To Fear or Not to Fear:

The role of the amygdala & prefrontal cortex in the regulation of fear.

By

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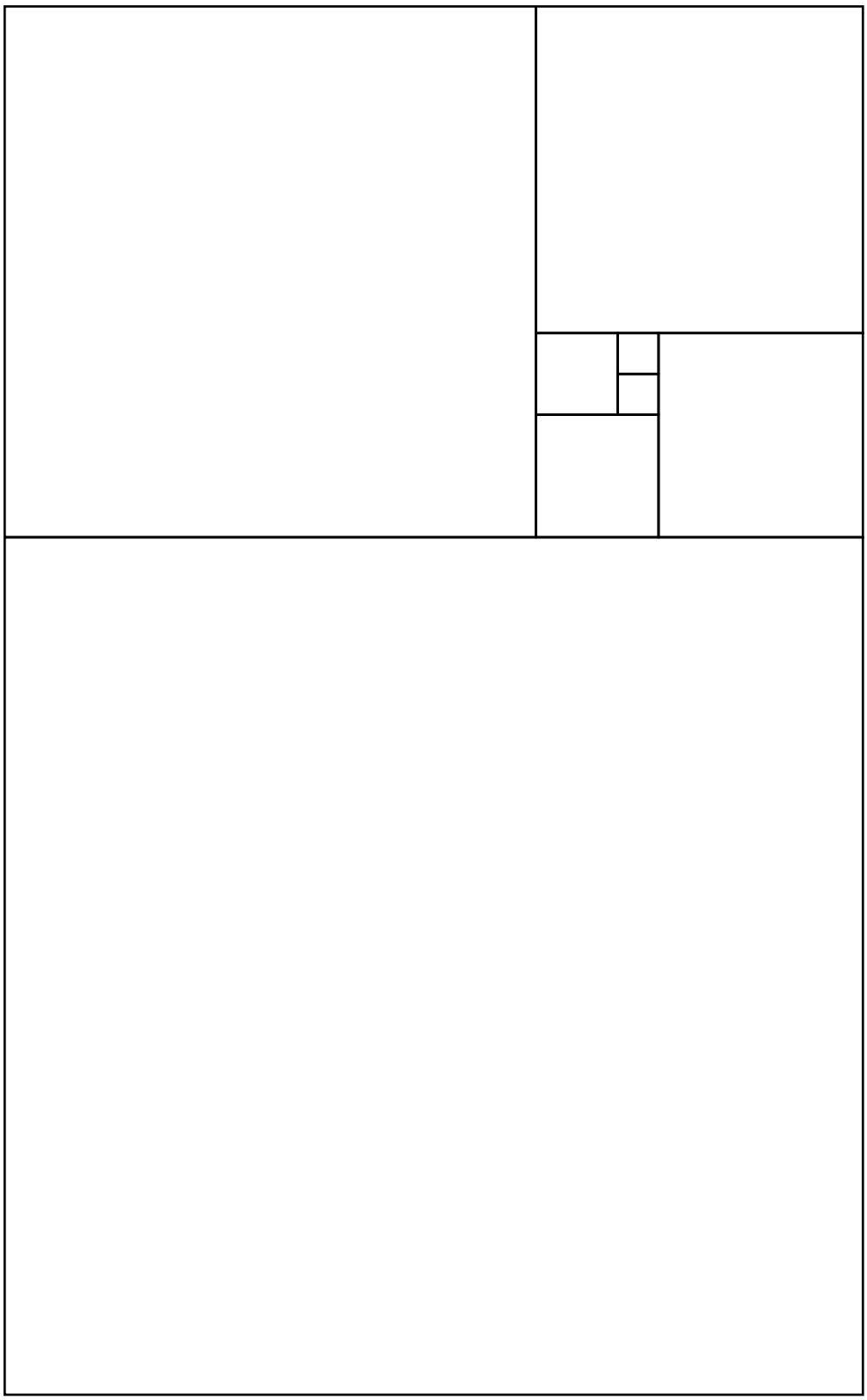

DEDICATION

To my family, for their continuous love and generosity

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Many students, faculty and staff at NYU contributed academically and technically to this thesis or supported me in other ways. In the LeDoux lab, Tad Blair, Marta Moita and Chris Repa helped get me started with unit recording. Yu Zhou built many of the electrodes used in this thesis. Claudia Farb ran a series of experiments to examine the distribution of cholecystokinin that did not make it into the final thesis. Peter Lau helped build some of the electronics that were used to run the experiments.

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Glimcher took special interest in my project and I would probably still be working on another analysis to perform on my data if not for his guidance. I'd like to thank Wendy Suzuki for helping me with the application for the Howard Hughes Medical Institute pre-doctoral fellowship, which funded my graduate education for five years, from 2000 to 2005. Jonathan Pillow and Brian Lau were extremely helpful as sounding boards for the logic and mathematics involved in the analyses of neural activity and behavior.

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PREFACE

The seeds of this dissertation were planted by the science fiction that I read in my childhood. Especially deserving of credit is Isaac Asimov and his positronic brain, which served as a context for the question of what it means to be human. Early biology lessons helped me realize that what differentiates man from mouse is almost entirely the brain. The rest of physiology, the heart, liver, kidney, etc. is fundamentally the same for all mammals. It is the brain and the mind it instantiates that makes us human. The wonder of the computational complexity of human cognition was illustrated beautifully in Douglas Hofstadter's Gödel, Escher, Bach: probably the singularly most influential book that I read before entering university. Armed with this fascination for the neurobiology of cognition, I set off to study computer science and neuroscience at McGill University. And it was there in a seminar on the neurobiology of memory that the seeds of this dissertation began to sprout.

My final paper for that course was on the topic of 'recovered memories'. It was (and remains) a controversial topic. After finding convincing evidence that indeed in extreme cases of trauma, memories could be repressed through dissociation and later recovered, I set out to develop a potential mechanism for this phenomenon. This was my first foray into the world of emotional modulation of memory. I was fascinated by the idea that a network of neurons could retrieve different memories

depending on the neuromodulatory state of the brain. I was sold on emotion. I decided to pursue a PhD in neuroscience, specifically studying emotion and memory. That is how I arrived in New York City, at the lab of Joseph LeDoux, ready to do science.

However, my plans to dive into research were hampered (thankfully) by course requirements. When I first realized the burden of the coursework I was frustrated. But this was quickly replaced with the joy of learning as I was exposed to the research and techniques of the world-class physiologists and theoreticians in the department. I was especially influenced by Michael Platt and Paul Glimcher's study of decision making and the parietal cortex. Analytically, I was impressed by the techniques used to extract the neural code in the early visual system, specifically spike-triggered averaging. Those ideas were the light and the water that helped the little seedling grow. While my botanical skills have been lax at times, I think that with the assistance of many gardeners the adult plant is healthy and ready to provide cuttings.

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1. General Introduction

1.1. Fear: a double edged sword

"The oldest and strongest emotion of mankind is fear"-- HP Lovecraft (Lovecraft, 1945).

H.P. Lovecraft, Edgar Allan Poe, Mary Shelley and Bram Stoker were visionaries who exploited and explored human fears. The ubiquity and longevity of horror myths, like Lovecraft's *Necromonicon*, Shelley's *Frankenstein* and Stoker's *Dracula*, demonstrate the power of fear in our minds. Why is fear so powerful?

"Fear is an emotion indispensable for survival," wrote Hannah Arendt, influenced by her experience as a Jewish philosopher who escaped from Nazi Germany. This sentiment may resonate with many of us on an intuitive level, but it echoes the evolutionary perspective of fear. According to Darwin's theory of natural selection, a trait persists because it provides an advantage in survival or reproduction (Darwin, 1859). What, in this view, is the evolutionary advantage of fear? For one thing, it provides motivation and resources to avoid injury and death. Without fear we fail to be cautious and consider the negative consequences of future actions. Unfortunately, the benefits of fear are obscured for people crippled by it. The double

edged sword of fear is that too little fear and too much fear are both life threatening (Nell, 2002; Tarrier and Gregg, 2004).

Phobias, social anxiety disorder and post-traumatic stress disorder (PTSD) can be thought of as pathologies of overwhelming fear: sufferers have exaggerated fear responses to everyday events. Social anxiety disorder is the third most prevalent psychiatric illness (after depression and alcoholism). It can interfere with the sufferer's ability to function in society, including difficulties with work. Considering the number of sufferers, this illness places a significant burden on all of society (Westenberg, 1998; Moutier and Stein, 1999; Pollack, 2001). PTSD is becoming a major health concern worldwide. This disorder results in significant occupational, psychiatric, and social disability, and its consequences are costly, not only to the survivors and their families, but also to the health care system and society (Brunello et al., 2001). The attack on the World Trade Center in New York City in 2001 ushered in an era of post-traumatic stress (LeDoux and Gorman, 2001; Silver et al., 2002), which has been perpetuated with the ongoing wars in Afghanistan and Iraq. Rates of post-traumatic stress disorder among veterans returning from Iraq may be as high as 17% (Hoge et al., 2004), more than twice the already high prevalence of PTSD in the general population (Kessler et al., 1995). While current therapies and drugs offer some respite (Brunello et al., 2001; Schoenfeld et al., 2004) there is an immediate need for a better understanding of the neurobiology of fear.

Fear can be generally classified as innate or learned. Innate fears are encoded into our genes. For example, a rat that has been born and raised in a lab will exhibit fear when presented with cat or fox odor (Wallace and Rosen, 2001). Being able to avoid cats and foxes is so important that detecting these smells became part of the genetic code of the rat. Learned fears are acquired through painful or traumatic life experiences. For example, being bitten by a dog during childhood can result in a lifelong fear of dogs (Terr, 1991). While it makes good sense to avoid the dog which attacked, hiding in the house to avoid all dogs is maladaptive. How does the child regulate the fear keeping him in the house, with his desire to go out and play with his friends? In order to transform this question into one more amenable to scientific inquiry we turn to fear conditioning, an animal model of learned fear.

1.2. Fear conditioning as an animal model

Much of what is known about the neurobiology of learned fear comes from Pavlovian fear conditioning (LeDoux, 2000; Maren, 2001; Barad, 2005; Phelps and LeDoux, 2005). During fear conditioning an initially neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US), so that the CS comes to predict the US. A rat exposed to a single pairing of a tone with a shock will show fear of the tone. In the lab, fear is defined behaviorally and physiologically. Behaviorally, there is a spectrum of defensive behaviors that are engaged when an animal is

threatened – from vigilance to suppression of appetitive behavior to freezing (Blanchard et al., 1989). Physiologically, fear is associated with activation of the autonomic nervous system: release of adrenaline, changes in heart rate, eyes widen, pupils open – the *fight-or-flight* response (Cannon, 1929; LeDoux, 1987). Thus, when applied to rats, the term *fear* does not necessarily refer to a feeling of being afraid, but instead refers to one or more behavioral or physiological responses mentioned above, which can be objectively measured.

In our lab, we focus on auditory fear conditioning. The CS is a tone or a series of pips and the US is an electrical shock. The general procedure of auditory fear conditioning consists of 3 sessions: habituation, acquisition and extinction (Quirk et al., 1995; Rogan et al., 1997; Maren, 1999). During *habituation* the CS is played a number of times with no reinforcement to eliminate the confounding effects of novelty (Acquas et al., 1996). During *acquisition* presentations of the CS are followed by a reinforcing US. The *extinction* session is the same as habituation: unreinforced presentations of the CS. If a rat exhibits more fear to the CS after acquisition we can infer that the CS-US association has been learned. After effective extinction training the CS no longer evokes fear behaviors. However, this is not due to the rat forgetting the CS-US association, but rather learning a new CS-'no US' association which regulates fear (Rescorla, 2001; Bouton, 2002).

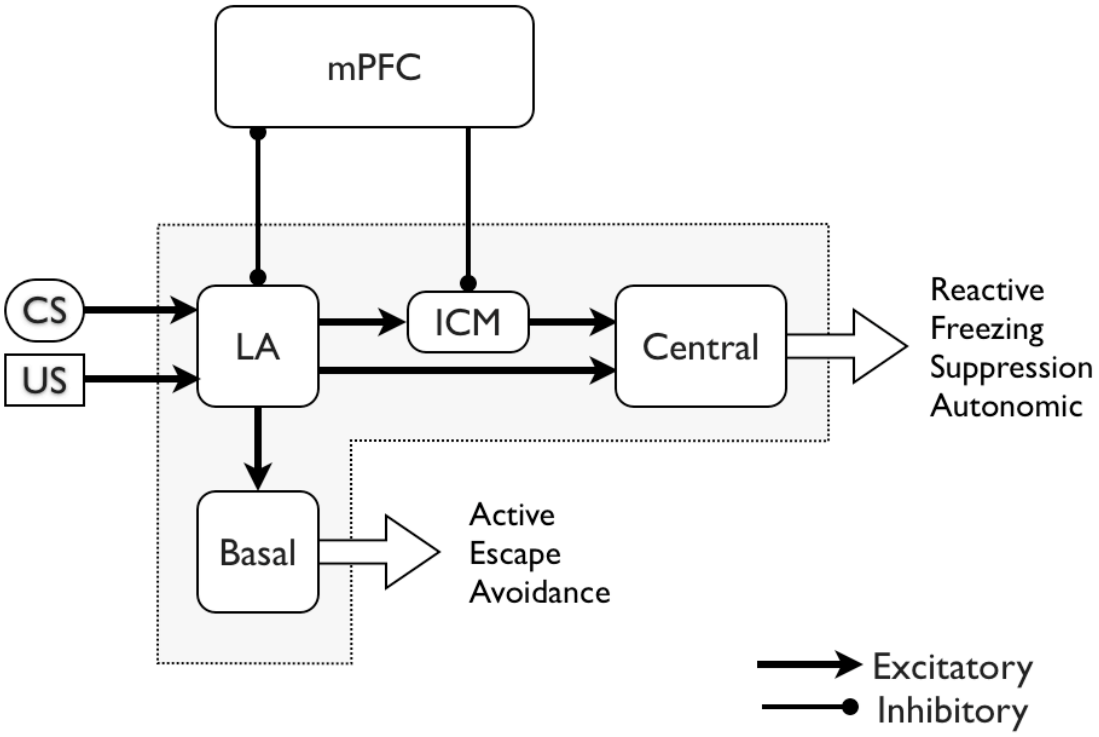


Figure 1.1 Fear conditioning circuitry.

In auditory fear conditioning, animals learn to fear an innocuous tone. By pairing a tone (conditioned stimulus, CS) and a shock (unconditioned stimulus, US), the tone acquires the capacity to elicit defensive reactions, such as freezing. Tone and shock stimuli converge in the lateral amygdala (LA), resulting in associative plasticity in the tone–LA pathway. Subsequent presentations of the tone can now activate LA neurons. The LA then communicates with the central nucleus (CE), which controls the expression of fear by way of connections to specific circuits that mediate freezing behavior. The LA connects with CE directly and by way of connections to other amygdala areas, including the intercalated cell masses (ICM), which gate the output, and the basal nucleus (B), which is important for active responses to fear. The medial prefrontal cortex (mPFC) can inhibit neurons in the LA directly and also gate the outputs of the LA via connections with the ICM. These projections seems to be the mechanism by which mPFC facilitates extinction.

1.2.1. Acquisition of fear

The amygdala is a key component in the neural circuit underlying fear conditioning (LeDoux, 2000; Maren and Quirk, 2004). The amygdala is made up of a dozen or so small nuclei (Pitkanen, 2000) but there are four that are important for auditory fear conditioning: the lateral, central, basal and intercalated nuclei(LeDoux, 2000; Pare et al., 2004) (Figure 1.1). Each of these nuclei performs a distinct function in fear conditioning. The lateral nucleus (LA) is the putative site of learning, where the CS and US input converge to form the CS-US association (Blair et al., 2001; Maren and Quirk; Rodrigues et al., 2004a). The central nucleus is necessary for automatic reactions to fear, like freezing (Killcross et al.; Amorapanth et al.; Nader et al., 2001). The intercalated cells are a gateway between the LA and central, and may be a key component in the regulation of fear (Quirk et al., 2003; Pare et al., 2004). The basal nucleus is necessary for active responses to a CS, like escape (Killcross et al., 1997; Amorapanth et al., 2000).

Before acquisition, the synapses between the auditory inputs which carry the information about the CS and the cells in the LA are weak. Thus, the CS does not drive the neurons in the LA to fire action potentials and does not illicit fear (Rogan et al., 1997; Repa et al., 2001). In contrast, the US, as an innately aversive stimulus, powerfully drives neurons in the LA (Rosenkranz and Grace, 2002b). Neurons in the LA act as coincidence detectors. When a neutral CS is presented with a US, the

auditory inputs that carry the CS are strengthened. This is referred to as Hebbian plasticity (Hebb, 1949; Blair et al., 2001).

The molecular mechanism of this Hebbian plasticity involves the influx of calcium into the post-synaptic cell via NMDA receptors (Maren et al., 1996b; Rodrigues et al., 2001) and L-type voltage gated calcium channels (Bauer et al., 2002). This calcium triggers downstream cascades of kinases (Schafe et al., 2000; Rodrigues et al., 2004b). These cascades results in rapid changes in cytoskeletal molecules (Lamprecht et al., 2002) and also trigger new protein synthesis (Schafe and LeDoux, 2000) which is essential for the plasticity to be long lasting. (For review, see Schafe et al., 2001; Lamprecht and LeDoux, 2004; Rodrigues et al., 2004a) . The end result of all these molecular changes is that the CS now can drive the cells in the LA to fire action potentials, or *spikes*.

These spikes in the LA affect behavior via the efferents from the LA to the central and basal nuclei (Stefanacci et al., 1992; Savander et al., 1997). The outputs of the central nucleus project to the periaqueductal gray to elicit freezing behavior (Oca et al., 1998; Amorapanth et al., 1999) and the hypothalamus to engage the autonomic nervous system (LeDoux et al., 1988). Lesions of the central nucleus block conditioned suppression as well (Killcross et al., 1997). In contrast, the basal nucleus is not required for strictly Pavlovian responses to an auditory CS (Nader et al., 2001). But it is important for fear conditioning when the CS or the outputs are more complex (Yaniv et al., 2004). For example, the basal nucleus is important for contextual fear

conditioning (Goosens and Maren, 2001) and for active responses to a CS, like escape (Amorapanth et al., 2000) or avoidance (Killcross et al., 1997).

Thus, there is a dichotomy between the behaviors elicited by the central and basal amygdala. If a rat is freezing it is not escaping, and intense fear interferes with an animals ability to learn how to escape (Grahn et al., 2002). But once an animal learns to how to use the CS to avoid danger (if it can) the CS no longer elicits automatic defense behaviors, including the autonomic responses associated with fear (Roozendaal et al., 1992). This is an important clinical concept. Perhaps people with PTSD, who are paralyzed by fear, can learn active coping strategies which will reduce the dominance of the LA to central amygdala pathway and enhance the LA to basal amygdala pathway (LeDoux and Gorman, 2001).

Extinction of fear

Extinction of the CS-US association is another way to reduce the paralyzing effects of fear. The neurobiology of extinction is poorly understood, compared to the neurobiology of acquisition. However, the clinical relevance of extinction has resulted in a recent surge in interest (Davis et al., 2006). The prevailing theory is that extinction involves the interaction of the medial prefrontal cortex (mPFC) and the amygdala (Morgan et al., 1993; Morgan and LeDoux, 1995; Sotres-Bayon et al., 2004b; Barad, 2005; Phelps and LeDoux, 2005).

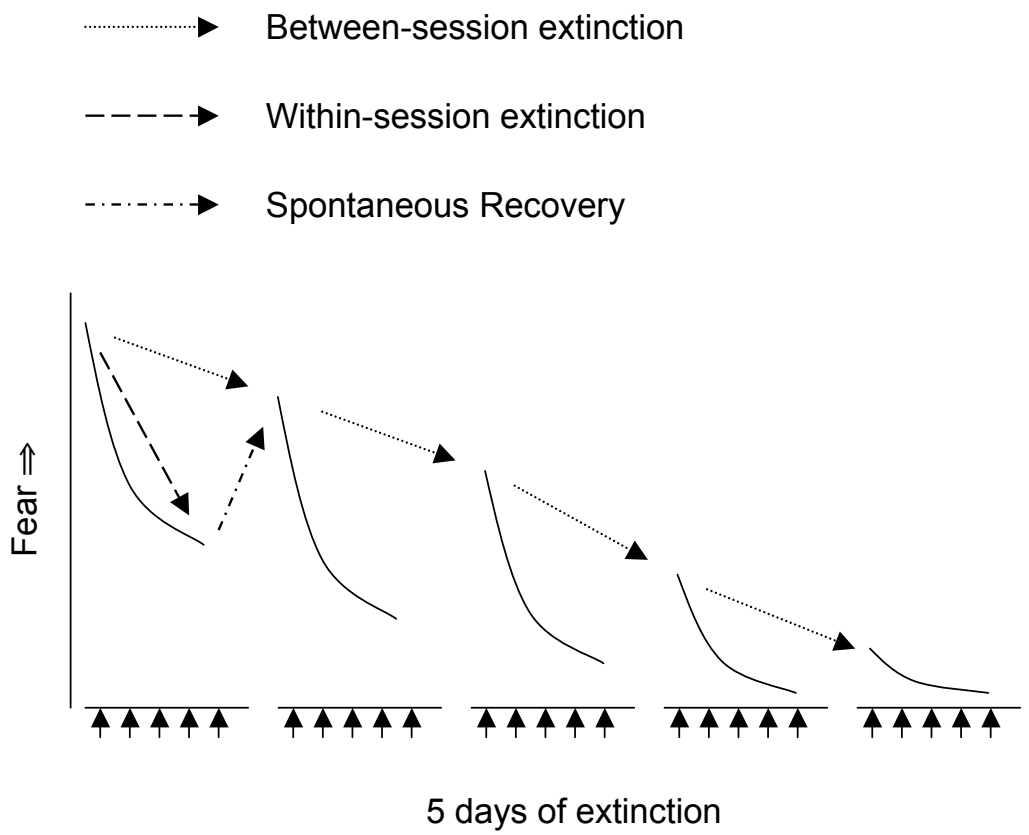


Figure 1.2 Extinction and Spontaneous Recovery.

Each arrowhead on the x-axis is a presentation of a CS. There are five presentations per day. On the first day, the CS evokes a strong behavioral response which diminishes over the course of the five presentations (within-session extinction). The following day the first CS presentation evokes more fear than the last CS presentation on day 1 (spontaneous recovery). Over days the fear evoked by the first presentation each day diminishes (between-session extinction).

When a CS is presented repeatedly without reinforcement, an animal learns that the CS no longer predicts the US and it loses its power to elicit fear. However, when an extinguished CS is presented at a later time it can spontaneously recover its ability to elicit fear (Fig. 1.2). This indicates that *within-session* extinction does not erase the memory of acquisition, but instead is a process of regulation of fear (Bouton, 2004). Further sessions of extinction can decrease the amount of spontaneous recovery (Denniston et al., 2003). Thus, there are two distinct processes in extinction: *within-session* and *between-session* extinction.

Within-session extinction begins with the first presentation of the CS in the absence of the US. At the beginning of extinction there is a subset of cells in the LA that respond vigorously to the CS (Repa et al., 2001). This burst of activity may trigger an enhancement of local inhibitory networks (Akirav et al., 2006) for an immediate reduction in CS-elicited fear. Across-session extinction involves many of the same amygdala mechanisms that underlie fear acquisition. Using the fear-potentiated startle paradigm, Davis and colleagues have demonstrated that blockade of NMDA receptors (Falls et al., 1992) or MAP kinase (Lu et al., 2001) in the amygdala block across-session extinction. As well, D-cycloserine, an agonist of the NMDA receptor, facilitates across-session extinction when infused systemically or directly into the amygdala (Walker et al., 2002).

The mPFC seems to be important for consolidating within-session extinction into between-session extinction. Lesions of the mPFC attenuate between-session

extinction but not within-session extinction (Quirk et al., 2000; Lebron et al., 2004). Using single-unit recording Milad and Quirk (2002) observed that neurons in the mPFC signal the degree of between-session extinction. Then, they demonstrated a causal link between this activity and extinction by facilitating extinction through stimulation of mPFC (Milad and Quirk, 2002; Milad et al., 2004). This effect is likely mediated by strong reciprocal connections with the amygdala (Sesack et al., 1989; Conde et al., 1995; McDonald et al., 1996; Pitkanen, 2000) since stimulation of mPFC inhibits neurons in the LA (Rosenkranz and Grace, 2002b, 2002a) and decreases the responsiveness of neuron in the central amygdala which project to the brainstem areas which control freezing (Quirk et al., 2003).

There are several other molecular mechanism involved in extinction that have not been pinpointed to a specific brain region. First, systemic injections of ifenprodil, which blocks calcium permeable NMDA receptors, attenuates within-session extinction (Sotres-Bayon et al., 2004a). Systemic injections and intra-amygdala infusions of ifenprodil block acquisition of fear conditioning as well (Rodrigues et al., 2001). Second, the endogenous cannibinoid system has been implicated in extinction via genetic knockouts (Marsicano et al., 2002) and pharmacological manipulation (Chhatwal et al., 2005). Animals lacking the type 1 cannabinoid receptor exhibited deficits in both within-session and between session extinction (Marsicano et al., 2002). Systemic injections of an antagonist of the type 1 cannibinoid receptor also attenuated

extinction. Likewise, pharmacologically inhibiting the degradation of endogenous cannibinoids enhanced extinction learning (Chhatwal et al., 2005).

1.2.2. From animals to humans

Can the knowledge that we learn from fear conditioning in rats be applied to humans? The data from humans with brain lesions and from neuroimaging show an amazing correspondence to what has been observed in rodent and primate (Phelps and LeDoux, 2005). For example, patients with amygdala damage show deficits in fear conditioning (LaBar et al., 1995). Functional magnetic resonance imaging (fMRI) investigations of fear conditioning in humans found that amygdala activity correlated with the fear response in acquisition and extinction (LaBar et al., 1998; Phelps et al., 2004) and that mPFC activity was negatively correlated with fear, especially during the recall of extinction (Phelps et al., 2004). This corresponds well with recordings from the LA (Rogan et al., 1997; Repa et al., 2001; Maren and Quirk, 2004) and from mPFC (Garcia et al., 1999; Milad and Quirk, 2002) during fear conditioning in rodents.

