information is then sent either directly from the BLA to the CEA or indirectly via the ITC to the CEA (Figure 1.1) (Royer et al., 1999, 2000). By this model, the BLA is essential for the associative

processing of the CS and US. It is therefore critical for the acquisition and expression of conditioned fear, whereas the CEA serves as a passive output structure critical for only the expression of conditioned fear. A large history of literature supports such roles. Pre-training lesions or temporary inactivation of the BLA block the acquisition of conditioned fear. Likewise, post-training lesions or pre-test inactivation of the BLA or CEA block the expression of conditioned fear (LeDoux et al., 1990; Helmstetter, 1992; Campeau and Davis, 1995; Maren et al., 1996a; Cousens and Otto, 1998; Goosens and Maren, 2001).

The Bed Nucleus of the Stria Terminalis

The bed nucleus of the stria terminalis (BNST) was first examined in the fear conditioning literature due to its connectivity with the amygdala and the hypothalamic and brainstem structures required for the production of various CRs. Specifically, the BNST, like the amygdala, receives projections from the thalamus and hippocampus in addition to projections from the BLA and CEA (Dong et al., 2001). In turn, the BNST projects to the same brainstem nuclei required for the productions of CRs as the CEA (Dong et al., 2001). Additionally, the BNST also projects to the hypothalamus whereby it can influence the Hypothalamic-Pituitary-Adrenal (HPA) axis. Because of this connectivity, the BNST is anatomically positioned to play a key role in the processing of conditioned fear. Furthermore, it may serve as a link between stress, anxiety, and conditioned fear.

The first evidence for the involvement of the BNST in stress and anxiety came from experiments studying corticotropin releasing hormone (CRH - a key element of the HPA axis) enhanced startle. Just as humans do, rats display an activity burst, or startle response to an unpredicted loud tone. CRH-enhanced startle is a paradigm in which systemic injection or intracerebroventricular infusion of CRH increases the startle response. Lee and Davis (1997) discovered that infusions of CRH directly into the BNST increased the startle response. Additionally, they demonstrated that NMDA lesions or infusions of α-helical CRH (αhCRH - a CRH antagonist) into the BNST blocked CRH enhanced startle (Lee and Davis, 1997). Interestingly, similar manipulation of the BLA, CEA, and HIPP had no effect on CRH enhanced startle suggesting a selective role for the BNST in anxiety and stress responses.

Additional evidence for the importance of the BNST for the production anxiety responses came from studies of light-enhanced startle (LES), a paradigm in which the startle response is enhanced in a brightly lit environment as compared to a dim or dark environment (Walker and Davis, 1997b). LES as a model for anxiety is supported by the findings that that LES is blocked by systemic administration of anxiolytic agents such as benzodiazepines (Walker and Davis, 1997b; de Jongh et al., 2002; Walker and Davis, 2002). Similar to CRH-enhanced startle, infusions of the AMPA receptor antagonist NBQX into the BNST blocked LES whereas infusions into the BLA and CEA once again had no effect (Walker and Davis, 1997a).

The anatomy of the BNST in conjunction with its role in anxiety suggests the BNST may play a critical role in conditioned fear as well. Specifically, the BNST may serve as a link between conditioned fear and the increased stress response associated with conditioning. The role of the BNST in conditioned fear was first investigated by Hitchcock and Davis (1991) using the fear potentiated startle (FPS) paradigm (a model of Pavlovian conditioned fear similar to fear conditioning) (Davis, 2001). They discovered that electrolytic lesions of the BNST had no effect on FPS. Likewise, FPS was unaffected by excitotoxic lesions or AMPA receptor antagonism of the BNST (Walker and Davis, 1997a; Gewirtz et al., 1998). Conversely, any of these manipulations made to the BLA or CEA completely block FPS (Hitchcock and Davis, 1986; Campeau et al., 1992; Campeau and Davis, 1995; Walker and Davis, 1997a, 2000). The results up to this point including the findings for CRH-enhanced startle, LES, and FPS suggest a selective role for the BNST in unconditioned fear or anxiety, while the amygdaloid nuclei are required for conditioned fear.

The Extinction of Conditioned Fear

Thus far, the neural circuitry supporting fear has only been discussed in terms of the acquisition and expression fear responses. However, of potentially more clinical relevance is the process of extinction, or the degradation of the relationship between the CS and the US by presenting the CS alone numerous times in the absence of the US (Chang et al., 2009). During extinction, animals learn that the CS no longer reliably predicts the US and form a new inhibitory

memory that suppresses fear. This suppression is labile, however, and fear CRs may return with the passage of time (spontaneous recovery) (Baum, 1988), changes in context (renewal) (Maren, 2005; Bouton et al., 2006), or the presentation of a single unpaired US (reinstatement) (Rescorla and Heth, 1975). Such findings suggest that similar to the acquisition of the original CS/US association, extinction is an active new learning process.

The neurobiological and molecular mechanisms underlying extinction are similar to those necessary for the acquisition of conditioned fear. Just like the acquisition of the original CS/US association, the acquisition of extinction is highly dependent upon the amygdala (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001a). Furthermore, just as NMDA receptor antagonism within the BLA blocks the acquisition of fear, it also blocks extinction (Miserendino et al., 1990; Campeau et al., 1992; Falls et al., 1992; Cox and Westbrook, 1994; Fanselow and Kim, 1994; Maren et al., 1996b; Lee and Kim, 1998; Rodrigues et al., 2001; Santini et al., 2001; Lin et al., 2003; Goosens and Maren, 2004; Maren and Quirk, 2004; Walker et al., 2005). Interestingly, infusions of the NMDA receptor agonist d-cycloserine (DCS) into the BLA facilitate extinction (Walker et al., 2002).

Additional evidence for the role of the amygdala in extinction comes from electrophysiological studies. In-vivo studies recording changes in single unit activity throughout extinction have discovered a population of LA neurons that decrease their spike firing rate in correlation with the animal's behavior (Quirk et al., 1997). Additionally, Hobin and colleagues (2003) have demonstrated that

individual neurons can code both conditioning and extinction memories. Furthermore, the changes in spike firing observed in these neurons are context dependent (Hobin et al., 2003). Such results suggest that extinction may involve an inhibitory network of cells within the amygdala capable of gating the context dependent expression of fear in response to hippocampal input (Figure 1.2). Through a better understanding of the specific circuitry and molecular mechanisms of extinction we have the potential to significantly improve the treatment of anxiety disorders such as PTSD.

Specific Aims and Hypotheses

The amygdala has long been held a key structure necessary for the acquisition, expression, and extinction of conditioned fear. Historically, the BLA has been viewed as the amygdaloid nuclei critical for the acquisition of fear while the CEA was believed to be only a passive relay structure necessary for expression. Recently however, findings that rats can acquire conditioned fear in the absence of the BLA (Maren, 1999; Goosens and Maren, 2003) suggest the CEA my play a broader role in fear learning and memory storage; specifically, we propose that the CEA plays a role in the acquisition, expression, and extinction of conditioned fear. The primary purpose of this dissertation is to explore the role of the CEA in the acquisition, expression, and extinction of Pavlovian conditioned fear and the molecular mechanisms underlying these processes.

The experiments discussed in Chapter 2 explore the role of the CEA in the acquisition and expression of overtrained conditioned fear. As previously

mentioned, rats with BLA lesions are still able to acquire and express conditioned fear, but require substantially more training to do so (overtraining) (Maren, 1999; Goosens and Maren, 2003). We hypothesize that the CEA is a critical component of the neural circuitry necessary to compensate for the loss of the BLA. Utilizing excitotoxic lesions of the BLA in conjunction with lesions or temporary inactivation of the CEA, we demonstrate that the CEA is necessary for the acquisition and expression of cued and context conditioned fear in rats regardless of the state of the BLA. Importantly, these results demonstrate that the CEA plays a critical role in the acquisition of conditioned fear even in cases where the BLA is still intact, and strongly suggest that the CEA is the locus of compensation in the absence of the BLA.

As discussed above, the BNST possesses connectivity very similar to that of the CEA (Dong and Swanson, 2004). While the BNST is not essential for the expression of FPS (Hitchcock and Davis, 1991; Walker and Davis, 1997a; Gewirtz et al., 1998), FPS is unable to explore contextually conditioned fear. Furthermore, the possibility remains that the BNST may be able to compensate for the loss of the BLA and mediate both contextual and cued conditioned fear following overtraining. To examine this possibility, Chapter 3 explores the role of the BNST in rats overtrained with BLA lesions in combination with post-training lesions or pretesting inactivation of the BNST. Similar to the findings of Sullivan and colleagues (2004), we discover that lesions or temporary inactivation of the BNST only block the expression of contextual fear, leaving auditory cued fear

intact. These results provide additional evidence that the CEA is the locus of compensation for conditioned fear in the absence of the BLA.

Similar to the original CS/US association obtained during fear conditioning, the process of extinction also requires new learning of a CS/no-US association. While the necessity of NMDA dependent synaptic plasticity within the BLA is well established in the formation of this extinction memory, the role of the CEA in extinction has never been explored. Furthermore, in light of our findings for a role of the CEA in the acquisition of conditioned fear it seems likely that plasticity within the CEA may also be important for the acquisition of an inhibitory extinction memory. To explore this possibility, Chapter 4 investigates the role of AMPA and NMDA receptors within the BLA or CEA on the extinction of conditioned fear. Interestingly, we found that while AMPA receptor antagonists infused into either the BLA or CEA blocked the expression of conditioned fear during the extinction session, rats still acquired an extinction memory as tested 24 hours later drug-free. Alternatively, antagonism of BLA or CEA NMDA receptors had no effect on the expression of fear during the extinction session. Surprisingly, only NMDA receptor antagonism within the BLA blocked the acquisition of extinction; NMDA receptor antagonists infused into the CEA had no effect. These results, in association with those discussed in Chapter 2, suggest a dissociation between the roles of the BLA and CEA in the acquisition and extinction of conditioned fear. Whereas the acquisition of fear is reliant upon the CEA, even in the absence of the BLA, extinction requires the

activation of NMDA receptors within BLA, not CEA.

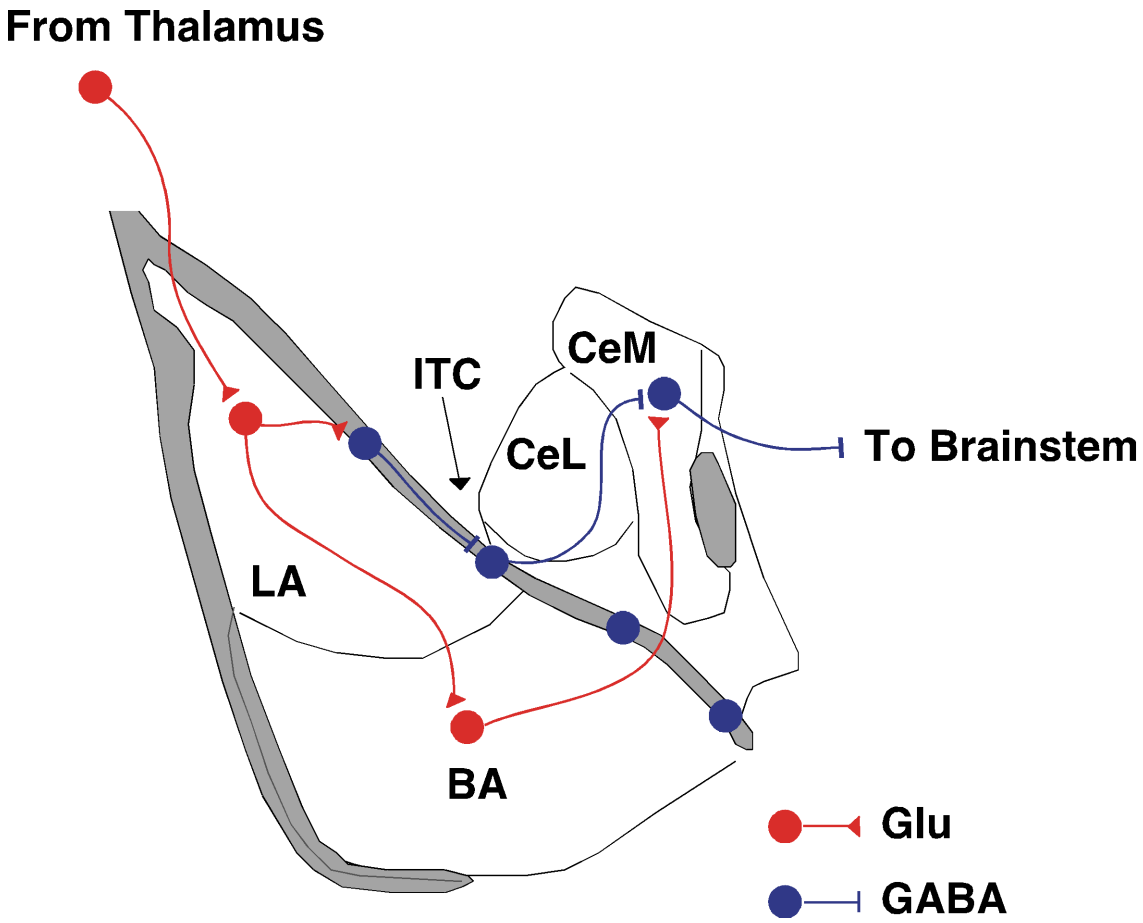

Figure 1.1. Intra-amygdaloid processing.
According to the serial processing theory CS and US information is transmitted to the lateral amygdala (LA) from the thalamus. That information then excites central amygdala (CEA) neurons via excitatory connections through the basal amygdala (BA) or feed-forward inhibition through the intercalated cell mass (ITC). The CEA acts as the primary output structure sending efferent connections to the brainstem nuclei required for the expression of CRs.

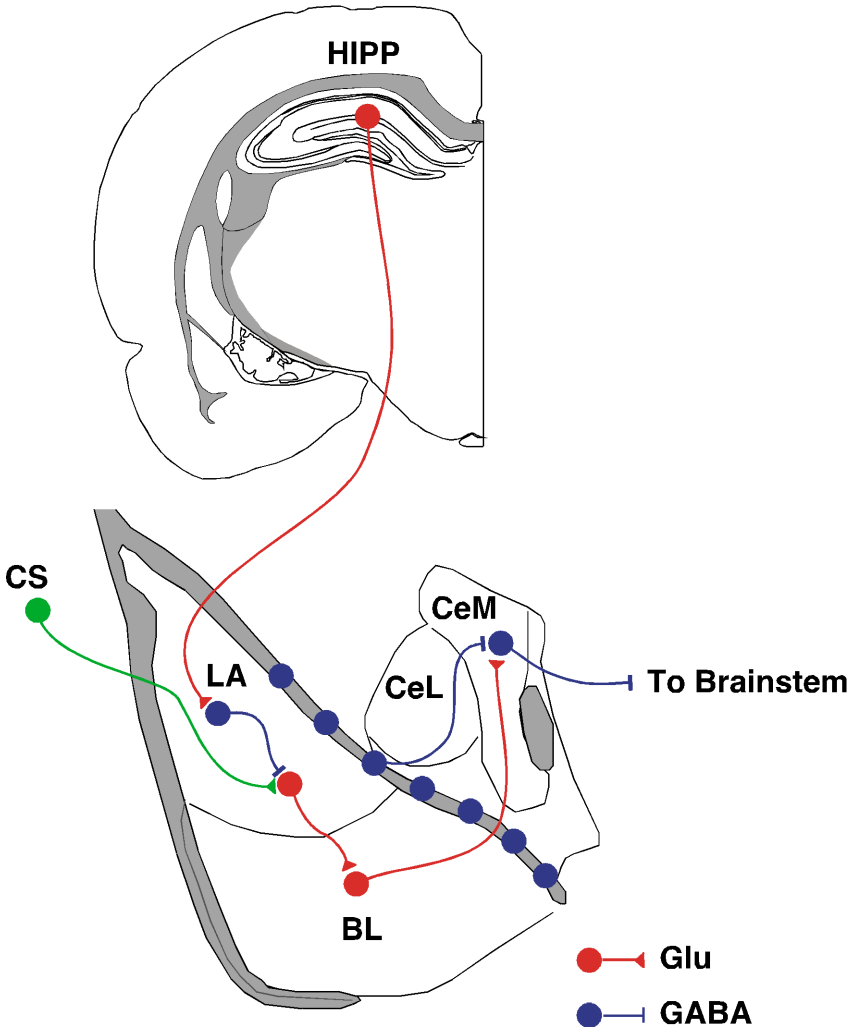

Figure 1.2. The neurobiology of extinction.
The extinction of Pavlovian conditioned fear is highly contextually dependent. The contextual modulation required for extinction is likely gated by the hippocampus (HIPP) via projections to the lateral amygdala (LA). Pathways through the LA reduce activity in the central nucleus (CEA) via the activation of inhibitory interneurons within LA.

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CHAPTER 2

THE CENTRAL NUCLEUS OF THE AMYDALA IS ESSENTIAL FOR ACQUIRING AND EXPRESSING CONDITIONAL FEAR AFTER OVERTRAINING

Pavlovian fear conditioning is an important model for studying the neural mechanisms contributing to emotional learning and memory (Davis 1992; LeDoux 2000; Maren 2001; Maren 2005). In this paradigm, a conditioned stimulus (CS), such as a tone, is presented with an aversive unconditional stimulus (US), such as a footshock. The pairing of the CS and the US comes to elicit conditioned fear responses (CRs) including increased heart rate, blood pressure, acoustic startle, and somatomotor immobility (i.e. freezing). It is now well established that the amygdala is critical for this form of learning (Davis and Whalen 2001; Fendt and Fanselow 1999; LeDoux 2000; Maren 2001). The majority of current work focuses on the role of the nuclei within the amygdala, specifically the basolateral complex [BLA: consisting of the lateral (LA), basolateral (BL), and basomedial (BM) nuclei] and the central nucleus (CEA), in this form of learning. Within the amygdala, the BLA is believed to be the site at which information regarding the CS (auditory and contextual cues) and the US first converge, although CEA neurons also receive auditory and somatic input. Afferents from the medial geniculate body (MGm) (Doron and Ledoux 2000), as well as various sensory cortices including the primary auditory cortex (Romanski

and LeDoux 1992), route information regarding the CS to the LA. Afferents from the hippocampus transmit multimodal information regarding the context and time of conditioning to the BLA (O'Reilly and Rudy 2001; Sanders et al. 2003); highly processed sensory information from cortical regions, including the prefrontal cortex (PFC), also converges in the LA (McDonald 1998).

In contrast, the medial division of the CEA (CEm) has been posited to be the primary output structure of the amygdala. The CEA receives information from the LA via the intercalated nuclei, and it also receives direct projections from the BL and thalamus. The CEm in turn projects to brain areas involved in the production of the CR, including the periaqueductal gray and the lateral hypothalamus, which mediate freezing and cardiovascular response, respectively (LeDoux et al. 1988). However, recent studies suggest that the CEA may also have a role in the acquisition of conditional fear (Goosens and Maren 2003; Maren 2005; Wilensky et al. 2006), and it is anatomically positioned to serve this role (Pare et al. 2004). These findings lend support to two competing models of information processing within the amygdala during learning. In the serial model, information about the CS and US enter and are associated within the BLA, and this information is then transmitted to the CEA for the expression of fear. Alternatively, the parallel model proposes that the BLA and CEA both perform associative functions (for reviews see Pare and colleagues (2004), Maren (2005), and Balleine and Killcross (2006)), suggesting that one nucleus might compensate for the loss of the other under certain conditions.

Lesions of either the BLA or the CEA produce deficits in both the acquisition and the expression of conditional fear (Campeau and Davis 1995; Cousens and Otto 1998; Goosens and Maren 2001; Helmstetter 1992; LeDoux et al. 1990; Maren et al. 1996). However, despite previous findings that overtraining (25 CS-US trials) does not mitigate the effects of excitotoxic BLA lesions (Maren 1998), rats with BLA lesions can acquire conditional freezing after extensive overtraining (75 CS-US trials) (Goosens and Maren 2003; Maren 1999). Given the important role for the CEA in fear conditioning, it is possible that CEA neurons are involved in the acquisition and expression of conditional freezing in rats without an intact BLA. The current experiments address this possibility and reveal an essential role for the CEA in the acquisition and expression of conditional fear after extensive overtraining.

Materials and Methods

Experiment 1: Neurotoxic CEA Lesions Prevent the Acquisition of Overtrained Fear

Subjects. The subjects were 74 male Long-Evans rats (200-224 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN). After arrival, the animals were individually housed in clear plastic cages hanging from a standard stainless-steel rack. The vivarium lights were on a 14/10 light/dark cycle (lights on at 7:00 am) and the rats had free access to food and tap water. After housing, the rats were handled (15-20 sec

each) for five days to acclimate them to the experimenter. All experiments were carried out in accordance with guidelines approved by the University of Michigan University Committee on Use and Care of Animals.

Behavioral apparatus. Eight identical observation chambers (30 x 24 x21 cm; Med-Associates, St. Albans, VT) were used for all phases of training and testing. The chambers were constructed from aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating chests located in an isolated room. The floor of each chamber consisted of 19 stainless-steel rods (4 mm diameter) spaced 1.5 cm apart (center to center). The rods were wired to a shock source and solid-state grid scrambler (Med-Associates) for delivery of the foot shock unconditioned stimulus (US) (1.0 mA, 2 sec). For “context A” (used for conditioning and context retention testing), background noise (65 dB) was provided by ventilation fans built into the chests, house lights within the chambers and fluorescent lights within the room provided illumination, the chest doors were left open, and the chambers were cleaned with a 1% ammonium hydroxide solution. For “context B” (used for tone retention testing), illumination was provided by fluorescent red lights, the chest doors were closed, the ventilation fans were inactive, and the chambers were cleaned with a 1% acetic acid solution. Stainless steel pans containing a thin film of the corresponding cleaning solutions were placed underneath the grid floors before the animals were placed inside the boxes.

Each conditioning chamber rested on a load cell platform that was used to record chamber displacement in response to each rats’ motor activity. To ensure

interchamber reliability, each load cell amplifier was calibrated to a fixed chamber displacement. The output of the load cell of each chamber was set to a gain that was optimized for detecting freezing behavior. Load cell amplifier output from each chamber was digitized and acquired on-line using Threshold Activity software (Med-Associates).

Surgery. The rats were randomly assigned to groups that received bilateral neurotoxic lesions of the BLA, CEA, or combined lesions of both the BLA and CEA (BLA + CEA); control rats received sham (SH) surgery. After handling for at least five days, rats were treated with atropine sulfate (0.4 mg/kg body weight, i.p.) and sodium pentobarbital (Nembutal, 65 mg/kg body weight, i.p.), and mounted in stereotaxic apparatus (David Kopf instruments, Tujunga, CA). The scalp was incised and retracted, and the head was positioned to place bregma and lambda in the same horizontal plane. Small burr holes (2 mm in diameter) were drilled bilaterally in the skull for the placement of 28-gauge cannula in the BLA (3.3 mm posterior to bregma, 5.0 mm lateral to the midline), CEA (2.3 mm and 2.7 mm posterior to bregma, 4.3 mm lateral to the midline), or both. Two 10 μl Hamilton syringes were mounted into an infusion pump (Harvard Apparatus, South Natick, MA) and connected to the injection cannula with polyethylene tubing. NMDA was dissolved in 100 mM PBS (20 mg/ml; ph 7.4; Sigma, St. Louis, MO). For BLA lesions, NMDA was infused (0.1 μl/min) at two sites: 8.0 mm ventral to brain surface (0.2 μl) and 7.5 mm ventral to brain surface (0.1 μl). For CEA lesions, NMDA was infused (0.1 μl/min) 7.9 mm ventral to brain surface (0.15 μl) at each of the anterior-posterior coordinates. Five minutes

were allowed after each infusion for diffusion of the drug. Sham animals received a similar surgery and had small burr holes drilled bilaterally in their skulls, but injectors were not lowered into the brain. After surgery, the incision was closed with stainless steel wound clips, and the rats were allowed to recover on a heating pad before returning to their home cage.

Procedure. Following surgery, the rats were allowed at least 7 days to recover. On the conditioning day, the rats were transported to the laboratory in squads of eight and placed in the conditioning chambers. The chamber position was counterbalanced for each squad and group. The rats received 75 tone (80 dB, 10 sec, 2kHz) shock (1.0 mA, 2.0 sec) pairings (70 sec intertrial interval) beginning 30 sec after being placed in the chamber (this interval was 3 min for Experiments 2-4) and ending 60 sec after the final shock (context A). Twenty-four hours after training, contextual fear was assessed by returning the rats to the conditioning chambers and measuring freezing behavior (somatomotor immobility except that necessitated by breathing) during an 8 minute extinction test (context A). Forty-eight hours following training, conditional fear to the tone CS was assessed by placing the rats in a novel context (context B) and measuring freezing behavior during an extinction test in which a 6-minute continuous tone was presented 2 minutes after the rats were placed in the chambers.

During both the conditioning and test sessions, each rat's activity was monitored continuously using the data acquisition software described above. For each chamber, load cell activity was digitized at 5 Hz, yielding one observation per rat every 200 msec (300 observations per rat per minute). Load cell values ranged between 0 and 100, and this value was used to quantify locomotor activity. Freezing was quantified by computing the number of observations for each rat that had a load cell value less than the freezing threshold (threshold = 10). The freezing threshold was determined in a separate group of pilot animals by comparing load cell output with an observer's rating of freezing behavior. To avoid counting momentary inactivity as freezing, an observation was only scored as freezing if it fell within a contiguous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec. For each session, the freezing observations were transformed to a percentage of total observations. In the present experiment, freezing was quantified before footshock during the pre-trial period and after footshock offset on the conditioning day, and during the 8 min context and tone extinction tests.

Histology. Histological verification of lesion location was performed after behavioral testing. Rats were perfused across the heart with 0.9% saline followed by 10% formalin. After extraction from the skull, the brains were post-fixed in 10% formalin for 2 days and 10% formalin and 30% sucrose until sectioning. Coronal sections (45 μm thick, taken every 135 μm) were cut on a cryostat (-20 ^{o}C) and wet mounted on glass microscope slides with 70% ethanol.

After drying, the sections were stained with 0.25% thionin to visualize neuronal cell bodies. Lesions were verified by visual inspection of the stained brain sections.

Data analysis. For each session, the freezing data were transformed to a percentage of total observations, a probability estimate that is amenable to analysis with parametric statistics. These probability estimates of freezing were analyzed using ANOVA. Post-hoc comparisons in the form of Fisher's PLSD tests were performed after a significant overall *F* ratio. All data are represented as means ± SEMs.

Experiment 2: Neurotoxic Lesions of the CEA or BLA Prevent the Expression of Overtrained Fear

Subjects. The subjects were 60 adult male Long-Evans rats (200-224 g; Blue Spruce) obtained and housed as described in Experiment 1.

Apparatus, surgery, and procedure. The behavioral apparatus and conditioning procedures were identical to those described in Experiment 1, except that rats received lesions 1-4 days after conditioning (rather than 1-week before conditioning as in Experiment 1). The rats were randomly assigned to groups that were to receive post-training lesions of the BLA, CEA, or combined lesions of both BLA and CEA (BLA + CEA); control rats received sham (SH) surgery. After surgery, the animals were allowed at least 7 days for recovery. Fear conditioning to the conditioning context and auditory CS was assessed as

described in Experiment 1. The rats' activity and freezing were measured and quantified as described in Experiment 1.

Histology. Rats were perfused and the intact brain was prepared as described in Experiment 1. Alternate coronal sections (45 μm thick slices, taken every 135 μm) were cut on a cryostat (-20° C). The first slice was wet mounted on glass microscope slides with 70% ethanol to be stained with 0.25% thionin for histological verification of lesions. The second slice was stored at 4°C in a cryoprotective buffer containing 25% ethylene glycol, 25% glycerin, and 0.05 M phosphate buffer. Myelin staining was preformed as described by Koo et al. (2004). Sections were washed free-floating 3 × 10 min in 0.02 M PBS (0.6% NaCl). Slices were then incubated in a 0.2% AuCl solution containing gold chloride trihydrate, 30% H_2O_2 and 0.02 M PBS (0.6% NaCl) until the fibers in the amygdala contrasted with the background tissue (approximately 2 hours). Rinsing the tissue for 10 min in normal saline stopped the reaction. Following the saline rinse the tissue was fixed for 5 min in a 5% sodium thiosulfate solution. Tissue was next rinsed 3 × 5 min in 0.02 M PBS (0.6% NaCl) and mounted on unsubbed glass microscope slides and dried overnight at 37°C. On the following day tissue was dehydrated and coverslipped. Fiber staining was assessed by visual inspection of the sections under a light microscope.

Data analysis. Data analysis was preformed as described in Experiment 1.

Experiment 3: Muscimol Inactivation of the CEA Prevents the Acquisition of Overtrained Fear

Subjects. The subjects were 48 adult male Long-Evans rats (200-224 g) obtained and housed as described in Experiment 1.

Surgery. After handling for at least five days, rats were treated with atropine sulfate (0.4 mg/kg body weight, i.p.) and sodium pentobarbital (Nembutal, 65 mg/kg body weight, i.p.), and mounted in stereotaxic apparatus (David Kopf instruments, Tujunga, CA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (2 mm in diameter) were drilled bilaterally in the skull for the placement of 28-gauge cannula in the BLA (3.3 mm posterior to bregma, 5.0 mm lateral to the midline) for rats receiving BLA lesions. In addition, all rats were implanted with Plastics One (Roanoke, VA) 26-gauge guide cannula (cut at 11 mm below the pedestal) into the CEA (2.5mm posterior to bregma, 4.3 mm lateral to the midline). BLA lesions were made as described in Experiment 1. Sham animals did not receive BLA lesions, but were implanted with guide cannulas in the CEA as described above. Following implantation dental acrylic was applied to the skull to hold the cannulas in place. After surgery, dummy cannulas (33-gauge, 16 mm; Plastics One, Roanoke, VA) were inserted into the guide cannulas, and the rats were returned to their home cages. The dummy cannulas were replaced every other day during the week of recovery.

Apparatus and procedure. The behavioral apparatus and training procedures were identical to those described in Experiment 1, except that

infusions of either saline or muscimol were made into the CEA before conditioning. All retention testing was preformed drug-free as described in Experiment 1 with context and tone tests 24 and 48 hours after conditioning, respectively. The rats were randomly assigned to groups in a 2x2 design (lesion x drug). This design yielded the following groups: rats with pre-training sham surgeries receiving saline in the CEA (SH-SAL), rats with pre-training sham surgeries receiving muscimol in the CEA (SH-MUS), rats with pre-training NMDA lesions of the BLA receiving saline in the CEA (BLA-SAL), and rats with pre-training NMDA lesions of the BLA receiving muscimol in the CEA (BLA-MUS).

After at least 7 days recovery from surgery, rats were acclimated to the infusion procedure by transporting them to the infusion room in identical white 5-gallon buckets in squads of eight (counterbalanced for each squad and group). Their dummy cannulas were replaced and the infusion pumps (Harvard Apparatus, South Natick, MA) were activated. After two minutes and 30 sec, the pumps were stopped and the animals were returned to their home cages. Twenty-four hours after acclimation, the rats were transported to the infusion room as described above and infused with either muscimol (0.125 µg in 0.25 µl of sterile saline at 0.1 µl/min) or sterile saline (0.9 %; 0.25 µl at 0.1 µl/min). After the infusion, one minute was allowed for diffusion before removing the internal cannulas. After removing the internal cannulas, clean dummy cannulas were inserted into the guide cannulas and rats were immediately transported to the conditioning chambers as described in Experiment 1, where they received

auditory fear conditioning. Fear to the conditioning context and auditory CS were assessed 24 and 48 hours later respectively, as described in Experiment 1.

Histology. Histological verification of lesions and cannula placement location was performed after behavioral testing and completed as described in Experiment 1.

Data Analysis. Data analysis was performed as described in Experiment 1.

Experiment 4: Muscimol Inactivation of the CEA Prevents the Expression of Overtrained Fear

Subjects. The subjects were 48 adult male Long-Evans rats (200-224 g; Blue Spruce) obtained and housed as described in Experiment 1.

Surgery. Surgeries were performed as described in Experiment 3.

Apparatus and procedure. The behavioral apparatus and contexts were identical to that described in Experiment 1. The rats were randomly assigned to groups in a 2x2 design (lesion x drug). This design yielded the following groups: rats with pre-training sham surgeries receiving saline in the CEA prior to testing (SH-SAL), rats with pre-training sham surgeries receiving muscimol in the CEA prior to testing (SH-MUS), rats with pre-training NMDA lesions of the BLA receiving saline in the CEA prior to testing (BLA-SAL), and rats with pre-training NMDA lesions of the BLA receiving muscimol in the CEA prior to testing (BLA-MUS). After 5 days of handling, rats underwent surgery. The rats were allowed

at least 7 days to recover from surgery. Rats were then acclimated to the infusion procedure as described in Experiment 3.

Twenty-four hours after acclimation the rats were conditioned in context A. On the conditioning day, the rats were transported to the conditioning chambers in squads of eight counterbalanced for each squad and group where they received auditory fear conditioning as described in Experiment 1. Twenty-four hours after conditioning, the rats underwent a context retention test. Prior to testing, squads of 8 rats were transported into the laboratory in individual white buckets for infusions of muscimol or saline as described in Experiment 3. After the infusion, one minute was allowed for diffusion before removing the internal cannulas. After removing the internal cannula, clean dummy cannulas were inserted into the guide cannulas and the rats were immediately placed in the conditioning context where fear was assessed as described in Experiment 1. Seventy-two hours after conditioning animals were once again transported to the infusion room and infused as described above. Immediately after the infusion the rats were transported to a novel context where auditory fear was assessed as described in Experiment 1.

Histology. Histological verification of lesions and cannula placement location was performed after behavioral testing and completed as described in Experiment 1.

Data Analysis. Data analysis was preformed as described in Experiment 1.

Results

Experiment 1: Neurotoxic CEA Lesions Prevent the Acquisition of Overtrained Fear

Rats with BLA lesions acquire conditional freezing after extensive overtraining in either contextual or auditory fear conditioning paradigms (Maren 1999). To determine the involvement of the CEA in conditional freezing after overtraining, we explored the effect of pre-training lesions of the BLA, CEA, or combined lesions of both the BLA and CEA on conditional freezing after a 75-trial auditory fear conditioning procedure. Short-term fear responses were assessed by measuring conditional freezing during the overtraining session. Long-term fear memory was assessed by independently measuring conditional freezing to the conditioning context and the auditory CS, which was presented in a novel context.

Histology. Based on the histological results, 19 of 74 rats were excluded. Rats were excluded if their lesions were larger than intended, misplaced, or largely unilateral. This yielded the following group sizes: CEA (n = 14), BLA (n = 8), CEA + BLA (n = 19), and SH (n = 15). The extent of the amygdala damage for rats included in the analyses is depicted in Figure 2.1. As can be seen, damage was generally confined to the targeted nucleus. For lesions targeting the BLA, there was some damage to the rostral entorhinal cortex. For lesions targeting the CEA, there was minor damage to the caudate putamen and

substantia innominata. Not surprisingly, the combined lesions of the CEA and BLA were more extensive than the individual CEA or BLA lesions.

Behavior. Post-shock freezing during the conditioning session is shown in Figure 2.2A. The data were analyzed using a two-way ANOVA with variables of lesion (SH, CEA, BLA, and CEA + BLA) and trial (fifteen 5-trial blocks). During the pre-trial period rats displayed minimal levels of freezing (<5%) before footshock. After the onset of conditioning, rats exhibited robust freezing. The ANOVA revealed a main effect of lesion [$F_{(3,47)} = 19.5$; $p < 0.0001$] and a main effect of training trial [$F_{(14, 658)} = 10.4$; $p < 0.0001$] without a significant interaction of lesions x training trial [$F_{(42, 658)} = 1.2$; $p = 0.22$]. This indicates that freezing differed among the groups across the training session. Post-hoc analysis of the main effect of lesion revealed a difference between SH rats and rats with either CEA lesions ($p < 0.0001$) or CEA + BLA lesions ($p < 0.05$), and there was a trend towards a significant difference between the SH and BLA groups ($p = 0.07$). Rats with BLA lesions showed significantly greater freezing than rats with either CEA lesions or CEA + BLA lesions ($p < 0.05$), which did not differ from one another.

The group differences in conditional freezing were apparent early in training. Further analysis of the first 10 training trials (first 2 blocks; shown as an inset to Figure 2.2A) with a two-way ANOVA with variables of lesions (SH, CEA, BLA, and CEA + BLA) and training trial (1 – 10) revealed a main effect of lesion [$F_{(3, 47)} = 23.9$; $p < 0.0001$], training trial [$F_{(9, 423)} = 5.5$; $p < 0.0001$], and an interaction of lesion x training trial [$F_{(30, 470)} = 3.1$; $p < 0.0001$]. This indicates that

freezing differed among the groups during the first 10 trials of training. Post-hoc analysis of the main effect of lesion shows that sham rats exhibited significantly greater freezing than rats in any other group, and rats with BLA lesions exhibited greater freezing than rats with CEA or CEA + BLA lesions ($p < 0.05$ for all comparisons). There was no difference between rats with CEA lesions and rats with CEA + BLA lesions. As we have previously reported (Maren 1998), amygdala lesions did not affect shock reactivity to the first conditioning shock [$F_{(3, 52)} = 0.7$; $p = 0.6$] (data not shown). Thus, these data indicate that CEA lesions (whether alone, or in combination with BLA lesions) significantly impaired the acquisition of conditional freezing. Rats with BLA lesions exhibited more freezing than rats with CEA lesions, but were also deficient relative to controls.

Long-term fear memories to the conditioning context and the auditory CS were assessed in separate retention tests conducted 24 and 48 hours after conditioning, respectively. Figure 2.2B shows the freezing data for the context test. A two-way ANOVA with variables of lesion (SH, CEA, BLA, and CEA + BLA) and time (minutes 1 - 8) revealed a significant main effect of lesion [$F_{(3,52)} = 27.8$; $p < 0.0001$] and time [$F_{(7,364)} = 11.5$; $p < 0.0001$] without a significant interaction of lesion and time [$F_{(21, 364)} = 1.4$; $p = 0.10$] during the context test. Post-hoc analysis of the main effect of lesion revealed that rats with CEA lesions (alone, or in combination with BLA lesions) exhibited impaired freezing compared to both BLA lesion rats and sham rats ($p < 0.05$ for all comparisons). As previously reported (Maren 1999), rats with only BLA lesions did not exhibit a significant impairment in contextual freezing when compared to sham rats, and

there was no significant difference between the CEA and CEA + BLA lesion groups. These data indicate that animals with BLA lesions acquire fear to a context after overtraining, and that this contextual fear is eliminated by CEA lesions.

Freezing during the tone test is shown in Figure 2.2C. A two-way ANOVA with variables of lesion (SH, CEA, BLA, and CEA + BLA) and time (minutes 3 - 8) revealed a significant main effect of lesion [$F_{(3,52)} = 7.2$; $p < 0.0004$], time [$F_{(5, 260)} = 8.8$; $p < 0.0001$], and a significant interaction of lesion X time [$F_{(15, 260)} = 3.3$; $p < 0.0001$]. Rats with CEA lesions (alone, or in combination with BLA lesions) exhibited impaired freezing compared to both BLA lesion rats and sham rats ($p < 0.05$). In contrast to an earlier report (Maren 1999), rats with BLA damage acquired freezing to the auditory CS (but see Experiment 4). Nonetheless, rats with CEA damage (either alone or in combination with BLA damage) had impaired memory to the tone CS, similar to their deficits in contextual fear.

Experiment 2: Neurotoxic Lesions of the CEA or BLA Prevent the Expression of Overtrained Fear

Experiment 1 replicated earlier reports that the BLA is not an essential structure for the acquisition or expression of Pavlovian fear when animals are overtrained in an auditory fear-conditioning paradigm. Indeed, the CEA appears to be critical for the acquisition of Pavlovian fear after overtraining. Because CEA lesions were made before conditioning in Experiment 1, however, it is not clear whether the effects of CEA lesions were due to impairments in the

acquisition or expression of the conditional fear memory. To further explore the nature of the deficit in rats with CEA lesions, we used post-training lesions in Experiment 2 to determine whether the CEA is required for the expression of fear when the amygdala is intact during the acquisition of fear conditioning. Because recent work by Koo and colleagues (2004) has suggested that deficits observed in rats with CEA lesions may be the results of damage to *en passant* axons rather than CEA neurons, we also examined myelin staining within the amygdala to characterize the influence of NMDA on fibers of passage.

Histology. Based on the histological results, 14 of the 60 rats were excluded from the analysis. This yielded group sizes of: CEA (*n* = 5), BLA (*n* = 12), CEA + BLA (*n* = 15), and SH (*n* = 14). The extent of the amygdala damage for rats included in the data analysis is depicted in Figure 2.3 and is similar to that described in Experiment 1. Figure 2.4 shows representative thionin- and AuCl-stained coronal sections from rats that received NMDA lesions in the CEA, BLA, or CEA + BLA, and SH rats. Relative to control tissue, myelin staining in rats with either CEA or BLA lesions appeared normal, suggesting that there was little or no damage to *en passant* axons. However, in rats with combined lesions of the CEA and BLA a loss of myelin staining in the region of the lesion was observed. Therefore, the larger volumes of NMDA used to create combined lesions of the CEA and BLA yielded far more extensive damage (including fibers of passage) than lesions of either the BLA or CEA alone. Although myelin staining was apparently normal in rats with CEA lesions, it is possible that undetectable damage to the CEA in these rats contributed to their deficits.

Moreover, reversible inactivation of the CEA (see Experiments 3 and 4), which presumably does not affect axonal conduction, reproduced the effects of CEA lesions.

Behavior. Post-shock freezing during the conditioning session did not differ among the groups prior to surgery (not shown). Data for the context and tone retention tests, which were conducted one week after surgery, are shown in Figure 2.5A and Figure 2.5B, respectively. For the context test, a two-way ANOVA with variables of lesion (SH, CEA, BLA, and CEA + BLA) and time (minutes 1 - 8) revealed a significant main effect of lesion [$F_{(3,42)} = 10.8$; $p < 0.0001$] and time [$F_{(7,294)} = 4.9$; $p < 0.0001$]. Rats with lesions of the BLA, CEA, or both CEA + BLA displayed significantly impaired freezing compared to control animals ($p < 0.05$) (Figure 2.5A). Similar results were observed during the tone test (Figure 2.5B). A two-way ANOVA with variables of lesion (CEA, BLA, CEA + BLA, and SH) and time (minutes 3 - 8) revealed a significant effect of lesion [$F_{(3,42)} = 10.9$; $p < 0.0001$], time [$F_{(5, 210)} = 12$; $p < 0.0001$], and lesion X time interaction [$F_{(15, 210)} = 4.3$; $p < 0.0001$]. All groups displayed low levels of freezing before tone onset (< 10%). After tone onset animals in all of the lesion groups exhibited a significant impairment in freezing to the auditory CS compared to sham rats ($p < 0.05$). These data indicate that both the CEA and the BLA are essential for the expression of conditional freezing when animals undergo fear conditioning with both structures intact.

Experiment 3: Muscimol Inactivation of the CEA Prevents the Acquisition of Overtrained Fear

Experiments 1 and 2 demonstrate that the expression of fear after overtraining is impaired in rats with lesions of the CEA, regardless of whether those lesions are made before or after training. However, pre-training lesions might influence performance by affecting either the acquisition and/or the expression of conditional freezing. Therefore, we used a temporary inactivation procedure in the following experiments to independently assess the role of the CEA in the acquisition and the expression of conditioned freezing after overtraining. Experiment 3 examined whether pre-training inactivation of the CEA with the $GABA_A$ receptor agonist muscimol impairs the acquisition of overtrained fear in rats with BLA lesions.

Histology. Based on the histological results 17 of 48 rats were excluded. This yielded the following: SH-SAL (*n* = 7), SH-MUS (*n* = 8), BLA-SAL (*n* = 10), and BLA-MUS (*n* = 6). The extent of the BLA lesions as well as CEA cannula placements for rats included in the data analyses are depicted in Figure 2.6. All cannula placements were located in the CEA. The BLA lesions were similar to those described in Experiment 1.

Behavior. Freezing during the conditioning session, which was conducted immediately after the intra-CEA infusions, is shown in Figure 2.7A. The data were analyzed using a three-way ANOVA with variables of lesion (SH, BLA), drug (SAL, MUS) and trial (fifteen, 5-trial blocks). During the pretrial period rats displayed minimal levels of freezing (< 10%) before footshock. After the onset of

conditioning, the rats exhibited robust freezing. There was neither a main effect of lesion [$F_{(1, 27)} = 0.3$; $p = 0.6$] or drug [$F_{(1, 27)} = 0.2$; $p = 0.6$], nor a lesion X drug interaction [$F_{(1, 27)} = 1.0$; $p = 0.3$] on conditional freezing after the onset of conditioning. However, the ANOVA revealed a significant main effect of trial [$F_{(14, 378)} = 17.5$; $p < 0.0001$], a significant lesion X trial interaction [$F_{(14, 378)} = 7.1$; $p = 0.01$], and a significant drug X trial interaction [$F_{(14, 378)} = 7.1$; $p < 0.0001$]. This indicates that although the overall levels of freezing were unaffected by either the lesion or drug, the rates of acquisition were affected by both lesion and drug treatment. Furthermore, as shown in Figure 2.8, muscimol treatment did not affect the activity burst elicited by the first conditioning shock as measured by ANOVA with variables of lesion (SH, BLA), drug (SAL, MUS), and shock reactivity (pretrial activity, shock activity). This analysis revealed only a significant main effect of shock reactivity [$F_{(1, 27)} = 1.0$; $p < 0.0001$] indicating an equivalent burst of activity for each group during the first footshock.