This same LA-mPFC circuit also seems to be altered in PTSD, further supporting fear conditioning as an animal model of clinical fear disorders. Similar to fear conditioning, symptom provocation in Vietnam veterans resulted in increases in amygdala activity and correlated decreases in mPFC activity, measured as cerebral

blood flow using positron emission tomography. Furthermore, symptom severity was positively related to amygdala activity and negatively related mPFC activity. Similar results have been found using symptom provocation in non-combat PTSD patients (Gilboa et al., 2004), in the neural response of PTSD patients to fearful faces (Shin et al., 2005) and to traumatic images (Protopopescu et al., 2005). These results suggest the existence of a reciprocal relationship between activity in the LA and mPFC in PTSD patients much like that observed in human and rodent fear conditioning.

1.3. Fear regulation

The previous section laid out the neural mechanisms of fear conditioning. I described how the pairing of a CS and US results in changes in the amygdala which underlie the new emotional power of the CS. I also described how the CS can lose its emotional significance through extinction, mediated by a combination of within-session inhibition and between-session plasticity in the amygdala and the mPFC. However, both of these process, acquisition and extinction, are driven by changes in the CS-US contingency. In other words, they reflect mechanisms by which we adapt to changes in our environment. But it is also important to understand how we regulate fear elicited by familiar dangers, like the neighbor's vicious dog.

Armed with the neurobiology of fear conditioning we can re-approach the question "How does the child regulate the fear keeping him in the house, so he can go

out and play with his friends?" I explained how Hebbian plasticity in the LA associates the dog with the pain of the bite. But does the LA play a continued role in the fear of dogs long after the initial attack? There are several lines of evidence that indicate it does. First, amygdala activity increases during symptom provocation in PTSD patients, long after the initial trauma (Gilboa et al., 2004). Second, people with amygdala damage have emotional deficits not related to learning, such as judging whether a stranger is trustworthy (Adolphs et al., 1998). Third, in rats, lesions of the LA four weeks after acquisition block the expression of fear elicited by the CS (Maren et al., 1996a). Together, these data suggest that the role of the LA is to detect danger and that learning about new dangers is just a tool for performing this task (LeDoux, 2001). Moreover, unlike the hippocampus, which is required for learning new episodic memories but not for retrieval of these memories weeks later (Kim and Fanselow, 1992; Squire et al., 2004), the amygdala seems to be required for emotional learning and retrieval of emotional memories long after initial learning.

Does the mPFC play a role in inhibiting fear to a familiar danger? Lesions of mPFC do not disrupt within-session extinction. This suggests that it is specifically involved in adapting to the new CS-'no US' contingency. However, there is direct evidence from humans and indirect evidence from animals that suggest it plays a role in regulation of fear. First, in symptom provocation in PTSD patients activity in the mPFC significantly decreases (Gilboa et al., 2004). Second, in studies of cognitive regulation of emotion, where subjects were told to reappraise negative images in

unemotional terms, mPFC activity is linked to the degree of regulation (Ochsner et al., 2002; Ochsner et al., 2004), although this is not precisely the same region of mPFC involved in extinction (Phelps et al., 2004; Milad et al., 2005). Third, in rats, unconditioned fear elicited by fox odor increases dopamine turnover in mPFC (Morrow et al., 2002). Together, these data provide incentive to further investigate the role of the mPFC in regulation of fear elicited by a familiar CS.

1.4. Experimental Approach

The strength of fear conditioning as an experimental approach lies in the ability to compare neural changes with the changes in fear due to acquisition and extinction. To study fear regulation I required a task that would also result in changes in fear, but not due to external changes. Rather, the changes would be evidence of an internal process of fear regulation. To this end, I modified the traditional fear conditioning task, so that a CS would only elicit a fear response about half the time. As well, to observe the onset and offset of fear during a single CS a precise measure of fear was required. Common measures of fear in rats, such as freezing and bar-press suppression are adequate for comparing behavior across trials but are not precise enough to measure changes in fear within a trial. When rats drink, they lick six to eight times per second. Thus, the suppression of licking can be timed with an

accuracy of about 150 milliseconds. Moreover, a sweet drink provides a background of appetitive motivation against which fear can be reliably measured.

The task was as follows. I placed rats in a chamber where they had access to a sweet drink (Orange Kool-Aid). After each rat consistently drank the Kool-Aid, an auditory CS was presented that was partially reinforced (PR) with a mildly aversive US. Partial reinforcement means that only some presentations of the CS are paired with the US. In pilot experiments I adjusted the strength of the US and the CS-US contingency so that the CS would elicit intermediate levels of lick suppression. Specifically, the CS generated enough fear so that the time spent drinking Kool-Aid was about equivalent to the time spent suppressing. Moreover, during the course of a single CS a rat would switch back and forth between drinking and suppression. Therefore, like a typical fear conditioning task I could compare the neural activity when the CS elicited fear to when it failed to elicit fear.

1.4.1. Experiment 1

Hypothesis: The lateral amygdala is essential for processing a fear conditioned stimulus even after it is well-learned and when the CS-US contingency is probabilistic.

Background: The evidence supporting the role of the LA in fear learning is overwhelming, but the evidence for its role in regulation of fear is weaker. In order to study the regulation of fear we modified the typical fear conditioning experiment in

three ways. First, I used a familiar CS. Second, I used a probabilistic CS so that the CS would elicit intermediate levels of fear. Third, I used lick suppression to provide a temporally precise measure of fear.

Approach: To test the hypothesis, I used a within subject design. Animals were well-trained on the task so that they knew the CS-US contingency. Then I tested the effect of local infusions of muscimol and pentagastrin into the LA on CS-evoked fear. Muscimol is a GABA-A agonist which temporarily inactivates neurons. Pentagastrin is an agonist of the cholecystochinin-B receptor. It has been shown to increase the excitability of neurons. By using both an inhibitory and excitatory compound we aimed to demonstrate that bidirectional modulation of neural activity in the LA results in a bidirectional modulation in fear behavior.

1.4.2. Experiment 2

Hypothesis: Single-unit activity in the lateral amygdala predicts moment-to-moment changes in fear behavior. Specifically, that activity in the lateral amygdala would increase before the onset of fear.

Background: The prevailing hypothesis regarding single-unit activity in the LA is that the spike rate of neurons in the LA encodes the strength of the CS-US relationship. However, there is no data regarding moment-to-moment correlations between fear behavior and unit activity in LA.

Approach: I implanted electrodes into the LA to record single-unit activity from behaving rats. Rats had free access to Kool-Aid and the CS-evoked suppression of drinking Kool-Aid was used as an index into the fear of the animal. Since rats licks at a rate of 6-8Hz, the time of the onset and offset of suppression could be measured with millisecond accuracy.

1.4.3. Experiment 3

Hypothesis: Single-unit activity in the medial prefrontal cortex predicts moment-to-moment changes in fear behavior. Specifically, that activity in the medial prefrontal cortex would increase before the offset of fear.

Background: Evidence is mounting that the mPFC plays a contrasting role to the LA in fear. However, there is little data from behaving rats regarding the role of neurons in mPFC in fear conditioning. My hypothesis is based on lesion data and human imaging data as well as a single study of single-unit activity that reported correlation between mPFC firing and the recall of extinction.

Approach: I implanted electrodes into the mPFC to record single-unit activity from behaving rats. Rats had free access to Kool-Aid and the CS-evoked suppression of drinking Kool-Aid was used as an index into the fear of the animal. Since rats licks at a rate of 6-8Hz, the time of the onset and offset of suppression could be measured with millisecond accuracy.

2. Conditioned suppression is attenuated by muscimol and potentiated by pentagastrin infusions into the lateral amygdala

2.1. Abstract

The lateral nucleus of the amygdala is the putative neural substrate for Pavlovian fear conditioning, an animal model of fear and anxiety. Most studies have focused on the role of the amygdala in learning the association between a neutral conditioned stimulus (e.g. a tone) and an aversive unconditioned stimulus (e.g. a shock). However, patients with fear disorders seek treatment well after the initial learning. Here we demonstrate the continued importance of the lateral amygdala in processing an auditory fear conditioned stimulus well after (1-5 weeks) initial learning by infusing drugs directly into the lateral amygdala. Muscimol, which blocks neural activity, attenuated conditioned stimulus-evoked fear behavior. Pentagastrin, which potentiates neural activity, resulted in persistence of conditioned stimulus-evoked fear behavior.

2.2. Introduction

The lateral amygdala is the putative neural substrate of Pavlovian fear conditioning–the pairing of a neutral conditioned stimulus (CS) with an aversive

unconditioned stimulus (US) so that the CS comes to elicit behaviors originally elicited by the US (LeDoux, 2000; Maren and Quirk, 2004). Great progress has been made in understanding the molecular (Rodrigues et al., 2004) and electrophysiological (Maren and Quirk, 2004) mechanisms of the synaptic plasticity required for learning the CS-US association. However, processing of previously learned threats is also important to understand, especially in a clinical context—patients suffering from anxiety or post-traumatic stress disorders typically seek help for problems stemming from previously learned associations rather than help in the prevention of new fear learning.

Here we ask about the role of LA in lick suppression in response to a well-learned partially reinforced fear conditioned auditory CS. We used a partial-reinforcement procedure (PR) to produce intermediate levels of fear. In a second study we used to the same task during single-unit recording of LA neurons (Erlich et al., 2002). Intermediate levels of fear allow us to observe the neural activity during both the engagement and disengagement of lick suppression.

Relatively few studies have examined the role of the LA in the generation of fear behaviors in response to a well-learned CS. Lesions of the LA after extensive overtraining in a single session produce similar deficits in the expression of contextual fear conditioning as pre-training lesions (Maren, 1998). As well, lesions made long after training (28 days) produce similar deficits to lesions made immediately after training in both fear conditioning (Maren et al., 1996) and fear potentiated startle (Lee

et al., 1996). In contrast, while muscimol inactivations of the LA disrupt initial learning of avoidance behavior, they do not disrupt its expression once it is well-learned (Poremba and Gabriel, 1999). The inconsistency between the effect of muscimol on expression of avoidance and the effect of lesions on fear conditioning is likely due to differences in the neural substrates of Pavlovian and instrumental behaviors. However, it could also be that the amygdala lesions disrupted fibers of passage. Indeed, there is evidence that even excitotoxic lesions can damage fibers of passage (c.f. Koo et al., 2004). Thus, there remains some doubt about the role of the LA in expression of well-learned fear conditioning.

Our study is the first to examine the effect of pharmacological manipulations of the LA on the expression of CS-evoked suppression after the CS-US association is well-learned. To demonstrate the role of the LA in this task we did two manipulations. Muscimol was infused to inactivate neural activity in the LA and pentagastrin was used to potentiate activity in LA. Muscimol binds to GABA-A receptors, which results in the hyperpolarization of neurons. Pentagastrin binds to the cholecystokinin-B (CCK_B) receptor, a g-protein linked receptor, which causes release of calcium from intracellular stores via the phospholipase C and A_2 second messenger systems (Pommier et al., 1999) resulting in an increase in the excitability of neurons (Boden and Woodruff, 1994). Intravenous pentagastrin causes panic attacks in humans (Geraci et al., 2002) and local infusion into the LA of rats potentiates acoustic startle (Frankland et al., 1997).

We find that local infusion of muscimol into the LA attenuates CS-evoked lick suppression and local infusion of pentagastrin results in the persistence of suppression after the termination of the CS. Thus, inactivation of neural activity via GABA attenuates, and potentiation via CCK_B increases, fear behavior in response to a partially-reinforced well-learned auditory CS, demonstrating that the LA continues to play a central role in processing an aversive stimulus even after it is well-learned. This lends support for further studies of amygdala function using this task as an animal model to better understand post-traumatic stress and anxiety disorders.

2.3. Methods

Subjects. Subjects were adult male Sprague Dawley rats (Hilltop, Scottdale, PA) weighing 350-400 g. They were housed individually in plastic Nalgene cages and maintained on a 12 hr light/dark cycle. Water was provided freely throughout the experiment. After recovery from surgery (see below) they were maintained on a restricted diet until they reached 90% of their original body weight. All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University. Ten rats underwent surgery. Two animals never met criteria for pre-habituation (described below) and one animal was excluded due to an erroneous cannula placement.

Surgery. Under ketamine (100 mg/kg, i.p.), xylazine (6.0 mg/kg, i.p.), and medetomidine (0.5 mg/kg, i.p.). anesthesia, rats were implanted bilaterally with 22-gauge stainless steel cannulas that terminated in the LA. Coordinates, taken from Paxinos and Watson (1986), were +5.7 anteroposterior, ±5.3 mediolateral, and +2.0 mm dorsoventral with reference to inter-aural zero. The cannulas were anchored to the skull with dental cement. A 28-gauge dummy cannula was inserted into each cannula to prevent clogging, and dummy cannulas were changed once per week. After surgery, rats were given buprenorphine HCl (0.2 mg/kg, s.c.) as an analgesic. Rats were given at least 5 d to recover.

Drugs. The vehicle (VEH) was 50mm sodium bicarbonate in physiological saline (0.9% NaCl). The pentagastrin (PENT) infusion was a 0.5 nmoles of pentagastrin in 0.25 μL of vehicle. The muscimol (MUSC) infusion was 1.1 nmoles of muscimol in 0.25 μL of physiological saline (4.4 mM) .

Behavior. All behavior took place in a Plexiglas chamber with dimensions 23 cm x 28 cm x 34 cm (Med-Associates, Inc.) with a recessed lick spout enclosed in a sound and light attenuating chamber. The CS was an auditory stimulus made up of 20 pips. The pip frequency was 12KHz and duration was 250ms. The interval between pip onsets was 1 second. Thus, the total duration of the CS was 20 seconds. The US was a brief footshock (0.5 mA, 200 ms) delivered 20 seconds after the onset of the CS. Fear behavior was measured as suppression ratio.

$$SuppressionRatio = \frac{(L_{PRE} - L_{CS})}{(L_{PRE} + L_{CS})}$$

Where L_{PRE} is the number of licks in the 20 sec pre-CS period and L_{CS} is the number of licks in the 20 sec CS period. When there is no CS-evoked suppression the suppression ratio is zero. When there is CS-evoked suppression the suppression ratio is positive. If the CS evoked facilitation the suppression ratio would be negative.

After recovery from surgery rats were pre-habituated to the behavioral chamber where they were trained to lick for a sugary orange drink (Kool-Aid®, Kraft Foods) to maintain a constant level of activity against which suppression could be reliably measured. Once the rats licked consistently the behavioral training began (Figure 2.1). The experiment lasted five weeks. The first week consisted of one habituation (HAB) session and a partial reinforcement session (PR 0) the following day. The first day of training (HAB) rats were placed in the chamber for a 20 trial session. Each trial was a presentation of the CS. The next day rats returned to the chamber for a 20 trial session (PR-0) with a 30% CS-US contingency. In the following weeks each animal had one PR session per day for four days (Monday-Thursday, PR 1-4. See Table 2.1 for exact reinforcement history). The first 3 of these days were maintenance days with no infusions and on the fourth day (of the 3rd, 4th, and 5th week) each animal received an intra-LA infusion of either vehicle, pentagastrin, or muscimol, counterbalanced across weeks. For example, one rat

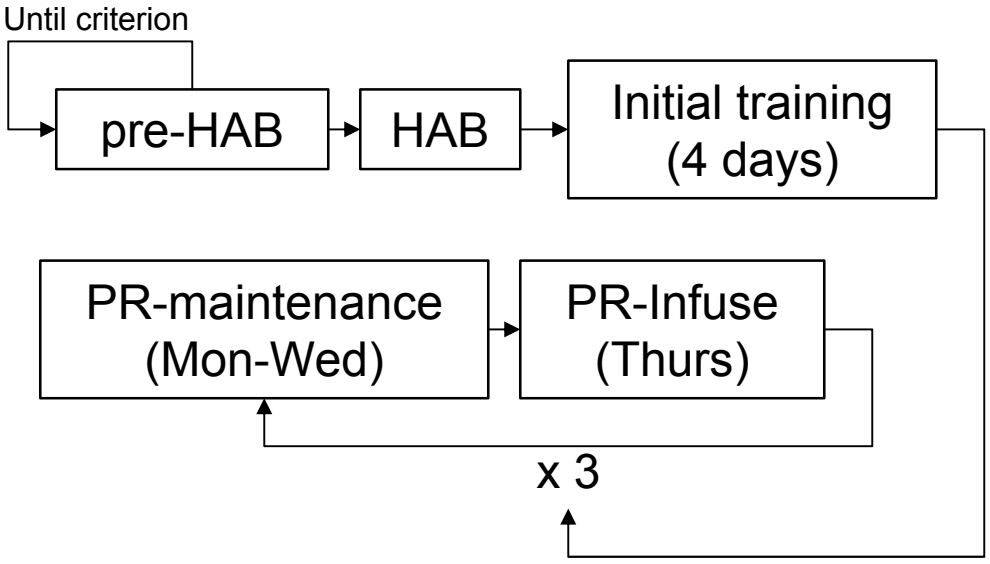


Figure 2.1. Schematic of behavioral protocol

Animals are pre-habituated (pre-HAB) to the behavioral apparatus and to the Kool-Aid. This continues until they reach a criterion of licking consistently for about an hour. Habituation (HAB) consists of 20 presentations of the CS with no reinforcement. The next day training begins. Each training session consists of 20 presentation of the CS with a 30% probability of the CS being followed by a footshock. (See methods for details)

received VEH, PENT, and MUSC and a second rat received PENT, MUSC, and VEH in the 3rd, 4th and 5th week respectively. Thus, over the course of the experiment each rat received infusions of all three drugs (Figure 2.1). Since there was no difference between maintenance days between drugs (Main Effects ANOVA; DRUG F_2=0.094, p>.05; DAY F_2=2.84, p>.05) we present the effect of drug and vehicle on suppression ratio without adjusting for the activity during maintenance days (Figure 2.3a).

Intracranial infusions. Rats were held in the experimenter's lap while dummy cannulas were replaced with 28 gauge infusion cannulas attached to 1.0 µl Hamilton (Reno, NV) syringes via polyurethane tubing. The tubing was back-filled with sesame oil, with a small air bubble separating the oil from the drug. Drugs were infused bilaterally by an infusion pump, and cannulas were left in place for an additional 2 min after infusion to allow diffusion of the drug away from the cannula tip before dummy cannula replacement. A total amount of 0.25 µl of drug or vehicle was infused into the LA bilaterally over 105 sec. This volume was chosen on the basis of autoradiographic studies of the spread of muscimol applied to the size and structure of the target (Martin et al., 1991). Although the LA was the main target, the infusions also likely affected the adjacent basal nucleus. Infusions preceded the behavioral session by 15 minutes.

Data Analysis. All statistical analyses were performed using Statistica 7.0 (Statsoft; Tulsa, OK). For all analyses, trials were excluded if the rat did not lick

during the pre-CS period for that trial. For all analyses, trials for each rat were averaged and effects were tested using a repeated measures design with each rat as a case and the drug as the factor. For the analysis of CS versus CS-US suppression one animal was excluded since there were no valid CS-US MUSC trials. For the analysis of persistence of suppression two animals were excluded because they did not lick during the pre-CS period on the CS-US trials.

Histology. To verify infusion cannula tip locations, rats were anesthetized with an overdose of chloral hydrate (600 mg/kg, i.p.) and perfused transcardially with 10% buffered formalin. The brains were post-fixed in 30% sucrose in formalin and subsequently blocked, sectioned on a cryostat at 50 μm, and stained for Nissl using 0.5% cresyl violet. Sections were coverslipped with Permount and examined under light microscopy for tip penetration into the amygdala (Figure 2.5a,b). The mean placement in mm with respect to inter-aural zero was (mean ± standard error) : AP, -5.59±0.04; ML, 5.19±0.09; DV, 2.17±0.10 (Paxinos & Watson, 2004).

2.4. Results

Consistent with lesions of the LA, muscimol infusions into the LA resulted in a profound attenuation of CS-evoked lick suppression (Repeated Measures ANOVA, $F_{(2,12)}=7.91$, $p<0.01$). In six out of seven rats lick suppression during the muscimol session was lower than both vehicle and pentagastrin suppression levels. Additionally,

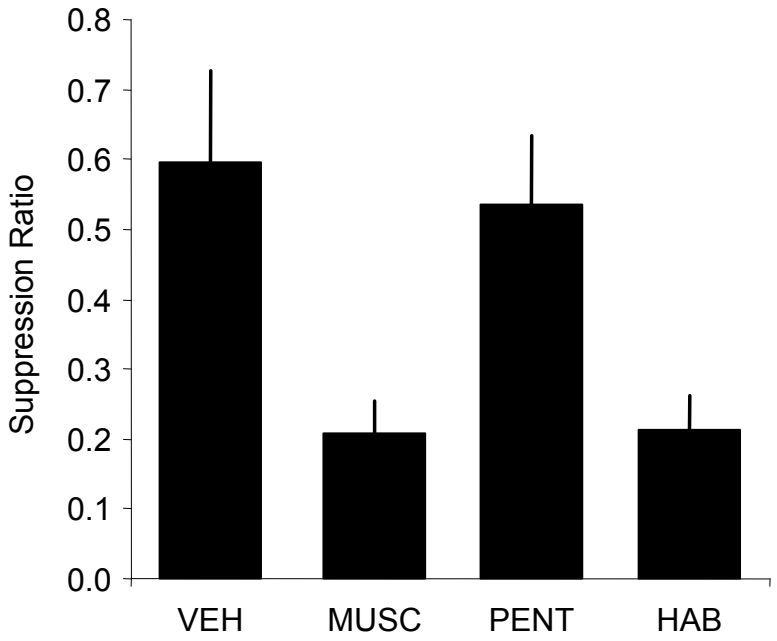


Figure 2.2. Effect of infusions into the LA on Suppression Ratio.

Mean ± SE CS-evoked suppression ratio in rats given 0.25 μL intra-LA infusions of 50 mm sodium bicarbonate in 0.9% NaCl (VEH), 1.1 nmoles of muscimol (MUSC), or 0.5 nmoles of pentagastrin (PENT). For comparison the suppression ratio from the habituation session is shown (HAB). Using a counter-balanced within-subject design all rats received all infusions (n = 7).

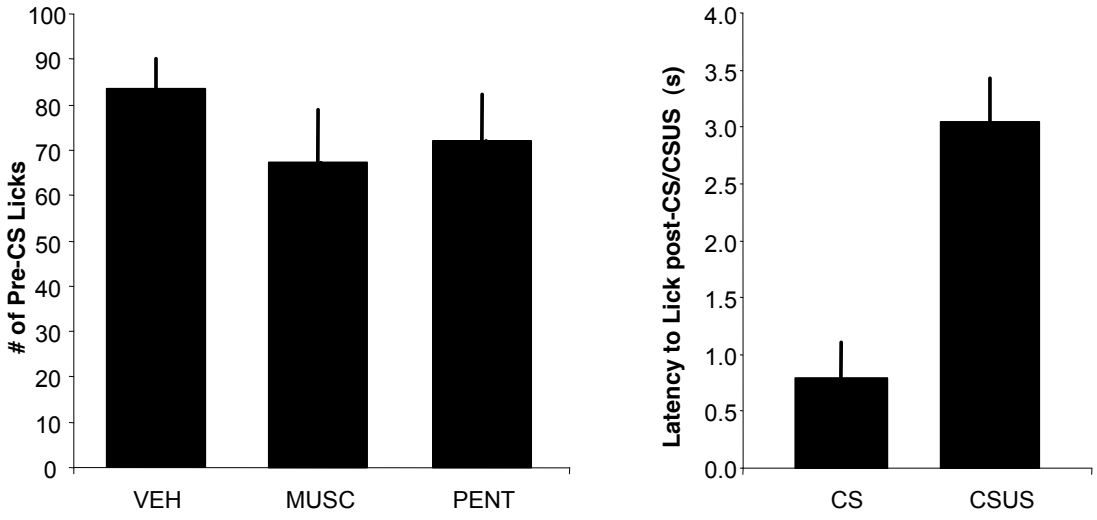


Figure 2.3. Effect of muscimol on suppression not due to general effect on behavior.

A. Drug-treatment did not affect pre-CS lick levels. Mean ± SE number of licks during the 20 second pre-CS interval in rats given 0.25 μL intra-LA infusions of 50 mm sodium bicarbonate in 0.9% NaCl (VEH), 1.1 nmoles of muscimol (MUSC), or 0.5 nmoles of pentagastrin (PENT). Using a counter-balanced within-subject design all rats received all infusions (n = 7). B. Muscimol does not eliminate post-shock suppression. Mean ± SE latency to lick (seconds) in rats given 0.25 μL intra-LA infusions of 1.1 nmoles of muscimol (MUSC) after a CS-alone trial (CS) or a CS-US trial (CSUS). (n = 6)

there was no significant difference between the suppression ratio for HAB and MUS (paired *t*-test, p=0.955; *n* = 7).

Since the suppression-ratio is a function of CS and pre-CS licking it is possible that the effect of muscimol on lick suppression was due to changes in pre-CS licking. However, an analysis of pre-CS licking in three conditions shows no difference between VEH, MUS, and PENT in the amount of pre-CS licking (Repeated Measures ANOVA, $F_{(2,12)}$=2.40, p>0.1) (Figure 2.3a). Since neither muscimol nor pentagastrin had a significant effect on pre-CS licking we can conclude that the change seen in suppression ration was due to a change in licking during the CS.