Data from the context and tone retention tests, which were conducted drug-free 24 and 48 hours after conditioning, are shown in Figure 2.7B and Figure 2.7C, respectively. For the context test, an ANOVA with variables of lesion (SH, BLA), drug (SAL, MUS), and time (minutes 1-8) revealed a significant main effect of drug [$F_{(1,27)} = 9.3$; $p < 0.01$] and a significant drug x time interaction [$F_{(7, 189)} = 2.2$; $p < 0.04$] without significant main effects of lesion [$F_{(1, 27)} < 0.01$; $p = 1.0$] or time [$F_{(7, 189)} = 1.0$; $p = 0.4$]. This indicates that rats for which the CEA was inactivated during training (SH-MUS, BLA-MUS) showed significantly less freezing than rats that received saline infusions into the CEA during training (SH-

SAL, BLA-SAL) regardless of whether the BLA was damaged (Figure 2.7B). A similar effect was observed during the tone test (Figure 2.7C). All groups displayed low levels of freezing before tone onset (< 5%), and, in this particular experiment, freezing to the tone CS was also unusually low. For this reason, we focused the analysis on the first two minutes of the tone test, a period during which freezing to the tone CS was greater than baseline. An ANOVA with variables of lesion (SH, BLA) and drug (SAL, MUS) revealed a significant main effect of drug [$F_{(1, 27)} = 4.1$; $p = 0.05$] without a significant main effect of lesion [$F_{(1, 27)} < 0.01$; $p = 0.93$] or a lesion X drug interaction [$F_{(1, 27)} < 0.01$; $p = 0.97$]. Thus, during the first two minutes of the tone CS, rats infused with muscimol during training (SH-MUS, BLA-MUS) showed significantly less freezing that saline-infused rats (SH-SAL, BLA-SAL). Although freezing to the tone CS was lower than normal in the saline-treated rats, it is clear that the muscimol infusion into the CEA impeded acquisition. These data indicate that the CEA is critical for the acquisition of conditioned fear in rats with BLA lesions and that CEA inactivation in intact rats also impairs the acquisition of fear conditioning.

Experiment 4: Muscimol Inactivation of the CEA Prevents the Expression of Overtrained Fear

Experiment 3 demonstrates the necessity of the CEA for the acquisition of conditioned fear following overtraining. Experiment 4 examined whether CEA inactivation also impairs the expression of conditioned freezing in rats overtrained after receiving BLA lesions.

Histology. Based on the histological results, 11 of the 40 rats were excluded. Exclusions were made because the lesions were smaller or larger than intended, misplaced, or cannula were improperly placed in the CEA. One animal that displayed a marked motor deficit after muscimol infusion was also excluded. This yielded samples sizes per group of: SH-SAL (n = 12), SH-MUS (n = 6), BLA-SAL (n = 5), and BLA-MUS (n = 6). The extent of the BLA lesions as well as CEA cannula placements for rats included in the data analyses are depicted in Figure 2.9. All cannula placements were located in the CEA. BLA lesions were similar to those described in Experiment 1.

Behavior. Post-shock freezing during the conditioning session is shown in Figure 2.10A. Similar to Experiment 1, post-shock freezing was significantly lower in rats with BLA lesions as compared to sham controls. Two-way ANOVA with variables of lesion (SH, BLA) and training trial block (fifteen, 5 trial blocks) confirmed this via a main effect of lesion [$F_{(1, 27)}$ = 10.4; p < 0.01] and training block [$F_{(14, 378)}$ = 3.2; p < 0.0001].

Conditional freezing during the context and tone retention tests is shown in Figure 2.10B and Figure 2.10C, respectively. Animals receiving muscimol infusions into the CEA showed a marked reduction in conditional freezing to the context. An ANOVA with variables of lesion (SH, BLA), drug (SAL, MUS), and time (minutes 1-8) revealed a main effect of drug [$F_{(1,25)}$ = 13.1; p < 0.01] indicating that rats receiving muscimol in the CEA exhibited significantly lower freezing in both intact rats and rats with BLA lesions. Importantly, neither a significant main effect of lesion [$F_{(1, 25)}$ < 0.02; p = 0.89] nor a significant lesion X

drug interaction [$F_{(1, 25)} < 0.03$; $p > 0.88$] was observed, indicating that BLA lesions did not prevent the acquisition of context fear after overtraining (Experiment 1; Maren 1999).

During the tone test, freezing was greatly reduced in animals receiving muscimol infusions into the CEA. An ANOVA with variables of lesion (SH and BLA), drug (SAL and MUS) and time (minutes 3 - 8) revealed a significant drug X time interaction [$F_{(5,120)} = 2.9$; $p < 0.02$]; no other effects reached significance. Post-hoc comparisons revealed higher levels of freezing in saline-infused rats compared to muscimol-infused groups in the first two minutes of the test. This result indicates that rats receiving muscimol infusions into the CEA exhibited impairments in the expression of conditional freezing to the auditory CS as compared to the SH-SAL group. Although there was not a statistically significant effect of the lesion in the overall ANOVA, it is apparent that rats with BLA lesions exhibited considerably less freezing than saline-infused SH controls. To assess this we separately analyzed freezing during the first two-minutes of the test in the saline-infused groups. During this period, BLA rats infused with saline exhibited significantly less freezing than SH rats [$F_{(1,15)} = 6.2$; $p < 0.05$]. The failure of BLA rats to acquire auditory freezing after overtraining has been observed in earlier reports (Maren 1999). Together, these data indicate that pharmacological inactivation of the CEA blocks the expression of overtrained fear to either contextual or auditory stimuli in both intact rats and rats with BLA lesions.

Discussion

The present experiments used an overtraining procedure to determine the role of the CEA in the acquisition and expression of Pavlovian fear conditioning in rats with neurotoxic BLA lesions. The main outcome of the experiments was that permanent or temporary inactivation of the CEA in rats with BLA lesions prevented both the acquisition and expression of conditioned freezing to the conditioning context and an auditory CS after a 75-trial overtraining procedure. Thus, the ability of rats with BLA lesions to acquire conditioned freezing (e.g., Maren 1999) appears to depend on the integrity of the CEA. Moreover, similar to the findings of Wilensky and colleagues (2006), the CEA appears to be involved in the acquisition of conditioned fear in rats with an intact BLA, insofar as reversible inactivation of the CEA impaired the acquisition of conditioned freezing in both intact rats and rats with BLA lesions. Both the BLA and CEA appear to be essential for the expression of conditioned freezing acquired after overtraining, because post-training lesions of either structure eliminated conditioned freezing.

These data replicate and extend earlier observations that extensive overtraining promotes conditioning in rats with neurotoxic BLA lesions (Lee et al. 2005; Maren 1999). Less extensive training (e.g., 25 trials) produces a modest level of fear conditioning in rats with BLA lesions (Lee et al. 2005; Maren 1998), but it is now clear that considerably more training is required to obtain the high levels of conditioned freezing that are typically observed in intact animals after only a few trials. Similar to the findings of Cahill and colleagues (2000), rats with

BLA lesions acquired conditioned freezing at a significantly lower rate than intact rats during the conditioning session. Despite a reduction in post-shock freezing, rats with BLA lesions exhibited robust conditioned freezing to the conditioning context (and in one experiment the auditory CS) during retention testing. In contrast, rats with CEA lesions, either alone or in combination with BLA lesions, expressed low levels of conditional freezing during both conditioning and retention testing despite overtraining.

Kim and Davis (1993) have also found that rats with CEA lesions are unable to acquire fear-potentiated startle even after extensive training. Because we found that reversible inactivation of the CEA prevented the acquisition and expression of conditional freezing in both intact rats and rats with BLA lesions, these results suggest that the CEA is necessary for both the acquisition and expression of conditional freezing, even after extensive overtraining. Interestingly, rats with CEA lesions can reacquire a fear-potentiated startle response if they are extensively trained prior to the lesion. This suggests that the BLA, which continues to be important for the expression of conditioned fear even after overtraining (Falls and Davis 1995; Maren 1998; Maren 1999), might be able to control the performance of fear CRs in the absence of the CEA.

The critical role for the CEA in the expression of conditional fear has been recognized in numerous studies (Campeau and Davis 1995; Goosens and Maren 2001). Our results, indicating that discrete lesions of the CEA block the expression of conditioned fear, extend this important role of the CEA in fear conditioning to overtrained fear. However, our results are at odds with a recent

report that found that rats with fiber sparing ibotenic acid lesions of the CEA exhibited normal contextual freezing and only slightly attenuated fear to an auditory CS (despite showing a deficit in conditioned ultrasonic vocalizations) after 10-trial fear conditioning (Koo et al. 2004). The reason for this discrepancy is not clear, insofar as we obtained reliable deficits in conditioned freezing with both fiber-sparing CEA lesions (see Experiments 1and 2) and muscimol infusions into the CEA (see Experiments 3 and 4), and have previously observed deficits in conditioned freezing with small ibotenic acid lesions in the CEA (Goosens and Maren 2001). It is possible that the precise locus or extent of the lesions within the CEA differ between these studies. Indeed, the lesions made by Koo and colleagues (2004) were made with iontophoretic methods that may have yielded smaller lesions than the pressure injections of NMDA used in the present study. Nevertheless, we also obtained deficits in conditional freezing after muscimol infusions into the CEA, suggesting that neurons in the CEA are not only critical for the expression, but also the acquisition of conditional fear. This finding is further supported by the work of Wilensky and colleagues (2006) using a 2-trial fear conditioning procedure. The fact that our deficits were obtained in rats with BLA lesions argues against the possibility that infusions of muscimol into the CEA produced its effects by diffusing to the neighboring BLA.

Despite exhibiting deficits in the expression of conditional freezing during retention testing, rats with CEA inactivation or lesions exhibited substantial levels of conditional freezing during the conditioning session (see Experiment 1 and Experiment 3). It has been argued that freezing behavior shortly after footshock

is a conditioned response to the conditioning context, rather than an unconditioned response to footshock (Fanselow 1990). If so, our data suggest that short-term conditioned fear responses may survive, at least in part, CEA lesions or inactivation. Alternatively, freezing during the conditioning session may represent an unconditioned response to footshock (Bevins et al. 1997). In either case, it may be that other brain areas that are essential for freezing behavior, such as the periaqueductal gray, are involved in the expression of fear responses (whether conditioned or unconditioned) shortly after footshock. However, our data suggest that the CEA is ultimately critical for the expression of conditioned freezing driven by the long-term memory of the conditioning experience.

Interestingly, the ability of the CEA to compensate for loss of the BLA appears to be engaged by overtraining. That is, rats with neurotoxic BLA lesions exhibit substantial deficits in conditioned freezing with either 1 or 25 conditioning trials (Maren 1998; Maren 1999). It is only when these rats are given extensive overtraining (50 or 75 conditioning trials) that they exhibit conditioned freezing that is similar in magnitude to that in control rats. It is important to note that the CEA only compensates for the loss of the BLA if conditioned fear is acquired in the absence of the BLA (Experiment 2); a finding similar to that of Anglada-Figueroa and Quirk (2005). Two possibilities might account for this pattern of results. First, the CEA might only be involved in the expression of fear driven by memory acquired in other brain areas. If so, then these other areas are either inefficient in acquiring fear memory in the absence of the BLA or insufficient to

drive the performance of fear responses via the CEA. However, Experiment 3 suggests that the CEA itself may be involved in the encoding of fear memory, because CEA inactivation in intact rats impaired the acquisition of conditional freezing, a finding consistent with the recent work of Wilensky et al. (2006). If the CEA is the primary site of memory encoding in the absence of the BLA, it would appear to be less efficient than the BLA in coding fear memories insofar as it required substantially more training to elicit conditioned freezing in rats with BLA lesion. It might also be the case that the associative representations mediated by the CEA and BLA are different (Balleine and Killcross 2006; Holland and Gallagher 2003; Killcross et al. 1997), and that those mediated by the CEA can come to generate conditioned freezing, but only after extensive training. Studies are underway to explore this possibility.

Our data suggests a role for the CEA not only in the expression, but also in the acquisition of long-term fear memory (or at least the fear behavior engendered by such memories), a role formerly limited to the BLA (Maren 2005; Pare et al. 2004). Intact animals receiving muscimol inactivation of the CEA during conditioning showed significant deficits in conditioned fear to both contextual and auditory cues 24 and 48 hours, respectively, following conditioning (Experiment 3). Additionally, we have recently reported that an infusion of an NMDA receptor antagonist into the CEA prevents the acquisition of conditional freezing (5 tone-footshock pairings), although these rats did exhibit some savings when they received additional training in a drug-free state (Goosens and Maren 2003). And although we have not observed impairments in

conditioned freezing after the infusion of a broad-spectrum protein kinase inhibitor (Goosens et al. 2000) or a Ras antagonist (Merino and Maren 2006) into the CEA, others have reported deficits after infusions of either a protein kinase A inhibitor or a protein synthesis inhibitor into the CEA (Wilensky et al. 2006). These data suggest that cellular mechanisms involved in long-term memory and synaptic plasticity might operate in the CEA to encode fear memory. Further work is necessary to explore this possibility.

In summary, we have found that the CEA is essential for the acquisition and expression of conditioned freezing after overtraining. Moreover, our results reveal that the capacity of rats with neurotoxic BLA lesions to acquire fear after overtraining is mediated by the CEA. These results therefore provide additional support for the parallel processing model within the amygdala through which associative functions are mediated by both the BLA and CEA, as animals are able to learn in the absence of the BLA (a structure essential for learning according the to serial processing model). Furthermore, our data suggest an unequal weighting in the parallel pathways, as rats trained in the absence of the BLA require substantially more training (extensive overtraining) to acquire substantial conditioned fear. Moreover, rats trained with an intact BLA lose their fear memory after the BLA is damaged, even after extensive overtraining. It is not clear whether the nature of the associations encoded by the CEA is similar to that of the BLA. Nonetheless, these data reveal an important role for the CEA in mediating overtrained fear and support the emerging view that associative

processes in the CEA might contribute to fear conditioning under some conditions.

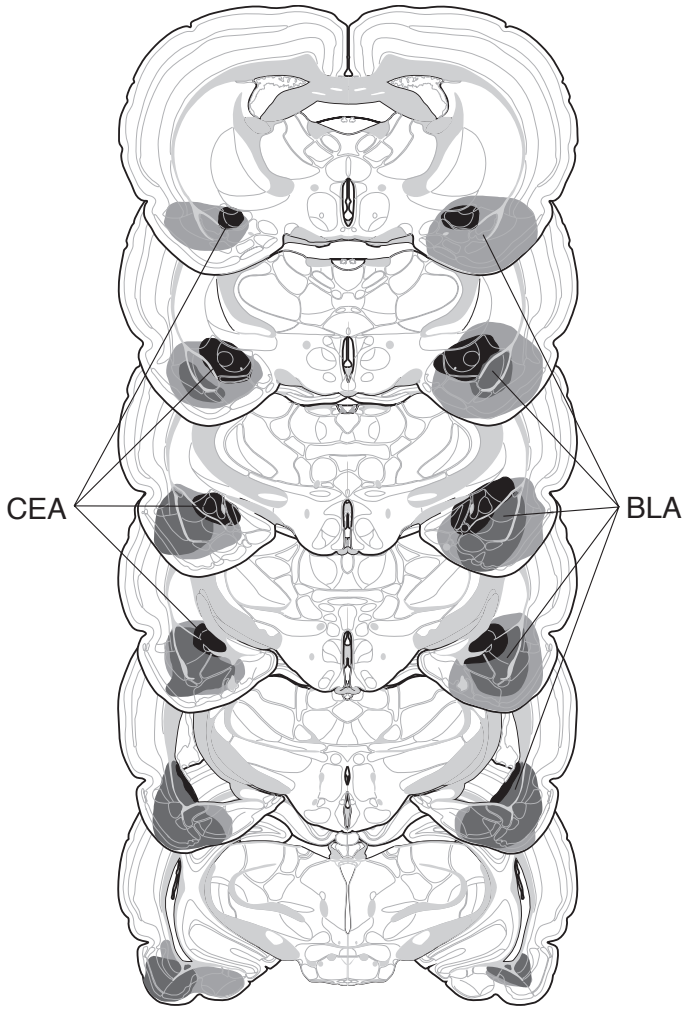

Figure 2.1. Schematic representation of the extent of pre-training NMDA lesions (Experiment 1).
Schematic representation of the extent of pre-training NMDA lesions (median lesion) of the CEA (black), BLA (dark-grey), and CEA and BLA lesions (light-grey) for Experiment 1. Coronal brain section images adapted from Swanson (1992).

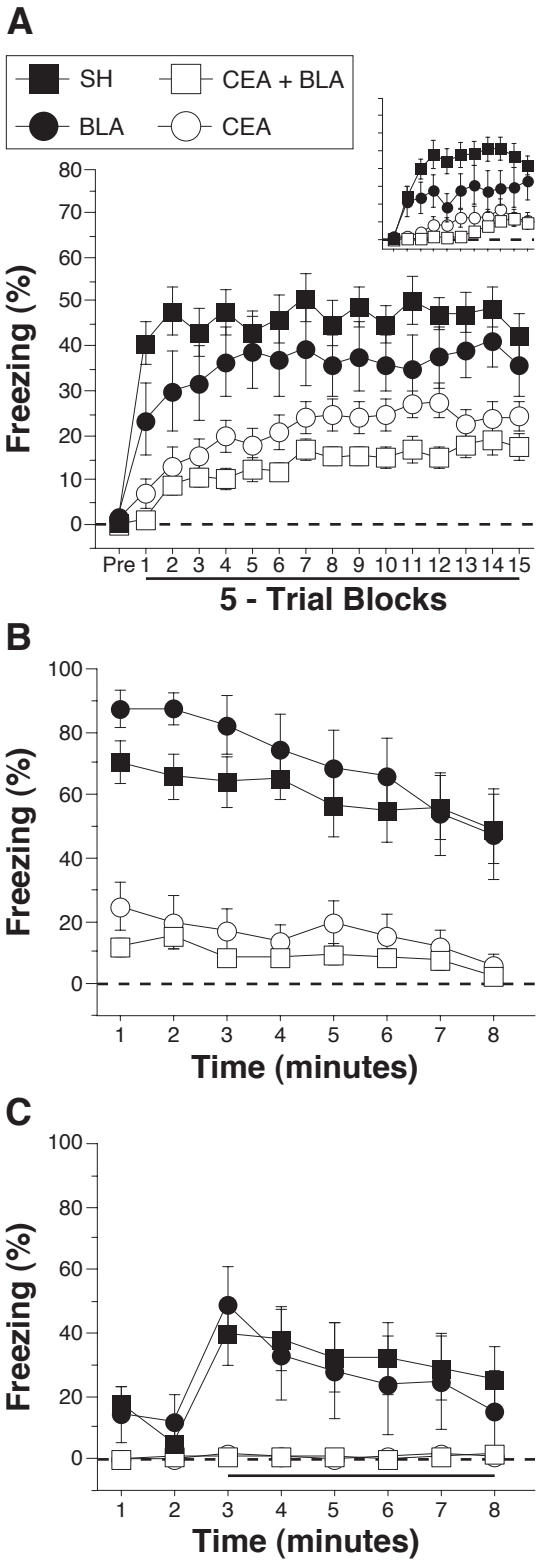

Figure 2.2. Conditioned freezing in rats with pre-training amygdala lesions (Experiment 1).

A, Mean percentage of freezing (± SEM) during the 75 trial training session (data are displayed with a 30 second pre-trial period followed by 15 bins consisting of 5 trials each). Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial; these values were averaged in 5-trial blocks. The inset shows the mean percentage of freezing (± SEM) during the pre-period through the first two training blocks expanded to show minutes 1-10. *B*, Mean percentage of freezing (± SEM) to contextual (8 min context extinction test) cues 24 hours following training. *C*, Mean percentage of freezing (± SEM) to the auditory CS in a novel context 48 hours following training. The auditory CS was initiated 2 min after rats were placed in the chambers (horizontal bar indicates the CS). Data are shown for rats with lesions of the BLA (closed circle), CEA (open circle), or CEA + BLA (open square); SH rats (closed squares).

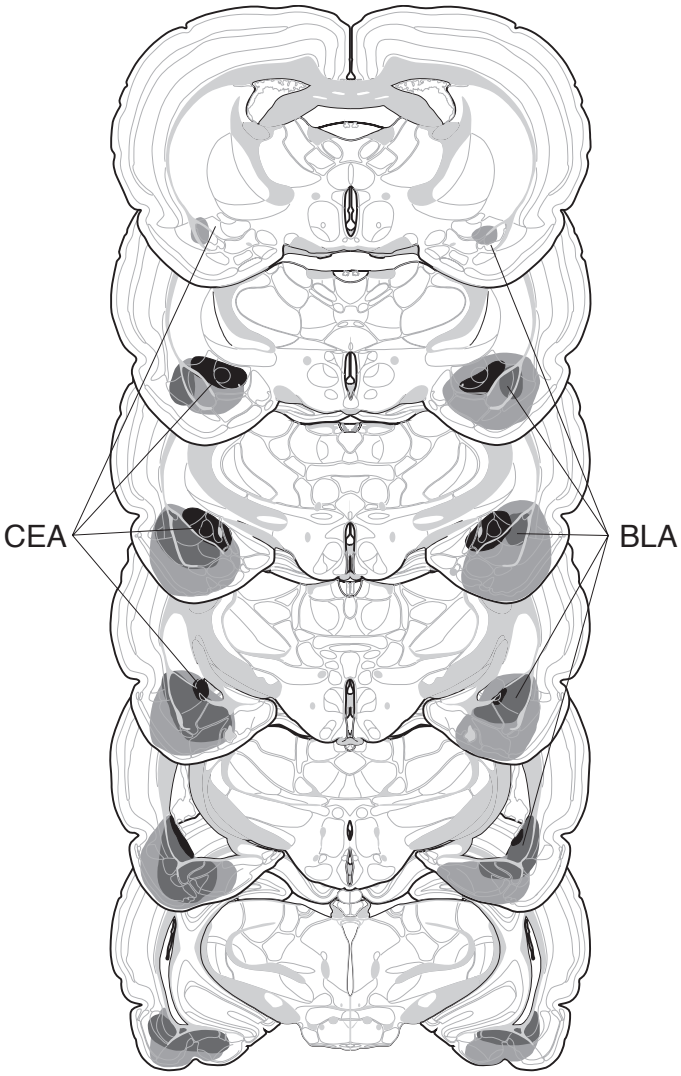

Figure 2.3. Schematic representation of the extent of post-training NMDA lesions (Experiment 2)
Schematic representation of the extent of post-training NMDA lesions (median lesion) of the CEA (black), BLA (dark grey), and CEA + BLA lesions (light grey) for Experiment 2. Coronal brain section images adapted from Swanson (1992).