It is also possible that rats show little, if any, lick suppression during the CS because they are incapable of suppressing or incapable of processing any sensory input. To verify that the effect of muscimol was specific to the CS-evoked suppression we examined whether the drug also attenuated US-evoked suppression. To quantify this we compared the latency to resume licking after CS-US versus CS-alone trials during the MUS session. Figure 2.3b demonstrates that there was a significant increase in the latency to resume licking after a CS-US trial compared with a CS-alone trial (Repeated Measures ANOVA, $F_{(1,5)}$=17.34, p<0.01). This effect was robust: all rats took longer to resume licking after a CS-US trial then a CS trial (*n* = 6). Since muscimol infusion into the amygdala does not disrupt lick suppression as an unconditioned response this suggests that the effect of muscimol infusions on CS-evoked lick suppression is due to a failure to evaluate the CS as aversive, not an

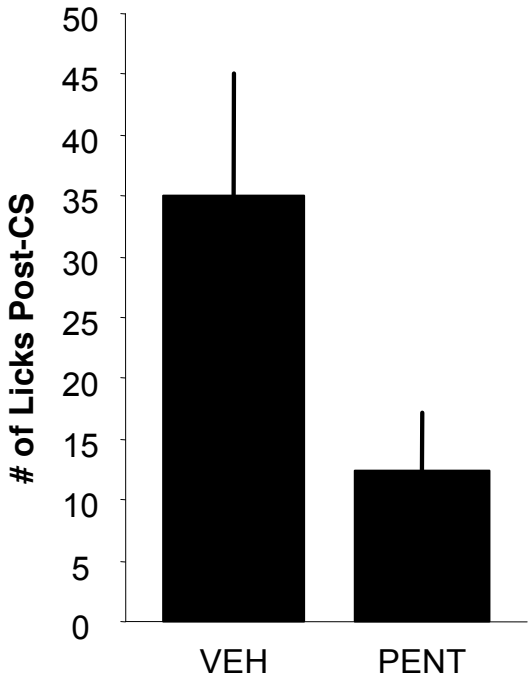


Figure 2.4. Pentagastrin results in persistence of suppression beyond the termination of the CS.

Mean ± SE number of licks during the 20 second post-CS interval in rats given 0.25 μL intra-LA infusions of 50 mm sodium bicarbonate in 0.9% NaCl (VEH) or 0.5 nmoles of pentagastrin (PENT) after CS-alone trials where the SR was greater than 0.4. (n = 5)

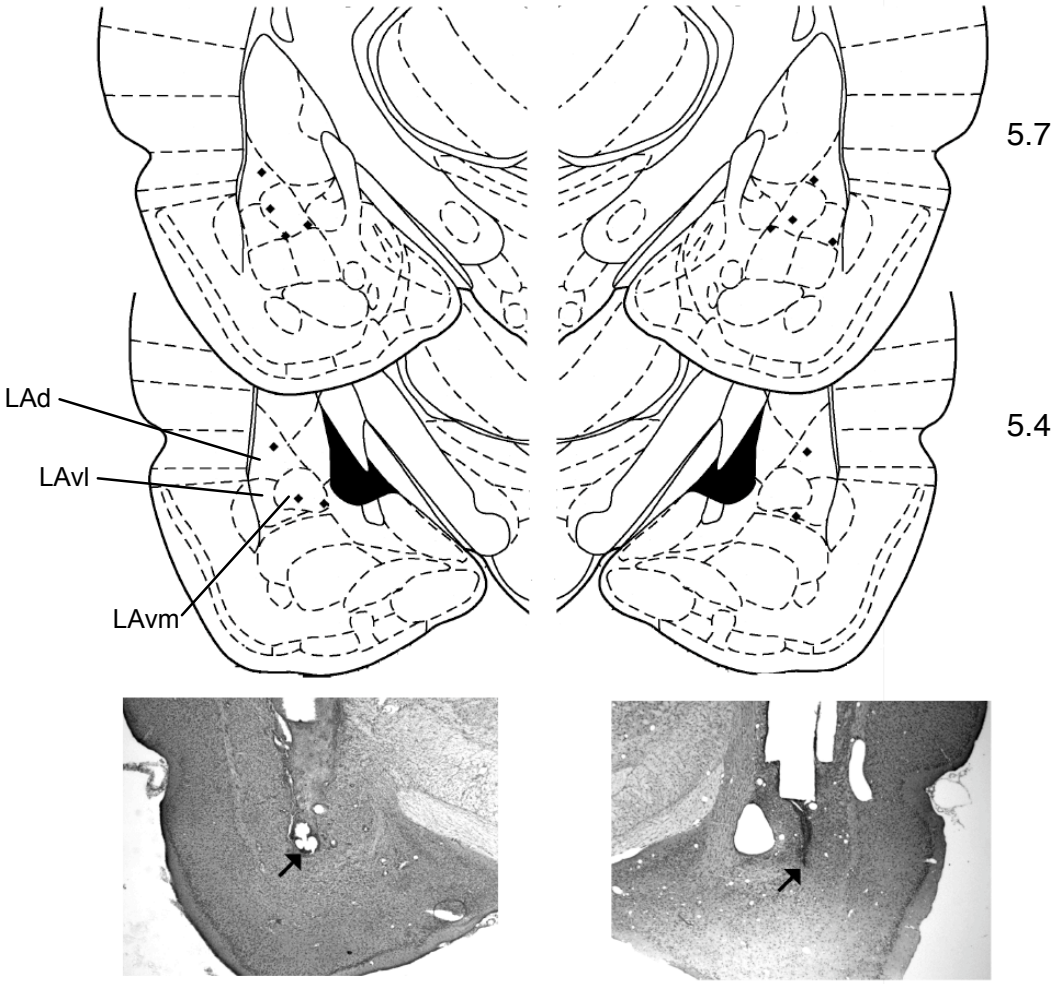


Figure 2.5. Histological verification of cannula placements.

A. Locations of cannula tip placements where infusions were delivered are indicated with diamonds. Numbers indicate anterior-posterior distance(mm) from inter-aural zero. (Dorsal LA - LAd; ventro-lateral LA- LAvl; ventro-medial LA – Lavm). B. Examples of nissl stained sections used to construct A.

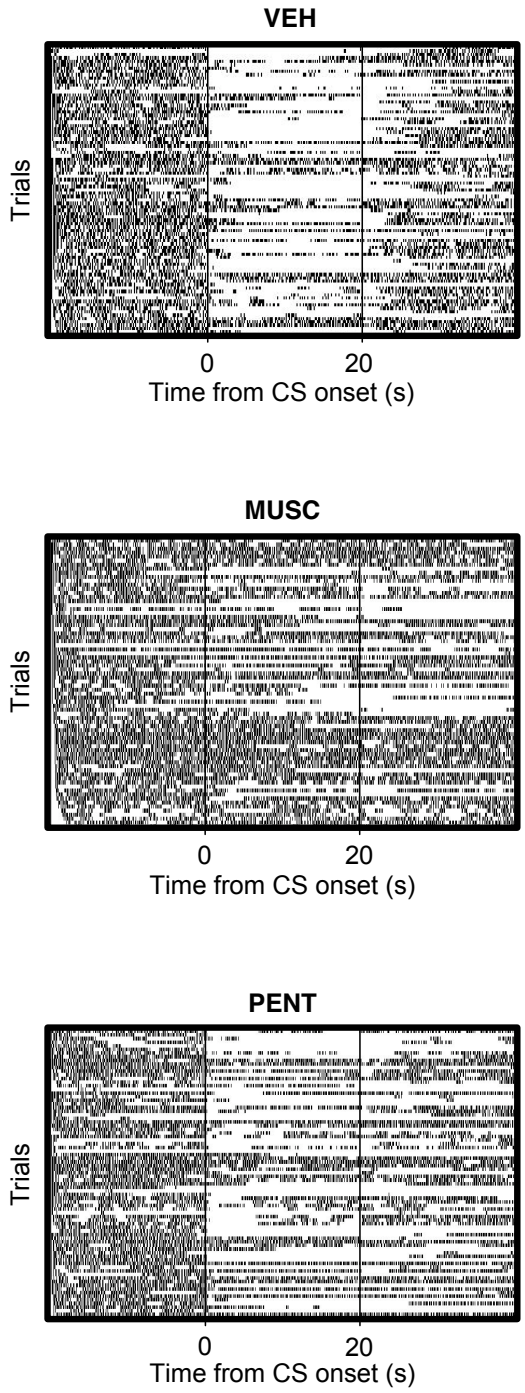


Figure 2.6. Raw Lick Rasters

Each line of the raster is an individual trial. Rasters include all trials from all animals.

inability to react to aversive stimuli.

We hypothesized that pentagastrin would facilitate lick suppression in response to the CS, but we did not see an effect of pentagastrin on suppression ratio (Figure 2.2). One reason for this may have been a ceiling effect: the conditioning procedure may have been too strong to observe an increase in suppression ratio with pentagastrin infusions. An inspection of the raw lick rasters (Figure 2.6) suggested to us that the PENT treatment did have an effect. During VEH sessions, animals suppressed during the CS, but resumed licking after the termination of the CS. In contrast, during PENT sessions, if the CS elicited suppression, the suppression was sustained passed the termination of the CS (Figure 2.4). To quantify this effect we analyzed the number of post-CS licks for trials where the CS elicited suppression (excluding CS-US trials). We did not include the MUS sessions in this comparison because very few trials satisfied the criteria of suppressing to the CS. We found that PENT resulted in a significant maintenance of suppression beyond the termination of the CS compared with VEH (Paired *t*-test, $T_4=3.35$, $p<0.05$; $n=5$).

2.5. Discussion

This study is the first demonstration that pharmacological manipulation of the LA can bi-directionally modulate the processing of a well-learned auditory CS. There are several advantages of this task as an animal model. One is that patients generally

seek help for anxiety, phobia or post-traumatic stress disorder for previously learned stimuli, so understanding the role of the amygdala in processing a well-learned CS may be a more accurate animal model of PTSD than models that examine the initial learning of CS-US associations. Second, there is a practical benefit to this task in that it allows the use of within subject design since the manipulations are done after the CS-US contingency is learned. The within-subject design requires fewer animals to be used and it controls for individual differences between animals.

Lick Suppression

This study differs from typical fear conditioning experiments in the use of lick suppression as a measure of fear instead of the more common measures of bar-press suppression or freezing. The main advantage of lick suppression over bar-press suppression is that licking occurs at 6-8 Hz, much faster than the rate of bar pressing (Repa et al., 2001). This gives a very accurate temporal measure of the onset and offset of bouts of suppression. This task was designed to facilitate the analysis of dynamics of neural activity and fear behaviors via single-unit electrophysiology (Erlich et al., 2005), so the ability to precisely measure the time of the onsets and offsets of suppression was imperative. It is important to note that we were not delivering shocks through the lick spout to produce Vogel conflict (van Gaalen et al., 2002) nor was the occurrence of the CS or US in any way dependent on the lick behavior of the rat.

The advantage of suppression of appetitive activity instead of freezing is that longer sessions can be used while still reliably measuring fear behavior since the rats have an incentive not to fall asleep. As well, licking is measured unambiguously by break in an infra-red beam (the time of each beam-break is recorded by a computer), so there is no question of whether the animal is licking or not. In contrast, the measure of freezing when it is scored by computer is based on a threshold of movement that is somewhat arbitrary and the improvement of automated scoring is an ongoing development but still offers advantages over manual scoring of freezing, such as lack of scorer biases (Marchand et al., 2003; Takahashi, 2004).

Probabilistic Reinforcement

This study differed from typical fear conditioning experiments in the use of a probabilistic contingency between the CS and US. Typically, the CS-US contingency is 100% in order to maximize learning. We chose to reduce the contingency in order to produce intermediate levels of fear behavior so that we could then study the dynamics of behavior and neural activity. Moreover, formal models (Sutton and Barto, 1998) of the role of amygdala in behavior should take into consideration the full range of CS-US contingencies which have rarely been examined in this system. While we demonstrate here that the LA plays a role in processing a well-learned CS with a CS-US contingency of 30%, future experiments will determine the precise role of the LA in tracking contingencies that are dynamic. For example, is plasticity in the amygdala required for an animal to learn a shift in contingency from 30% to 50%?

For a rewarding US, this kind of learning seems to be mediated by an amygdalar-nigral-prefrontal circuit (Holland and Gallagher, 2004). The task used in this experiment is a first step towards asking more quantitative questions about the precise role of amygdala activity in processing aversive stimuli.

Effect of Muscimol

Our results are consistent with the finding that muscimol infusions into the amygdala (Muller et al., 1997; Blair et al., 2005) as well as lesions of the amygdala (Maren et al., 1996; Maren, 1998; LeDoux, 2000) prevent the acquisition of fear conditioning.

Even though licking may be considered an instrumental response (Cannon and Palmiter, 2003), the CS-US contingency in this study is strictly Pavlovian. This is the likely reason why our data differs from the finding that well-learned avoidance behavior is not amygdala dependent (Poremba and Gabriel, 1999). It is established that there are multiple memory systems in the brain that are essential for different kinds of learning (Packard and Cahill, 2001; White and McDonald, 2002) and across different time scales (Dudai, 2004). So it is not surprising that manipulation of amygdala activity affects our Pavlovian task and not an instrumental avoidance task.

The theory that the amygdala is the substrate for the memory of fear conditioning is the subject of much debate (Cahill et al., 1999; Vazdarjanova, 2000). An alternate hypothesis is that the primary role of the amygdala is to affect the

consolidation of diverse memory systems through its control of neuromodulators (McGaugh, 2004). Nonetheless, in our task the CS-US contingency was well-learned, and therefore was consolidated, before any pharmacological manipulation of the LA took place. So it seems unlikely that the results observed could be due to an effect of memory consolidation.

Since lick suppression is defined as the absence of licking, as opposed to freezing which is a specific behavior, it can be thought of as an umbrella measurement. Any conditioned response—orienting, vigilance, freezing, escape—will result in lick suppression. Thus, it is parsimonious to hypothesize that the lack of CS-evoked lick suppression with muscimol in the LA is due to the processing of the CS as opposed to an effect on the expression of a specific motor response. Studies investigating unconditioned fear responses have found that responses to predatory odors do not require the LA (Wallace and Rosen, 2001; Fendt et al., 2003; Chen et al., 2006) (But see Blair et al., 2005) supporting our hypothesis that the LA is required for processing a well-learned auditory CS.

Effect of Pentagastrin

The effect of pentagastrin, suppression that persists beyond the termination of the CS, is analogous to a finding from human imaging. It has been reported that one manifestation of fear pathology in humans is amygdala activity that is sustained long after the termination of an aversive stimulus (Siegle et al., 2002). This suggests that it

could be helpful to understand what turns the amygdala off. And indeed there is a rich research program into the extinction of fear (Myers and Davis, 2002; Richardson et al., 2004; Sotres-Bayon et al., 2004; Cammarota et al., 2005).

We chose pentagastrin because local infusions of this drug into the BLA had previously been demonstrated to have a facilitating effect on acoustic startle without affecting baseline activity (Frankland et al., 1997). It was crucial that our drug manipulations did not affect pre-CS licking since the measurement of CS-evoked lick suppression critically depends on stable pre-CS activity. Although it was not the goal of this experiment to investigate the mechanism of pentagastrin, our results suggest that future work towards understanding the role of the CCK system in the behavioral phenomena of persistent fear behavior is warranted.

Spread of Infusion

We cannot rule out the possibility that the drug infusions spread to other brain regions near the injection sites. Based on the putative role of the central nucleus of the amygdala (Nader et al., 2001; Fendt et al., 2003) we might expect similar results with muscimol infusions there. However, there are several reasons why we are fairly confident that the observed effects were due to the LA. One is that we chose a small infusion volume, 0.25μL, based on autoradiography of muscimol (Martin, 1991) and other studies which performed muscimol inactivations of the lateral amygdala (Muller et al., 1997; Poremba and Gabriel, 1999; Fendt et al., 2003; Blair et al., 2005). As

well, histological analysis (Figure 2.5) reveals that injections were made across the dorsal-ventral and medial-lateral extent of the LA without any significant correlation between infusion site and drug effect.

Summary

The purpose of this study was to test whether the LA plays a continuing role in processing an auditory fear conditioned stimulus long after initial learning. By infusing muscimol and pentagastrin directly into the LA we demonstrated that neural activity in the LA is functionally tied to the expression of conditioned suppression. Inhibiting activity in the LA attenuated conditioned suppression. Exciting activity in the LA enhanced the persistence of conditioning suppression. This paves the way for future experiments which examine how the dynamics of neural activity in the LA affect fear behavior.

Trial #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
HAB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PR day 0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0
PR day 1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0
PR day 2	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0
PR day 3	1	0	0	0	0	1	1	0	0	1	0	0	1	0	1	0	0	1	1	1
PR day 4 (infuse)	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0

Table 2.1. Reinforcement History

A ‘0’ indicates a CS-alone trial and a ‘1’ indicates a CS-US trial. After initial training each week consisted of PR days 1-4.

3. Turning fear on: contribution of spikes in the lateral amygdala

3.1. Abstract

The lateral nucleus of the amygdala (the LA) is an essential component of the neural circuitry that mediates fear responses to threats. The focus of most investigations of neuronal activity in the LA has been the connection between fear learning and synaptic plasticity. To investigate the role of the active, real-time influence of the LA on fear regulation directly, we designed a task in which single neuron responses in the LA were recorded while the engagement and disengagement of fear responses was measured on a millisecond timescale. We find that increases in the LA single-unit activity precede the engagement of fear responses, while decreases in activity precede the disengagement of fear responses.

3.2. Introduction

An animal's survival depends on its ability to detect and react to stimuli that predict danger, even when engrossed in other activities, like eating or social interaction. The ability to respond to threats can be thought of as encompassing two distinct processes: first, a stimulus is determined to be a threat and second, an appropriate response is initiated. Although animals are innately averse to some stimuli (Rosen, 2004) (predatory odors, intense stimulation and pain), they also have the

ability to learn about novel stimuli that predict danger (LeDoux, 2000; Maren, 2001). We focus on learned threats here.

Learning about new dangers is often modeled in the laboratory as Pavlovian fear conditioning, a procedure in which the pairing of a neutral CS, like a tone, with an aversive US, typically a shock, converts the CS into a threatening stimulus (LeDoux, 2000; Maren, 2001). Overwhelming anatomical (Pitkanen, 2000), pharmacological (Rodrigues et al., 2004), lesion (Goosens and Maren, 2001; Nader et al., 2001), genetic (Shumyatsky et al., 2002) and electrophysiological (Rogan et al., 1997; Maren, 2000; Pare and Collins, 2000; Repa et al., 2001; Rosenkranz and Grace, 2002; Tsvetkov et al., 2002; Goosens et al., 2003) evidence suggests that the neural substrate for auditory fear conditioning includes the LA (LeDoux, 2000; Maren, 2001; Pare et al., 2004; Rosen, 2004). Lesion (LaBar et al., 1995) and imaging (LaBar et al., 1998; Buchel and Dolan, 2000; Phelps et al., 2004) studies confirm the role of the amygdala in fear conditioning in humans, and also show that an overactive amygdala correlates with symptoms of depression (Thomas et al., 2001) and anxiety (Davidson et al., 1999; Shin et al., 2005) in people suffering from clinical fear disorders (such as panic, phobic, or post-traumatic stress disorders).

The focus of most research on fear conditioning has been on the role of the the LA in learning the association between the CS and the US (Maren, 2001; Pare et al., 2004; Rodrigues et al., 2004). Relatively few studies have examined the role of the LA in processing the CS once it is familiar (Poremba and Gabriel, 1999; Maren,

2000). However, processing of familiar threats is also important to understand, especially in a clinical context. Patients typically seek help for problems stemming from previously learned associations rather than help in the prevention of new fear learning.

The focus of the present study was on the role of the LA in the regulation of fear elicited by a familiar threat, as measured by the interruption (i.e. suppression) of ongoing behavior. To do this, we assessed single-unit activity in the LA during moment-to-moment engagement and disengagement of conditioned fear responses using a procedure where the contingency between the CS and US is probabilistic. In order to get a precise measure of the engagement and disengagement of fear we measured lick suppression. Rats lick at a frequency of 6-8 Hz, so the suppression of licking provided a temporal accuracy of about 150 ms for the onset and offset of suppression: an order of magnitude faster than other common measures of fear, such as freezing or bar-press suppression (Repa et al., 2001). Lick suppression is a sensitive measure of fear since any fear response—including orienting, risk assessment, vigilance or freezing (Blanchard et al., 1993)—results in suppression of licking. Additionally, lick suppression may involve the tri-synaptic connection from the central amygdala, the main output region of the amygdala, to the hypoglossal motor nucleus, which controls licking (Ugolini, 1995).

The ability to measure the onset and offset of suppression with millisecond accuracy allowed the direct comparison of fear behavior and amygdala activity on a

shorter timescale than has previously been possible (Rogan et al., 1997; Repa et al., 2001; Goosens et al., 2003). We report that firing in the LA does not simply encode the associative strength of the CS; it adaptively encodes moment-to-moment changes in lick supporession, and thus presumably in fear. Specifically, increases in the LA single-unit activity precede the engagement of fear responses while decreases in activity precede the disengagement of these same fear responses.

3.3. Methods

Subjects. Male Sprague–Dawley rats (n=30) weighing 350–400 g were maintained on a restricted diet until they reached 90% of their original body weight. They were trained to lick for a sugary orange drink (Kool-Aid®, Kraft Foods) to maintain a constant level of activity against which suppression could be reliably measured. All behavior and recording took place in a Plexiglas recording chamber with dimensions 23 cm x 28 cm x 34 cm (Med-Associates, Inc.).

Surgery and histology. The surgical procedures performed were similar to those previously described (Repa et al., 2001). Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.), xylazine (6.0 mg/kg, i.p.), and medetomidine (0.5 mg/kg, i.p.). A ten-channel non-movable microelectrode was implanted in the right LA, with coordinates -3.3 mm posterior, 5.3 mm lateral and 7.5 mm ventral to bregma. An identical bundle was implanted in the right medial pre-frontal cortex (data reported elsewhere). Wires were arranged either individually or twisted in pairs to form

stereotrodes with a tip impedance of 1–2MΩ. Silver wires (75 μm diameter, stripped of insulation 2 mm from the tip) were threaded through the skin of the right eyelid for delivery of a periorbital shock, which served as the US. Rats were allowed to recover from suregery for at least 5 days before the beginning of training. At the conclusion of the experiment, recording sites were marked with small current injections before perfusion with ferrocyanide (Gomori, 1936). Electrode locations were reconstructed with standard histological techniques. Nine recording sites were located in the dorsal LA and 10 were in the ventral LA (Fig. 3.1c). There were no observable differences between neurons in dorsal and ventral the LA, thus all cells were treated as one group. The mean number of cells that reached criterion (see Single-Unit Recording, below) per recording site was 4.47 (Range 1-32).

Behavior. Behavioral data was collected from 30 animals that were surgically implanted with electrodes. Rats were pre-habituated (pre-HAB): placed in the recording chamber on each day and given free access to orange Kool-Aid® until they licked consistently for an hour (Fig. 3.1a). After reaching criterion, there was a habituation session (HAB) which consisted of 20 CS trials with no US presentations. The next day the subject began the partial reinforcement (PR) task, which consisted of 20 trials with a random 1-5 minute inter-trial interval. Each day the animal returned to the recording chamber for a minimum of five days of PR and as long as cells could be isolated (Range 5-12 days). Each trial consisted of a CS followed probabilistically (30 or 50%) by a US so that on any trial the subject did not know whether or not a US

would occur but could learn over sessions the CS-US contingency. The CS was an auditory stimulus made up of 20 pips (Repa et al., 2001). The pip frequency was 12KHz and duration was 250ms, with an inter-pip interval of 1 second. The US was series of 5 weak periorbital shocks delivered to the right eyelid 1 second after the onset of the final pip. Each shock was 2mA in intensity and 2ms in duration. The suppression ratio is defined as:

$$SuppresionRatio = \frac{Licks_{PRE} - Licks_{CS}}{Licks_{PRE} + Licks_{CS}}$$

Where $Licks_{PRE}$ is the number of licks in the 20 s pre-CS period and $Licks_{CS}$ is the number of licks in the 20 s CS period (Repa et al., 2001). The time of individual licks were recorded by a computer via detection of a break in an infra-red photo-beam (Med-Associates, Inc.). Instantaneous suppression ratio is defined as the instantaneous lick rate normalized to the pre-CS lick rate as in suppression ratio.

Single-Unit Recording. We recorded single-unit activity for 60 seconds during each trial beginning 20 seconds before the onset of the CS. Single-unit spikes were identified using online and offline cluster analysis software (Neuralynx, Tuscon, AZ). Single-units had to meet several criteria for inclusion in the study. First, spike waveforms had to remain stable and well discriminated throughout the experiment. Second, ISI histograms had to exhibit a refractory period of at least 1 ms, so that high-frequency multiunit spike waveforms would not be included in the data set. Third, cells had to fire a minimum spike rate of 0.25 Hz to be included in the analysis. 84

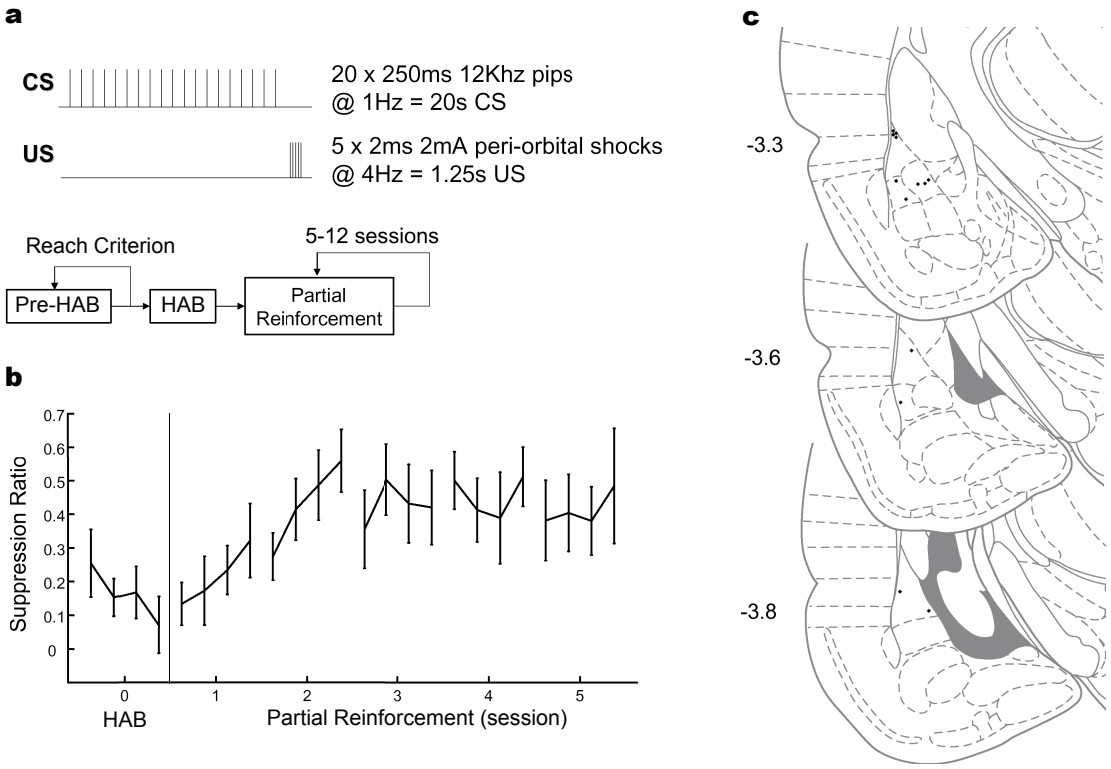


Figure 3.1. Behavior & Histology

A. The top of figure describes the CS and US used in the experiment. B. Each point is the average suppression ratio of 5 trials across all animals (n=23). Error bars are standard error across animals. C. Histology. Black diamonds are recording sites. The three coronal sections of a rat brain are at -3.3, -3.6 and -3.8 mm from bregma going from top to bottom. (Adapted from Paxinos & Watson, 2004)

cells, which met criteria for inclusion in the study, were successfully recorded from 13 of the 30 implanted animals (Fig. 3.1c).

Data Analysis. Rasters and histograms were generated in MATLAB (R14, Mathworks, Inc) using custom software (J.C.E). Data was imported into Statistica (7.0, StatSoft, Inc) for ANOVA, t-tests, and regression analysis. For figures 3.2 and 3.3 the lick times were converted to a lick rate by taking the reciprocal of the interlick interval (Lansky, 2004). The instantaneous suppression ratio is simply the lick rate normalized by the mean pre-CS lick rate using the same formula as the suppression ratio.

To calculate the correlation between CS-evoked neural response and suppression ratio for each trial, we took the total number of spikes fired during the 20 second CS and normalized the response to the trial with the highest number of spikes. After normalizing all the neural responses, we excluded those trials where the rat didn't lick during the pre-CS period.

To normalize each neuron's activity in figure 3.4 we bootstrapped (1000 iterations) the distribution of firing rates during the CS period by randomly shifting the spike times on each interation and generating the lick onset- and offset-triggered spike histograms with these shifted spikes.

3.4. Results

Behavior

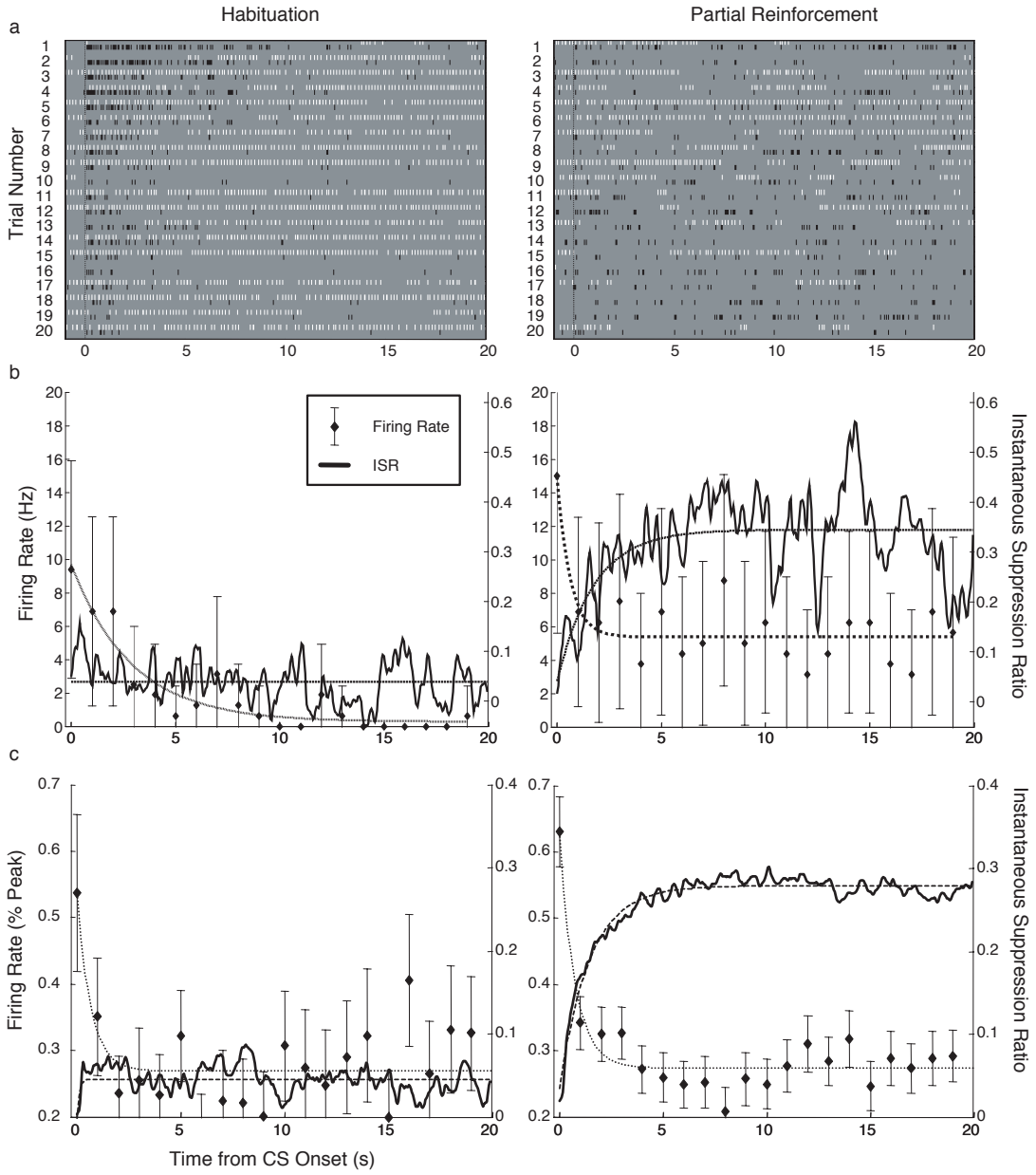
Rats began to show fear responses to the CS, measured as lick suppression (Chapman and Sears, 2003), after the first CS-US pairing on the first day of PR, and reached asymptotic levels of suppression on the second day of PR. This level of performance was sustained for the duration of the experiment (5-12 days) (Fig. 3.1b). The suppression ratio, which measures the degree of behavioral inhibition induced by the CS, and thus presumably the level of fear, did not vary systematically by trial number (ANOVA, $p>0.8$). Note that suppression ratio reached asymptote (around .45) by the second day of PR. Thus, subjects were expressing steady intermediate levels of CS-induced fear for the duration of the experiment. The key feature of this task is that the rats switch back and forth between licking and suppression over the course of a single CS (Fig. 3.2a—right). This within-trial behavioral variability cannot be explained by any external variables, like reinforcement history. Rather, it is an expression an internal process of fear regulation.

Unit Activity

A total of 84 cells recorded from the LA of 13 rats were included in the analyses. The spontaneous firing rate of these neurons was 1.489 (0.954-2.127; 5-95% c.i.), with a geometric mean of 0.516 Hz (0.404-0.662; 5-95% c.i.), consistent with previous studies (Quirk et al., 1995; Repa et al., 2001; Goosens et al., 2003). The

Figure 3.2. Within CS comparison of the LA activity and behaviour.

A. Raster plots (black–spikes; white–licks). B. CS-onset peri-event time histograms of the rasters above. Each diamond represents the 100 ms of the neuron's pip response. Error bars are standard error across trials. The solid line graphs the instantaneous suppression ratio. C. Population PETHs from HAB (left) and PR (right). Each cell was normalized to its peak activity. Error bars indicate standard error across cells. Note that the within-CS neural response has an exponential shape in HAB and PR. But only in PR does the behavior come to mirror the neural activity. (ISR, Instantaneous Suppression Ratio). The ISR in B & C was smoothed using a Gaussian with a standard deviation of 50 ms. Neural activity and behavior (B &C) were fit with decaying and rising exponential functions, respectively.


a
Habituation
Partial Reinforcement
Trial Number
b
Firing Rate
ISR
Firing Rate (Hz)
Instantaneous Suppression Ratio
c
Firing Rate (% Peak)
Instantaneous Suppression Ratio
Time from CS Onset (s)

low spontaneous firing rates suggest that the neurons recorded were pyramidal-like projection neurons, as opposed to interneurons (Pare and Gaudreau, 1996). There was neither a change in the spontaneous rate of the neurons across days of the experiment (ANOVA, $p>0.3$) nor across duration of a 20 trial session (ANOVA, $p>0.9$).

Within-CS Analysis

The neural responses to the CS diminished rapidly after the first pip of the CS (Fig. 3.2b,c; diamonds). This degree of adaptation to an auditory stimulus with a relatively long (750 ms) period of silence between pips is not seen in cortex (Fishman et al., 2001; Erlich et al., 2002), inferior colliculus (Finlayson, 2002), hippocampus (M. Moita, personal communication) or the posterior intralaminar nucleus of auditory thalamus (Bordi and LeDoux, 1994), which is the main auditory thalamic input to the LA (Doron and Ledoux, 2000). This suggests that the rapid change in response to the first and second pip was a result of computation in the LA, and not simply a reflection of the inputs to the LA. This rapid adaptation of the neural response was in stark contrast to the CS-evoked lick suppression. During partial reinforcement, after the CS-US association was learned, the CS-evoked lick suppression was sustained for the duration of the CS. (Fig. 3.2c, right).

Previous results have suggested that the LA activity may encode the magnitude of conditioned fear, based on a comparison of CS evoked activity in the LA before and after conditioning (Quirk et al., 1995; Rogan et al., 1997; Repa et al., 2001; Goosens et

al., 2003). However, the within-CS unit activity (Fig. 3.2c, right) clearly does not encode the magnitude of fear response, since the asymptotic level of suppression (in the second half of the CS) corresponds to low levels of unit activity. Indeed, the population neural activity and behavior are significantly negatively correlated ($r=-0.914$, $p<0.00001$). One way to reconcile this was to posit that the unit activiy in the LA was signaling a change in suppression. This hypothesis could explain how a transient neural signal was connected to a sustained behavior response. Moreover, this hypothesis was more consistent with the putative role of the LA in fear conditioning than the view that spikes in the LA encode the inverse of the magnitude of fear.

Spike-Triggered Analysis

In order to test the hypothesis that neural activity in the LA was encoding changes in suppression we generated spike-triggered averages of lick rate for the simultaneously acquired neuronal and behavioral activity. Even though the suppression was asymptotic on average (Figure 2c) it was highly variable on each trial (Figure 2a). The spike-triggered lick rate would show whether the timing of suppression was linked to the timing of unit activity in the LA. Licking behavior was converted from a point-process to a rate by taking 1/inter-lick interval (ILI) as rate. Figure 3.3a shows example cells from HAB (left) and PR (right). To generate statistics for the population of cells we calculated the difference between the spike-triggered average during the 2-sec period before and after spikes for each cell, and

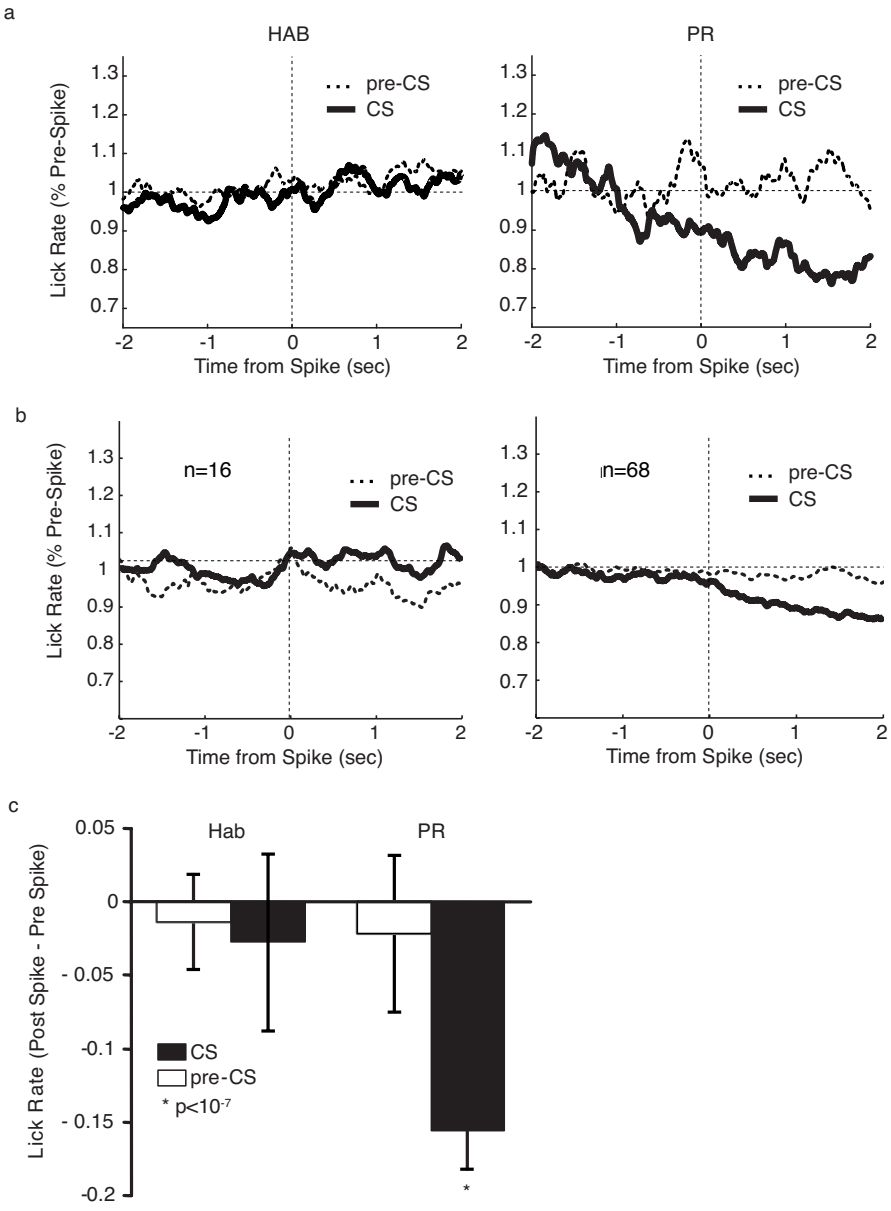


Figure 3.3. Spike-triggered lick rate.

A. Example cells from HAB (left) and PR (right). B. Population averages of the spike-triggered lick rate for HAB (left) and PR (right). C. Summary of differences of pre-post spike lick rate for each phase of experiment. Two paired t-tests demonstrated that only in PR sessions and only during the CS there was a significant correlation between spikes in the LA and lick suppression [PR (t_{67}=2.8, p<.005); HAB (t_{15}=.07, p=.9)]. A & B. Lick rate was smoothed using a Gaussian with a standard deviation of 100ms.

compared the difference between the pre-CS and CS periods using paired *t*-tests. Comparing the pre- versus post-spike portions of the spike-triggered average gives a basic measure of the relationship between firing rate and lick behavior. If the post-spike lick rate was less than the pre-spike lick rate this would indicate that spikes had a suppressive effect. The analysis (Fig. 3.3c) showed a significant difference in spike-triggered lick rate between pre-CS and CS periods during PR (t_{67}=2.8, $p<0.005$) but not during HAB (t_{15}=0.07, p=0.9), supporting the hypothesis the LA activity correlates with changes in suppression.

On average, for the population, each spike from a single neuron was predictive of a 10% reduction in lick rate (Fig. 3.3b—right). (Alternatively, this could be considered a 10% increase in the likelihood of suppression). Notice that this is the average effect *per spike.* To illustrate how this effect scales, assuming a simple multiplicative code, a burst of 30 consecutive spikes increases the likelihood of suppression by 95%. This calculation cannot be performed across neurons since there are thousands of neurons in the LA of which we record a few at a time. Each spike we record from a single neuron is likely occurring in close temporal proximity to thousands of other spikes in the LA. Thus the effect per spike reported actually reflects the effect of the spike recorded and also the effects of all the simultaneously occurring spikes from neurons that were not recorded. The contribution of the unrecorded neurons is probably substantial given that fear conditioning increases the functional connectivity between neurons in the LA (Quirk et al, 1995).

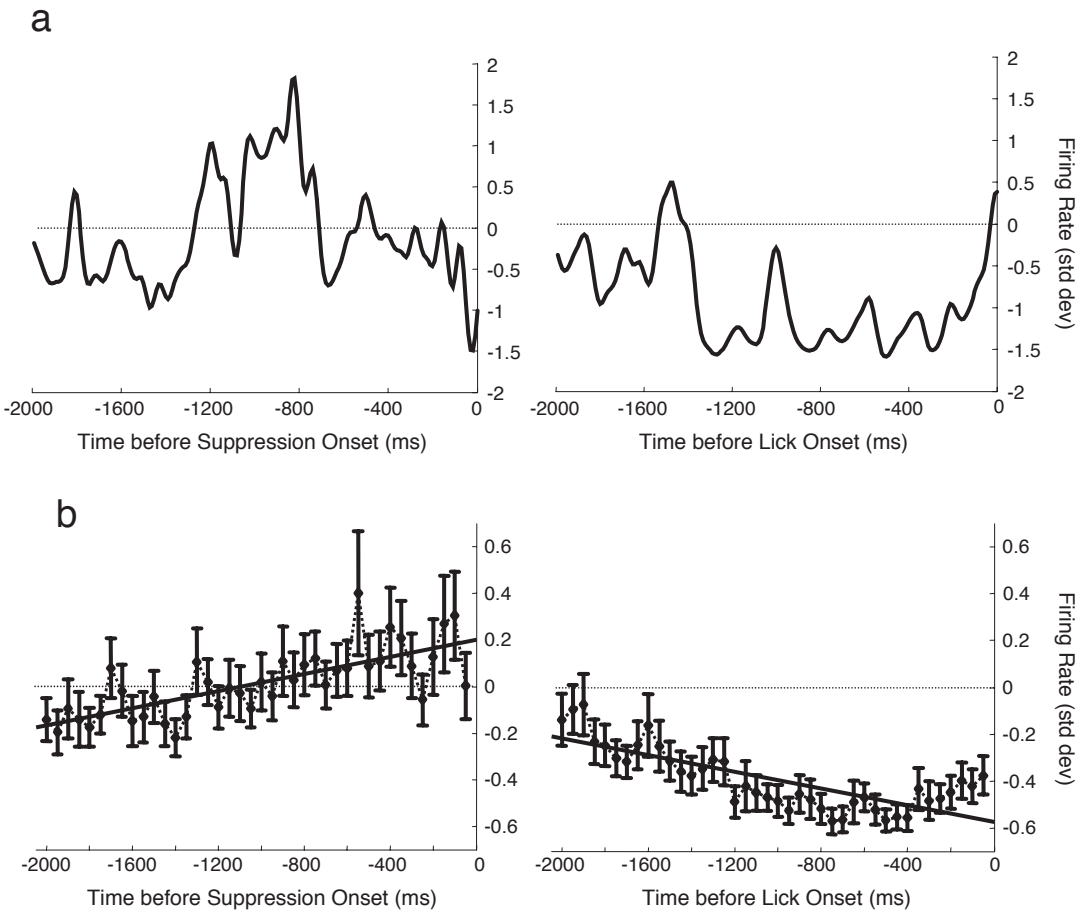


Figure 3.4. Behavior-triggered spike rate.

A. Behavior-triggered spike histograms of an example cell from day 3 of PR (Left – suppression onset; right – lick onset). Firing rate was smoothed using a Gaussian with a standard deviation of 20ms B. For the population data each diamond is a 50ms bin. Error bars are the standard error across cells. The solid line is the least squares fit of the data. Neural activity ramps up before the onset of suppression and ramps down before the onset of licking. A & B. Each cells activity was normalized to its own activity during the CS epoch using a bootstrap technique.

Note, in figure 3.3a, that in the example from PR the change in lick rate seems to decrease before time zero. At first glance, this suggests that the spikes are not driving the change in lick-rate. In fact, the spike-triggered analysis is not an indicator for or against causality. Imagine a train of spikes that begins at time zero and lasts for two seconds. If lick suppression began at 500ms then the first 500ms of the spike train occurred before the change in behavior and the last 1.5 sec of the spike train occurred after the change in behavior. The spike-triggered average of this event will look much like figure 3.3a (right). Even though the spike train began before the change in behavior the spikes in the train that come after the change in behavior obscure the temporal relationship. Therefore, we need another analysis to address the temporal relationship between changes in behavior and changes in neural activity.

Behavior-Triggered Analysis

In order to test whether the neurons are driving behavior or following behavior we generated 'behavior-triggered' averages of spike trains. We only analyzed behavioral events that occurred within the CS during partial reinforcement since it is only during this phase that the LA activity is correlated with behavior (Fig. 3b). We defined 'behavior-triggers' by finding pauses in licking that were greater than the 99% confidence interval in the distribution of ILIs during the pre-CS period (1200 msec). We took the first lick after a pause as a lick-onset event, and we took the last lick before a pause as a lick-offset event. However, the beginning of suppression is actually the time of the first missing lick. Since licking is rhythmic, we can estimate

the time of the missing lick by taking the mode of the ILI histogram for each behavioral session and adding that time to the lick-offset times to create suppression-onset events. We then generated suppression-onset-triggered spike histograms and lick-onset-triggered spike histograms (Fig. 3.4).

Figure 3.4 illustrates that changes in the LA activity preceded changes in behavior. Since this analysis focused on the relative peaks and troughs of activity during the CS, all cells were normalized, using a bootstrap method (Bradley, 1993), to their own activity during the CS period. Figure 3.4a shows suppression-onset triggered spike histogram (left) and lick-onset triggered spike histogram (right) for a single cell recorded on day 3 of PR. Comparing the left and right graphs it is clear that the cell signaled changes in behavior about 1000 msec before the event occurs. For suppression-onset the signal was a significant (>95% CI) increase in spike rate. For lick-onset, the signal was a relative depression in firing.

The results illustrated by the example cell were confirmed by the population analysis (Fig. 3.4b). For the population we binned the spike data from the 2 seconds preceding 'behavior-triggers' into 50 ms bins. Figure 3.4b shows that spike activity ramped up before the onset of suppression and ramped down before the offset of suppression. We performed a two-factor (EVENT x TIME) repeated measures analysis of variance (ANOVA) with EVENT coding lick-onset versus suppression-onset. The ANOVA revealed that there was not a significant main effect of TIME (p=. 29), but that there was a significant main effect of EVENT ($F_{1,67}$=37.13, $p<10^{-7}$), and

also a significant EVENT x TIME interaction ($F_{39,2613}$=4.285, $p < 10^{-9}$). The interaction was followed up with an analysis of the simple effects of TIME for each EVENT condition. This revealed a significant TIME effect for both suppression onset ($p<10^{-4}$) and lick onset ($p<10^{-7}$). To summarize, there were significantly distinct spiking patterns before lick-onset and suppression onset. High firing rates preceded the onset of suppression and low firing rates preceded the onset of licking.

We used linear regression to provide a statistical measure of the direction of the change in activity. Significant regression lines were found for both suppression onset ($F_{1,2718}$=29.19, $p<10^{-7}$) and lick onset ($F_{1,2718}$=62.53, $p<10^{-7}$). Notably, at 2 sec prior to each event onset (i.e., intercept ± standard error of estimate) the population cell firing was highly similar for each event (suppression onset: -0.163 ± 0.042; lick onset: - 0.211 ± 0.027). However, the slope for each line (± standard error of estimate) was opposite in direction, although almost equal in magnitude (suppression onset: 0.192 ± 0.035; lick onset: -0.182 ± 0.023).

3.5. Discussion

We developed a task that would allow us to observe the ongoing engagement and disengagement of fear behavior in order to investigate the specific role of the LA in fear regulation. By combining single-unit recording with lick suppression we were able to align neural activity to changes in behavior with high temporal resolution.

This approach provided several novel insights about what the firing rates of the LA neurons encode.

What Does the LA Activity Encode?

The mainstream view, which has persisted for over a decade, is that the firing rate, of neurons in the LA, encodes the associative strength between the CS and the US (Rogan et al., 1997; Repa et al., 2001; Goosens et al., 2003; Pare et al., 2004). Our data show that single-unit activity in the LA encodes changes in moment-to-moment behavior even when learning is asymptotic. The lack of correlation between the CS-evoked response and the suppression ratio on a trial-to-trial comparison in our data set ($r^2<0.01$) highlights the need for an update to the current model. The reason for this lack of correlation is that averaging suppression over a trial does not provide information about the temporal dynamics of the behavior within a trial. For example, a trial with a suppression ratio of 0.333 means the rat licks half as many times during the CS as during the pre-CS period, but it does not tell us whether the rat alternated between licking and suppression each second or whether the rat licked for the first half of the CS and suppressed for the second half. According to our data different patterns of the LA neural activity would occur for these different patterns of within-trial behavior. In the former case, the neural activity will fluctuate up and down to drive the changes in behavior. In the latter case, the neuron only fires at one point to trigger

the single onset of suppression. Thus, by examining the short timescale dynamics this novel connection between brain and behavior emerges.

Gating of the LA activity

In both HAB and PR the CS-evoked response diminishes rapidly within a trial (figure 3.2). However, the behavior only reflects this activity during PR. This data was confirmed by spike-triggered averaging (figure 3.3): the LA activity is only correlated with lick suppression during PR and during the CS. Intuitively, behavior and neural activity in the amygdala should only be correlated during fear (and other amygdala dependent behaviors). During HAB and during the pre-CS period where rats are engaged in the appetitive behavior of drinking Kool-Aid®, it would be surprising if there was a significant correlation between the LA unit-activity and behavior. The data from chapter 2 support this intuition. Muscimol and pentagastrin infusions into the LA modulated CS-evoked suppression without affecting pre-CS licking. But what is the mechanism by which the LA comes to control behavior during the CS?

There are two possible mechanisms. The first is that the LA activity is only linked to behavior after conditioning (in PR but not HAB) and only during the CS. This could possibly be due to some threshold of activity that has to be reached before the LA drives behavior. Given that the CS results in increased spike activity after conditioning (Quirk et al., 1995; Rogan et al., 1997; Repa et al., 2001; Goosens et al.,

2003), the threshold may then be crossed. The other possibility is that another region, like the medial prefrontal cortex, plays a role in gating the output of activity in the LA via projections to the intercalated nuclei (Pare et al., 2004). Of course, these two mechanisms may be operating simultaneously.