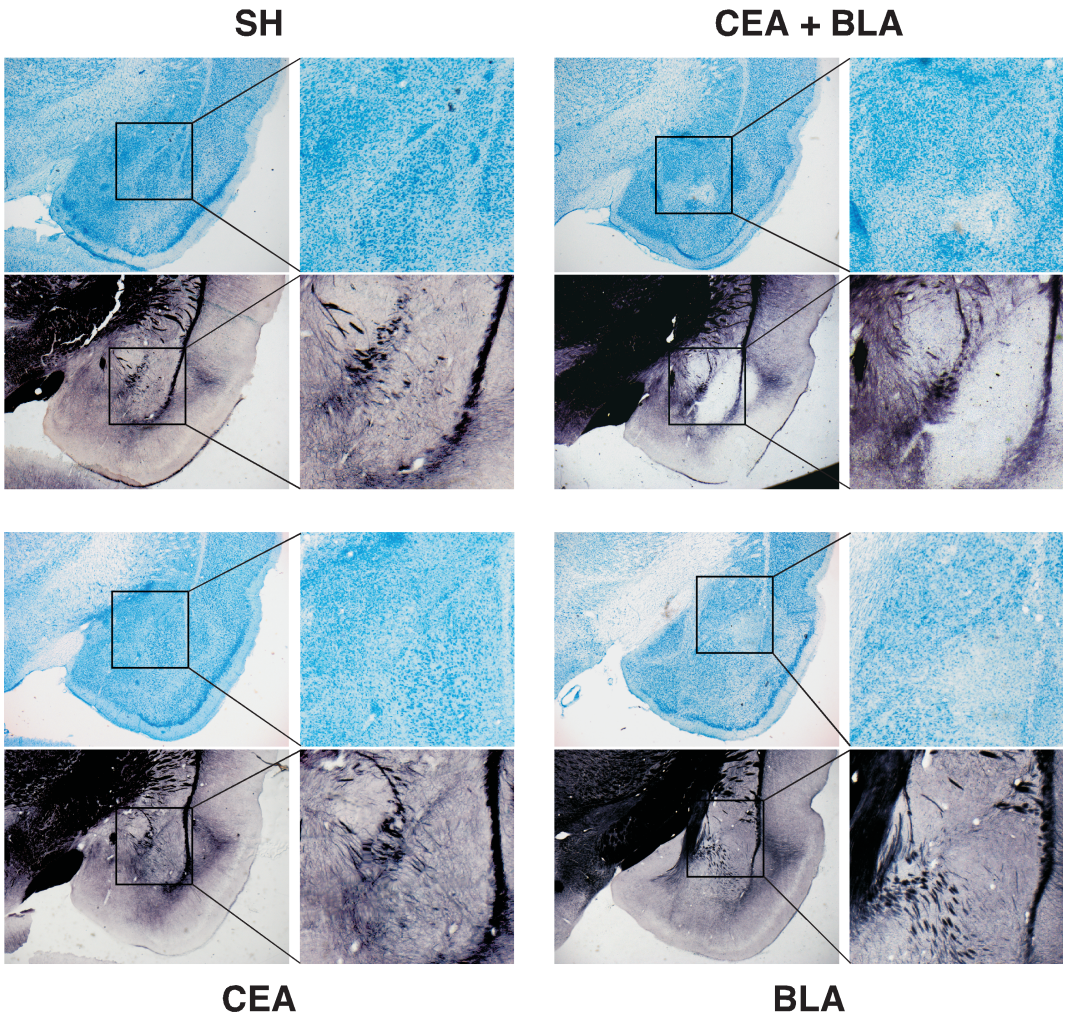

Figure 2.4. Representative slices stained with thionin and AuCl.
Slices shown from rats that received lesions of the CEA + BLA (upper right), CEA (lower left), or BLA (lower right); SH rats (upper left). Both the thionin and the AuCl stained slices for each group are taken from the same representative animal at approximately the same A/P level with a magnification of the amygdala shown to the immediate right.

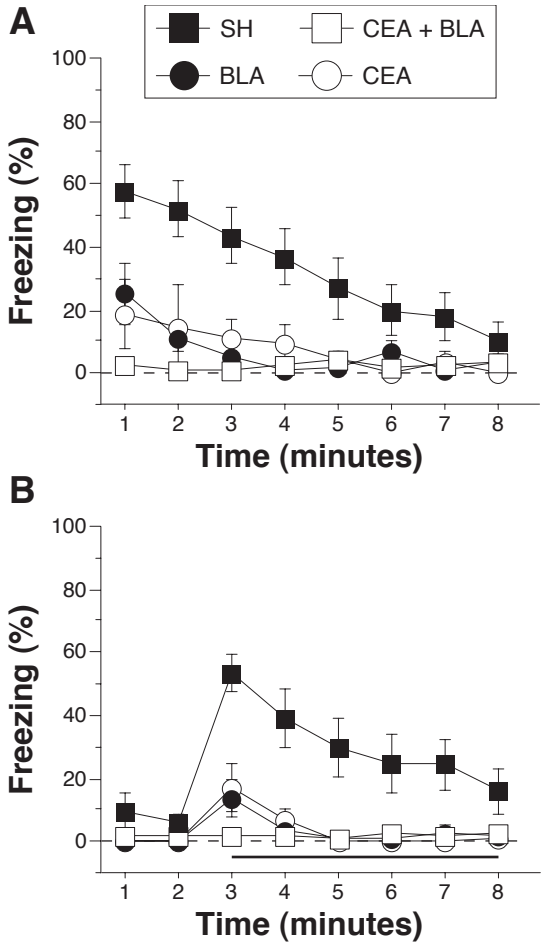

Figure 2.5. Conditioned freezing in rats with post-training amygdala lesions (Experiment 2).
A, Mean percentage of freezing (± SEM) to contextual (8 min context extinction test) cues after at least 7 days of recovery from post-training surgery. *B*, Mean percentage of freezing (± SEM) to the auditory CS in a novel context 24 hours after contextual testing. The auditory CS was initiated 2 min after rats were placed in the chambers (horizontal bar indicates the CS). Data are shown for rats with lesions of the BLA (closed circle), CEA (open circle), or CEA + BLA (open square); SH rats (closed squares).

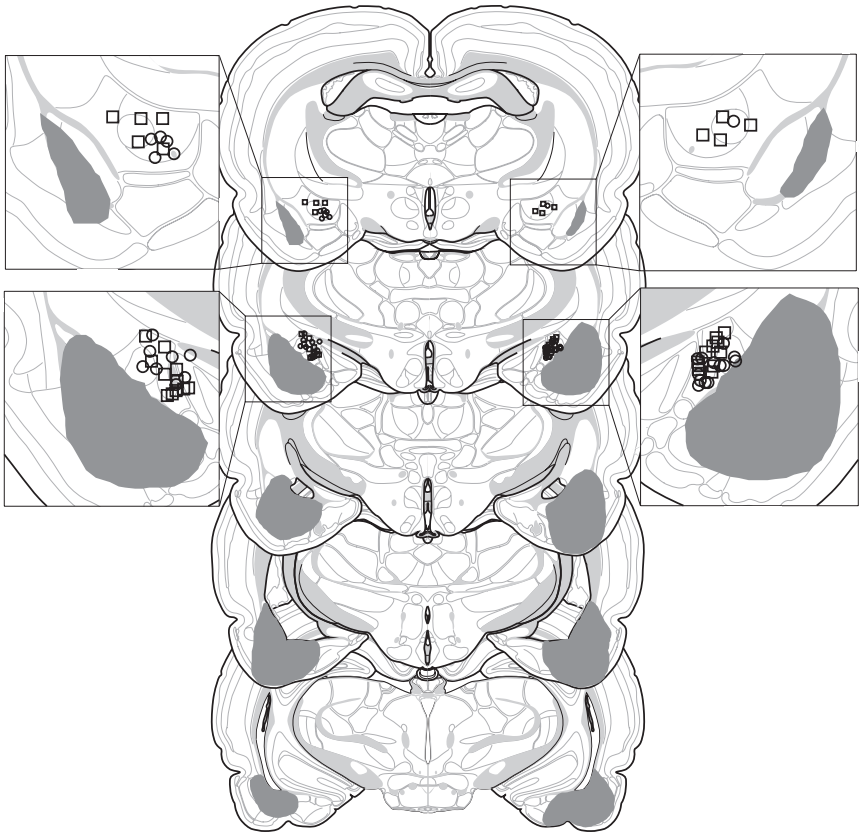

Figure 2.6. Schematic representation of the extent of pre-training NMDA lesions (median lesion) of the BLA (dark grey) and the locations of included cannula placements (Experiment 3).
Schematic representation of the extent of pre-training NMDA lesions (median lesion) of the BLA (dark grey) and the locations of included cannula placements for the infusion of muscimol (circles) or 0.9% sterile saline (squares) in the CEA (Experiment 3). A magnification of the amygdala is shown in the insets adjacent to the coronal brain sections. Coronal brain section images adapted from Swanson (1992).

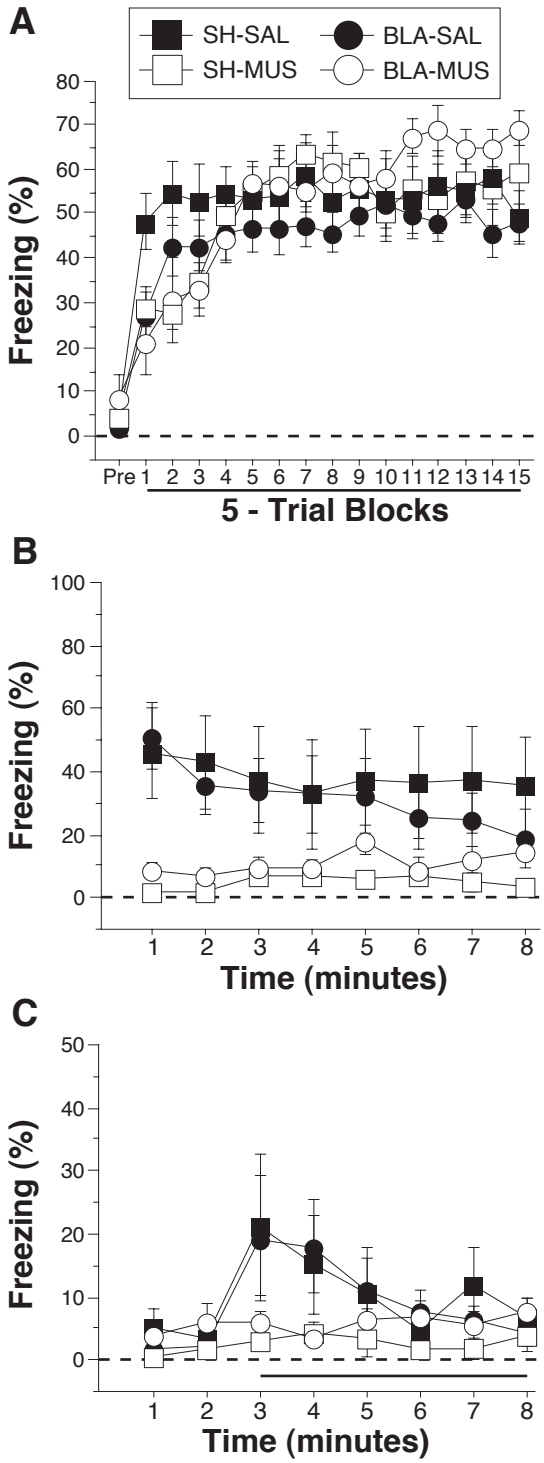

Figure 2.7. Conditioned freezing in rats with pre-training BLA lesions and temporary inactivation of the CEA during training (Experiment 3).
A, Mean percentage of freezing (± SEM) during the 75 trial training session (data are displayed with a 3 min pre-trial period followed by 15 bins consisting of 5 trials each) after infusion of 0.9% saline or muscimol into the CEA. Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after

each conditioning trial; these values were averaged in 5-trial blocks. *B*, Mean percentage of freezing (± SEM) to contextual (8 min context extinction test) cues 24 hours following training. *C*, Mean percentage of freezing (± SEM) to the auditory CS in a novel context 48 hours following training. The auditory CS was initiated 2 min after rats were placed in the chambers (horizontal bar indicates the CS). Data are shown for rats with pre-training sham surgeries receiving saline in the CEA prior to training (SH-SAL: closed squares), pre-training sham surgeries receiving muscimol in the CEA prior to training (SH-MUS: open squares), pre-training NMDA lesions of the BLA receiving saline in the CEA prior to training (BLA-SAL: closed circles), or pre-training NMDA lesions of the BLA receiving muscimol in the CEA prior to training (BLA-MUS: open circles).

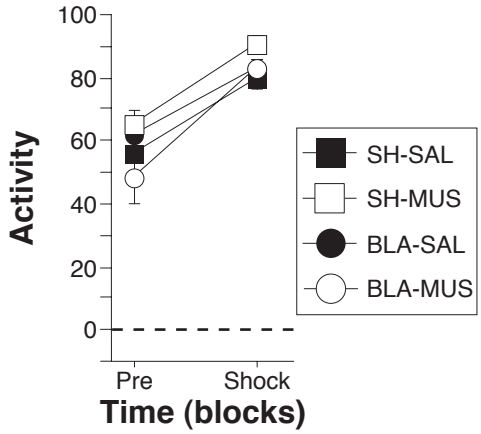

Figure 2.8. Shock reactivity in rats with pre-training BLA lesions and temporary inactivation of the CEA before training (Experiment 3). Mean percentage of activity (± SEM) before the first conditioning trial (Pre) and during the first 2 sec shock (Shock) during the conditioning session. Data are shown for rats with pre-training sham surgeries receiving saline in the CEA prior to training (SH-SAL: closed squares), pre-training sham surgeries receiving muscimol in the CEA prior to training (SH-MUS: open squares), pre-training NMDA lesions of the BLA receiving saline in the CEA prior to training (BLA-SAL: closed circles), or pre-training NMDA lesions of the BLA receiving muscimol in the CEA prior to training (BLA-MUS: open circles).

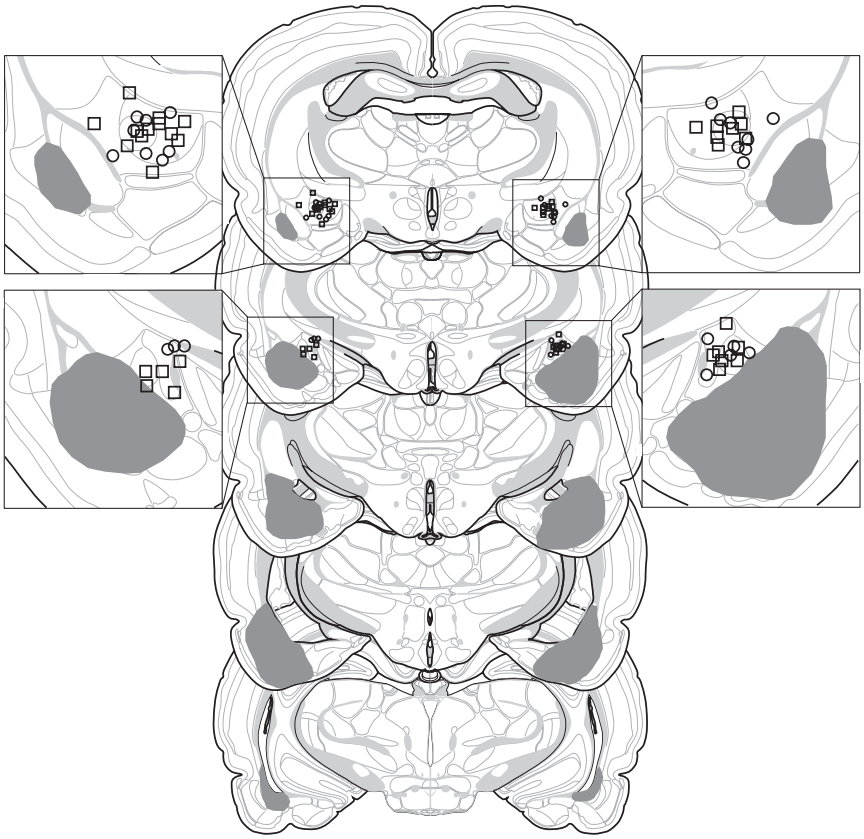

Figure 2.9. Schematic representation of the extent of pre-training NMDA lesions of the BLA and the locations of included cannula placements (Experiment 4).
Schematic representation of the extent of pre-training NMDA lesions (median lesion) of the BLA (dark grey) and the locations of included cannula placements for the infusion of muscimol (circles) or 0.9% sterile saline (squares) in the CEA (Experiment 4). A magnification of the amygdala is shown adjacent to the coronal brain sections. Coronal brain section images adapted from Swanson (1992).

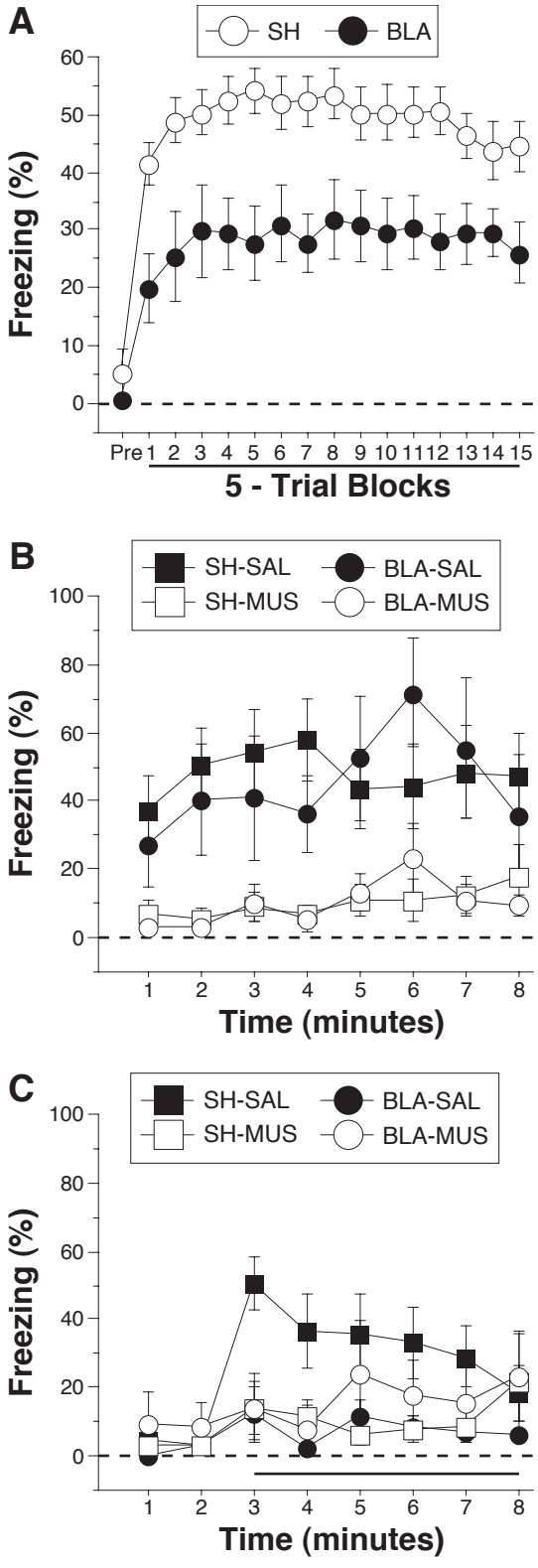

Figure 2.10. Conditioned freezing in rats with pre-training BLA lesions and temporary inactivation of the CEA during testing (Experiment 4).

A, Mean percentage of freezing (± SEM) during the 75 trial training session (data are displayed with a 3 min pre-trial period followed by 15 bins consisting of 5 trials each). Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial; these values were averaged in 5-trial blocks. *B*, Mean percentage of freezing (± SEM) to contextual (8 min context extinction test) cues after infusion of 0.9% sterile saline or muscimol into the CEA. *C*, Mean percentage of freezing (± SEM) to the auditory CS in a novel context 48 hours following training after infusion of 0.9% sterile saline or muscimol into the CEA. The auditory CS was initiated 2 min after rats were placed in the chambers (horizontal bar indicates the CS). Data are shown for rats with pre-training sham surgeries receiving saline in the CEA prior to testing (SH-SAL: closed squares), pre-training sham surgeries receiving muscimol in the CEA prior to testing (SH-MUS: open squares), pre-training NMDA lesions of the BLA receiving saline in the CEA prior to testing (BLA-SAL: closed circles), or pre-training NMDA lesions of the BLA receiving muscimol in the CEA prior to testing (BLA-MUS: open circles).

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CHAPTER 3

THE BED NUCLEUS OF THE STRIA TERMINALIS DOES NOT COMPENSATE FOR THE BASOLATERAL AMYGDALA TO MEDIATE OVERTRAINED FEAR IN RATS

The amygdala is a brain structure widely believed to be involved in the acquisition and expression of Pavlovian fear memories (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001). Specifically, the basolateral complex of the amygdala (BLA) is believed to be the critical site of CS-US convergence underlying the acquisition and of Pavlovian fear memories. However, we have discovered that deficits in fear conditioning in rats with BLA lesions can be overcome with overtraining (Maren, 1999a; Zimmerman et al., 2007). The capacity for fear learning in rats with BLA lesions suggests that a brain area other than the BLA is sufficient for the acquisition and expression of conditional fear. Indeed, we have recently shown that the amygdaloid central nucleus (CEA), which also receives CS and US information, is essential for the acquisition and expression of conditional fear in rats with BLA lesions. These findings suggest that the CEA mediates the acquisition of fear in rats with BLA lesions, although this memory requires many more trials to acquire (Maren, 1999a) and is short-lived (Poulos et al., 2009).

Another brain structure that might mediate fear conditioning in the absence of the BLA is the bed nucleus of the stria terminalis (BNST). The BNST

possesses similar afferent and efferent connectivity to that of the CEA (Dong et al., 2001; Walker et al., 2003). Furthermore, Sullivan and colleagues (2004) recently demonstrated a role for the BNST in the expression of conditioned fear. Specifically, they found that lesions of the BNST block the expression of contextual, but not auditory cued fear (Sullivan et al., 2004; Waddell et al., 2006). The possibility remains that the bed nucleus of the stria terminalis (BNST), like the CEA (Zimmerman et al., 2007), may be able to compensate for the loss of the BLA following overtraining and mediate the expression of both auditory and contextually cued fear. The following experiments address this possibility. Rats received bilateral BLA lesions prior to overtraining, and then received either post-training lesions of the BNST or pre-testing infusions of the AMPA receptor antagonist NBQX into the BNST. We report that although BNST lesions or inactivation disrupt the expression of context freezing in rats with BLA lesions, they did not effect the expression of fear to the auditory CS. These results reveal that although the BNST is critical for the expression of contextual fear, it is not the locus of compensation for fear learning in the absence of the BLA.

Materials and Methods

Experiment 1: BNST lesions and the expression of overtrained fear in rats without a BLA

Subjects. The subjects were 56 male Long-Evans rats (200-224 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague Dawley,

Indianapolis, IN). After arrival, the animals were individually housed in clear plastic cages hanging from a standard stainless-steel rack. The vivarium lights were on a 14/10 light/dark cycle (lights on at 7:00 am) and the rats had free access to food and tap water. After housing, the rats were handled (15-20 sec each) for five days to acclimate them to the experimenter. All experiments were carried out in accordance with guidelines approved by the University of Michigan University Committee on Use and Care of Animals.

Behavioral apparatus. Eight identical observation chambers (30 x 24 x21 cm; Med-Associates, St. Albans, VT) were used for all phases of training and testing. The chambers were constructed from aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating chests located in an isolated room. The floor of each chamber consisted of 19 stainless-steel rods (4 mm diameter) spaced 1.5 cm apart (center to center). The rods were wired to a shock source and solid-state grid scrambler (Med-Associates) for delivery of the foot shock unconditioned stimulus (US) (1.0 mA, 2 sec). For “context A” (used for conditioning and context testing), background noise (65 dB) was provided by ventilation fans built into the chests, house lights within the chambers and fluorescent lights within the room provided illumination, the chest doors were left open, the chambers were cleaned with a 1% ammonium hydroxide solution, and the rats were transported in white 5-gallon buckets with bedding. For “context B” (used for tone testing), illumination was provided by incandescent red lights, the chest doors were closed, the ventilation fans were inactive, the chambers were cleaned with a 1% acetic acid

solution, the floors were covered with black plastic panels, and the rats were transported in white 5-gallon buckets with bedding. Stainless steel pans containing a thin film of the corresponding cleaning solutions were placed underneath the grid floors before the animals were placed inside the boxes.