Activity Drives Behavior

The spike-triggered average of licking (figure 3.3) demonstrates the correlation between the LA unit-activity and suppression but it does not clearly demonstrate the directionality of the correlation. In figure 3.3a (right panel) it seems like the decrease in lick rate begins before time 0: before the reference spike. The reason for this is that a burst of spikes might lead to a pause in licking and the first few spikes of that burst may precede onset of suppression, but the rest of the burst may follow the onset. If the spikes encode change why does the cell continue to fire after the onset of suppression? As mentioned, lick suppression is an extremely sensitive measure of fear. The continued firing may reflect the rat's progression into a further state of fear, beyond suppression. That is why we also performed the analyses using onset and offset of suppression (figure 3.4) as reference events to align the spike data. This clearly demonstrates that increased activity precedes suppression-onset and decreased activity precedes suppression offset.

The suppression-onset (Fig. 3.4b-left) and lick-onset (Fig. 3.4b-right) seem to have different time courses. Activity before suppression-onset seems to peak within a

few hundred msec of the behavioral event; activity preceding suppression offset reaches its nadir 800 msec before the event. This suggests that LA's link to suppression-onset is more direct—activity in the LA, via its output connections, puts the brakes on licking. In contrast, the link to lick-onset is indirect—the decrease in activity takes the brakes off, allowing other brain regions to re-initiate licking.

Effect Size

One apparent limitation to our findings is that while the effects we report are statistically strong, the size of the effects we observe are small. For the spike-triggered average (figure 3.3) the difference in pre- versus post-spike lick rate was on the order of 10%. For the behavior-triggered analysis (figure 3.4) the regression lines had slopes on the order of 20%. There are several reasons why we might expect these small effect sizes.

The first is that we are using a fixed auditory stimulus to drive the neurons in the amygdala. Since we are examining behavior in response to a familiar CS we cannot use a novel optimal stimulus for each recording session. We used a 12KHz pip because most neurons in the LA respond best to high frequency sounds (Bordi & LeDoux, 1992), but this certainly was not the optimal stimulus for all the neurons we recorded. Since we are unlikely to be presenting optimal stimuli, we are not generating the maximal or most reliable spike responses, and this means that any

attempt to model behavior to these sub-optimal responses will be quite noisy, since the neural responses themselves are highly variable.

The second reason why the effect sizes might be expected to be small is that the goal of the task was to create a motivational situation where the fear and appetitive drive compete so that we could observe engagement and disengagement of suppression. If we used a stronger shock or higher probability of CS-US pairing, we might have observed more reliable CS-evoked responses, but the rats would have produced too much suppression. To demonstrate this 3 rats were trained with 100% CS-US pairing and had an asymptotic suppression ratio > 0.9. During the CS there was no alternation between licking and suppression, they were simply suppressing the entire duration of the CS. Therefore, we would not have observed the variability in behavior which allowed us to analyze the dynamics of neural activity in the LA and moment-to-moment changes in fear expression.

Associative View

The predominant view of single-unit activity in the LA is that the firing rate of the LA neurons encodes the associative history of a CS (Repa et al., 2001; Goosens et al., 2003; Maren and Quirk, 2004). The theory that neurons in the LA encode the value of the CS seems inconsistent with our findings, since we found that during a single CS, where the value of the CS is stable, the neural activity in the LA predicted the timing of the behavioral variability. However, our study differed in several

important ways from this other study. They used a 2-sec auditory CS and measured freezing behavior as an index of fear. Changes in freezing cannot be reliably measured within a single 2-sec CS. Also, they used 100% reinforcement while we used a partial reinforcement task over many days. It was the use of a long CS, and the high temporal precision for detecting the onset and offset of lick suppression, and the large amount of behavioral variability produced by partial reinforcement that allowed us to observe the effects reported here.

It is important to note that our data do not refute the associate view, but only show that it is incomplete as an explanation for the amygdala's role in fear behavior. We propose that the amygdala is a real-time processor of emotional events, consistent with work from other species and tasks (Anderson and Phelps, 2001; Sander et al., 2003). Part of its role in processing emotional events is to learn about new stimuli that predict positive and negative outcomes, to act as the substrate for associative learning (LeDoux, 2000; Maren, 2004). But our data suggest that its role encompasses the regulation fear elicited by a familiar CS.

Our data also suggest that activity in the LA encodes changes in fear. Encoding change provides several advantages over encoding the absolute level of fear *per se* (Fairhall and Bialek, 2002). This eliminates the continual transmission of redundant information and increases the sensitivity of a neuron to changes in the environment. Adaptive coding also extends the range of possible threats that a single neuron in the LA can encode. We argue that this principle, observed in sensory

(Hosoya et al., 2005), motor (Sparks, 2002), and reward systems (Tobler et al., 2005), can now be extended to fear regulation.

While our findings suggest the need to update the view that neural activity in the LA encoded the CS-US association, they also provide further evidence to refute criticisms of that model. Specifically, our data directly challenge the view that the primary role of the amygdala is to modulate memory consolidation in other brain regions (McGaugh, 2004). We acknowledge that the amygdala plays an important role in regulating neuromodulators and hormones that have a myriad of physiological effects, including the modulation of memory consolidation in other brain regions. However, we demonstrate here that changes in lick suppression over the course of seconds are predicted by changes in activity in the LA. Thus, the LA continues to play an active role in the encoding of the stimulus, even when the behavior has reached asymptotic levels. This is corroborated by the finding that inactivation of the LA attenuates CS-evoked lick suppression in the same task (Erlich et al., 2005), and that post-training lesions of the LA disrupt expression of fear even after overtraining (Maren, 1998) or after a long delay between training and the lesion (Maren et al., 1996). These data are inconsistent with the view that the amygdala only modulates memory consolidation.

Conclusion

Our data provide physiological evidence towards the hypothesis that the LA is a key component of a circuit that regulates fear. Importantly, we show that spikes in the LA do not simply encode the strength of the CS-US association (Repa et al., 2001; Goosens et al., 2003), since changes in the LA activity precede behavioral changes within a single CS. Still, encoding of the change in fear subsumes an encoding of CS-US associative strength since the regulation of fear must take into account contingency and contiguity between the CS and US, key dimensions in associative learning (Rescorla and Wagner, 1972). By elucidating the circuits underlying fear regulation we hope to contribute to the understanding of the neurophysiology of phobia and post-traumatic stress, which seem to be pathologies whereby innocuous stimuli elicit inappropriate fear responses.

4. Turning fear off: contribution of spikes in the medial prefrontal cortex

4.1. Abstract

The medial prefrontal cortex (mPFC) is emerging as a key component of the neural circuitry that regulates fear. Destruction of the mPFC can lead to deficits in extinction of conditioned fear and failure to inhibit unconditioned fear. However, no clear connection between neural activity and fear behavior has emerged from recordings in mPFC. We modified the classic fear conditioning paradigm to produce intermediate, variable levels of fear, measured as lick suppression, in response to a conditioned auditory stimulus (CS). This allowed us to test the hypothesis that single-unit activity in medial prefrontal cortex would predict the moment-to-moment variability in suppression. More specifically, we predicted that increases in single-unit activity in the mPFC would precede the reduction of fear, consistent with the view of mPFC as a regulator of fear. We found that there are two classes of neurons in mPFC, those that are excited by the CS and covary positively with suppression, and those that are inhibited by CS the and covary negatively with suppression. We then assessed the temporal relationship between changes in neural activity and changes in behavior. Consistent with our hypothesis that the mPFC would inhibit fear, we found a class of neurons in mPFC which increase their firing rate before the switch from suppression to licking.

4.2. Introduction

Classical fear conditioning has been an invaluable animal model for elucidating the neural circuitry underlying the processing of aversive stimuli (LeDoux, 2000; Maren and Quirk, 2004). Much of the focus has been on the role of synaptic plasticity in the amygdala in the acquisition of fear conditioning: the pairing of a neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US). In recent years, there has been an increase in interest in the neural substrate for the extinction of fear conditioning: the presentation of a fearful CS alone so that it no longer predicts an aversive US. The medial prefrontal cortex (mPFC) has emerged as a key component of the neural substrate of extinction (Morgan et al., 1993; Morgan and LeDoux, 1995; Sotres-Bayon et al., 2006).

The mPFC consists of several functionally distinct subregions (Sesack et al., 1989; Kolb and Tees, 1990; Conde et al., 1995; Uylings et al., 2003). With respect to extinction of fear the prelimbic and infralimbic regions have received the most attention (Garcia et al., 1999; Milad and Quirk, 2002; Sotres-Bayon et al., 2004). These two regions (sometimes with the more ventral medial orbital region (Milad and Quirk, 2002)) are referred to as ventral mPFC with the more dorsal cingulate cortex being referred to as the dorsal mPFC (Morgan and LeDoux, 1995). Here, we use the term mPFC to refer to the prelimbic and infralimbic cortices (Figure 4.1). (as in Baeg et al., 2001; Sotres-Bayon et al., 2004).

There are several lines of evidence that the mPFC is involved in extinction of fear conditioning. First, lesions of mPFC lead to retardation of extinction (Morgan and LeDoux, 1995; Quirk et al., 2000; Lebron et al., 2004) (but see Garcia et al., 2006). Second, recordings of neural activity during fear extinction suggest that the mPFC signals the memory of extinction (Milad and Quirk, 2002) and stimulation of mPFC can facilitate extinction of fear (Milad et al., 2004). Third, mPFC has strong reciprocal connections with several amygdalar nuclei (Pitkanen, 2000) and stimulation of mPFC inhibits lateral amygdala neurons (Rosenkranz and Grace, 2002b). Fourth, the differential response to conditioned versus neutral stimuli in mPFC is eliminated by destruction (Garcia et al., 1999) or inactivation of the lateral amgydala (Laviolette et al., 2005). Thus, there is substantial evidence that the mPFC plays an important part in the extinction of fear via its interaction with the amygdala (Sotres-Bayon et al., 2004).

Extinction of fear involves the inhibition of fear driven by a change in the CS-US contingency. However, it is also important to understand how fear is regulated independent of changes in external statistics. For example, when making decisions about known risks, like playing the stock market or walking home alone at night, does the mPFC play a role in inhibiting our fear? Imaging data from human studies suggest that the mPFC and the amygdala play opposing roles in the regulation of fear, with the amygdala as a generator of fear and the mPFC as an inhibitor of fear (Hull, 2002;

Ochsner et al., 2002; Gilboa et al., 2004; Ochsner et al., 2004; Phelps et al., 2004; Shin et al., 2004; Bremner et al., 2005; Protopopescu et al., 2005; Shin et al., 2005).

To directly test the hypothesis that activity in the mPFC inhibits fear behavior we modified the typical fear conditioning paradigm to produce variable fear responses to an auditory CS. To this end, we trained rats in a partial reinforcement auditory fear conditioning task using lick suppression as a measure of fear. The use of partial reinforcement means that, after an initial phase of learning the CS-US association, the rats expressed asymptotic, but variable, levels of CS-evoked lick suppression. That is, rats switched back and forth between licking and suppression during the course of a single CS. Rats lick at a frequency of 6-8 Hz, so the suppression of licking provided a temporal accuracy of about 150 ms for the onset and offset of suppression: an order of magnitude faster than other common measures of fear, such as freezing or bar-press suppression (Repa et al., 2001). Lick suppression provided a sensitive measure of fear since any fear response-including orienting, risk assessment, vigilance or freezing (Blanchard et al., 1993)-resulted in suppression of licking. The ability to time-stamp lick suppression with millisecond accuracy allowed comparison of behavior and mPFC activity on a shorter timescale than in previous experiments (Baeg et al., 2001; Milad and Quirk, 2002). We hypothesized that increases in activity in the mPFC would precede the switches from suppression to licking.

4.3. Methods

Subjects. Male Sprague-Dawley rats (n=30) weighing 350-400 g were maintained on a restricted diet until they reached 90% of their original body weight. They were trained to lick for a sugary orange drink (Kool-Aid ®, Kraft Foods) to maintain a constant level of activity against which suppression could be reliably measured. All behavior and recording took place in a Plexiglas recording chamber with dimensions 23 cm x 28 cm x 34 cm (Med-Associates, Inc.). All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University.

Surgery and histology. The surgical procedures performed were similar to those previously described (Repa et al., 2001). Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.), xylazine (6.0 mg/kg, i.p.), and medetomidine (0.5 mg/kg, i.p.). A ten-channel non-movable microelectrode was implanted in right mPFC, with coordinates 3.0 mm anterior, 0.5 mm lateral and 3.5 mm ventral to bregma. An identical bundle was implanted in the right lateral amygdala (data reported elsewhere). Wires were arranged either individually or twisted in pairs to form stereotrodes with a tip impedance of 1-2MΩ. Silver wires (75 μm diameter, stripped of insulation 2 mm from the tip) were threaded through the skin of the right eyelid for delivery of a periorbital shock, which served as the US. Rats were allowed to recover for at least 5 days before the beginning of training. At the conclusion of the experiment, recording

sites were marked with small current injections before perfusion with ferrocyanide (Gomori, 1936). Electrode locations were reconstructed with standard histological techniques. Thirteen recording sites were in prelimbic and two were in infralimbic cortex (Fig. 4.1). There were no observable differences between neurons in prelimbic and infralimbic cortex, thus all cells are treated as one group. The mean number of cells that reached criterion per recording site was 10.13 (Range 1-42).

Behavior. Behavioral data was collected from 30 animals that were surgically implanted with electrodes. Rats were pre-habituated (pre-HAB): placed in the recording chamber on each day and given free access to orange Kool-Aid® until they licked consistently for an hour. After reaching criterion, there was a habituation session (HAB) which consisted of 20 CS trials with no US presentations. The next day the subject began the partial reinforcement (PR) paradigm which consisted of 20 trials with a random 1-5 minute inter-trial interval. Each day the animal returned to the recording chamber for a minimum of five days of PR and for as long as cells could be isolated (Range 5-12 days). Each trial consisted of a CS which was followed probabilistically by a US so that on any trial the subject did not know whether or not a US would occur but could learn over sessions the CS-US contingency (30 or 50%). The CS was an auditory stimulus made up of 20 pips (Repa et al., 2001). The pip frequency was 12KHz and duration was 250ms. The interpip interval was 1 second. The US was series of 5 weak periorbital shocks delivered to the right eyelid one second after the onset of the final pip. Each shock was 2mA in intensity and 2ms in

duration delivered at 4Hz. The suppression ratio (SR) is defined as

$$SR = \frac{Licks_{PRE} - Licks_{CS}}{Licks_{PRE} + Licks_{CS}}$$

Where *Licks*$_{PRE}$ is the number of licks in the 20 s pre-CS period and *Licks*$_{CS}$ is the number of licks in the 20 s CS period (Repa et al., 2001). Individual licks were time-stamped by a break in an infra-red photo-beam (Med-Associates, Inc.). Instantaneous suppression ratio is defined as the instantaneous lick rate normalized to the pre-CS lick rate as in suppression ratio. Trials where Licks$_{PRE}$=0 were not included in analyses.

Single-Unit Recording. We recorded single-unit activity for 60 seconds of each trial beginning 20 seconds before the onset of the CS. Single-unit spikes were identified using online and offline cluster analysis software (Neuralynx, Tuscon, AZ). Single-units had to meet several criteria for inclusion in the study. First, spike waveforms had to remain stable and well discriminated throughout the experiment. Second, ISI histograms had to exhibit a refractory period of at least 1 ms, so that high-frequency multiunit spike waveforms would not be included in the data set. Third, the autocorrelation of each cell was examined to rule out a peak around 125ms which was evidence of contamination from licking. Fourth, cells had to have minimum average spike rate of 0.25 Hz to be included in the analysis. The 142 cells which met criteria for inclusion in the study were successfully recorded from 15 of the 30 implanted animals (Fig. 4.1).

Data Analysis. Rasters and histograms were generated in MATLAB (R14, Mathworks, Inc) using custom software (J.C.E). Regression, *t*-tests and ANOVA were performed in MATLAB using the statistics toolbox. For averaging data across cells, we used Z-score normalization, as follows. For each cell, CS and pre-CS spiking data were averaged across trials at a specific bin width. Then, the mean and standard deviation of the spikes/bin pre-CS activity were calculated. Each bin of unit activity was converted to Z-score by subtracting the mean and dividing by the standard deviation of the pre-CS activity.

To parameterize the CS response we generated the CS response index:

$$CSRI = \ln\left(\frac{Spikes_{CS}}{Spikes_{PRE}}\right)$$

Where $Spikes_{CS}$ is the number of spikes during the CS interval and $Spikes_{PRE}$ is the number of spikes in the pre-CS interval. This measure is similar to the suppression ratio in that no change between pre-CS and CS activity is scored as zero. We used this instead of the suppression ratio because neural activity has a wider dynamic range than behavior in this task. The CS response index is better at capturing CS-evoked increases in neural activity than the suppression ratio.

Even though the CS-US contingency is stable on average, the partial reinforcement sessions consists of CS-alone trials and CS-US trials. It could be that there are trial-to-trial changes in behavior that are driven by the reinforcement history.

For example, rats might express higher levels of CS-evoked suppression if they received a shock on the previous trial. This is evidence of fear learning. Fear regulation, the focus of this study, is characterized by within-trial variability in behavior that cannot be explained by reinforcement history since all variance within a trial is subject to the same reinforcement history. In order to regress out the effects of reinforcement history, that is, the effects of learning, on behavior we fit the suppression ratio in each session with an associative learning model (Rescorla & Wagner, 1972) using the following algorithm.

$$
\begin{aligned}
V_1 =& V_{init} \\
&for\ n \in [1, \#\ of\ trials) \\
&\quad \Delta V_n = \alpha \cdot (\lambda_n - V_n) \\
&\quad V_{n+1} = V_n + \Delta V_n \\
&end
\end{aligned}
$$

$$
\lambda_n = \begin{cases} 0 & \text{if } trial_n \text{ is CS-alone,} \\ \Omega & \text{if } trial_n \text{ is CS-US.} \end{cases}
$$

The free parameters in the model were: α, the learning rate parameter; Ω, the strength of the US; and V_{init}, the CS-US association from previous sessions. Thus, for each session there were three free parameters to fit the suppression ratio on the twenty trials in a session. The optimal parameters were found using 'fminsearch' which instantiates the Nelder-Mead simplex search algorithm (Matlab 7.1, Mathworks, 2005). The result of the fit for each session was $\hat{V}$, a vector of the CS-US associative strength on each

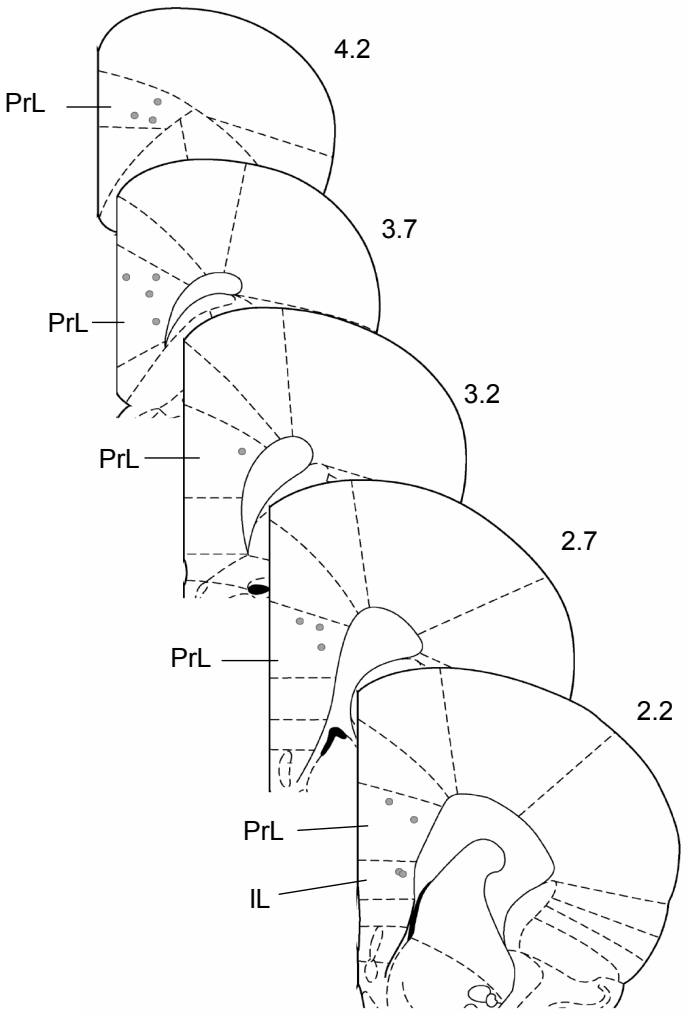


Figure 4.1. Histology

Schemata of coronal slices from rat brain. Dots indicate recording sites. Numbers indicate distance from Bregma in mm. 12 sites were in PrL and 2 sites in IL (PrL, prelimbic cortex; IL, infralimbic cortex). (Adapted from Paxinos & Watson, 2004).

trial. This vector was then used as the independent variable in the first step of the regression analyses.

The stepwise regression for each neuron was performed as follows:

$$SR = (\beta_{RW} \cdot \hat{V}) + \rho_1 \quad (4.7)$$

$$\rho_1 = (\beta_{between} \cdot X_{between}) + \rho_2 \quad (4.8)$$

$$\rho_2 = (\beta_{within} \cdot X_{within}) \quad (4.9)$$

The first step was the regression of suppression ratio (SR) using $\hat{V}$ as the independent variable (Eq. (4.7)). The residuals of the regression, ρ_1, were then used as the dependent variable in the second regression in which the trial averaged neural activity ($X_{between}$) was the independent variable. The residuals of second regression, ρ_2, were then used as the dependent variable in the third regression in which the neural activity (X_{within}) in 1 second bins as the independent variable.

To generate behavior-triggered spike histograms we defined 'behavior-triggers' by finding pauses in licking that were greater than the 99% confidence interval in the distribution of inter-lick intervals during the pre-CS period in each session. We took the first lick after a pause as a lick-onset event, and we took the last lick before a pause as a lick-offset event. However, the beginning of suppression is actually the time of the first missing lick. Since licking is rhythmic, we estimated the time of the missing lick by taking the mode of the inter-lick interval distribution for each behavioral session and added that time to the lick-offset times.

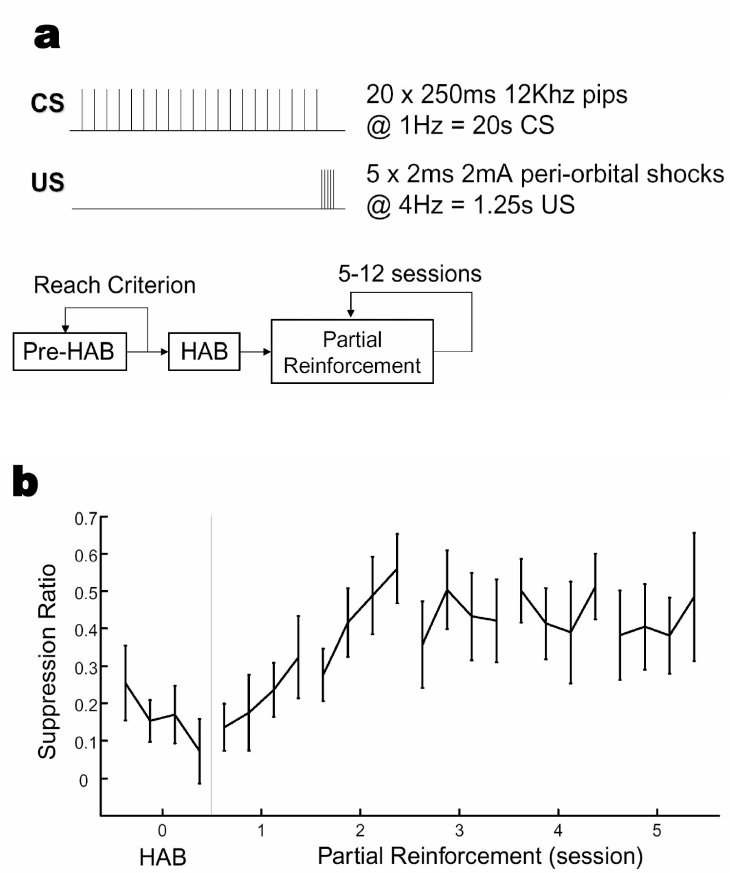


Figure 4.2. Behavior

A. Describes the conditioned (CS) and unconditioned (US) stimulis in the task. The CS was a train of 20 12Khz pips. Each pip was 250ms in duration and delivered at 1Hz. The US was a train of 5 2mA peri-orbital shocks delivered at 4 Hz. During pre-habituation (Pre-HAB) rats were placed in the recording chamber and given free access to a flavored sucrose beverage until they reached the criterion of one hour of consistent licking. The next day they returned to the chamber and received 20 CS-alone presentations. The following day partial reinforcement (PR) training began. During each session of PR there are 20 trials. Each trial consists of a CS followed by US either 30 or 50% of the time. B. Rats (n=29) showed asymptotic fear learning by the 2nd session of PR. Each point is the average of 5 trials and error bars indicate the standard error across animals. We only show the first 5 days of training, as all rats experienced 5 days, with extra days (up to 12) depending on the availability of neurons.

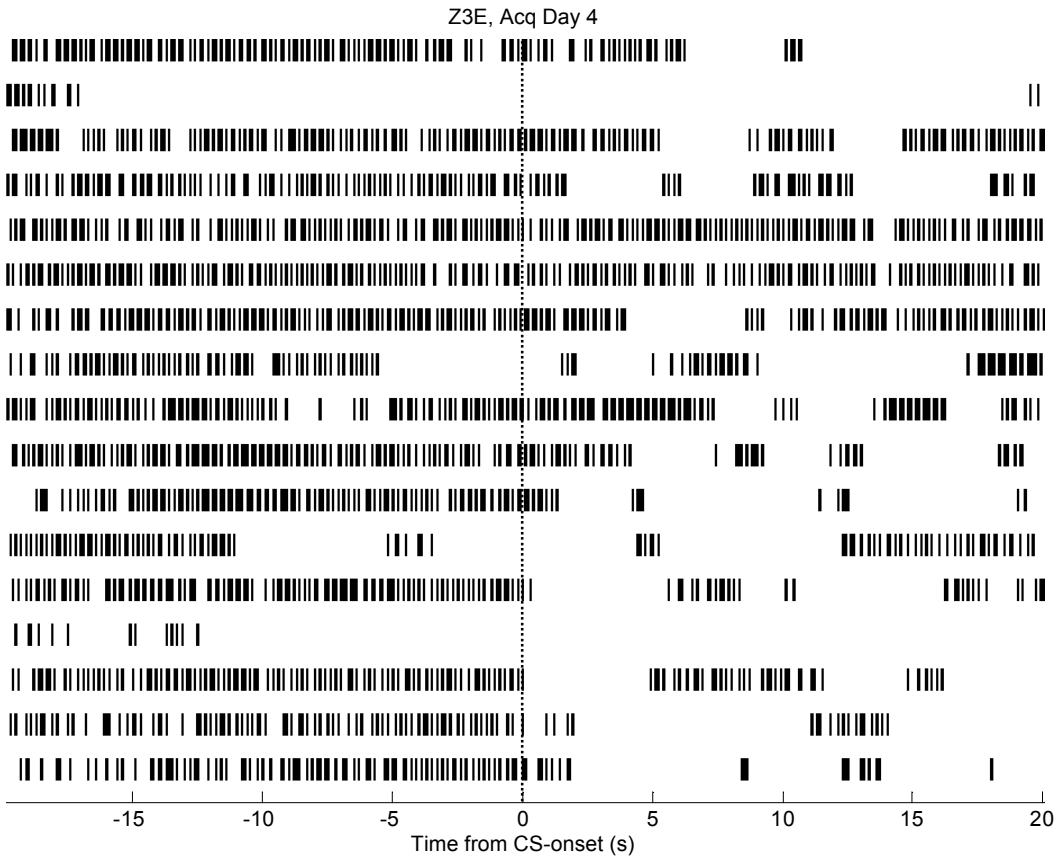


Figure 4.3. Lick Raster

An example lick raster from rat Z3E on the 4th day of PR. Black marks indicate individual licks. Each line of the raster is a trial. The dotted line indicates the onset of the CS. Note that there is clear CS-evoked suppression, but it is not 100% suppression. The animal switches back and forth from licking to suppression over the course of the 20 second CS.

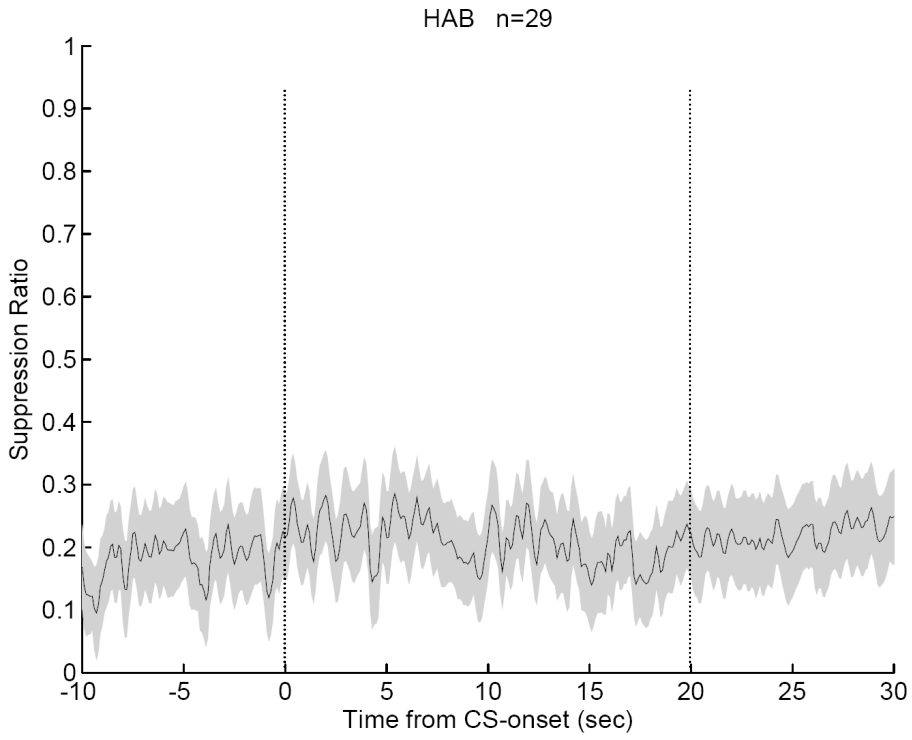


Figure 4.4. No suppression to the CS during habituation

Peri-stimulus time histogram of suppression ratio from 29 rats that underwent habituation training. Note that there is no change in suppression ratio at the onset or offset of the CS. Dotted lines indicate the onset and offset of the CS. Shaded area is the standard error across animals. Instantaneous suppression ratio was binned at 100ms and then smoothed with a gaussian kernel with a standard deviation of 100ms. (HAB, habituation)

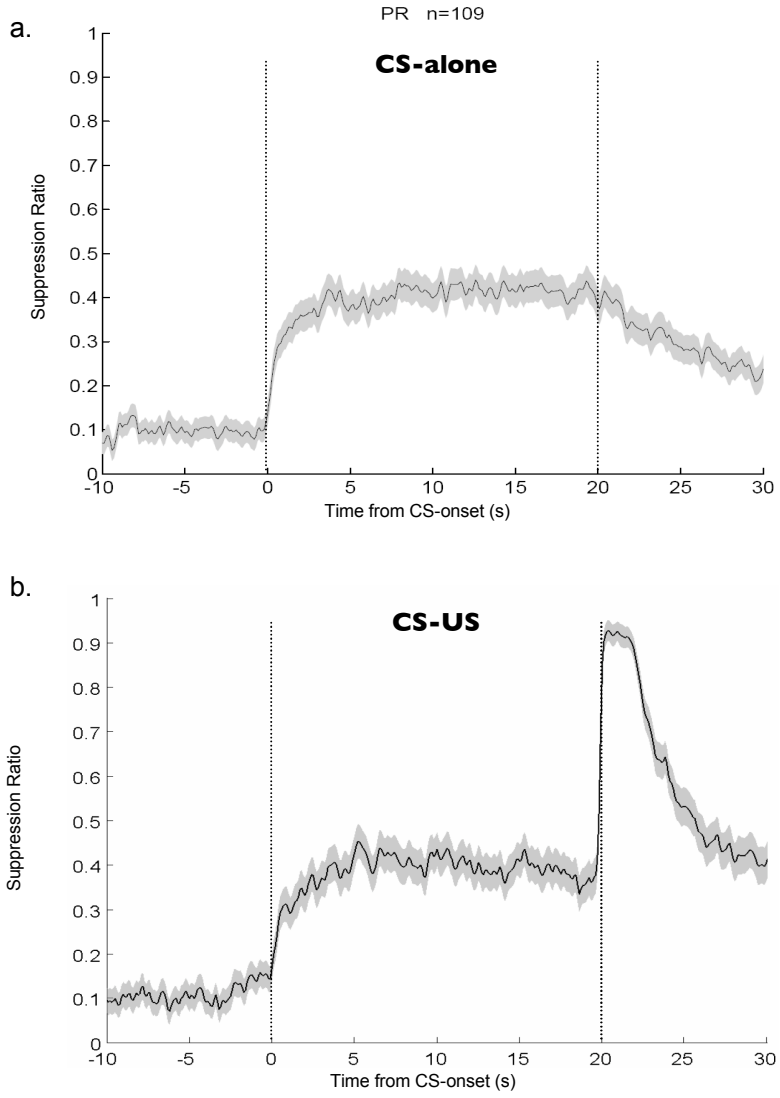


Figure 4.5. CS-and CS-US evoked suppression

a. Peri-stimulus time histogram of suppression ratio for all CS-alone trials. b. Peri-stimulus time histogram of suppression ratio for all CS-US trials. The US (delivered at the end of the CS) elicited total suppression for a few seconds. a.&b. Dotted lines indicate CS onset and offset. Line is suppression ratio binned at 100ms and then smoothed with a gaussian kernel with a standard deviation of 100ms. Shaded area is standard error across sessions. (PR, partial reinforcement)

4.4. Results

Behavior

Rats began to show fear responses to the CS, measured as lick suppression (Chapman and Sears, 2003), after the first CS-US pairing on the first day of PR, and reached asymptotic levels of suppression on the second day of PR. This level of performance was sustained for the duration of the experiment (5-12 days) (Fig. 4.2b). The suppression ratio, which measures the degree of behavioral inhibition induced by the CS, and thus presumably the level of fear, did not vary systematically by trial number (ANOVA, $p>0.8$). Suppression ratio reached asymptote, around 0.45, by the second day of PR. Thus, subjects were expressing intermediate levels of CS-induced fear. The key feature of this task is that the rats switch back and forth between licking and suppression over the course of a single 20-sec multi-pip CS (Fig. 4.3).

During habituation the CS did not evoke any suppression (figure 4.4). This was in contrast to the profound lick suppression evoked by the CS after conditioning (Fig. 4.5). Importantly, the CS-evoked suppression is indistinguishable between CS (figure 4.5a) and CS-US (figure 4.5b) trials verifying that the animals could not predict when the CS-US trials would occur. The US elicited substantial post-shock suppression (figure 4.5b), demonstrating the difference between the intermediate suppression evoked by a partially reinforced CS and the extreme suppression evoked by a periorbital shock.

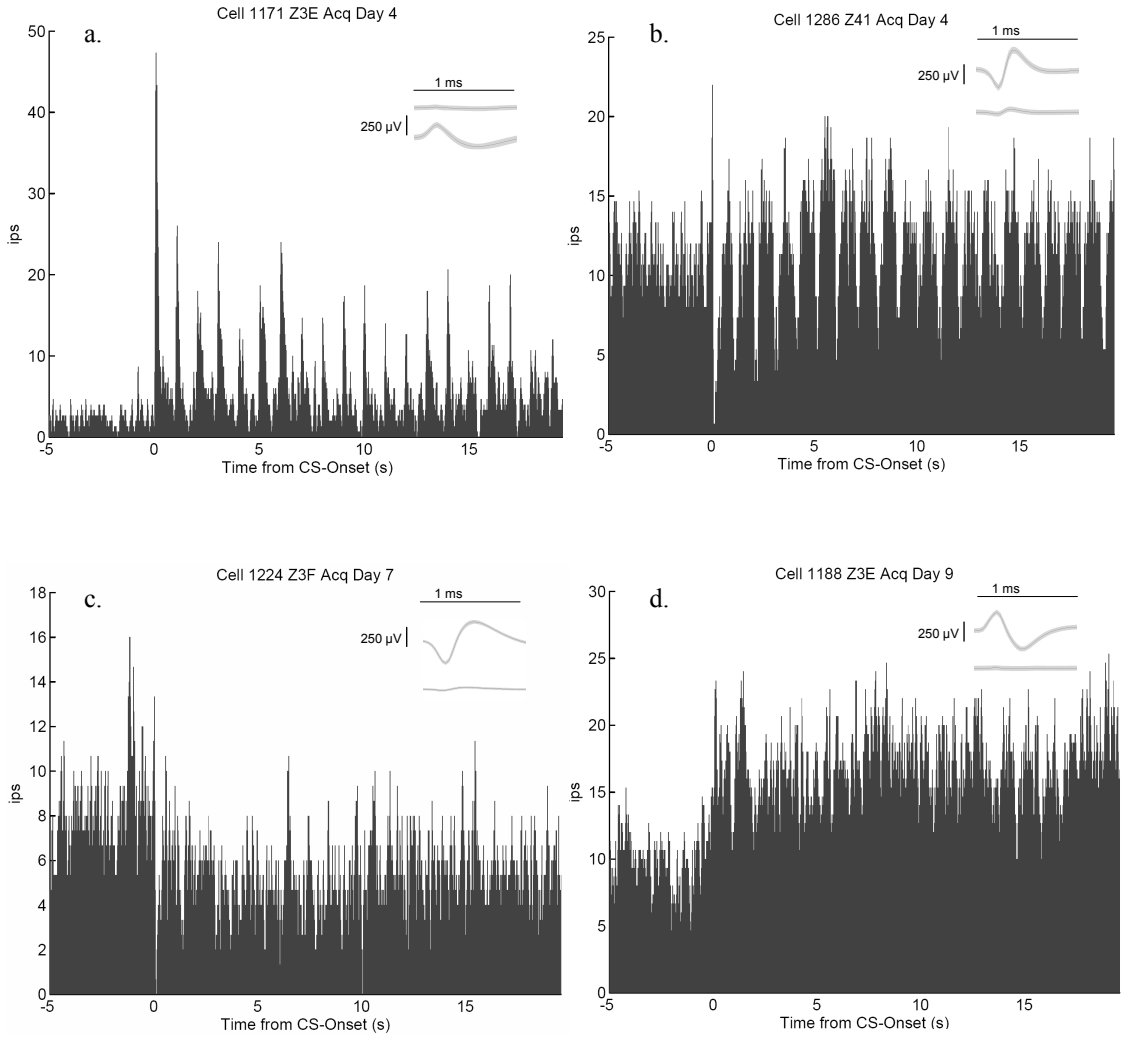


Figure 4.6. CS-evoked unit-activity

A-D. Examples of CS-evoked responses in mPFC. Time from CS-onset (in seconds) on the x-axis. Neuronal activity (impulses per second, ips) on the y-axis. Notice the different scales on the Y axis in each panel. Individual spikes were binned at 25ms and then smoothed with a 75 ms boxcar. Inset. Waveform (mean ±std. dev). Each graph is the average of 20 trials.

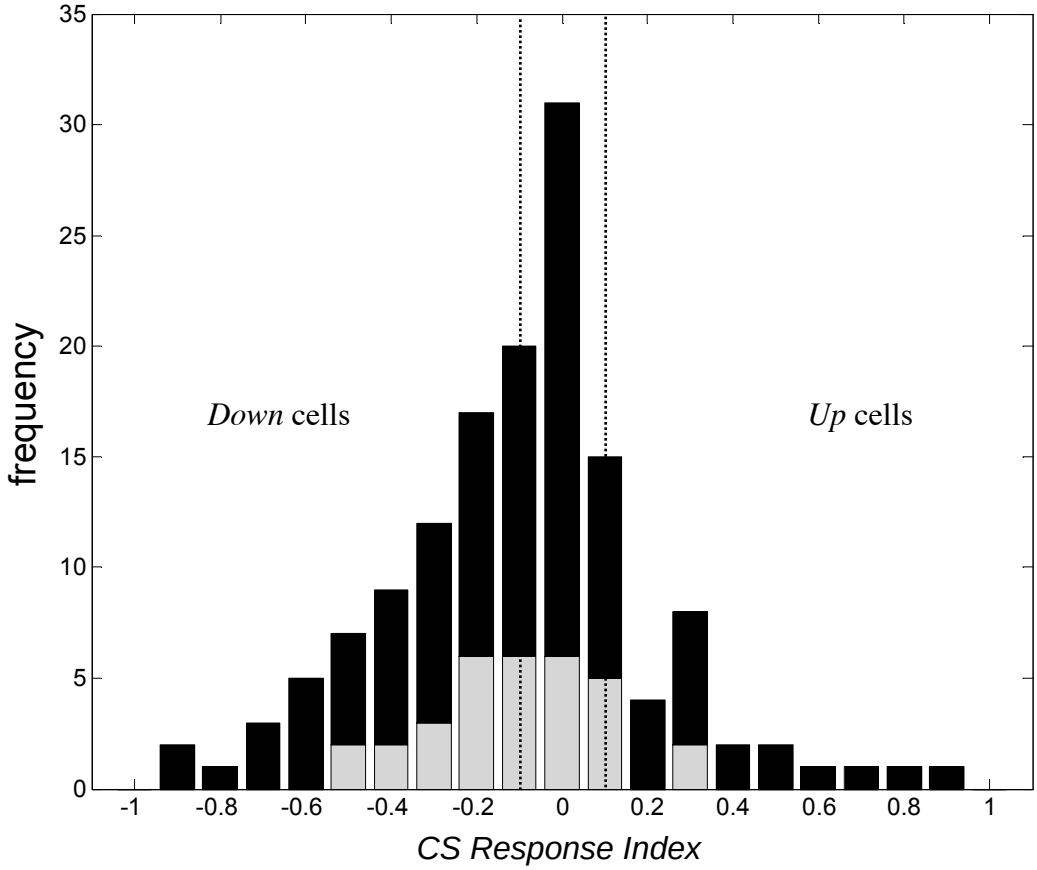


Figure 4.7. Distribution of CS response index

To parameterize the various CS-reponses we generated a CS response index (csri) for each cell. The CS-response index is positive for cells excited by the CS and negative for cells that are inhibited the CS. There are more cells that are inhibited by the CS. (*t*-test, T_{141}=-2.10, $p<0.05$). Light bars indicate distribution of cells from habituation. Black bars are cells from partial reinforcement. The variance of the distribution of csri for partial reinforcement is higher than the variance of the distribution for habituation indicating that fear conditioning increases the magnitude of the CS-response ($p<0.05$). The mean of the two distributions are not significantly different suggesting that fear conditioning does not change the direction of the CS-evoked neural response in mPFC, only the magnitude.

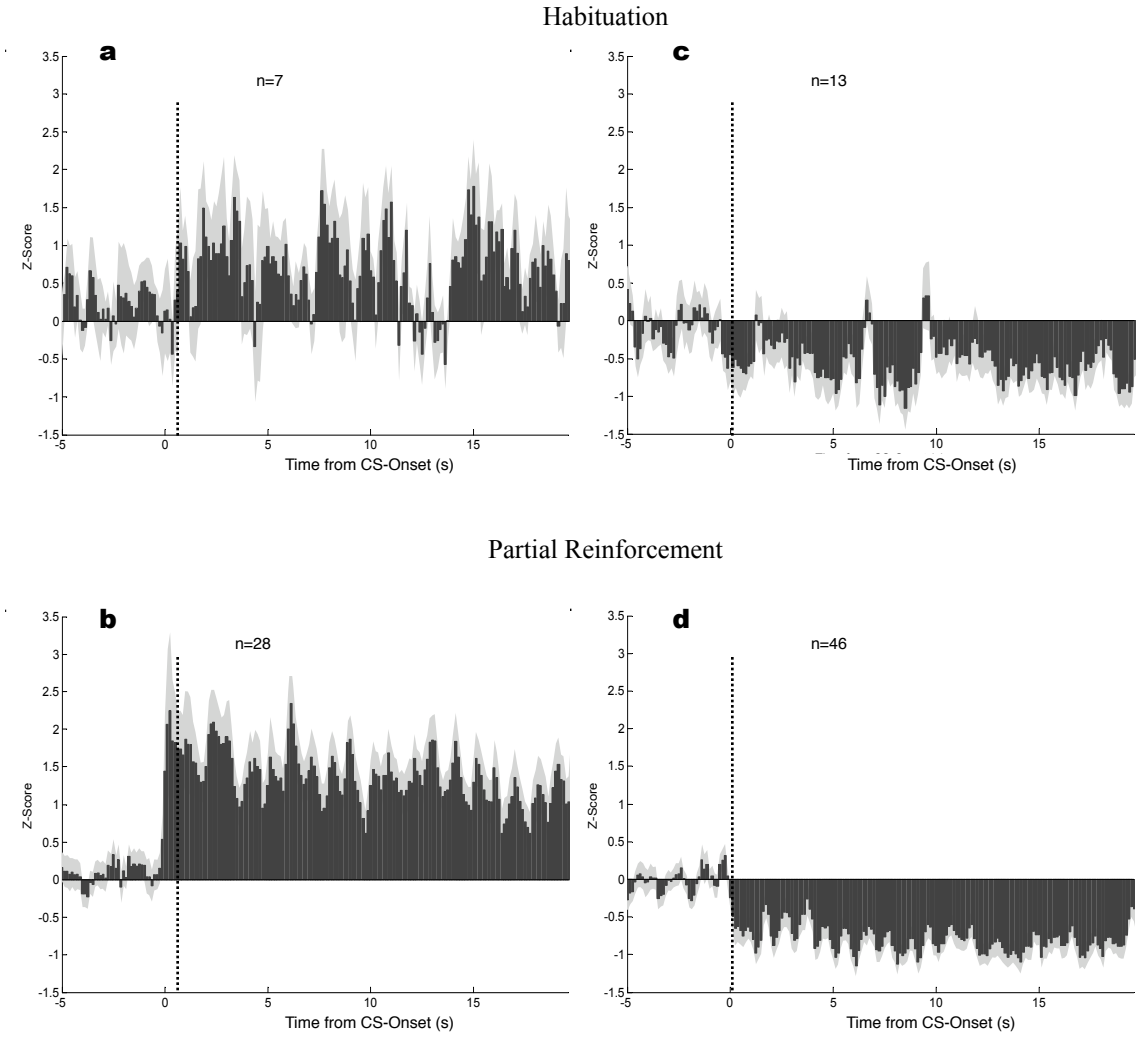


Figure 4.8. *Up* and *down* cells

A & B. *Up* cells from habituation (A, n=7) and from partial reinforcement (B, n=28). C & D. *down* cells from habituation (C, n=13) and from partial reinforcement (D, n=46). a-d,. Spikes were binned at 125ms then smoothed with a boxcar of 375ms. Individual neurons were Z-score normalized based on their pre-CS activity. The shaded area indicated standard error across cells.

Unit Activity

A total of 142 cells recorded from the mPFC of 15 rats were included in the analyses. The spontaneous firing rate of these neurons was 2.56 Hz with a range of 0.26-27.98 Hz, and a geometric mean of 1.50 Hz (1.31-1.71 Hz; 5-95% c.i.), consistent with previous studies (Baeg et al., 2001).

CS Response

The existing data on single-unit responses in the mPFC are scarce. Therefore, before proceeding to compare neural activity and behavior we characterized the CS-evoked activity in mPFC. The CS-evoked responses of mPFC neurons exhibited a high degree of heterogeneity. Figure 4.6 shows examples of different CS-responses. It is significant to note that some cells were strongly entrained to the individual pips (Fig. 4.6a, b), while other cells responded to the CS overall (Fig. 4.6c,d). In order to quantify the significance of the CS response in a manner that was unbiased with regard to the precise shape of the response, we generated a CS response index by taking the natural log of the ratio of the number of spikes in the CS and pre-CS period. If the cell fired more during the CS, then the index was positive and the cell was categorized as an *up* cell. If the cell fired less during the CS, then the index was negative and the cell was categorized as a *down* cell. Figure 4.7 shows the distribution of the CS response index for all cells, with cells recorded during habituation sessions

in light grey and cells recorded from partial reinforcement sessions in black. The distribution is significantly shifted toward negative values (*t*-test, T_{141}=-2.10, $p<0.05$).

To test whether fear conditioning had an affect on the CS-evoked response of mPFC neurons we compared the variance of the distribution of CS response index from HAB and PR. We found that the variance of the PR distribution is significantly larger (HAB variance=0.038; PR variance=0.110). We determined the 95% confidence intervals for each distribution using a bootstrap technique. The 95% confidence intervals of the variance for HAB were [0.022, 0.054]. The 95% confidence intervals of the variance for PR were [0.077, 0.143]. There was no overlap, thus we concluded that these distributions are significantly different ($p<0.05$) indicating that fear conditioning increased the variance of the CS-evoked responses. That is to say, after conditioning the responses of the mPFC neurons to the CS were more pronounced.

We divided the neurons into *up*-cells and *down*-cells based on their CS response. We picked a threshold CS response index of ±0.092. A third of the population, centered around zero, failed to exceed this threshold. Neurons with a CS-response index > 0.092 were classified as *up*-cells (Fig. 4.8a,b). Neurons with a CS-response index < -0.092 were classified as *down*-cells (Fig. 4.8c,d).

Suppression as a function of spike rate

We were interested in testing the relationship between neural activity and behavior over two distinct temporal windows: between trials and within trials. The variability in behavior between trials might be explained by the reinforcement history. However, the behavior during a single CS is subject to the same reinforcement history, so the within-trial variability cannot be accounted for by reinforcement history and learning. In this way, the trial-to-trial correlation between neural activity and behavior corresponds somewhat to the findings from experiments focused on fear learning (Baeg et al., 2001; Milad and Quirk, 2002; Phelps et al., 2004), whereas the within-trial correlation corresponds to experiments of regulation of fear that is unrelated to learning (Giorgi et al., 2003).

To address the relative contribution of individual neurons to behavior we performed stepwise regression with three independent variables: the reinforcement

% cells , p<0.05	$\beta_{between}$	β_{within}
HAB	66% (21/32)	13% (4/32)
PR	56% (62/110)	27% (30/110)

Table 4.1 Percent of cells that accounted for a significant portion of behavior variability

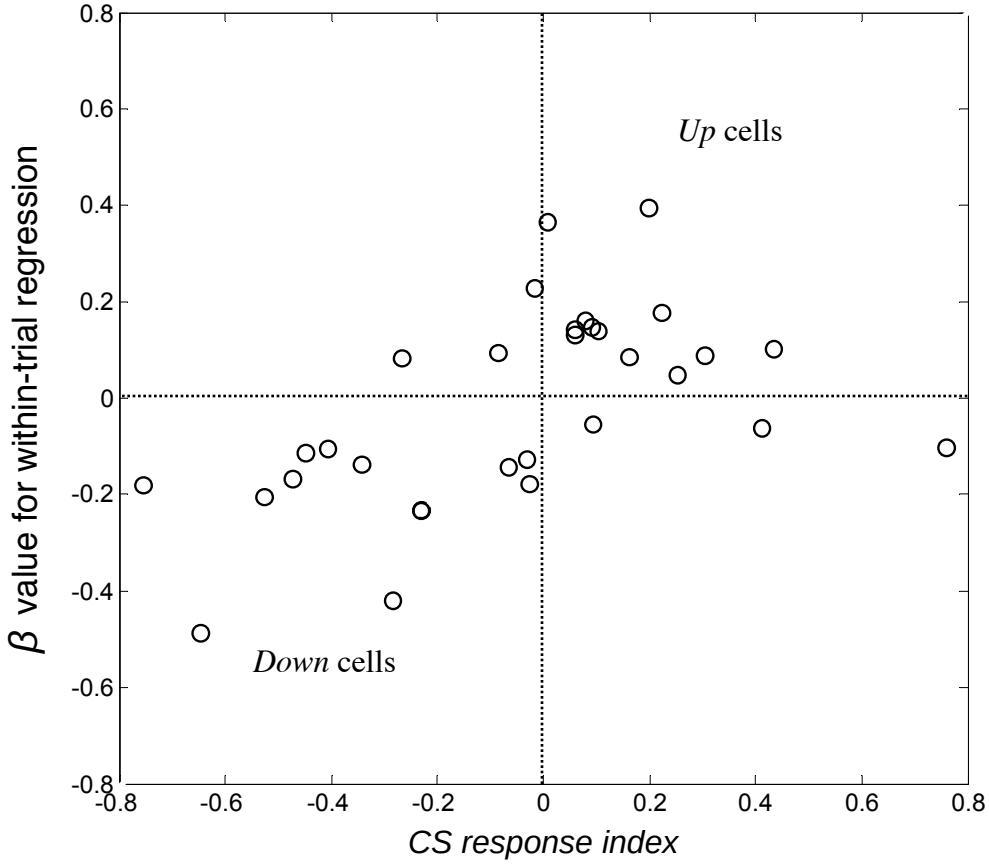


Figure 4.9. Scatterplot of β_{within} vs. *CS response index*

Each dot represents a cell from partial reinforcement whose within-trial neural activity accounted for significant amount of variance in within-trial behavior. β_{within} is the regression coefficient, plotted against the *CS response index* of that neurons. The CS-evoked response is correlated with the covariance between behavior and neural activity ($r=0.547$, $p<0.005$, $n=30$). *Up* cells covary positively with suppression and *down* cells covary negatively with suppression.