Each conditioning chamber rested on a load cell platform that was used to record chamber displacement in response to each rats' motor activity. To ensure interchamber reliability, each load cell amplifier was calibrated to a fixed chamber displacement. The output of the load cell of each chamber was set to a gain that was optimized for detecting freezing behavior. Load cell amplifier output from each chamber was digitized and acquired on-line using Threshold Activity software (Med-Associates).

Surgery. After handling for at least five days, rats were treated with atropine sulfate (0.4 mg/kg body weight, i.p.) and sodium pentobarbital (65 mg/kg body weight, i.p.), and mounted in stereotaxic apparatus (David Kopf instruments, Tujunga, CA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (2 mm in diameter) were drilled bilaterally in the skull for the temporary placement of 28-gauge cannula in the BLA (3.3 mm posterior to bregma, 5.0 mm lateral to the midline). Two 10 μl Hamilton syringes were mounted into an infusion pump (Harvard Apparatus, South Natick, MA) and connected to the injection cannula with polyethylene tubing. NMDA was dissolved in 100 mM PBS (20 mg/ml; ph 7.4; Sigma, St. Louis, MO). For BLA lesions, NMDA was infused (0.1 μl/min) at two sites: 8.0 mm ventral to brain

surface (0.2 μl) and 7.5 mm ventral to brain surface (0.1 μl). Five minutes were allowed after each infusion for diffusion of the drug. Sham animals received a similar surgery and had small burr holes drilled bilaterally in their skulls, but injectors were not lowered into the brain. Additionally, five additional small burr holes were drilled in the skull for the bilateral placement of two 26-gauge guide cannula (cut at 11 mm below the pedestal; Plastics One, Roanoke, VA) in the BNST (0.5 mm posterior to bregma, 2.7 mm lateral to the midline, 7.4 mm ventral to bregma at a10 degree angle from vertical) and 3 small screws. Following implantation, dental acrylic was applied to the skull to hold the cannula in place. After surgery, dummy cannulae (33-gauge, 16 mm; Plastics One, Roanoke, VA) were inserted into the guide cannula, and the rats were allowed to recover from the anesthesia before being returned to their home cages. The dummy cannulae were replaced every other day during the week of recovery.

Procedure. After at least 7 days recovery from surgery, rats were transported to the laboratory in squads of eight and placed in the conditioning chambers for fear conditioning. The chamber position was counterbalanced for each squad and group. The rats received 75 tone (80 dB, 10 sec, 2kHz) shock (1.0 mA, 2.0 sec) pairings (70 sec intertrial interval) beginning 3 min after being placed in the chamber and ending 60 sec after the final shock (context A). The rats were then transported back to their home cages. Twenty-four hours after conditioning the rats were anesthetized as described above in order to receive an intracranial NMDA infusion (3.5 μg in 0.175 μl of 100 mM PBS at 0.1 μl/min; pH 7.4; Sigma) into the BNST. Bilateral BNST infusions were made using 10 μl

Hamilton syringes mounted into an infusion pump (Harvard Apparatus, South Natick, MA) and connected to injection cannula (28 gauge; 16 mm; Plastics One, Roanoke, VA) with polyethylene tubing. After the infusion, five minutes was allowed for diffusion before removing the injection cannula. Rats receiving sham BNST lesions were anesthetized but received no infusions. After removing the internal cannula, clean dummy cannulae were inserted into the guide cannula and rats were allowed to recover from the anesthesia before being returned to their home cages. After 3 days of recovery rats, were placed in the conditioning chambers in the absence of tones or foot-shocks for 10 min (context A) to test the level of conditioned fear to the conditioning context. Twenty-four hours after the context test, rats were transported back to the chambers and placed in a novel context (context B) for a tone test. Two minutes after placement in the chambers, the rats were presented with an 8-min continuous tone (80 dB, 2kHz).

During the training and test sessions, each rat's activity was monitored continuously using the data acquisition software described above. For each chamber, load cell activity was digitized at 5 Hz, yielding one observation per rat every 200 msec (300 observations per rat per minute). Load cell values ranged between 0 and 100, and this value was used to quantify locomotor activity. Freezing was quantified by computing the number of observations for each rat that had a load cell value less than the freezing threshold (threshold = 10). The freezing threshold was determined in a separate group of pilot animals by comparing load cell output with an observer's rating of freezing behavior. To avoid counting momentary inactivity as freezing, an observation was only scored

as freezing if it fell within a contiguous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec. For each session, the freezing observations were transformed to a percentage of total observations. In the present experiment, freezing was quantified before footshock during the pre-trial period and after footshock offset on the conditioning day, and throughout the entirety of the tests.

Histology. Histological verification of lesions and cannula placements were performed after behavioral testing. Rats were perfused across the heart with 0.9% saline followed by 10% formalin. After extraction from the skull, the brains were post-fixed in 10% formalin for 2 days and 10% formalin and 30% sucrose until sectioning. Coronal sections (45 μm thick, taken every 135 μm) were cut on a cryostat (-20 ^{o}C) and wet mounted on glass microscope slides with 70% ethanol. After drying, the sections were stained with 0.25% thionin to visualize neuronal cell bodies. Lesions and cannula placements were verified by visual inspection of the stained brain sections.

Data analysis. For each session, the freezing data were transformed to a percentage of total observations, a probability estimate that is amenable to analysis with parametric statistics. Freezing to the tone CS was normalized to the pre-CS baseline. These probability estimates of freezing were analyzed using ANOVA. Post-hoc comparisons in the form of Fisher's PLSD tests were performed after a significant overall F ratio. All data are represented as means ± SEMs.

Experiment 2: BNST inactivation and the expression of overtrained fear in rats without a BLA

Subjects. The subjects were 28 male Long-Evans rats (200-224 g; Blue Spruce) obtained and housed as described in Experiment 1.

Behavioral apparatus and surgery. The behavioral apparatus and surgical procedures are identical to those described in Experiment 1.

Procedure. After at least 7 days recovery from surgery, rats were acclimated to the infusion procedure by transporting them to the infusion room in identical white 5-gallon buckets in squads of eight (counterbalanced for each squad and group). Their dummy cannulas were replaced and the infusion pumps (Harvard Apparatus, South Natick, MA) were activated. After 3 minutes, the pumps were stopped and the animals were returned to their home cages. Twenty-four hours after acclimation, on the conditioning day, the rats were transported to the laboratory in squads of eight and placed in the conditioning chambers. Training was identical to that described in Experiment 1. Twenty-four hours after training, the rats were transported to the infusion room as described above. Infusions were delivered using 10 μl Hamilton syringes mounted into an infusion pump (Harvard Apparatus, South Natick, MA) and connected to the injection cannula (28 gauge; 16 mm; Plastics One, Roanoke, VA) with polyethylene tubing. Rats were infused with the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 3.0 μg in 0.3 μl of 100 mM PBS at 0.1 μl/min) or 100 mM PBS (VEH; 0.3 μl at 0.1 μl/min). After the infusion, one minute was allowed for diffusion before removing the

internal cannula. After removing the internal cannula, clean dummy cannula were inserted into the guide cannula and rats were immediately transported to the conditioning chambers for a context test as described in Experiment 1. Seventy-two hours after conditioning the rats were transported back to the infusion room where they received a second BNST infusion identical to that described above. The rats were then immediately transported back to the conditioning chambers for a tone test as described in Experiment 1.

Histology and Data Analysis. Histology and data analysis were performed as described in Experiment 1.

Results

Experiment 1: BNST lesions and the expression of overtrained fear in rats without a BLA

Histology. Based on the histological results, 10 of 66 rats were excluded. Rats were excluded if their lesions were larger than intended, misplaced, or largely unilateral. This yielded the following group sizes: BLA-BNST (*n* = 13), BLA-SH (*n* = 14), SH-BNST (*n* = 11), and SH-SH (*n* = 18). The extent of the amygdala and BNST damage for rats included in the analyses are depicted in Figure 3.1. As can be seen damage was generally confined to the targeted nucleus.

Behavior. Post-shock freezing during the conditioning session is shown in Figure 3.2A. Note that at this point in the experiment, some of the rats had received BLA lesions (and others sham surgery), but none had received a BNST lesion. The data were analyzed using a three-way ANOVA with variables of pre-training lesion (SH or BLA), post-training lesion (SH or BNST) and trial (fifteen 5-trial blocks). During the pre-trial period rats displayed minimal levels of freezing (<5%) before footshock. After the onset of conditioning, rats exhibited robust freezing. The ANOVA revealed a main effect of trial [$F_{(14,728)} = 7.9$; $p < 0.0001$] without a significant main effect or interaction for any other variable ($p > 0.05$ for all comparisons). This indicates that all rats acquired similar levels of conditioned fear at similar rates.

Long-term fear memories to the conditioning context and the auditory CS were assessed in separate retention tests conducted 4 and 5 days after conditioning, respectively. Figure 3.2B shows the freezing data during the context test. A three-way ANOVA for the 10 min context test with variables of pre-training lesion (SH and BLA), post-training lesion (SH and BNST), and time (min 1-10) revealed significant main effects of post-training lesion [$F_{(1,52)} = 13.2$; $p < 0.001$] and time [$F_{(9,468)} = 5.8$; $p < 0.0001$] without a significant main effect of pre-training lesion [$F_{(1,52)} = 0.2$; $p > 0.6$]. The ANOVA also revealed a significant three-way interaction of pre-training lesion X post-training lesion X time [$F_{(9,468)} = 2.0$; $p < 0.05$]. No other interactions were significant ($p > 0.56$ for all comparisons). These data indicate that rats with BLA lesions exhibited similar

degrees of conditioned fear to sham rats, and lesions of the BNST impaired the expression of contextual freezing in both sham rats and rats with BLA lesions.

Freezing during the tone test is shown in Figure 3.2C. A three-way ANOVA on conditional freezing during the tone with variables of pre-training lesion (SH and BLA), post-training lesion (SH and BNST), and time (min 3-10) revealed a significant main effect of time [$F_{(7,364)} = 18.9$; $p < 0.0001$] without significant main effects of pre-training lesion (SH or BLA) [$F_{(1,52)} = 1.4$; $p = 0.25$] or post-training lesion (SH or BNST) [$F_{(1,52)} = 0.4$; $p = 0.52$]. The three-way ANOVA revealed no significant interactions ($p > 0.19$ for all comparisons). These data indicate that neither pre-training BLA lesions nor post-training lesions of the BNST blocked the expression of auditory cued fear.

Experiment 2: BNST inactivation and the expression of overtrained fear in rats without a BLA

Histology. Based on the histological results, 43 of 71 rats were excluded. Rats were excluded if their cannulae were misplaced or lesions were larger than intended, misplaced, or largely unilateral. This yielded the following group sizes: BLA-NBQX ($n = 6$), BLA-VEH ($n = 3$), SH-NBQX ($n = 11$), and SH-VEH ($n = 8$). The extent of the amygdala damage and cannulae placements for rats included in the analyses is depicted in Figure 3.3. As can be seen, cannula placements

and damage were generally confined to the targeted nucleus. For lesions targeting the BLA, there was some damage to the rostral entorhinal cortex.

Behavior. Post-shock freezing during the conditioning session is shown in Figure 3.4A. The data were analyzed using a three-way ANOVA with variables of lesion (SH or BLA), drug (VEH or NBQX) and trial (fifteen 5-trial blocks). During the pre-trial period rats displayed minimal levels of freezing (<5%) before footshock. After the onset of conditioning, rats exhibited robust freezing. The ANOVA revealed a main effect of lesion [$F_{(1,24)} = 5.6$; $p < 0.05$] and a main effect of training trial [$F_{(14, 336)} = 4.0$; $p < 0.0001$] without a significant main effect of drug [$F_{(1, 24)} = 1.2$; $p = 0.290$] or significant interactions across all variables ($p > 0.29$ for all comparisons). This indicates that rats with pre-training BLA lesions froze significantly less than intact rats during the training session.

Long-term fear memories to the conditioning context and the auditory CS were assessed in separate retention tests conducted 24 and 72 hours after conditioning, respectively (Figure 3.4B). As in Experiment 1, there was no difference between BLA and SH rats in either contextual or auditory freezing, therefore this variable was collapsed in the analysis. Figure 3.4B shows the average freezing data for the first 2 min of the context test and the first 2 min of the tone test following tone onset. A two-way ANOVA for the first 2 min of the context test with variables of lesion (SH and BLA) and drug (VEH and APV) revealed a significant main effect of drug [$F_{(1,24)} = 5.0$; $p < 0.035$] without a significant main effect of lesion [$F_{(1,24)} = 1.3$; $p > 0.26$]. As previously reported, although rats with BLA lesions showed significant impairments in freezing during

the training session, there was no significant effect of lesion during the context test (Zimmerman et al., 2007). However rats receiving NBQX infusions in the BNST immediately before the context test showed significantly less freezing than those receiving vehicle. These data indicate that animals with BLA lesions acquire fear to a context after overtraining, and that the expression of this contextual fear is dependent upon the activation of AMPA receptors within the BNST.

Freezing during the tone test is also shown in Figure 3.4B. A two-way ANOVA for the first 2 min of the tone test following tone onset with variables of lesion (SH and BLA) and drug (VEH and APV) revealed no significant effects or interactions ($p > 0.30$ for all comparisons). These data indicate that the expression of contextual, but not auditory, fear requires the BNST.

Discussion

The results of the present study indicate that BNST lesions or inactivation have a selective effect on the expression of contextual fear after overtraining. This impairment was not observed to an auditory CS, and was manifest in both intact rats and rats with BLA lesions. These results are consistent with previous studies that have demonstrated that lesions of the BNST selectively disrupt contextual fear after limited training (Sullivan et al., 2004; Waddell et al., 2006). Similarly, lesions of the BNST do not effect fear-potentiated startle, a paradigm in which a discrete light CS paired with a shock unconditioned stimulus increases the acoustic startle reflex (Lee and Davis, 1997). Collectively, these data

suggest that the BNST is critically involved in the expression of contextual fear after both limited and extensive training. Because BNST lesions or inactivation did not influence the expression of fear to an auditory CS in rats with BLA lesions, it does not serve a general role in compensating for the absence of the BLA to mediate fear conditioning. Indeed, we have previously reported that lesions of the central nucleus of the amygdala prevent the acquisition and expression of both contextual and auditory fear in rats with BLA lesions (Zimmerman et al., 2007). Together, these data reveal that the CEA compensates for the loss of the BLA to mediate fear conditioning.

The present data add to a growing body of evidence that the BNST has a special role in the expression of conditioned anxiety, rather than conditioned fear *per se*. For example, Walker and Davis (1997) have demonstrated that lesions of the BNST prevent light-enhanced startle, a model for unconditioned fear in which the presence of a continuous anxiogenic stimulus (bright light) enhances fear to a loud noise burst. Moreover, Waddell and colleagues (2006) have found that BNST lesions effect fear conditioning to long duration CSs relative to short duration CSs. It has been argued that shock-associated contexts, long duration CSs, and ambient bright light yield a state of conditioned anxiety because they signal that an aversive event is likely to occur, but not when it will happen (Walker et al., 2003; Davis, 2006; Waddell et al., 2006). The BNST is highly interconnected with hypothalamic nuclei involved in coordinating the release of stress hormones, and therefore may engage conditioned and unconditioned anxiety responses that prepare animals for potential threats in the environment.

In contrast, the CEA is anatomically connected to brain stem systems involved in organizing conditioned fear responses, such as freezing, that anticipate imminent insult.

Much like the amygdala, the BNST receives input from the ventral hippocampus and ventral subiculum (primary output of the hippocampus) (Dong et al., 2001). Interestingly, these hippocampal subregions are implicated in the expression of conditioned fear and anxiety responses. Excitotoxic or electrolytic lesions of the ventral subiculum impair the acquisition and expression of conditioned fear (Maren, 1999b), much like lesions of the amygdala. Additionally, lesions of the ventral hippocampus produce an anxiolytic effect on tests of unconditioned anxiety (McHugh et al., 2004), an effect not seen in rats with amygdala lesions, but similar to the effects discussed above in rats with lesions of the BNST. Such findings suggest that the contextual information necessary for the expression of conditioned anxiety is likely mediated via input from the ventral subiculum and ventral hippocampus directly to the BNST.

In contrast to the BNST, lesions or inactivation of the CEA completely block the expression of conditioned freezing to both auditory CSs and shock-associated contexts (Zimmerman et al., 2007), as well as eliminating fear-potentiated startle to a visual CS (Walker and Davis, 1997). This suggests that the CEA is necessary for the acquisition and expression of conditioned fear in both intact rats and rats with BLA lesions. Of particular interest however, Kim and Davis (1993) have shown that rats with CEA lesions can reacquire fear potentiated startle after extensive training as long as the CEA was intact during

the initial acquisition of fear. In this case it is unclear whether the brain is compensating for the loss of the CEA during the reacquisition of fear, expression of fear, or both.

Importantly, inactivation of the CEA does not affect light-enhanced startle, whereas inactivation of either the BLA or BNST impairs this effect (Walker and Davis, 1997; Walker et al., 2003). These data suggest that the CEA is essential for mediating conditional fear, while the BNST is required for conditioned anxiety (Walker et al., 2003; Davis, 2006; Waddell et al., 2006). Interestingly, lesions of either the CEA or BNST block the expression of contextual fear indicating that freezing to the conditioning context encompasses aspects of both fear and anxiety. Moreover, these data imply that both freezing and startle can index different psychological states (fear or anxiety) and that behavior under these different states is mediated by different neural systems.

In summary, our findings indicate that the BNST is necessary for the expression of contextual fear even after overtraining. Importantly, BNST lesions did not prevent the expression of freezing to an auditory CS in either intact rats or rats with BLA lesions. Hence, it does not appear that the BNST functions as a surrogate for a damaged BLA. Rather, our previous work suggests that the CEA plays such a role, compensating for the BLA to mediate both context and CS fear after overtraining (Zimmerman et al., 2007). As has been previously suggested (Walker et al., 2003; Davis, 2006; Waddell et al., 2006), our data are consistent with a role for the CEA and BNST in mediating conditioned fear and anxiety, respectively. However, these systems are not mutually exclusive and interact to

mediate contextual freezing, for example. Understanding the precise circumstances under which each system is utilized will require additional research.

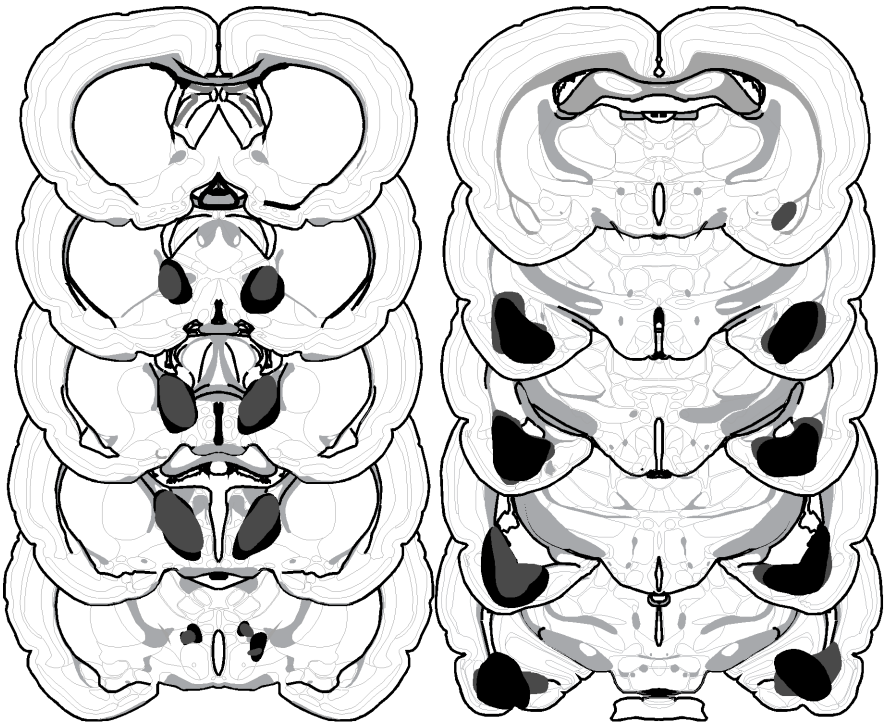

Figure 3.1. Schematic representation of the extent of NMDA lesions (Experiment 1).
Schematic representation of the extent of pre-training NMDA lesions of the BLA and post-training lesions of the BNST (BLA-BNST, represented in gray) and pre-training lesions of the BLA with Sham lesions of the BNST (BLA-SH, represented in black) or Sham lesions of the BLA with post-training lesions of the BNST (SH-BNST, represented in black) for Experiment 1. Coronal brain section images adapted from Swanson (1992).

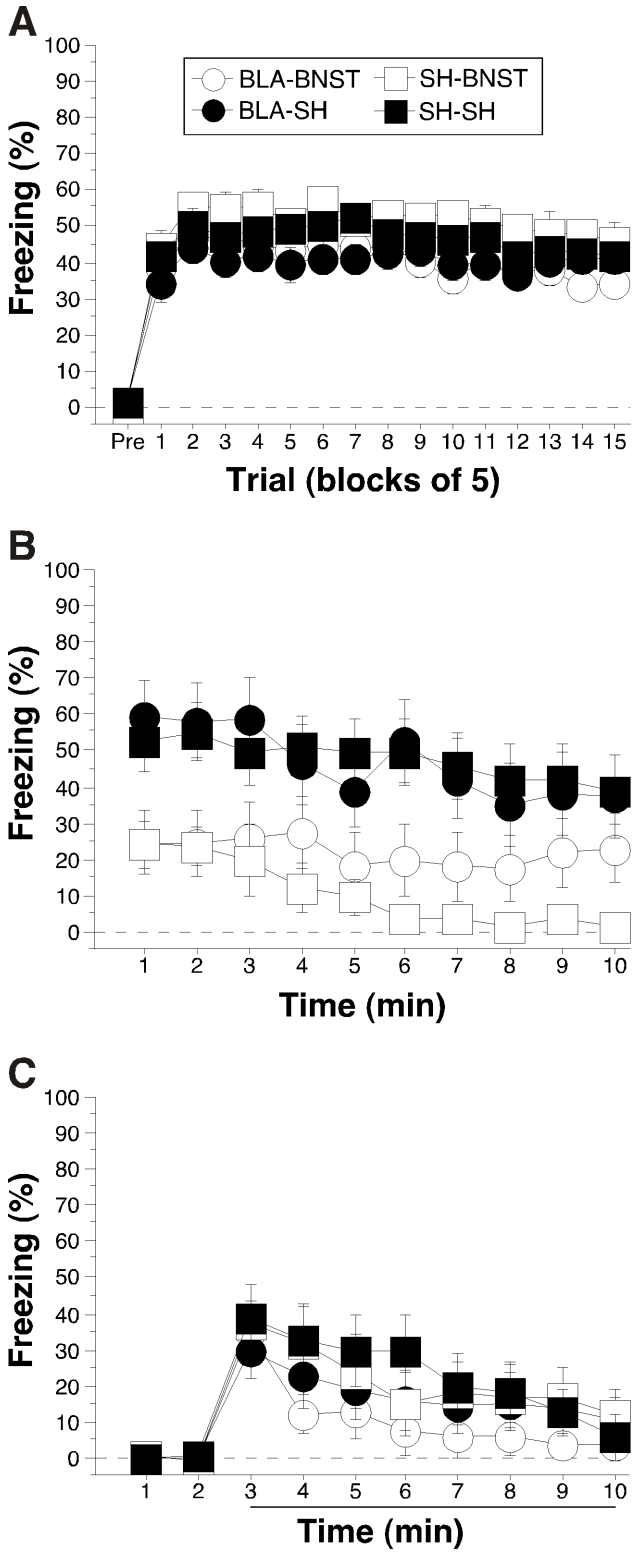

Figure 3.2. Conditioned freezing in rats with pre-training BLA and post-training BNST lesions (Experiment 1).

A, Mean percentage of freezing (± SEM) during the 75 trial training session (data are displayed with a 3 min pre-trial period followed by 15 bins consisting of 5 trials each). Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial; these values were averaged in 5-trial blocks. *B*, Mean percentage of freezing (± SEM) to contextual (10 min context extinction test) cues 4 days following training. *C*, Mean percentage of freezing (± SEM) to the auditory CS in a novel context 5 days following training. The auditory CS was initiated 2 min after rats were placed in the chambers (horizontal bar indicates the CS). Data are shown for rats with pre-training lesions of the BLA (closed circle), pretraining lesions of the BLA and post-training lesions of the BNST (open circle), post-training lesions of the BNST (open square), and intact rats (SH-SH; closed squares).

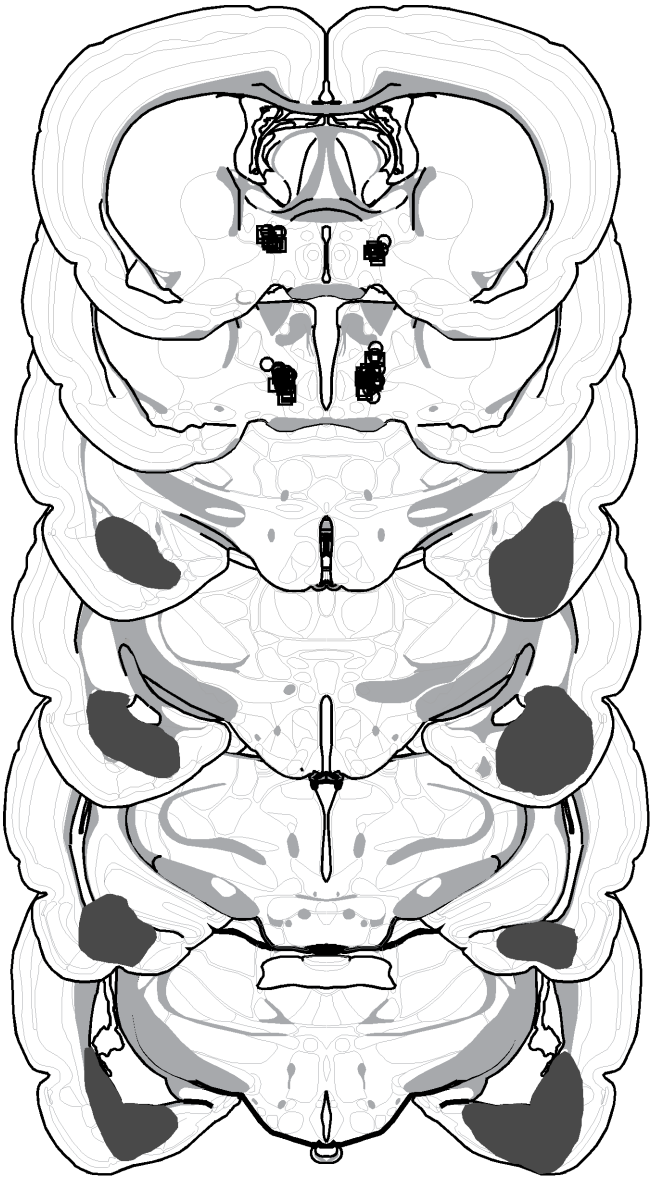

Figure 3.3. Schematic representation of the extent of pre-training NMDA lesions of the BLA (dark grey) and the locations of included cannula placements (Experiment 2).
Schematic representation of the extent of pre-training NMDA lesions (median lesion) of the BLA (dark grey) and the locations of included cannula placements for the infusion of NBQX (circles) or VEH (squares) in the BNST (Experiment 2). Coronal brain section images adapted from Swanson (1992).

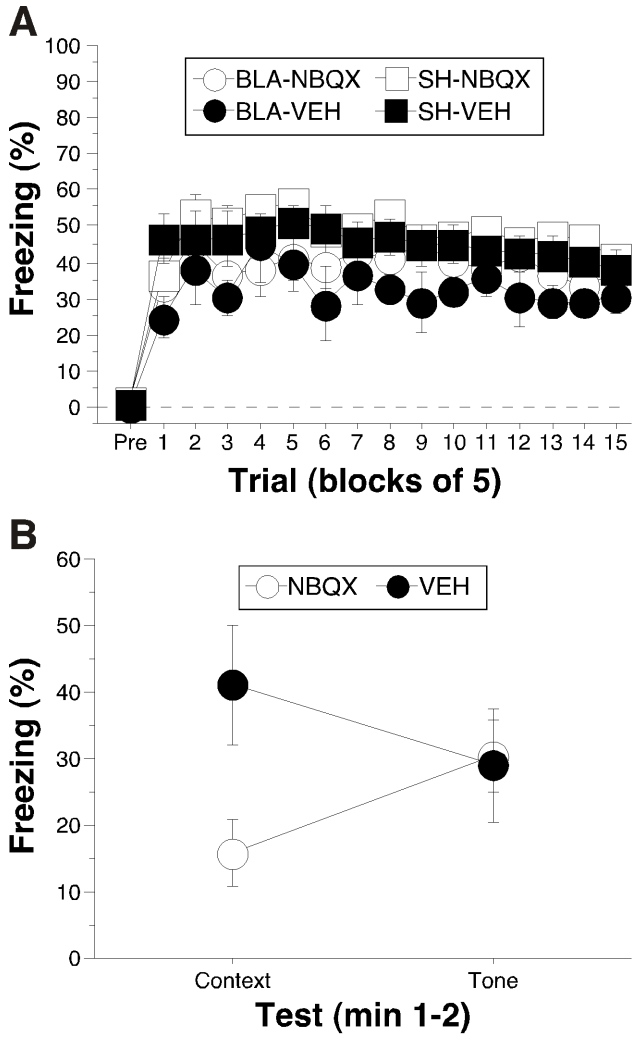

Figure 3.4. Conditioned freezing in rats with pre-training BLA lesions and pre-test NBQX infusions into the BNST (Experiment 1).
A, Mean percentage of freezing (± SEM) during the 75 trial training session (data are displayed with a 3 min pre-trial period followed by 15 bins consisting of 5 trials each). Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial; these values were averaged in 5-trial blocks. *B*, Mean percentage of freezing (± SEM) to first 2 min of the context test and tone test (following tone onset) after infusion of NBQX into the BNST. Data are shown for rats with pre-training sham surgeries receiving VEH in the BNST prior to testing (SH-VEH: closed squares), pre-training sham surgeries receiving NBQX in the BNST prior to testing (SH-NBQX: open squares), pre-training NMDA lesions of the BLA receiving VEH in the BNST prior to training (BLA-VEH: closed circles), or pre-training NMDA lesions of the BLA receiving NBQX in the BNST prior to training (BLA-NBQX: open circles).

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CHAPTER 4

NMDA RECEPTOR ANTAGONISM IN THE BASOLATERAL BUT NOT CENTRAL AMYGDALA BLOCKS THE EXTINCTION OF PAVLOVIAN FEAR CONDITIONING IN RATS

Pavlovian fear conditioning is an important behavioral paradigm used to study the neurobiological mechanisms of emotional learning and memory (Davis, 1992; Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001, 2005a). In this form of conditioning, an animal learns that a neutral conditioned stimulus (CS), such as a tone, predicts an aversive unconditioned stimulus (US), such as a footshock. After conditioning, the CS alone elicits a variety of conditioned fear responses (CRs), including increases in blood pressure, potentiated acoustic startle, and freezing behavior. Degrading the relationship between the CS and the US by presenting the CS alone numerous times results in an extinction of fear to the CS. During extinction, animals learn a new inhibitory memory that suppresses fear. This suppression is labile, however, and fear CRs may return with changes in context (renewal), for example (Maren, 2005a; Bouton et al., 2006).

In recent years, there has been a growing interest in understanding the neurobiological mechanisms of fear extinction (Maren and Quirk, 2004; Bouton et al., 2006; Corcoran and Quirk, 2007; Myers and Davis, 2007; Quirk and Mueller, 2008). It is now well established that the basolateral complex of the amygdala

(BLA) is crucial for the acquisition, expression, and extinction of conditioned fear (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001). Within the BLA, considerable work has revealed an important role for glutamate receptors in these processes. Specifically, infusions of AMPA (AMPAR) or NMDA receptor (NMDAR) antagonists into the BLA impair the expression of conditioned fear (Miserendino et al., 1990; Campeau et al., 1992; Maren et al., 1996; Lee and Kim, 1998; Fendt, 2001; Lee et al., 2001; Rodrigues et al., 2001; Goosens and Maren, 2004; Walker et al., 2005), whereas NMDAR antagonists prevent the acquisition and extinction of fear (Miserendino et al., 1990; Campeau et al., 1992; Falls et al., 1992; Cox and Westbrook, 1994; Fanselow and Kim, 1994; Maren et al., 1996; Lee and Kim, 1998; Rodrigues et al., 2001; Santini et al., 2001; Lin et al., 2003; Goosens and Maren, 2004; Maren and Quirk, 2004; Walker et al., 2005). Interestingly, infusions of NMDAR agonists into the BLA facilitate extinction (Walker et al., 2002).

In addition to the BLA, there is a growing appreciation for the role played by the central nucleus of the amygdala (CEA) in Pavlovian fear conditioning. For example, recent work has shown that lesions, temporary inactivation, or NMDAR antagonism of the CEA block the acquisition of conditioned fear (Goosens and Maren, 2003; Wilensky et al., 2006; Zimmerman et al., 2007). Furthermore, protein synthesis inhibition within the CEA immediately after the acquisition of conditioned fear blocks the consolidation of the fear memory (Wilensky et al., 2006). Consistent with the role of the CEA in the acquisition of fear, Samson and Pare (2005) have demonstrated NMDAR dependent plasticity within the CEA *in*

vitro. These findings suggest the possibility that CEA glutamate receptors, and NMDARs in particular, have a role in the extinction of fear. Here we address this issue by comparing the effects of glutamate receptor antagonism in the BLA and CEA on the extinction of fear to an auditory CS in rats.

Materials and Methods

Experiment 1: AMPA receptor antagonism in the BLA or CEA and fear extinction

Subjects. The subjects were 53 male Long-Evans rats (200-224 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN). After arrival, the animals were individually housed in clear plastic cages hanging from a standard stainless-steel rack. The vivarium lights were on a 14/10 light/dark cycle (lights on at 7:00 am) and the rats had free access to food and tap water. After housing, the rats were handled (15-20 sec each) for five days to acclimate them to the experimenter. All experiments were carried out in accordance with guidelines approved by the University of Michigan University Committee on Use and Care of Animals.

Behavioral apparatus. Eight identical observation chambers (30 x 24 x21 cm; Med-Associates, St. Albans, VT) were used for all phases of training and testing. The chambers were constructed from aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating chests located in an isolated room. The floor of each chamber

consisted of 19 stainless-steel rods (4 mm diameter) spaced 1.5 cm apart (center to center). The rods were wired to a shock source and solid-state grid scrambler (Med-Associates) for delivery of the foot shock unconditioned stimulus (US) (1.0 mA, 2 sec). For “context A” (used for conditioning), background noise (65 dB) was provided by ventilation fans built into the chests, house lights within the chambers and fluorescent lights within the room provided illumination, the chest doors were left open, the chambers were cleaned with a 1% ammonium hydroxide solution, and the rats were transported in black carriers. For “context B” (used for Drug and Drug-Free Extinction), illumination was provided by incandescent red lights, the chest doors were closed, the ventilation fans were inactive, the chambers were cleaned with a 1% acetic acid solution, the floors were covered with black plastic panels, and the rats were transported in white 5-gallon buckets. Stainless steel pans containing a thin film of the corresponding cleaning solutions were placed underneath the grid floors before the animals were placed inside the boxes.

Each conditioning chamber rested on a load cell platform that was used to record chamber displacement in response to each rats’ motor activity. To ensure interchamber reliability, each load cell amplifier was calibrated to a fixed chamber displacement. The output of the load cell of each chamber was set to a gain that was optimized for detecting freezing behavior. Load cell amplifier output from each chamber was digitized and acquired on-line using Threshold Activity software (Med-Associates).

Surgery. After handling for at least five days, rats were treated with atropine sulfate (0.4 mg/kg body weight, i.p.) and sodium pentobarbital (65 mg/kg body weight, i.p.) and mounted in stereotaxic apparatus (David Kopf instruments, Tujunga, CA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes were drilled bilaterally in the skull for the placement of 26-gauge guide cannula (cut at 11 mm below the pedestal; Plastics One, Roanoke, VA) in the BLA (2.8 mm posterior to bregma, 5.0 mm lateral to the midline, 6.3mm ventral to dura) or CEA (2.5 mm posterior to bregma, 4.3 mm lateral to the midline, 6.9 mm ventral to the skull surface) and 3 small screws. Following implantation dental acrylic was applied to the skull to hold the cannula in place. After surgery, dummy cannulae (33-gauge, 16 mm; Plastics One, Roanoke, VA) were inserted into the guide cannula, and the rats were allowed to recover from the anesthesia before being returned to their home cages. The dummy cannulae were replaced every other day during the week of recovery.

Procedure. After at least 7 days recovery from surgery, rats were acclimated to the infusion procedure by transporting them to the infusion room in identical white 5-gallon buckets in squads of eight (counterbalanced for each squad and group). Their dummy cannulas were replaced and the infusion pumps (Harvard Apparatus, South Natick, MA) were activated. After five minutes, the pumps were stopped and the animals were returned to their home cages. Twenty-four hours after acclimation, on the conditioning day, the rats were transported to the laboratory in squads of eight and placed in the conditioning

chambers. The chamber position was counterbalanced for each squad and group. The rats received 5 tone (80 dB, 10 sec, 2kHz) shock (1.0 mA, 2.0 sec) pairings (70 sec intertrial interval) beginning 3 min after being placed in the chamber and ending 60 sec after the final shock (context A). The rats were then transported back to their home cages. Twenty-four hours after training, the rats were transported to the infusion room as described above and infused with the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 2.5 µg in 0.25 µl of 100 mM PBS for the CEA or 5.0 µg in 0.5 µl of 100 mM PBS for the BLA at 0.1 µl/min) or 100 mM phosphate buffered saline (PBS) (VEH; 0.25 µl for the CEA or 0.5 µl for the BLA at 0.1 µl/min). After the infusion, one minute was allowed for diffusion before removing the internal cannula. After removing the internal cannulae, clean dummy cannulae were inserted into the guide cannula and rats were immediately transported to the conditioning chambers for Drug Extinction. Extinction consisted of 45 CS-alone presentations (80 dB, 10 sec, 2kHz) with a 30 sec intertrial interval beginning 3 min after being placed in the chamber and ending 3 min after the final CS presentation for rats in the extinction groups (BLA-NBQX-E, CEA-NBQX-E, VEH-E) (Context B). During the Drug Extinction session, rats in the no-extinction group (VEH-NE) were placed in the conditioning chamber for the same amount of time as the extinction groups in the absence of any CS presentations (Context B). Forty-eight hours after Training the rats were transported to the conditioning chambers for Drug-Free Extinction (Context B). Drug-Free Extinction was

identical to the Drug Extinction performed 24 hours prior (all groups received CS presentations).

During the training and extinction sessions, each rat's activity was monitored continuously using the data acquisition software described above. For each chamber, load cell activity was digitized at 5 Hz, yielding one observation per rat every 200 msec (300 observations per rat per minute). Load cell values ranged between 0 and 100, and this value was used to quantify locomotor activity. Freezing was quantified by computing the number of observations for each rat that had a load cell value less than the freezing threshold (threshold = 10). The freezing threshold was determined in a separate group of pilot animals by comparing load cell output with an observer's rating of freezing behavior. To avoid counting momentary inactivity as freezing, an observation was only scored as freezing if it fell within a contiguous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec. For each session, the freezing observations were transformed to a percentage of total observations. In the present experiment, freezing was quantified before footshock during the pre-trial period and after footshock offset on the conditioning day, and throughout the entirety of the extinction tests.

Histology. Histological verification of cannula placements was performed after behavioral testing. Rats were sacrificed with CO_2 asphyxiation followed by decapitation. After extraction from the skull, the brains were fixed in 10% formalin for at least 2 days followed by 10% formalin and 30% sucrose until

sectioning. Coronal sections (45 μm thick, taken every 135 μm) were cut on a cryostat (-20 ^{o}C) and wet mounted on glass microscope slides with 70% ethanol. After drying, the sections were stained with 0.25% thionin to visualize neuronal cell bodies. Placements were verified by visual inspection of the stained brain sections.

Data analysis. For each session, the freezing data were transformed to a percentage of total observations, a probability estimate that is amenable to analysis with parametric statistics. These probability estimates of freezing were analyzed using analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's PLSD tests were performed after a significant overall *F* ratio. All data are represented as means ± SEMs.

Experiment 2: NMDA receptor antagonism in the BLA or CEA and fear extinction

Subjects. The subjects were 60 male Long-Evans rats (200-224 g; Blue Spruce) obtained and housed as described in Experiment 1.

Apparatus, surgery, procedure, histology, and data analysis. All materials and methods are as described in Experiment 1 except that rats were infused with the NMDA receptor antagonist D,L-2-amino-5-phosphonopentanoic acid (APV; 2.5 μg in 0.25 μl of 100 mM PBS for the CEA or 5.0 μg in 0.5 μl of 100 mM PBS for the BLA at 0.1 μl/min) or 100 mM PBS (VEH; 0.25 μl for the CEA or 0.5 μl for the BLA at 0.1 μl/min).

Results

Experiment 1: AMPA receptor antagonism in the BLA or CEA prevents the expression, but not extinction, of conditioned fear

Histology. Based on histological results, 53 rats were included in this experiment. Rats were excluded if their guide cannula were located outside the intended structure. This yielded the following group sizes: rats receiving NBQX in the BLA during extinction (BLA-NBQX-E; *n*=12), rats receiving NBQX in the CEA during extinction (CEA-NBQX-E; *n*=12), rats receiving PBS in the BLA or CEA during extinction were not statistically different and were collapsed into a single group (VEH-E; *n*=18), and rats receiving PBS in the BLA or CEA that did not receive extinction were not statistically different and were collapsed into a single group (VEH-NE; *n*=11). BLA and CEA cannula placements for rats included in the analysis are depicted in Figure 4.1. All cannula placements were located within the intended structures (BLA or CEA).

Behavior. Post-shock freezing during the conditioning session is shown in Figure 4.2A. Freezing was not statistically different across groups. The data were analyzed using two-way ANOVA with variables of group (BLA-NBQX-E, CEA-NBQX-E, VEH-E, VEH-NE) and trial (1-5). During the pre-trial period rats displayed minimal levels of freezing (<10%). After the onset of conditioning rats displayed increased levels of freezing. The ANOVA revealed no main effect of group ($F_{(3,49)}$ = 0.97; P = 0.42) or a group x trial interaction ($F_{(12,196)}$ = 1.11; P = 0.35). Additionally, the ANOVA revealed a main effect of trial ($F_{(4,196)}$ = 12.38; P

< 0.0001). This indicates that the average level of freezing across the training session was not significantly different between the groups. However, the groups increased their freezing as the training session proceeded.

Twenty-four hours after training rats were infused with either VEH or NBQX immediately before Drug Extinction. Freezing during the Drug Extinction test is shown in Figure 4.2B. Before CS onset, all groups showed low levels of freezing (similar to those seen during the Pre-period of training). A two-way ANOVA with variables of group (BLA-NBQX-E, CEA-NBQX-E, VEH-E, VEH-NE) and trial (1-45) revealed a significant main effect of group ($F_{(3,49)}$ = 6.89; P = 0.0006), trial ($F_{(44,2156)}$ = 2.80; P < 0.0001), and a group x trial interaction ($F_{(132,2156)}$ = 5.02; P < 0.0001). Post-hoc analysis of the main effect of group revealed that rats receiving NBQX in the BLA or CEA (BLA-APV-E, CEA-NBQX-E) froze significantly less than the rats receiving VEH during extinction (VEH-E; P < 0.02 for both comparisons). Additionally, rats receiving VEH before extinction (VEH-E) froze significantly more than rats receiving VEH without extinction (VEH-NE; P = 0.0003). There were no significant differences between rats receiving NBQX in the BLA (BLA-NBQX-E), rats receiving NBQX in the CEA (CEA-NBQX-E) or rats receiving vehicle without extinction (VEH-NE). Importantly, these results demonstrate that rats receiving NBQX in the BLA or CEA were unable to express conditional fear to the auditory CS earned 24 hours earlier.

The long-term extinction memory acquired during the Drug Extinction session was tested 24 hours later by exposing the rats to a second Drug-Free Extinction session. The results from the Drug-Free Extinction session are shown

in Figure 4.2C. A two-way ANOVA with variables of group (BLA-NBQX-E, CEA-NBQX-E, VEH-E, VEH-NE) and trial (1-45) revealed a significant main effect of group ($F_{(3,49)} = 3.13$; $P < 0.04$), trial ($F_{(44,2156)} = 7.06$; $P < 0.0001$), and group x trial interaction ($F_{(132,2156)} = 2.12$; $P < 0.0001$). Post-hoc analysis of the main effect of group revealed that rats receiving NBQX in the BLA (BLA-NBQX-E) or CEA (CEA-NBQX-E) froze significantly less that rats receiving VEH without extinction (VEH-NE; $P < 0.03$ for both comparisons). Further analysis of the first 10 trials of the Drug-Free Extinction session via two-way ANOVA with variables of group (BLA-APV-E, CEA-APV-E, VEH-E, VEH-NE) and trial (1-10) revealed a significant main effect of group ($F_{(3,49)} = 4.56$; $P < 0.007$) and trial ($F_{(9,441)} = 8.04$; $P < 0.0001$). The group x trial interaction was not significant ($F_{(27,441)} = 1.36$; $P = 0.11$). Post-hoc analysis of the main effect of group revealed that rats that did not receive extinction during the Drug Extinction session (VEH-NE) froze significantly more than all other groups ($P \leq 0.008$ for all comparisons). These results indicate that while rats receiving NBQX in the BLA or CEA were unable to express freezing during the Drug Extinction session (Figure 4.2B) they were still able to acquire an extinction memory as tested during the Drug-Free Extinction session (Figure 4.2C).