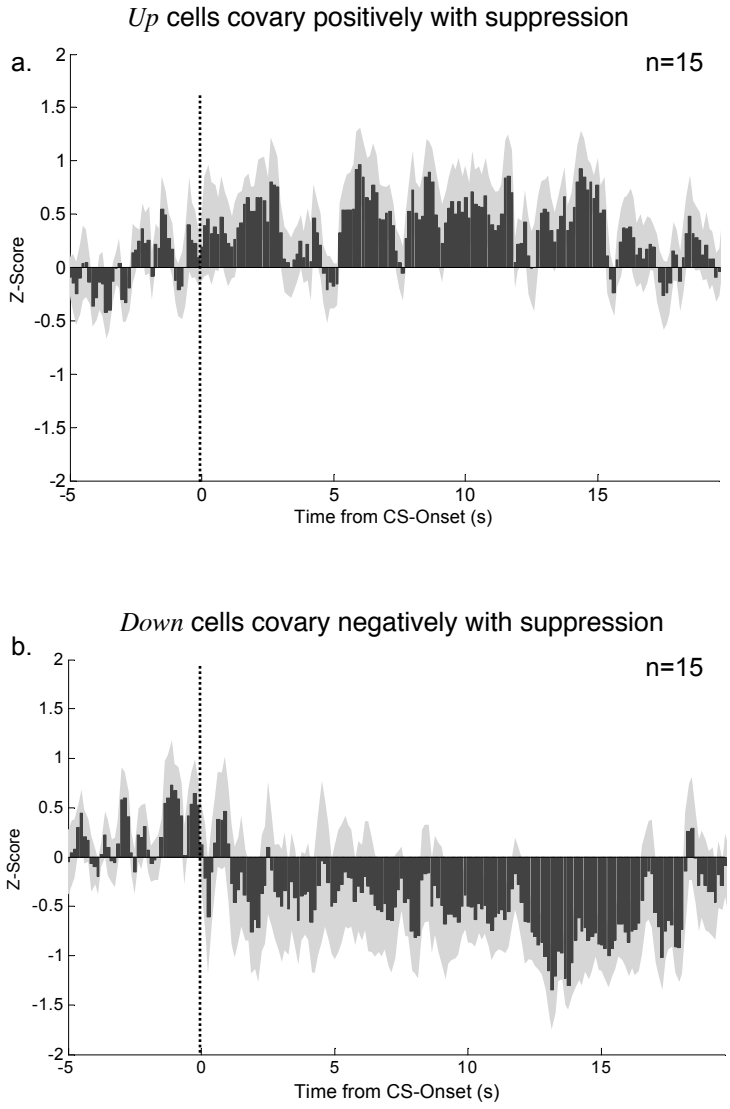


Figure 4.10. CS-Response of cells with significant β_{within}

A. Averaged CS-response of cells where $\beta_{within} > 0$ recorded during partial reinforcement (n=15). B. Averaged CS-response of cells where $\beta_{within} < 0$ recorded during partial reinforcement (n=15). A&B. Spikes were binned at 125ms then smoothed with a boxcar of 375ms. Individual neurons were Z-score normalized based on their pre-CS activity. The shaded area indicates standard error across cells. The dotted line indicates the onset of the CS

history, the average spike rate per trial and the spike rate per second. We performed regression analysis on each of these predictors in order, using the residuals of the previous regression (Eq. (4.6-4.8)). The reason for the stepwise regression is that an apparent correlation between neural activity and behavior could be a correlation between neural activity and the CS and between the CS and behavior. By first regressing out the effects of reinforcement history (Eq. (4.7)) and then of between-trial neural activity (Eq. (4.8)) we increased the confidence that a correlation between within-trial neural activity and behavior is not due to any spurious correlation generated by an external variable, like reinforcement history.

We found that in 56% (62/110) of cells recorded during PR and in 66% (21/32) of cells recorded during HAB the between-trial neural activity ($X_{between}$) accounted for a statistically significant proportion ($\beta_{between}$) of the variance in the behavioral measurements after removing variance accounted for by reinforcement history (ρ_1). In 27% (30/110) of cells recorded during PR and 13% (4/32) of cells recorded during HAB within-trial neural activity (X_{within}) accounted for a statistically significant proportion (β_{within}) of the remaining variance in the behavior (ρ_2) . In 21% (23/110) of cells from PR and 9% (3/32) cells from HAB the neural activity accounted for a statistically significant proportion of the variance for both between-trial (ρ_1) and within-trial variability (ρ_2) in behavior (Table 4.1). There was a significant correlation between $\beta_{between}$ and β_{within} for cells in PR ($r=0.54$, $p<0.00001$), but not in HAB ($p>0.1$).

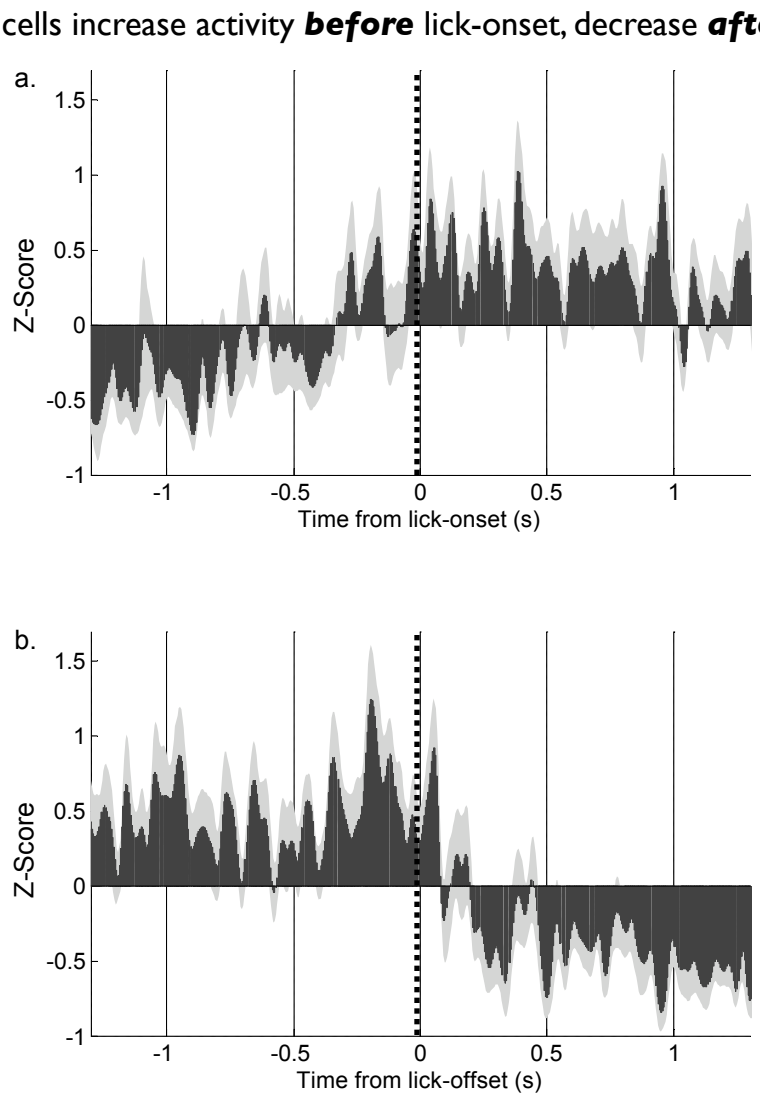


Figure 4.11. Behavior-Triggered Spike Histograms - $\beta_{within} < 0$

A. Population lick-onset spike histogram. Neural activity is aligned to lick-onset. Neural activity increases well before behavior change. B. Population lick-offset spike histogram. Neural activity is aligned to lick-offset. Neural activity decreases after behavior change. A&B. Spikes were binned at 5ms then smoothed with a Gaussian with std dev of 15ms. Individual neurons were Z-score normalized based on their pre-CS activity. The shaded area indicates standard error across cells. The subset of neurons used to generate this figure were those in figure 4.10b (n=15). The heavy dotted line indicates the time of the behavioral trigger.

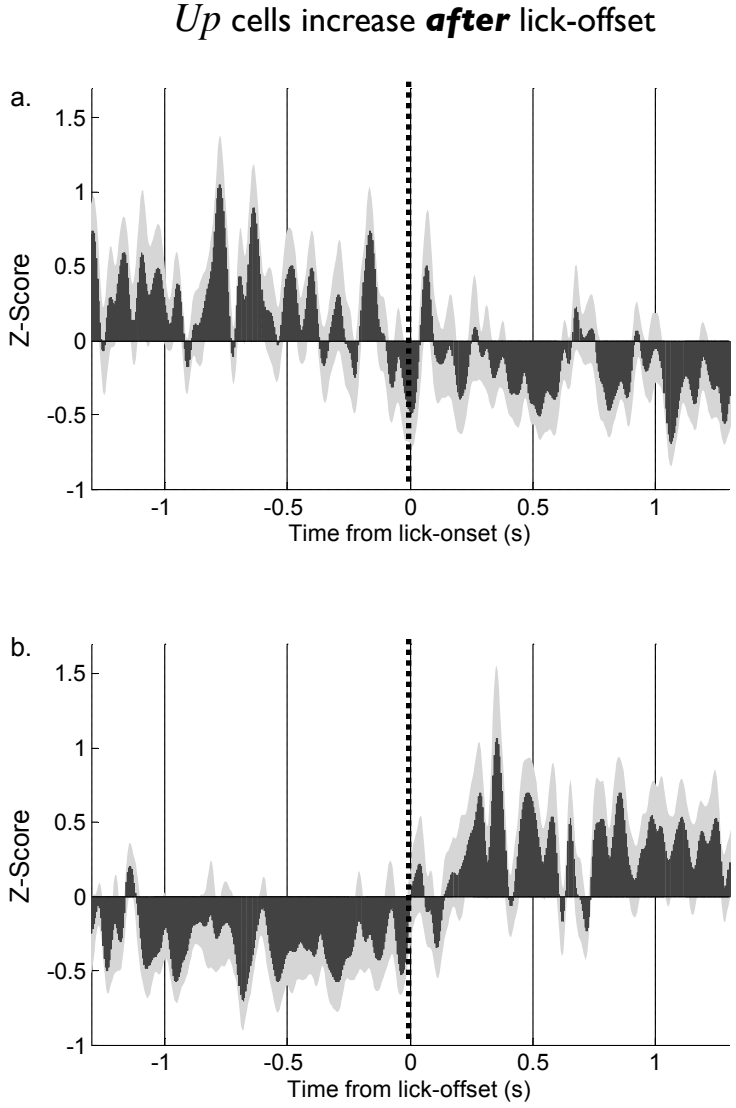


Figure 4.12. Behavior-Triggered Spike Histograms - $\beta_{within} > 0$

A. Population lick-onset spike histogram. Neural activity is aligned to lick-onset. Neural activity is not well aligned to the behavior change. B. Population lick-offset spike histogram. Neural activity is aligned to lick-offset. Neural activity increases after behavior change. A&B. Spikes were binned at 5ms then smoothed with a Gaussian with std dev of 15ms. Individual neurons were Z-score normalized based on their pre-CS activity. The shaded area indicated standard error across cells. The subset of neurons used to generate this figure were those in figure 4.10a (n=15). The thick dotted line indicates the time of the behavioral trigger.

We then tested if the covariance between activity and behavior was related to whether a cell was an *up* cell or *down* cell. Indeed, the direction of the CS response was recapitulated by the cells relationship with behavior ($r=0.547$, $p<0.005$) (Fig. 4.9). The spike rate of *down* cells was inversely correlated with suppression (Fig. 4.10b). The spike rate of *up* cells was positively correlated with suppression (Fig. 4.10a). Thus, *up* and *down* cells mirror each other. For all cells, larger excursions of neural activity from baseline are correlated with lick suppression. In other words, *up* cells fire more during suppression and *down* cells fire less during suppression.

Behavior-triggered spike histograms

Now that the cells whose activity accounted for a significant amount of the variance in behavior have been identified, the main hypothesis of this paper, that changes in neural activity will precede inhibition of suppression, can be tested. To test the hypothesis we generated lick-offset-triggered spike histograms and lick-onset-triggered spike histograms for all the cells with significant β_{within}. For simplicity, we refined the definition of *up* and *down* cells to include only those cells that accounted for a significant portion of the within-trial behavioral variance (Figure 4.9). The neural activity of *down* cells, those inversely correlated with suppression ($\beta_{within} < 0$), increased preceding the lick-onset event (Fig. 4.11a). In contrast, the change in firing rate of these neurons lagged the lick-offset event (Fig. 4.11b). This suggests that these

cells could have contributed to the resumption of licking, but not the generation of suppression.

For *up* cells, those positively correlated with suppression ($\beta_{within} > 0$), the data was more ambiguous (Fig. 4.12). Up until 400ms before lick-onset the normalized neural activity was substantially positive, and 400ms after the lick-onset event the neural activity was substantially decreased, but the change was not strongly time locked to lick-onset. For lick off-set the change in relative firing rate was over a shorter period. The shift from relatively low spike rate to relatively high spike rate occurred 0-200ms after lick-offset. Thus, even though the same number of neurons covaried positively and negatively with behavior, the downstream effect of the mPFC was the inhibition of suppression, since the timing of the change in spike rate for both *up* and *down* cells preceded lick-onset and followed lick-offset.

4.5. Discussion

We found two classes of neurons in mPFC, *up* cells and *down* cells, based on the cells responses to the CS and also their covariance with behavior. We provided the first evidence that fear conditioning increased the variance of these responses across the population. Specifically, fear conditioning increased the inhibition in cells inhibited by the CS and increased excitation in cells excited by the CS (Fig. 4.7). We then performed a series of regressions to select a sub-population of neurons whose

responses exhibited significant correlations with behavior. We found that the strength of the interaction, β_{within}, of within-trial neural with behavioral activity was significantly correlated with the CS response for each cell, further justifying the classification of cells as *up* and *down* cells.

The strongest evidence in support of the hypothesis that the mPFC is indeed a key structure in the regulation of fear was the demonstration that single-unit activity changed before lick-onset events but after lick-offset events (Figs 4.11 & 4.12). This was clearest in the *down* cells which accounted for a significant portion of the within-trial variance. The firing rate of these cells was inversely correlated with the expression of fear and these cells switched from low activity to high activity before lick-onset (Fig. 4.11a). This last analysis broke down the apparent symmetry between the *up* and *down* cells. This symmetry refers both to the CS-evoked unit responses and also the results of the regression analysis where we found an equal number of cells that covaried positively and negatively with behavior (Figure 4.9). While this symmetry is an interesting phenomena it does not support the hypothesis that the mPFC inhibits fear expression. It was only by analyzing the dynamics of the neural activity with respect to the behavioral switches that the symmetry broke down, since lick-onset but not lick-offset was preceded by an increase in activity in the mPFC.

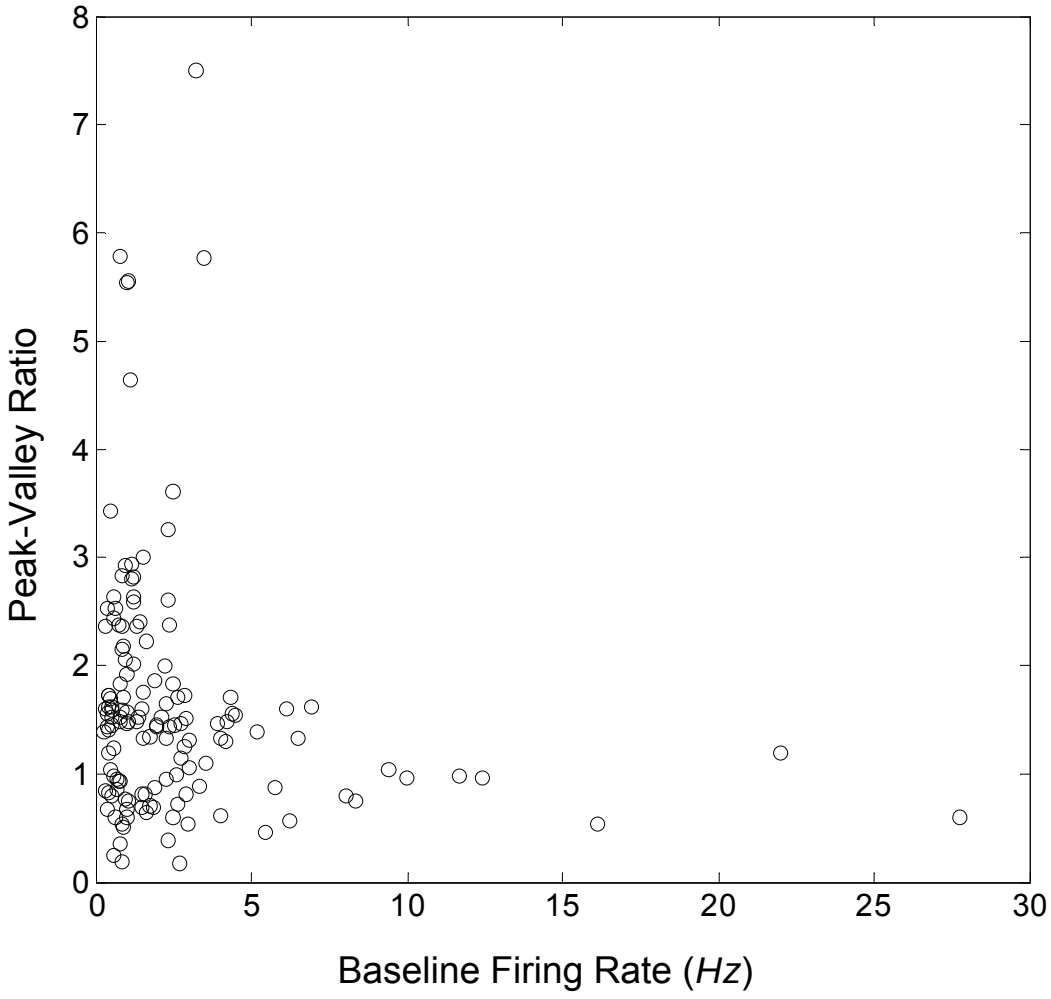


Figure 4.13. Peak-Valley Ratio vs. Baseline Firing Rate

There is weak significant correlation between firing rate and the peak- valley ratio of the waveform ($r=-0.176$, $p<0.05$). Cells with large after-hyperpolarizations tend to be faster spiking. However, we do not see a basis for distinct classes of cells based on this scatterplot. Moreover, we do not see correlations between baseline firing rate and any other measure of the CS-response or relation to fear behavior.

CS-evoked responses to fear conditioned stimuli

Only two previous studies recorded single-unit activity during fear conditioning in awake rats (Baeg et al., 2001; Milad and Quirk, 2002). The first study, from Baeg et al, also used partial reinforcement and recorded neurons after the CS-US relationship was well-learned (after a minimum of 4 days of training). They found cells that were excited and cells that were inhibited by the CS, consistent with the data here. They classified mPFC units as fast and regular spiking, based on the baseline firing rate and the peak-valley ratio of the waveforms. We also found a correlation between baseline firing rate and peak-valley ratio ($r=-0.176$, $p<0.05$), but there was no clear separation between fast and regular spiking cells (Figure 4.13). Interestingly, with our pulsiform CS, we could distinguish the response to individual pips from the response to the CS overall. For example, we observed cells that were inhibited by the pip but were excited by the CS (Fig. 4.6b). We found that the peak-valley ratio was significantly correlated with the pip response ($r=0.219$, $p<0.01$). We did not focus on this since we did not find any meaningful relationship between pip response and behavior.

Baeg et al. did not find any correlations between trial-to-trial unit activity and behavior. Perhaps the reason for this was their behavioral paradigm. In our task, the rats had access to kool-aid, which was a strong incentive and produced robust differences between pre-CS and CS behavior (Figure 4.5). Baeg et al. measured freezing and their data indicated substantial pre-CS freezing which was only slightly

modulated by the CS. This could explain why we found strong connections between neural activity and behavior while they found none. Moreover, this pattern of results suggest that the auditory afferents and the affective afferents to mPFC are not strictly correlated. That is, the sensory response does not define the affective response for a neuron in mPFC. We used a pulsiform CS to avoid sensory adaptation over the 20 second CS period. Fortuitously, it allowed for an interesting distinction between sensory and affective responses of mPFC neurons, which may have been obscured by a continuous tone CS.

The second study of single-unit activity and fear conditioning found a very different pattern of results based a very different training paradigm. Milad & Quirk (2002) recorded neurons during a 2-day experiment with acquisition and extinction on the day one, followed by a second extinction session on day two. In this task they did not report any significant CS responses on day one of the experiment. Only on day 2 did they observe responses that correlated with the memory of extinction, and only in infralimbic cortex. Our population of neurons was mainly in prelimbic cortex (114/142) with some recordings in infralimbic cortex (28/142). However, we did not find any analysis that distinguished cells in the two adjacent regions, therefore it is unlikely this discrepancy is due to different recording sites.

Milad & Quirk (2002) did not present the distribution of CS-evoked responses, only the averaged population response. It is possible that by viewing the population as unimodal they averaged out their excitatory and inhibitory responses. Nonetheless,

our finding that changes in population activity preceded lick-onset is consistent with Milad & Quirk's finding that increased activity in infralimbic cortex corresponded to decreased fear behavior.

There are two other studies that have recorded neural activity from the mPFC during fear learning. In one, fear conditioning was reported to result in CS-evoked increases in firing rates of single-unit activity in the mPFC of anesthetized rats (Laviolette et al., 2005). It is possible that the anesthesia used, chloral hydrate, affected the population in a biased manner, possibly via agonism of the 5-HT3 receptor (Solt and Johansson, 2002), such that only the increase in excited cells was observed. Agonists of the 5-HT3 receptor have been shown to be positive modulators of fear in the mPFC (Yoshioka et al., 1995). The second study reported only CS-evoked inhibition of multi-unit activity in awake mice (Garcia et al., 1999). In our sample, *down* neurons significantly dominate the distribution (Figure 4.6), so it is conceivable that multi-unit recording would only observe this feature of the mPFC activity. Consistent with this, the multi-unit activity in the mPFC of mice was negatively correlated with fear behavior, analogous to the *down* cells presented here.

Relation of mPFC single-unit activity to behavior

We observed that for the majority of neurons in mPFC neural activity accounted for a significant amount of the variance in behavior even after removing variations in behavior that were due to reinforcement history. Taking the

reinforcement history into account was essential for a clear interpretation of the correlation. Without regressing out this factor one could not distinguish whether an apparent correlation between neural activity and behavior was actually a correlation between neural activity and the CS and between the CS and behavior.

By aligning the single-unit activity with the lick-onset and -offset events we presented evidence that suggests that as a population the *down* cells were driving the lick-onset event. This is supported by previous experiments that have demonstrated that microstimulation of mPFC accelerates extinction (Milad and Quirk, 2002; Milad et al., 2004), stimulation of mPFC inhibits the amygdala (Rosenkranz and Grace, 2002a, 2002b) and that behavioral consolidation of extinction depends on long-term potentiation in the mPFC (Herry and Garcia, 2002).

Summary

Human and animal studies suggest that the mPFC plays an important role in the extinction of fear conditioning. Here, we examined the role of the mPFC in the regulation of fear. We specifically performed analyses to eliminate the effects of learning on our examination of how the dynamics of mPFC activity related to the expression of fear behavior, specifically lick suppression. We found *up* cells, that were excited by the CS and that covaried positively with fear and *down* cells, that were inhibited by the CS and covaried negatively with fear. We then aligned the unit activity in the mPFC with the behavioral switches from licking to suppression and

observed that changes in neural activity preceded the onset of licking and followed the onset of suppression. This is consistent with the view that the mPFC contributes to the inhibition of fear expression.

5. General Discussion

5.1. In a nutshell

I have modified the typical fear conditioning paradigm to study the neural basis of the regulation of fear. This modified task pits fear against the desire to drink Kool-Aid. By carefully adjusting the degree of fear elicited by the conditioned stimulus, I observed behavior switches between suppression and drinking. This behavioral switch is assumed to be a proxy for an internal process of fear regulations.

Chapter 2 illustrated, using pharmacological manipulation, that inactivation of the lateral amygdala resulted in a failure of the conditioned stimulus to elicited suppression. As well, excitation of the lateral amygdala resulted in persistence of elicited suppression. Moreover, the data in chapter 2 provided further evidence against the view that the amygdala is primarily dedicated to modulation of the consolidation of memory. The finding is significant insofar as it is the first to show the continued involvement of the amygdala in processing a fear conditioned stimulus well after the initial acquisition of fear conditioning using pharmacological manipulation of neural activity.