Experiment 2: NMDA receptor antagonism in the BLA, but not the CEA, prevents the extinction of conditioned fear

Histology. Based on histological results, 60 rats were included in this experiment. Rats were excluded if their guide cannula were located outside the

intended structure. This yielded the following group sizes: rats receiving APV in the BLA during extinction (BLA-APV-E; *n*=9), rats receiving APV in the CEA during extinction (CEA-APV-E; *n*=10), rats receiving PBS in the BLA or CEA during extinction were not statistically different and were collapsed into a single group (VEH-E; *n*=23), and rats receiving PBS in the BLA or CEA that did not receive extinction were not statistically different and were collapsed into a single group (VEH-NE; *n*=18). BLA and CEA cannula placements for rats included in the analysis are depicted in Figure 4.1. All cannula placements were located within the intended structures (BLA or CEA).

Behavior. Post-shock freezing during the conditioning session is shown in Figure 4.3A. Freezing was not statistically different across groups. The data were analyzed using two-way ANOVA with variables of group (BLA-APV-E, CEA-APV-E, VEH-E, VEH-NE) and trial (1-5). During the pre-trial period rats displayed minimal levels of freezing (<10%). After the onset of conditioning rats displayed potentiated freezing. The ANOVA revealed no main effect of group ($F_{(3,56)}$ = 1.9; P = 0.14). Additionally, the ANOVA revealed a main effect of trial ($F_{(4,224)}$ = 20.1; P < 0.0001) and a group x trial interaction ($F_{(12,224)}$ = 2.25; P = 0.01). This indicates that the average level of freezing across the training session was not significantly different between the groups. However, the groups increased their freezing as the training session proceeded and did so at different rates.

Twenty-four hours after training rats were infused with either VEH or APV immediately before Drug Extinction. Freezing during the Drug Extinction test is

shown in Figure 4.3B. Before CS onset, all groups showed low levels of freezing (similar to those seen during the pre-period of training). A two-way ANOVA with variables of group (BLA-APV-E, CEA-APV-E, VEH-E, VEH-NE) and trial (1-45) revealed a significant main effect of group ($F_{(3,56)} = 4.42$; $P < 0.008$), trial ($F_{(44,2464)} = 1.58$; $P < 0.01$), and a group x trial interaction ($F_{(132,2464)} = 2.41$; $P < 0.0001$). Post-hoc analysis of the main effect of group revealed that rats receiving APV in the BLA (BLA-APV-E) froze significantly more than all other groups ($P < 0.03$ for all comparisons). There were no significant differences between the other groups.

The long-term extinction memory acquired during the Drug Extinction session was tested 24 hours later by exposing the rats to a second Drug-Free Extinction session. The Drug-Free Extinction session is shown in Figure 4.3C. A two-way ANOVA with variables of group (BLA-APV-E, CEA-APV-E, VEH-E, VEH-NE) and trial (1-45) revealed a significant main effect of group ($F_{(3,56)} = 10.23$; $P < 0.0001$), trial ($F_{(44,2464)} = 18.07$; $P < 0.0001$), and group x trial interaction ($F_{(132,2464)} = 4.67$; $P < 0.0001$). Post-hoc analysis of the main effect of group revealed that rats in the BLA-APV-E group froze significantly more than rats in the CEA-APV-E group ($P < 0.02$). Additionally, rats in the CEA-APV-E and VEH-E groups froze significantly less than rats in the VEH-NE group ($P < 0.0001$ for both comparisons). Further analysis of the first 10 trials of the Drug-Free Extinction session via two-way ANOVA with variables of group (BLA-APV-E, CEA-APV-E, VEH-E, VEH-NE) and trial (1-10) revealed a significant main effect of group ($F_{(3,56)} = 17.74$; $P < 0.0001$), trial ($F_{(9,504)} = 13.43$; $P < 0.0001$), and

a group x trial interaction ($F_{(27,504)}$ = 2.87; P < 0.0001). Post-hoc analysis of the main effect of group revealed that rats in the BLA-APV-E and VEH-NE groups froze significantly more than rats in the CEA-APV-E and VEH-E groups (P < 0.0001 for all comparisons. Importantly, rats in the BLA-APV-E group and VEH-NE group were not significantly different (P = 0.71) indicating that rats with BLA NMDA receptor antagonism had no memory of the extinction session that occurred 24 hours earlier. Additionally, rats in the CEA-APV-E groups and VEH-E were not significantly different (P = 0.37) from one another. This indicates that NMDA receptor antagonism within the CEA had no effect on the formation of a long-term memory for extinction.

Discussion

The present experiments demonstrate distinct roles for AMPARs and NMDARs in the BLA and CEA in the expression and extinction of conditioned fear. We show that AMPARs within both the BLA and CEA are necessary for the expression of conditioned fear, but are not required for fear extinction. In contrast, NMDAR antagonism in the amygdala did not influence the expression of fear but did impair the acquisition of extinction. Importantly, the effect of NMDAR antagonism on extinction learning was only obtained with intra-BLA infusions of APV; antagonism of CEA NMDARs did not affect the expression or extinction of fear.

Consistent with previous reports, we found that AMPARs in the BLA are involved in the expression of conditional fear (Falls et al., 1992; Kim et al., 1993;

Walker et al., 2005). We now show that AMPARs in the CEA are also involved in the expression of conditioned freezing. Interestingly, NMDARs in the BLA and CEA were not involved in the expression of conditioned fear. This is consistent with other studies in which normal fear responses were reported after NMDAR antagonism in the BLA (Miserendino et al., 1990; Campeau et al., 1992; Rodrigues et al., 2001; Walker et al., 2005). However, these data stand in contrast to several reports, including an earlier report from our laboratory, that NMDAR antagonism in the BLA prevents the expression of fear (Maren et al., 1996; Lee and Kim, 1998; Fendt, 2001; Lee et al., 2001; Goosens and Maren, 2004). In our earlier report, we used a contextual conditioning paradigm, whereas in the present study we assessed fear to an auditory CS. It is possible that amygdala NMDARs are differently involved in the expression of fear to contexts and cues.

Alternatively differences in the contribution of NMDAR subtypes to fear expression (Walker and Davis, 2008) and the influence of different APV enantiomers on these subtypes (Matus-Amat et al., 2007) might contribute to the variable effects of NMDAR antagonists in the expression of fear. While both the BLA and CEA contain NMDARs, the NMDAR subunit composition within these areas is different and therefore differentially susceptible to various NMDAR antagonists. NMDARs form heteromultimers containing an NR1 subunit and a combination of NR2A and/or NR2B subunits (Cull-Candy et al., 2001; Prybylowski and Wenthold, 2004). A recent in vitro electrophysiological study of NMDARs in the BLA and CEA reveals that the NMDAR mediated current in CEA

neurons have slow kinetics and are blocked by NR2B specific antagonists, suggesting that they are composed of the NR1/NR2B subunits (Lopez de Armentia and Sah, 2003). In contrast, NMDAR currents in BLA neurons demonstrate much faster kinetics and are less sensitive to NR2B specific antagonists. This suggests that they are composed mostly of the NR1/NR2A subunits (Lopez de Armentia and Sah, 2003). Consistent with this, Walker and Davis (2008) have demonstrated that infusions of an NR2A antagonist (NVP-AAM077) into the BLA blocked both fear conditioning and expression whereas an NR2B antagonist (CP101,606) disrupted conditioning but not expression. It is therefore possible that a more selective NR2B specific antagonist, such as ifenprodil, would lead to an extinction impairment when infused into the CEA.

Although APV did not impair the expression of fear, it did produce a robust attenuation of extinction learning when infused into the BLA. This outcome confirms numerous reports indicating the importance of BLA NMDARs in extinction learning (Falls et al., 1992; Cox and Westbrook, 1994; Santini et al., 2001; Walker et al., 2002; Lin et al., 2003; Maren and Quirk, 2004; Sotres-Bayon et al., 2007). Together, these data provide strong support to the view that NMDAR-dependent plasticity in the BLA is involved in both the acquisition and extinction of fear conditioning (Davis, 2002; Maren, 2005b; Quirk and Mueller, 2008). To our surprise, however, APV infusions into the CEA did not impair extinction learning, even though they severely attenuate fear conditioning when infused prior to training (Goosens and Maren, 2003). This finding provides unique insight into the specific neurocircuitry underlying extinction and draws a stark

contrast between the role of NMDARs in the BLA and CEA in conditioning and extinction. Indeed, our findings here suggest a dissociation between the role of the CEA in the acquisition and extinction of fear. While NMDAR antagonism within CEA blocks the acquisition of conditioned fear (Maren et al., 1996; Goosens and Maren, 2003, 2004), it had no effect on the acquisition of extinction (Experiment 2). While this result is surprising, it is not without precedent. Bahar and colleagues (2003) found that infusion of a protein synthesis inhibitor into the CEA, blocked the acquisition of CTA while having no effect on the extinction of CTA. Collectively, these data suggest that NMDAR-dependent plasticity in the CEA (Wilensky et al., 2006) has a selective role in fear acquisition, whereas BLA plasticity has a broader role in acquiring both fear and extinction memories.

Although NMDAR-dependent plasticity in the CEA is not involved in extinction learning, there is considerable evidence that the regulation of neuronal activity in the CEA is importantly involved in the expression of extinction. Indeed, recent data indicate that a network of GABAergic inhibitory interneurons in the amygdala are involved in the expression of extinction (Likhtik et al., 2008). These intercalated neurons (ITC) receive input from both the lateral amygdala and the medial prefrontal cortex and strongly inhibit the CEA. Given the evidence linking the medial prefrontal cortex to the expression of extinction (Quirk et al., 2000; Santini et al., 2001; Maren and Quirk, 2004; Pare et al., 2004; Santini et al., 2004; Sotres-Bayon et al., 2007; Quirk and Mueller, 2008; Knapska and Maren, 2009), it is widely believed that medial prefrontal cortical projections

to the ITC and consequent inhibition of CEA activity is involved in the expression of extinction.

In conclusion, while both the CEA and BLA are necessary for the acquisition and expression of Pavlovian conditioned fear (Wilensky et al., 2006; Zimmerman et al., 2007) we now show distinct roles for AMPA and NMDARs within the BLA and CEA in the expression of conditional fear and the acquisition of extinction. AMPARs in both the BLA and CEA are involved in the expression of fear, but are not required for fear extinction. In contrast, NMDARs are necessary for the extinction, but not expression, of fear. Importantly, only BLA NMDARs are involved in extinction learning. These findings provide important insight into the molecular mechanisms that underlie extinction and help to further refine the intra-amygdaloid circuitry that underlies conditioned fear.

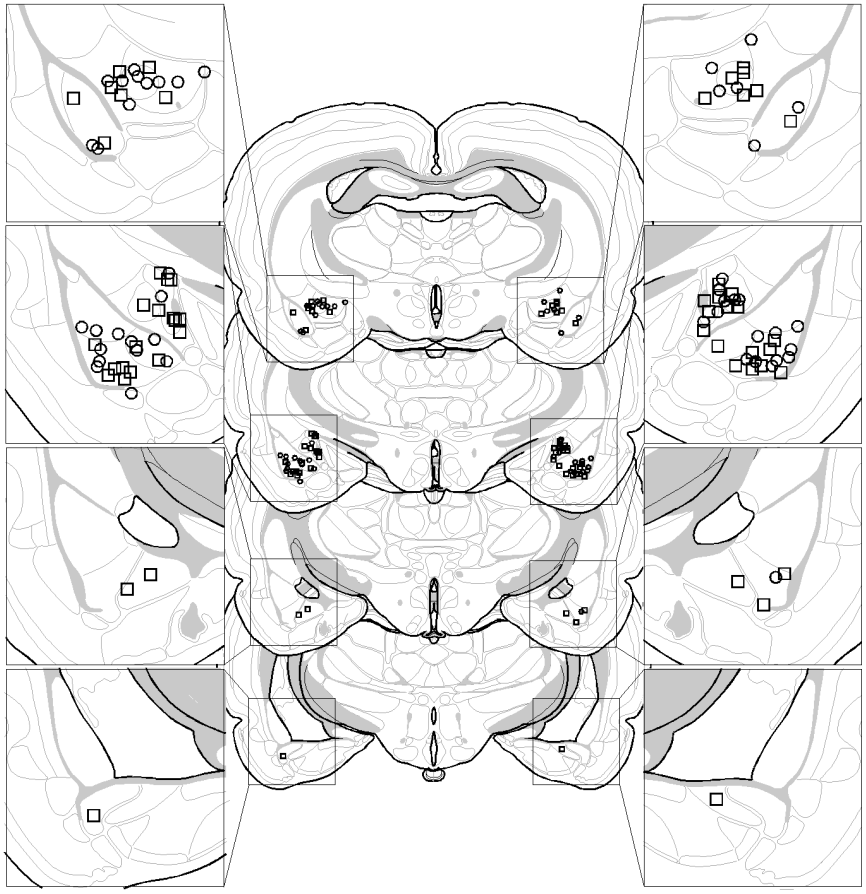

Figure 4.1. Schematic representation of the locations of the included cannula placements.
Schematic representation showing the discrete locations of the internal cannula used to infuse saline (squares) or drug (APV or NBQX; circles). Coronal brain section images adapted from Swanson (1992).

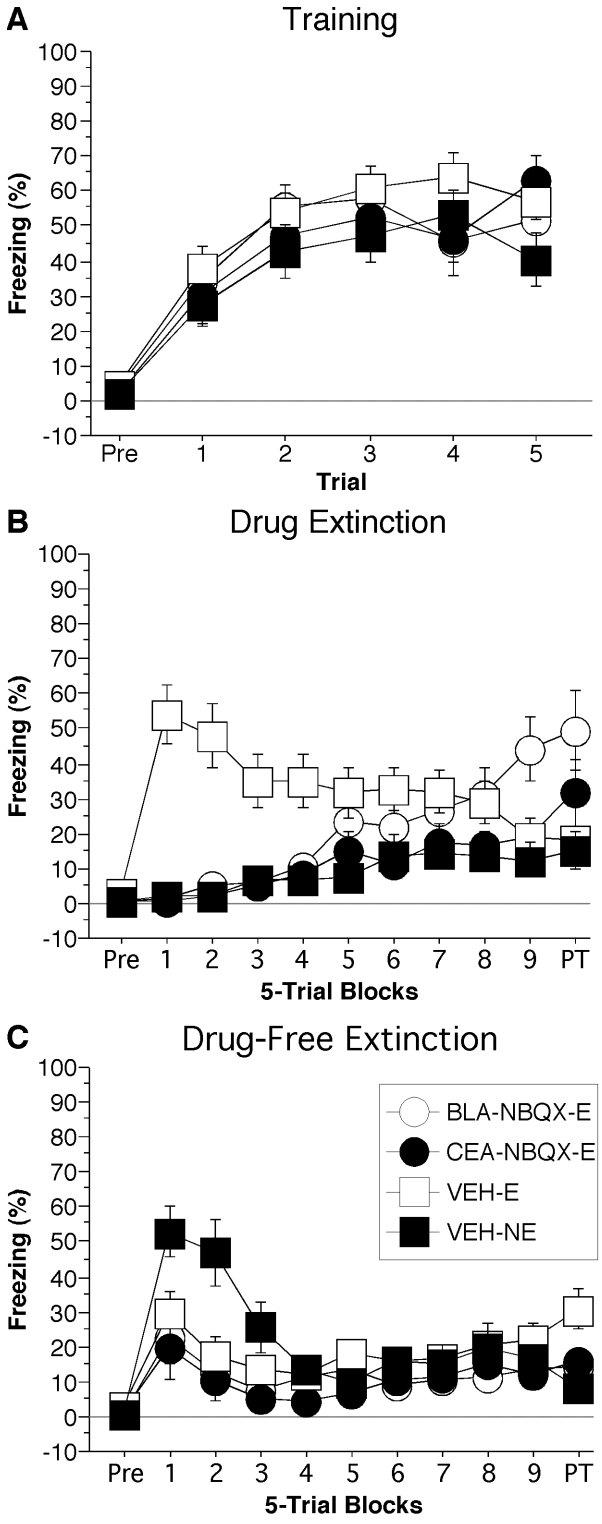

Figure 4.2. Conditioned freezing in rats receiving AMPA receptor inactivation during extinction (Experiment 1).
A, Mean percentage of freezing (± SEM) during the 5 trial training session (data are displayed with a 3 min pre-trial period followed by 5 tone-shock pairings).

Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial. *B*, Mean percentage of freezing (± SEM) during the drug extinction session immediately after drug infusions. Data are displayed with a 3 min pre-trial period followed by 9 bins consisting of 5 CS alone presentations and a 2 min post-trial period. Data was quantified before the first CS presentation (Pre), during each subsequent trial consisting of the 10 sec tone presentation and 30 sec inter-trial interval, and during the 2 min post-trial period. Rats in the no-extinction group (NE) were placed in the chambers for the same time period as all other rats however received no CS presentations. *C*, Mean percentage of freezing (± SEM) during the drug-free extinction session. Data is displayed and quantified as described in *B* with the exception of the NE rats, which received the same CS presentation as all other rats. Data are shown for rats receiving NBQX in the BLA and CS presentations during the drug extinction session (open circle), rats receiving NBQX in the CEA and CS presentation during the drug extinction session (closed circle), rats receiving VEH in the BLA or CEA and CS presentations during the drug extinction session (open square), and rats receiving VEH in the BLA or CEA and no CS presentations during drug extinction (closed square).

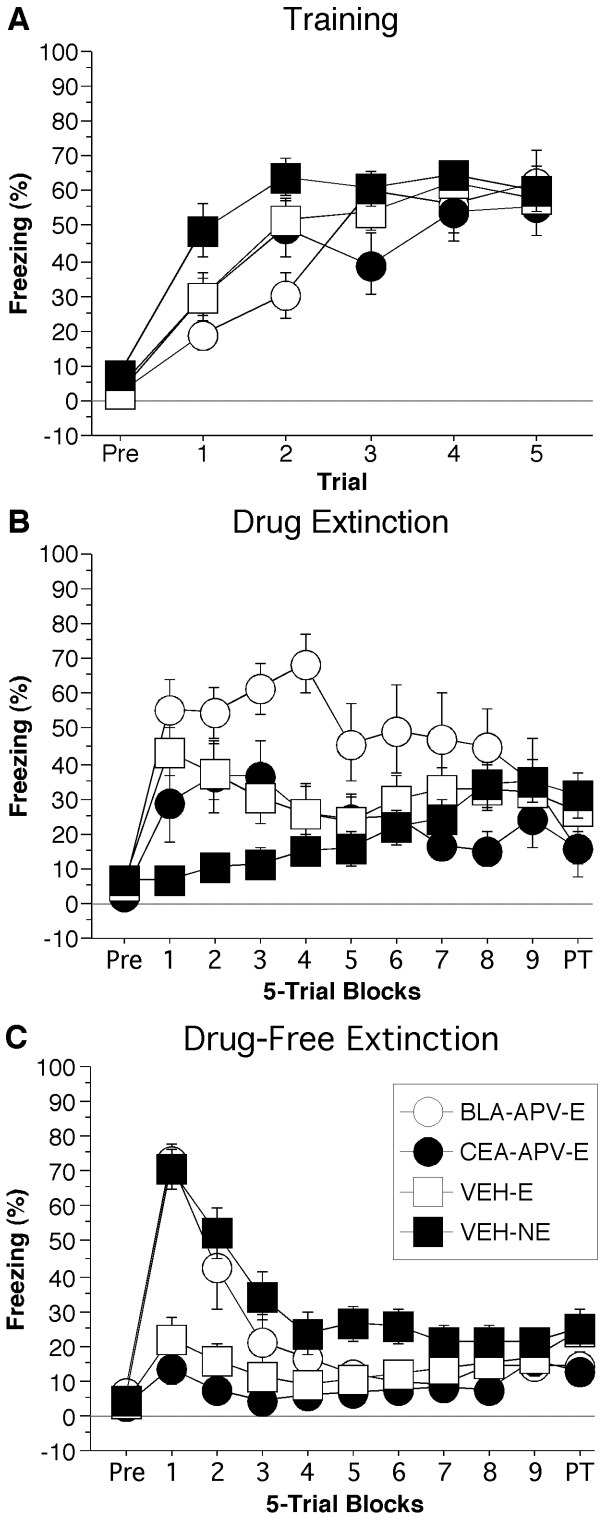

Figure 4.3. Conditioned freezing in rats receiving NMDA receptor inactivation during extinction (Experiment 2).
A, Mean percentage of freezing (± SEM) during the 5 trial training session (data are displayed with a 3 min pre-trial period followed by 5 tone-shock pairings).

Freezing was quantified before the first conditioning trial (Pre) and during the 1 min period after each conditioning trial. *B*, Mean percentage of freezing (± SEM) during the drug extinction session immediately after drug infusions. Data are displayed with a 3 min pre-trial period followed by 9 bins consisting of 5 CS alone presentations and a 2 min post-trial period. Data was quantified before the first CS presentation (Pre), during each subsequent trial consisting of the 10 sec tone presentation and 30 sec inter-trial interval, and during the 2 min post-trial period. Rats in the no-extinction group (NE) were placed in the chambers for the same time period as all other rats however received no CS presentations. *C*, Mean percentage of freezing (± SEM) during the drug-free extinction session. Data is displayed and quantified as described in *B* with the exception of the NE rats, which received the same CS presentation as all other rats. Data are shown for rats receiving APV in the BLA and CS presentations during the drug extinction session (open circle), rats receiving APV in the CEA and CS presentation during the drug extinction session (closed circle), rats receiving VEH in the BLA or CEA and CS presentations during the drug extinction session (open square), and rats receiving VEH in the BLA or CEA and no CS presentations during drug extinction (closed square).

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CHAPTER 5

CONCLUSIONS

Summary of Findings

Chapter 2 we used an overtraining procedure to explore the role of the CEA in the acquisition and expression of Pavlovian conditioned fear in rats with BLA lesions. We discovered that rats with lesions or temporary inactivation of the CEA were unable to acquire or express conditioned fear to both contextually cued and auditory cued CSs. Such findings reveal that the ability of rats with BLA lesions to acquire conditioned fear is dependent upon the CEA. Furthermore, our results demonstrate that the CEA is necessary for the acquisition of conditioned fear even when the BLA is intact; a result supported by the finding of Wilensky and colleagues (2006) in rats with limited training (5 trials). Importantly, post-training lesions of the BLA blocked the expression of conditioned fear even after overtraining, indicating that it plays an essential role in the acquisition of fear in the intact animal. Overall, the findings presented in Chapter 2 suggest that the CEA is necessary for both the acquisition and expression of Pavlovian conditioned fear. Additionally, the CEA is able to compensate for the loss of the BLA, but only if the BLA is ablated prior to conditioning.

While the findings in Chapter 2 clearly demonstrates the critical role of the CEA in conditioned fear, Chapter 3 explored the function of the BNST in these

processes. The interconnectivity between the thalamus, amygdala, and BNST discussed in Chapter 1 suggests the possibility that the BNST may have the ability to compensate for the loss of the BLA after overtraining. In Chapter 3 we demonstrate that permanent or temporary inactivation of the BNST only blocks freezing to the conditioning context, leaving fear responses to the auditory CS intact even after overtraining. Together with the work presented in Chapter 2, these results suggest that although the BNST is critically involved in the expression of contextual fear, in is not the locus of compensation for fear learning in the absence of the BLA.

Just as the BLA is critically involved in the acquisition, expression, and extinction of conditioned fear with limited training (5 trials), we now understand that the CEA is also essential for the acquisition and expression of conditioned fear. However, the role of the CEA in the extinction of conditioned fear had been left unexplored until the work presented in Chapter 4. Utilizing pharmacological inactivation of specific glutamate receptors, Chapter 4 explored the function of AMPA and NMDA receptors in the BLA and CEA on the extinction of conditioned fear. We discovered that infusions of the AMPA receptor antagonist, NBQX, into either the BLA or CEA impaired the expression of conditioned freezing to the auditory CS during extinction, but did not impair the formation of a long-term extinction memory to that CS. In contrast, infusion of the NMDA receptor antagonist APV into the amygdala spared the expression of fear to the CS during extinction training, while impairing the acquisition of a long-term extinction memory when infused into the BLA, but interestingly not the CEA. These results

reveal that AMPA and NMDA receptors within the amygdala make dissociable contributions to the expression and extinction of conditioned fear, respectively. Moreover, this outcome indicates that the amygdalar NMDA receptor-dependent processes involved in extinction learning are localized to the BLA. Taken together with the previous findings of Wilensky and colleagues (2006) and those presented in Chapter 2, these results reveal that NMDA receptors in the CEA have a selective role in the acquisition of fear memory.

Amygdalar Networks Mediating Conditioned Fear

The BLA is an important component of the amygdalar circuitry responsible for fear conditioning, because it is the first site at which CS and US information converge. Lesions or temporary inactivation of the BLA block both the acquisition and expression of conditioned fear suggesting that the BLA may be the locus of fear memory storage (LeDoux et al., 1990; Helmstetter, 1992; Campeau and Davis, 1995; Maren et al., 1996; Cousens and Otto, 1998; Goosens and Maren, 2001). As discussed in Chapter 1, the formation and storage of fear memories is believed to be dependent upon synaptic plasticity, a process requiring the activation of intracellular second messenger systems and protein synthesis. Immunohistochemical staining for phosphorylated mitogen-activated protein kinase (MAPK), the active form of the second messenger protein involved in synaptic plasticity, shows increased phosphorylation within the BLA after fear conditioning (Schafe et al., 2000). Likewise, microinfusions of MAPK pathway inhibitors or protein synthesis inhibitors into the BLA block the acquisition of

conditioned fear (Schafe et al., 2000; Schafe et al., 2001; Merino and Maren, 2006; Schafe et al., 2008; Di Benedetto et al., 2009). Additionally, Han and colleagues (2007) have shown that active cyclic-AMP response element binding protein (CREB), a transcription factor known to play a key role in learning processes (Silva et al., 1998), is upregulated in BLA neurons following fear conditioning. In a more recent paper, selective ablation of these neurons with upregulated active-CREB resulted in the removal of the neurons containing the fear memory trace and successfully erased the fear memory from the BLA (Han et al., 2009). Studies such as these clearly demonstrate the importance of the BLA in fear memory storage.

While the BLA has a demonstrated role in the acquisition and consolidation of fear memories, the CEA has long been thought to act as a passive relay necessary only for the expression of fear memory through it's output to the downstream nuclei necessary for the production of CRs. By this view, fear memories are processed serially, whereby CS/US information arrives in the BLA from the thalamus, is processed then transmitted to the CEA, which then passively sends the information to downstream brainstem nuclei for the production of CRs. However, the discovery that animals with BLA lesions could still acquire conditioned fear following overtraining suggested that another structure, possibly the CEA, must be able to compensate for the loss of the BLA (Maren, 1999; Goosens and Maren, 2003).

The finding in Chapter 2 that inactivation of the CEA blocks both the acquisition and expression of conditioned fear even in rats with an intact BLA

(Zimmerman et al., 2007) suggests a much more active role for the CEA in the acquisition and storage of fear memories. Our findings taken with those of Wilensky and colleagues (2006) that CEA inactivation and protein synthesis inhibition blocks the consolidation of fear memories, provide evidence for a second model of fear memory processing within the amygdala, a parallel processing model (Killcross et al., 1997; Pare et al., 2004; Balleine and Killcross, 2006) (Figure 5.1). According to this model, CS/US information from thalamic nuclei reaches both the BLA and CEA directly. Then the CS/US information received by the CEA either directly from the thalamus or indirectly from the BLA is output from the CEA. Such a model accounts for the importance of plasticity in both the BLA and CEA.

Providing further refinement to the parallel processing model is the finding in Chapter 2 that rats with post-training BLA lesions are unable to express conditioned fear, even after overtraining with an intact CEA (Zimmerman et al., 2007). Such a finding suggests that the indirect pathway (CS → BLA → CEA), requiring plasticity in both the BLA and CEA is preferentially utilized. In other words, while the BLA is intact, plasticity in the CS → BLA pathway and the BLA → CEA is favored over the CS → CEA direct pathway. However, in the absence of the BLA, the CS → CEA direct pathway is capable of supporting the acquisition and expression of fear with overtraining. So, while the CEA is critical for the acquisition of fear memories, plasticity in the BLA is necessary for the rapid acquisition of fear (Figure 5.1), and as Poulos and colleagues (Poulos et al., 2009) suggest, the persistence of the fear memory.

Involvement of the BNST in Fear and Anxiety

The interconnectivity of the BNST with the other fear processing circuitry necessary for Pavlovian fear conditioning suggests that it is also anatomically positioned to play a role in conditioned fear. While Davis and colleagues have previously demonstrated that lesions of the BNST had no effect on FPS, Sullivan and colleagues (2004) suggest that the BNST plays a selective role in contextual fear, as one limitation of the FPS paradigm is its inability to examine context as a CS. To investigate this possibility Sullivan and colleagues (2004) performed electrolytic lesions selective for either the CEA or the BNST after conditioning and discovered that lesions of the BNST prevented the expression of conditioned fear (freezing) only to the conditioning context. Lesions of the CEA on the other hand, attenuated freezing to both the contextual and cued CSs, just like our findings in Chapter 2.

The interconnectivity of the BNST still provides the distinct possibility that the BNST may be able to compensate for the absence of the BLA with additional training. However, while the results presented in Chapter 3 clearly demonstrate a role for the BNST in contextually conditioned fear, they also show that lesions and temporary inactivation of the BNST had no effect on the expression of auditory cued fear even after overtraining. These findings rule out the possibility that the BNST is the locus of compensation for fear conditioning in the absence of the BLA.

So what then is the role of the BNST as it relates to fear, anxiety, and stress? As discussed in Chapter 1, lesions of or AMPA antagonism within the

BNST block CRH-enhanced startle (Lee and Davis, 1997) and LES (Walker and Davis, 1997), leaving FPS intact. Conversely, amygdala lesions, specifically lesions of the CEA block fear conditioning and FPS (Walker and Davis, 1997), while leaving CRH-enhanced startle and LES intact. Such findings led Walker and Davis (1997) to conclude that the amygdaloid nuclei were import for conditioned fear while the BNST was involved in the production of unconditioned fear responses. However, our findings in Chapter 3 along with those of Sullivan and colleagues (2004) clearly demonstrate that lesions of the BNST block the expression of context fear conditioning, a behavior that is clearly conditioned.

Because the BNST is necessary for the expression of both conditioned and unconditioned fear, perhaps the involvement of the BNST is dependent upon the predictability of the cue. Waddell and colleagues (2006) investigated this theory and have found that BNST lesions effect fear conditioning to long duration (diffuse) auditory CSs relative to short duration CSs. It has been argued that shock-associated contexts, long duration CSs, and ambient light yield a state of conditioned anxiety because they signal that an aversive event is likely to occur, but not when it will happen (Walker et al., 2003; Davis, 2006; Waddell et al., 2006). As discussed in Chapter 1, the BNST is highly interconnected with hypothalamic nuclei involved in coordinating the release of stress hormones, and therefore may engage conditioned and unconditioned anxiety responses that prepare animals for potential threats in the environment. More specifically, fear to a highly predictable CS, such as a short-latency tone immediately followed by a US (shock or loud noise burst) requires the amygdala, not the BNST, whereas

more diffuse cues (e.g. context, brightly lit chamber, or a state of increased anxiety via injected CRH) that don't immediately predict an aversive event are dependent upon the BNST.

Mechanisms of Fear Extinction

As previously discussed, the acquisition of fear requires the potentiation of glutamatergic synapses within the amygdala receiving CS and US information. Specifically, the activation of NMDA receptors within both the BLA and CEA is necessary for acquisition of fear. During extinction, repeated presentations of the CS in the absence of the US reduces conditioned responding to subsequent presentations of the CS. Interestingly, antagonism of NMDA receptors within the BLA, but not the CEA, block this phenomenon, suggesting that the acquisition of extinction and the acquisition of the original CS/US association formed during conditioning rely on discrete neural circuitry. Specifically, the BLA facilitates fear acquisition, but unlike the CEA is not required (Chapter 2). Alternatively, while the BLA is critical for extinction, the CEA is surprisingly only involved in the expression of fear and has no apparent role in the acquisition of extinction (Chapter 4).

While Chapter 4 in conjunction with previous work clearly demonstrates BLA involved in the acquisition of extinction (Falls et al., 1992; Lu et al., 2001; Lin et al., 2003; Quirk and Mueller, 2008), the mPFC is strongly implicated in the consolidation and expression of long-term extinction memories. Rats with lesions of the mPFC require significantly more extinction to abolish responses to the CS

(Morgan et al., 1993). Additionally, temporary inactivation, NMDA receptor antagonism, and protein synthesis inhibition of the mPFC during extinction leads to impaired extinction retrieval (Santini et al., 2004; Sierra-Mercado et al., 2006; Burgos-Robles et al., 2007). Electrophysiological correlates also suggest a role of the mPFC in extinction. The mPFC shows increased evoked responses to CSs following extinction and LTP correlated with the learning of extinction (Herry and Garcia, 2002; Milad and Quirk, 2002; Hugues and Garcia, 2007).

While both the mPFC and the BLA have demonstrated roles in the extinction of fear, how do these two structures interact to decrease activity amongst CEA output neurons following extinction? Interestingly, the mPFC provides direct projections to the inhibitory GABAergic neurons of the ITC (McDonald et al., 1996) and stimulation of the mPFC decreases the responsiveness of CEA output neurons (Quirk et al., 2003). Furthermore, recall that the ITC can gate neuronal activity between the BLA and the output of the amygdala, the CEA (Royer et al., 1999, 2000). Since extinction requires the inhibition of the original CS/US association, the GABAergic projections from the ITC to the CEA are prime candidates to provide such inhibition.

Recent work demonstrated that selective lesions of the ITC do indeed block the expression of extinction, providing for first time direct evidence for the involvement of the ITC in extinction (Likhtik et al., 2008). Until a very recent report by Amano and colleagues (2010) however, it was still unclear what role the interaction between the mPFC and BLA played in this process. In amygdala slices prepared from rats that were previously fear conditioned or rats that

received fear conditioning and extinction, the authors first demonstrated that extinction reduced the responsiveness of CEA neurons to BLA stimulation due to enhanced inhibitory post-synaptic currents (Amano et al., 2010). Next, while recording from ITC cells during the stimulation of the BLA in similar groups of rats Amano and colleagues (2010) discovered that ITC neurons were more responsive to BLA inputs in extinguished rats. Interestingly, this potentiation was at least partially dependent upon an increase of non-NMDA glutamate receptors at the synapse (e.g. AMPA receptors), suggesting a possible mechanism of action for intra-amygdala APV infusions. Finally, the authors report that temporary inactivation of the mPFC during extinction blocks the enhanced responsiveness of ITC neurons to BLA stimulation. Overall this study demonstrates that extinction results in the mPFC dependent potentiation of BLA to ITC neuronal synapses. Furthermore, this potentiation results in decreased neuronal responsiveness of CEA neurons to BLA stimulation Figure 5.2. The findings of Amano and colleagues (2010) taken together with the results discussed in Chapter 4, suggest that the NMDA dependent synaptic plasticity underlying the extinction of conditioned fear operates upstream of the CEA and provides a possible explanation as to why NMDA dependent plasticity within the CEA is not necessary for the acquisition of extinction.

While the findings in Chapter 4 demonstrate the critical nature of NMDA receptor activation in the BLA, but not the CEA, for the acquisition of extinction, the possibility remains that other forms of plasticity within the CEA may be important for the acquisition of extinction (i.e. NMDA independent plasticity) such

as voltage gated calcium channel (VGCC) dependent plasticity. In-vitro studies using an amygdala slice preparation have demonstrated a role for VGCC in both the BLA and CEA (Bauer et al., 2002; McKinney et al., 2009). Furthermore, VGCC have been suggested to play a role in both the consolidation (Bauer et al., 2002; McKinney and Murphy, 2006; McKinney et al., 2008) and extinction (Cain et al., 2002, 2005; Suzuki et al., 2004) of conditioned fear (but see McKinney and Murphy, 2006; McKinney et al., 2008). As such, the role that VGCCs within the CEA play in the extinction of conditioned fear warrants further investigation.

Future Directions

From the work presented in Chapters 2 and 3, we suggest that the associative plasticity required for the acquisition of conditioned fear resides between CEA neurons and their thalamic afferents (Figure 5.1), while plasticity within the BLA supports the rapid acquisition of fear (i.e. overtraining is not required) and persistence of the fear memory (Poulos et al., 2009). For this theory to be accurate, thalamic-BLA and thalamic-CEA synapses must be to support synaptic plasticity independently. Indeed, NMDA dependent LTP within the thalamic afferents to the BLA has been demonstrated (Chapman et al., 1990; Rogan and LeDoux, 1995; Huang and Kandel, 1998). Likewise, fear conditioning induces LTP like changes as thalamo-amygdaloid synapses within the LA (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997), suggesting plasticity between thalamic inputs onto BLA neurons does indeed contribute the acquisition of fear. Additionally, Samson and Pare (2005) have demonstrated

NMDA dependent LTP in the thalamo-CEA pathway as well. Importantly, they show that plasticity within this pathway is independent of input from the BLA (Samson and Pare, 2005), suggesting that the thalamo-CEA pathway is capable of supporting the associative plasticity necessary to acquire conditioned fear with or without input from the BLA. Furthermore, Fu and Shinnick-Gallagher (2005) have demonstrated LTP between the BLA and CEA, providing a possible pathway by which the thalamo-BLA plasticity could influence the induction of thalamo-CEA plasticity. While these studies provide strong support for our theory by demonstrating that plasticity within the necessary pathways within the amygdala is possible in response to thalamic input, the nature of the interaction between the BLA and CEA necessary to support the acquisition of conditioned fear with or without the BLA is not clear. For example, I hypothesize that activity and plasticity in the thalamo-BLA and BLA-CEA pathways help to facilitate plasticity in the thalamo-CEA pathway, thereby facilitating the rapid acquisition of fear. Understanding the important interactions between these parallel pathways requires further investigation.

In Chapter 4 we demonstrate that the CEA is not necessary for the acquisition of extinction. Specifically, we demonstrate that APV antagonism of NMDA receptors within the BLA blocks extinction but similar infusions into the CEA have no effect. Interestingly, Laurent and colleagues (2008) recently demonstrated that while inactivation of the BLA or antagonism of NMDA receptors within the BLA blocks the extinction of contextual fear, identical manipulations made within the BLA during the re-extinction of contextual fear

have no effect. These findings suggest a couple of interesting possibilities. First, that following the consolidation of the original extinction memory, the plasticity necessary to reacquire extinction is completely independent of the BLA. Alternatively, similar to the ability of the CEA to compensate for the loss of the BLA following overtraining (Chapter 2), the additional extinction session allows another structure to compensate for the absence of the BLA during re-extinction. Regardless of which hypothesis is correct, the mPFC and CEA are both strong candidates for structures that my mediate this interesting effect. However, as the study of reacquisition, and re-extinction especially, are in the very early stages of exploration, a significant amount of future work will be necessary to fully understand the distinct neural mechanisms of these advanced processes.

In conclusion, through the knowledge of the structures, circuitry, and molecular mechanisms necessary to support the acquisition, expression, and extinction of conditioned fear, we gain a better understanding of the mechanisms underlying emotional disorders and contribute to the future of their clinical treatment.

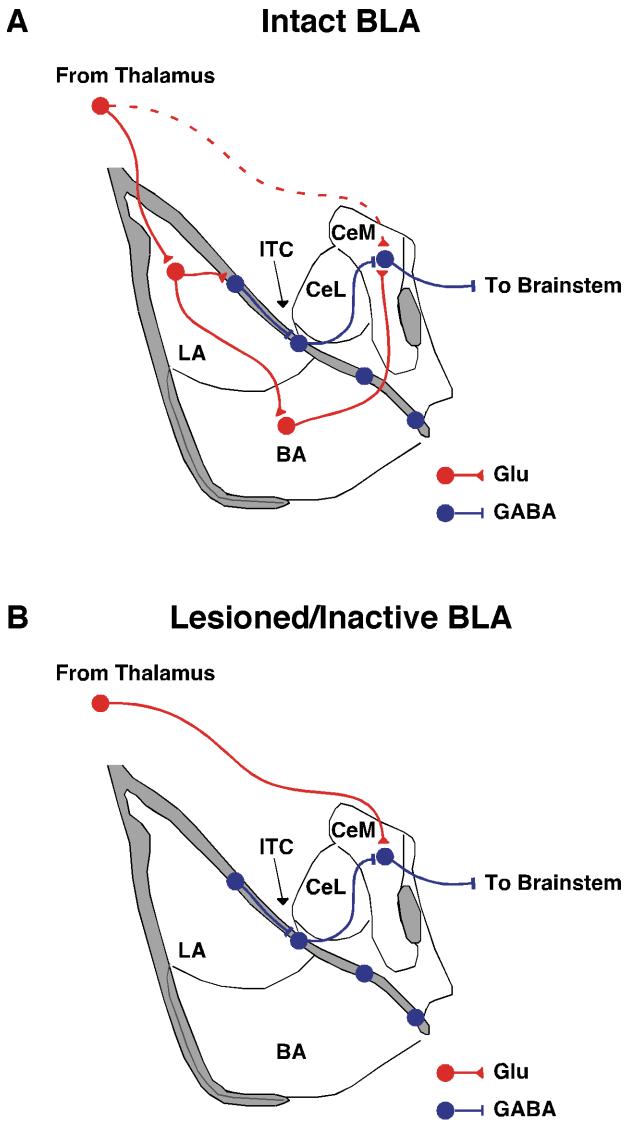

Figure 5.1. Intra-amygdaloid processing of fear.
A. With an intact BLA, according to the serial processing theory (solid lines) CS and US information in transmitted to the lateral amygdala (LA) from the thalamus. That information then excites central amygdala (CEA) neurons via excitatory connections through the basal amygdala (BA) or feed-forward inhibition through the intercalated cell mass (ITC). Alternatively, the parallel processing theory (dotted line) suggests that in addition to the connectivity discussed above, CS and US information is also directly received by the CEA. In both models the CEA acts as the primary output structure sending efferent connections to the brainstem nuclei required for the expression of CRs.
B. In the absence of the BLA, only the direct pathway from thalamus to the CEA remains intact and requires overtraining to support the acquisition of conditioned fear.

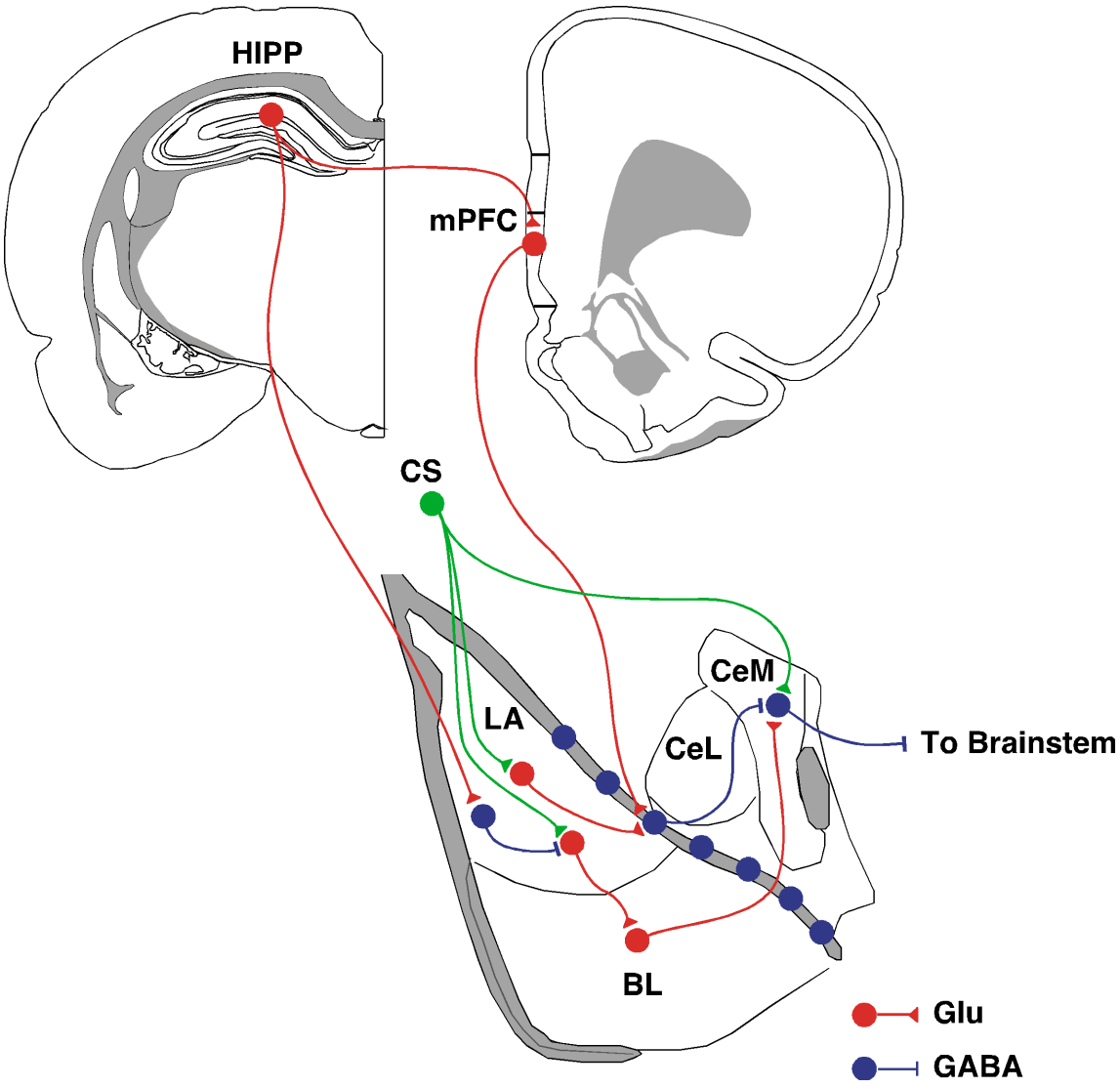

Figure 5.2. The neurobiology of Extinction
The acquisition of extinction requires the acquisition of a new context specific inhibitory memory capable of suppressing the neuronal activity necessary for the production of CRs. Current research suggests two possible intra-amygdaloid pathways capable of such inhibition, both under the contextual modulation of the hippocampus. The first pathway relies on inhibitory interneurons within the BLA to suppress glutamatergic input to the CEA. The second relies on the potentiation of synapses between LA glutamatergic neurons and GABAergic ITC cells facilitated by glutamatergic input from the mPFC.

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