Chapter 3 illustrated, using single-unit recording from behaving rats, that neural activity in the lateral amygdala covaried with fear behavior at sub-second timescales. Specifically, the neural activity of individual neurons increased before the onset of suppression and decreased before the onset of licking. This suggests that the

neurons in LA signaled changes in fear rather than fear *per se*. While electrophysiological recording is strictly observational, the demonstration that neural activity in the lateral amygdala ramped up before the onset of suppression suggests that these neurons were triggering that event.

Chapter 4 illustrated, using single-unit recording from behaving rats, that neural activity in the medial prefrontal cortex covaried with fear behavior at sub-second timescales. In contrast to neurons in the lateral amygdala, which seemed to encode change in fear, the neurons in the medial prefrontal cortex covaried directly with the behavioral state of the animal, which suggests that they encoded the level of fear *per se*. There were two classes of neurons in the medial prefrontal cortex. *Up* cells were excited by the fear conditioned stimulus. The spike rate of these cells was positively correlated with suppression. *Down* cells were inhibited by the fear conditioned stimulus. The spike rate of these cells was negatively correlated with suppression. In the population of cells recorded, there were significantly more *down* cells. Aligning the neural activity to the onset and offset of fear suggested that as a population the medial prefrontal cortex may be driving the offset of suppression, but not the onset of suppression.

Together, these data support the use of this task as a tool for studying the neurobiology of fear regulation. The use of a conditioning paradigm which resulted in intermediate levels of lick suppression provided a precise temporal measure of the onset and offset of fear which proved effective at probing the role of the LA and

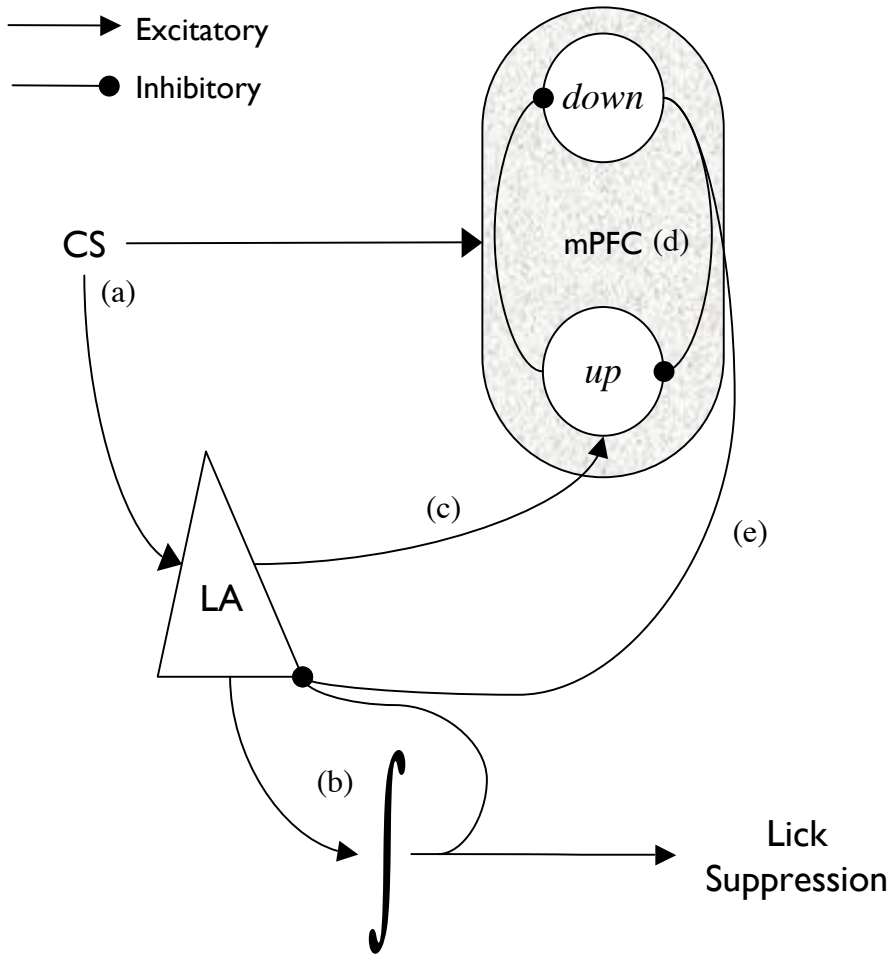


Figure 5.1 Model of LA-mPFC interactions in fear regulation

A. The CS drives activity in the LA and the mPFC. B. The activity in LA encodes changes in suppression. Modulation of this activity results in a concomitant modulation of suppression. Integration of this signal maintains suppression for the duration of the CS. C. The activity in the *up* cells reflects the moment-to-moment behavior. D. The activity of *down* cells in mPFC mirror the activity of *up* cells. E. The activity in *down* cells inhibits suppression either via inhibition of LA or via an unknown mechanism.

mPFC in the regulation of fear.

5.2. A model of the LA-mPFC interaction in fear regulation

Put together, the results presented in this thesis suggest that the role of the LA-mPFC circuit (Fig. 5.1) in fear regulation fits well with the extensive literature from fear conditioning (Fig. 1.1). In the introduction, I described the reciprocal relationship between the LA and the mPFC in fear learning and fear regulation. However, the individual chapters dealt exclusively with one or the other structure. The circuit in figure 5.1 outlines a potential model of LA-mPFC interaction in fear regulation that puts the data from the individual chapters together. In the model, a familiar CS evokes activity in the LA (Fig. 3.2-right) and in the mPFC (Fig. 4.6a,b). The activity in LA triggers changes in suppression (Fig. 3.3). The CS-evoked response in the LA was transient (Fig. 3.2c) but the suppression was sustained for the duration of the CS. Therefore, in the model, the LA output is integrated to maintain suppression for the duration of the CS. In the model, the activity in LA drives activity in the mPFC excitation in *up* cells and inhibition in *down* cells (Fig. 4.6c,d). This connection in the model is supported by lesion and inactivation experiments that demonstrate that conditioned responses in the mPFC depend on the integrity of the LA (Garcia et al., 1999; Laviolette et al., 2005). Finally, in the model increases in activity in *down* cells inhibit suppression via inhibition of the LA (Fig. 5.1e). This may be via direct

inhibition of cells in the LA (Rosenkranz and Grace, 2002) or via projections to the intercalated cell masses (Quirk et al., 2003). The rest of this section (Section 5.2) will discuss specific aspects of the model in greater depth.

Adaptive coding in LA

I have proposed that the LA and the mPFC play reciprocal roles in the regulation of fear. However, the relationship between behavior and neural activity is not mirrored in these two structures. Rather, activity in the LA encodes changes in suppression as opposed to activity in the mPFC, which encodes the degree of suppression *per se*. This implies that the neurons in the lateral nucleus utilize an adaptive code for fear. Adaptive codes have been observed in sensory (Hosoya et al., 2005), motor (Sparks, 2002), and reward systems (Tobler et al., 2005). This is the first indication that the LA also uses an adaptive code in the regulation of fear. The advantage of an adaptive code is that it is efficient (Barlow, 1961). It is efficient in the energetic sense, since a neuron need only fire to signal a change in fear, as opposed to having an increased firing rate for the entire duration of the fear (Fig. 3.2c-right). An adaptive code for fear is efficient also in the sense that it can effectively signal a change across a theoretically infinite range of fear states. In contrast, a linear code for fear would be capped by the biophysical limitations of a neuron.

There are several possible reasons why adaptive coding in the LA has not been previously observed. Studies that use a continuous tone as a conditioned stimulus generally observe strictly onset responses (Maren, 2004; Goosens et al., 2003). The

onset response could be interpreted as sensory adaptation, since some neurons in the auditory thalamus that project to the lateral amygdala also show this phenomenon (Bordi and LeDoux, 1994; Doron and Ledoux, 1999; Doron and Ledoux, 2000). Previous studies that have used a pulsiform stimulus, like the one used here, have used measures of behavior (freezing and bar-press suppression) that do not allow for the fine-grained analysis of changes in behavior during the conditioned stimulus. Using lick suppression was a technical advance that permitted the fine grained analysis of behavior. This was essential for the observation that short time scale variability in neural activity triggers the onset of the suppression.

Since the CS-response of neurons in LA was transient, the model proposes that this signal was be integrated somewhere to drive sustained behavior (Fig. 5.1b). The central nucleus of the amygdala is an obvious candidate. The LA projects to the central nucleus (Pitkanen, 2000), which projects to the brainstem areas that control freezing (LeDoux et al., 1988). Inactivation or lesions of the central nucleus attenuate expression of freezing and conditioned suppression (Killcross et al., 1997; Amorapanth et al., 2000; Goosens and Maren, 2001; Nader et al., 2001). Unfortunately, there are few recordings from the central nucleus *in vivo,* and all of the studies used short (less than one second) stimuli (Applegate et al., 1982; Pascoe and Kapp, 1985; Rorick-Kehn and Steinmetz, 2005). Future experiments recording the responses of central amygdala neurons in awake rate using a long CS, like the one

used here, are required for evaluating whether the central amygdala is the site of integration.

Sustained activity in the mPFC

The CS-evoked responses in mPFC were sustained (Fig. 4.6, 4.8b). This was in stark contrast to the responses in the LA, which were transient. Given that the CS-evoked responses in the mPFC were sustained, why does the input to the mPFC, in the model, come from the LA and not from the integrated signal? The answer to this is related to the redundant encoding of suppression in the mPFC: *up* cells and *down* cells apparently encoded the same signal, only with a change in sign.

The observation that some cells, *up* cells, covaried positively with suppression and other cells, *down* cells, covaried negatively with suppression is similar to another finding from prefrontal cortex. Romo et al. (1999) described two classes of neurons in monkey prefrontal cortex which had opposite encodings of the frequencies of mechanical vibrations. The firing rates of one class, like the *up* cells, covaried positively with the frequency of the stimuli. The firing rates of the second class, like the *down* cells, covaried negatively with the frequency of the stimuli. More recently, the same group developed a model that demonstrated that those two classes of cells can be configured as an attractor network that maintains sustained firing rates in response to an transient input (Miller et al., 2003; Machens et al., 2005).

Building on the work by Machens et al. (2005), my hypothesis is that the *up* and *down* cells also instantiate an attractor network via mutual inhibition (figure 5.1d)

which transform the transient signal from the LA into one that is sustained. In this case, the network has a single attractor which represents the firing rate of the neurons in the mPFC during the absence of suppression, for example, in the pre-CS period. Following the model, when the CS is presented it drives activity in the LA which drives activity in the *up* cells in the mPFC, pushing the network away from the attractor. The increase in activity in *up* cells causes an inhibition in *down* cells which attenuates the inhibition of the LA (figure 5.1e). The dynamics of the network are slow, assuming the time to resume licking after the termination of the CS can be used as an estimate (figure 4.5a). Nonetheless, as the network moves back towards the attractor the activity of the *down* cells increase which inhibits the activity in the LA, allowing for the resumption of licking.

Fine-tuning the model

If the mPFC can transform a transient response in LA into a sustained response, why do we need a separate integrator (Fig. 5.1b)? Why not apply Occam's razor and simply have the output of mPFC control behavior? This simplified model would be satisfactory for explaining the data in this thesis. However, previous work indicates that acquisition of fear conditioning is unaffected by lesions of the mPFC (Morgan et al., 1993; Morgan and LeDoux, 1995). Similarly, the mPFC receives auditory input from sensory cortex (Conde et al., 1995) so why does the model require connection from the LA to the mPFC? First, there was a clear difference in the sensory aspect and the affective aspect of the CS-evoked response in individual

neurons in the mPFC (Fig. 4.6). The sensory aspect was entrained to the individual pips of the CS and was not related to suppression. The affective aspect was not entrained to the pips and covaried with suppression. Second, lesions or inactivation of LA eliminate the difference in activity evoked by a CS and activity evoked by a neutral stimulus in the mPFC (Garcia et al., 1999; Laviolette et al., 2005).

There are two known mechanisms by which the mPFC can inhibit the output of LA. The first mechanism is direct inhibition of neurons in the LA (Rosenkranz and Grace, 2002). The second is modulation of the intercalated cells of the amygdala to gate the functional connection between the LA and the central amygdala (Quirk et al., 2003). The intercalated cell masses and the central amygdala are not explicitly in the model but may be involved in the integration of the output of LA. Using a spiking neuron model we could test how the addition of an inhibitory connection from the *down* neurons to the integrator affects the behavioral output of the model.

Learning versus Regulation

This thesis was devoted to the study of fear regulation. However, the majority of studies of fear conditioning have focused on fear learning (Maren & Quirk, 2004; Ledoux, 2000). How does the model presented in figure 5.1 fit with the role of the LA and the mPFC in fear learning? During fear learning the strength of the connections in figure 5.1 change. In acquisition, the connections from the CS inputs to the LA (figure 5.1a) becomes stronger (Repa et al., 2001; Rogan et al., 1997). Possibly, the connections from the adaptive signal in the LA to the integrator also become stronger.

In extinction, the strength of the CS inputs to the LA weaken (Repa et al., 2001) and the inhibitory connection from the mPFC to the amygdala may strengthen (Herry & Garcia, 2002). Thus, understanding the neurobiolgy of fear learning is understanding how synaptic strength is modified by experience. Understanding the neurobiology of fear regulation, on the other hand, is understanding how the dynamics of the system, that are set in place by fear learning, relate to behavior in real-time.

Licking

The task used in the thesis took advantage of the fact that a sweet drink provided a background of appetitive motivation against which CS-evoked suppression could be reliably measured. However, appetitive motivation is not included in the model because it was not manipulated experimentally. Future experiments could manipulate appetitive motivation either by altering the degree of food deprivation or by altering the concentration of the sugar in the sweet drink. It would be interesting to see how much these manipulations would affect suppression and whether the neurons in the fear regulation circuit would reflect these effects. An in depth analysis of the neurobiology of appetitive motivation is outside the scope of this thesis (for review, see Kelly et al., 2005). Nonetheless, the dopaminergic system, which plays an important role in the neurobiology of reward (Schultz, 2004; Schultz, 1997), is an effective modulator of amygdala activity (Rosenkranz & Grace, 2002).

To analyze the relative timing of changes in behavior and neural activity, I treated licking as a binary variable: a rat was licking or suppressing. However, the

model of fear regulation (figure 5.1) does not threshold the output of the integrator, which would be necessary to produce binary behavior. The model was intended to be a conceptual framework for future work of instantiating a rigorous computation model that could actually simulate neural activity and behavior for comparison with experimental data. The function that would transform a continuous neural variable into realistic real-time licking behavior would not be a simple thresholding function. Rather, this function would have to take into account the distribution of lengths of lick-bouts and the distribution of time between bouts. This could probably be modeled as a two-state system with the transition probabilities being functions of the neural activity. Modeling the average lick rate as a continuous linear function of the integrated signal would be a simpler first step.

5.3. Variability in behavior and neurons

On average, the CS-evoked suppression was reliable in the sense that a steady level of suppression was maintained across days (Fig. 4.2b). However, the trial-to-trial and moment-to-moment behavior was highly variable (Fig. 4.3). It may seem counter-intuitive, but being unpredictable is an important trait for survival. The key insight is to appreciate that in nature animals are competing against each other. If a rat always reacted the same way to a cat, a cat could learn to predict a rat's response; a lose for the rat (reviewed in Glimcher, 2005). Animals, from pigeons (Blough and Blough, 1968), to rats (Grunow and Neuringer, 2002), to monkeys (Dorris and

Glimcher, 2004), to humans (Miller and Neuringer, 2000) produce almost perfectly unpredictable behavior in tasks where unpredictable behavior is reinforced. In order to produce unpredictable behavior the neural circuits driving behavior must themselves appear unpredictable. For the fear regulation task the variability in the rats' behavior was accounted for by underlying variability in the LA and the mPFC. What was the source of the variability in neural activity?

The most parsimonious hypothesis is that the variability in neural activity came from the noise in synaptic connections. At each synapse, when an action potential reaches the terminal there is a chance (as high as 80%) that neurotransmitter will not be released (Calvin and Stevens, 1967; Hubbard et al., 1967; Auger and Marty, 2000; Stevens, 2003). Even the transmission of the CS from the speaker to the nervous system is affected by Brownian noise in the hair cells that transform sounds into electrical signals (Harris, 1968). There are two obstacles in connecting synaptic noise to behaviorally meaningful fluctuations in neural activity. The first obstacle is that there are thousands of neurons and millions of synapses involved in the LA-mPFC circuit. One would expect that averaging synaptic noise across millions of synapses would negate any small random effects. However, the synaptic architecture of pools of neurons results in small correlations in the noise of neurons within these pools. These small correlations mean that instead of noise averaging out across neurons, the noise produces a surprisingly large effect (Zohary et al., 1994; Cai et al., 2005).

The second obstacle is that the variability in behavior observed here is on the order of seconds (Fig. 4.3), while synaptic noise is on the order of milliseconds (Brunel et al, 2001). Several mechanisms may contribute to a temporal amplification of synaptic variability into behavioral variability (Cai et al., 2005). First, synaptic transmission from thalamus and cortex to the LA depends on NMDA receptors containing the NR2B subunit (Szinyei et al., 2003), which have relatively slow dynamics (Chen et al., 1999). Second, these NMDA receptors allow calcium to flow into the cell and these calcium transients have long decay times, on the order of hundreds of milliseconds (Stosiek et al., 2003). Third, neural activity in the LA signals changes in behavior, so an increase in activity in LA would not have to be sustained. If these mechanisms were playing a role in generating neural fluctuations then local infusions of drugs into the LA that modify calcium dynamics should change the dynamics of conditioned suppression. This could be accomplished either by targeting the kinetics of the NMDA receptor or by changing the kinetics of the molecules that buffer calcium in the post-synaptic cell.

These synaptic mechanisms explain possible sources of neural variability in general. An important question for fear regulation is whether there were independent fluctuations in both LA and mPFC, or whether the fluctuations in one were simply reflecting fluctuations in the other. Some evidence can be gleaned from the behavior-triggered spike histograms. In the LA, activity ramped up before the onset of suppression. In the mPFC, the change in neural activity occurred after the onset of

suppression. Thus, for the onset of suppression, activity in the LA may have driven activity in the mPFC.

For the offset of suppression the data is less clear as to whether LA activity affected mPFC activity or vice-versa. Activity in the LA ramped down before the offset of suppression and activity in *down* cells in the mPFC increased before the offset of suppression. The evidence from the literature is equivocal regarding the direction of influence in the LA-mPFC circuit. Stimulation of the mPFC inhibits neurons in the LA (Rosenkranz and Grace, 2002) and CS-evoked responses in mPFC are eliminated by lesions or inactivation of the LA (Garcia et al., 1999; Laviolette et al., 2005). The answer may emerge from ongoing work analyzing simultaneously recorded activity in the LA and the mPFC. If neural activity in the LA and the mPFC is statistically independent then a model of behavior that uses both neural signals should account for more variance in behavior than a model using either signal alone. Realistically, the result of this analysis will probably indicate that there is some correlation in the signals from the mPFC and the LA. Examination of the cross-correlation of simultaneously recorded neurons should provide some insight as to which brain structure is initiating the offset of suppression.

5.4. New targets for intervention

The model of fear regulation (figure 5.1) features two main differences from the model of fear learning (figure 1.1) that reflect the novel findings from chapters 2-4.

They are the existence of an integrator of the adaptive code in LA and the existence of a mutually inhibitory network in mPFC. Either of these features could be possible targets for intervention in clinical fear disorders.

The integration of activity in LA (Fig. 5.1b) has several features that can be inferred from a comparison of the activity in LA (Fig. 3.2) and the CS-evoked suppression (Fig. 4.5). Specifically, the latency of the CS-evoked response was 20-60 milliseconds, but the suppression had an exponential rise to asymptote, with a time-constant of 1.8 seconds. At the end of the CS, suppression decayed exponentially with a time constant of 7.6 seconds. We saw in chapter 2 that pentagastrin resulted in persistence of suppression after the termination of the CS, which could be thought of as increasing the time constant of the decay of suppression. This suggests that other pharmacological manipulations could alter these time constants so that fear behaviors are slower to ramp up and faster to ramp down.

The two pools of neurons in mPFC, *up* and *down* cells, may have distinct pharmacalogical or genetic signatures. For example, both serotonin (Abi-Saab et al., 1999) and dopamine (Grobin and Deutch, 1998) neurotransmission in the mPFC increase GABA release, and have overlapping but distinct effects on mPFC mediated behaviors (Ogren et al., 1985; Morrow et al., 1999; Robbins, 2005). The model of mPFC (Fig. 5.1d) posits the existence of distinct pools of inhibitory neurons. It could be that dopamine and serotonin specifically target one of the pools. Consistent with this, only a subset of the interneurons in the mPFC are activated by serotonergic

agonists (Leslie et al., 1993). To further test whether dopamine and serotonin specifically modulate *up* or *down* cells, we could perform *in vivo* intracellular recordings of neurons in mPFC during presentation of a CS to identify *up* and *down* cells and then fill these cells and use antibodies for dopamine and serotonin receptors to analyze whether *up* and *down* cells have distinct receptor pharmacology. To perform a genomic analysis after identifying and labeling the cell, the labeled cells could be extracted using laser capture and analyzed using a DNA microarray (Yao et al., 2005).

5.5. Regulation versus consolidation

One of the long-standing debates amongst amygdala researchers is whether the amygdala acts to modulate learning and memory in other structures (Cahill et al., 1999; McGaugh, 2004) or whether the amygdala is itself a site of learning and memory (LeDoux, 2000; Maren and Quirk, 2004). I have presented the latter view in some depth. The former view is based on many experiments that demonstrate that activation of the amygdala can result in enhanced learning in tasks that are known to be dependent on other structures, like the hippocampus or striatum (McDonald and White, 1993; Packard et al., 1994; Akirav and Richter-Levin, 1999; Roozendaal et al., 1999; Setlow et al., 2000; Frey et al., 2001; Kim et al., 2001; Roozendaal et al., 2001). There is no question that activation of the amygdala modulates brain circuitry that is involved in learning in memory. Adrenergic (Cahill et al., 1994), dopaminergic (Davis

et al., 1994; Cahill, 1998), cholinergic (Greba et al., 2000; Holland et al., 2000), and other neuromodulatory systems (Barros et al., 2001; Carrasco and Van de Kar, 2003) are affected by amygdala activity. This is intuitive because the amygdala is the core of a neural circuit for fear which engages systems that will enhance performance in a life and death situation and also learn from the experience to avoid danger in the future. However, this also means the level of stress or arousal of experimental subjects will affect neuromodulatory systems and the circuit properties of neurons (Flamm and Harris-Warrick, 1986). Critics of the hypothesis that LA is a site of plasticity could argue that changes in neuromodulatory state due to different stress levels before and after training could be mistaken for synaptic plasticity.

The effect of arousal or stress on neural activity is well illustrated by electrophysiological investigation of burst and tonic firing modes in the thalamus. When animals are asleep or in a low attentional state, the thalamus tends to fire in burst mode. During attention it switches to tonic mode (for review see Sherman, 2001). One property of this phenomenon is that neurons have higher firing rates during tonic mode. Cain et al. (2000) recorded from neurons in visual thalamus during presentation of an auditory CS+ and CS- and found that during the CS+ the firing rate of the neurons in visual thalamus increased. These neurons have no response to auditory stimuli, but the CS+ increases the level of arousal, which was observed in visual thalamus as a switch from burst to tonic mode (Cain et al., 2000). Therefore, we must be wary in interpreting changes in firing rate as a sign of plasticity.

Fear conditioning experiments attempt to control for this by presenting shocks explicitly unpaired with the CS. The unpaired control results in robust contextual conditioning. These animals freeze, but neurons in the lateral amygdala do not show enhanced CS responses in these animals. This suggests that the increase CS-evoked response is not due a general arousal effect. However, this control is not perfect, since a paired CS provides temporal information about when a shock will occur, while the unpaired CS does not provide any temporal information. This means that the kind of uncertainty in cued and unpaired fear is different, and it has been argued recently that different aspects of uncertainty are specifically linked to adrenergic and cholinergic systems (Yu and Dayan, 2005).

No such discrepancy exists in the comparison of neural activity before the onset and offset of fear during a single CS, since there is no change in the uncertainty about the outcome of the trial during a single presentation of the CS. Indeed, the primary design principle in the task was that all external variables are the same during each CS presentation, so that intra-trial variability in behavior cannot be explained by a difference in stimulus property (as in differential conditioning) or a difference in the neuromodulatory state due to differences in the task (as in unpaired controls). In this way, the finding that moment-to-moment changes in neural activity in LA predict the moment-to-moment changes in behavior is particularly strong evidence that the LA is not simply playing a role as a modulator of memory, but plays an active role in the detection and processing of aversive stimuli.

5.6. Not just fear

Based on the data presented here, along with extensive evidence from studies investigating fear conditioning (LeDoux, 2000; Maren and Quirk, 2004), fear potentiated startle (Walker and Davis, 2002; Davis et al., 2003), unconditioned fear (Rosen, 2004), post-traumatic stress (Hull, 2002; Gilboa et al., 2004) and anxiety (Davidson, 2002; Koster et al., 2005) we might think that the LA-mPFC circuit is primarily dedicated to the processing of fear. However, a great body of literature from rodents (de Bruin et al., 1994; Kruzich and See, 2001; See et al., 2003; Holland and Gallagher, 2004; Sun and Rebec, 2005), primates (Baxter et al., 2000; Miller and Cohen, 2001; Baxter and Murray, 2002) and humans (Bechara et al., 1996; Childress et al., 1999; Bechara, 2005) indicate that the interaction between the amygdala and prefrontal cortex plays an important role in reinforcement learning and decision making in general.

The emerging view is that the evolutionarily old amygdala is the substrate for detecting salient environment information and for driving the instinctual responses to these cues. As mammals evolved and developed a more complicated behavioral repertoire, including social behavior, the mPFC developed to control and refine the instinctual behaviors elicited by the amygdala (Miller and Cohen, 2001). In drug addiction, the amygdala is the neural substrate for cue-elicited craving (See et al., 2003) and the mPFC is important for inhibiting drug-seeking behavior (Bechara, 2005). In fear regulation, the amygdala is the neural substrate for CS-evoked

suppression and the mPFC is important for inhibiting that suppression. Thus, the amygdala drives both defensive and appetitive behavior while the mPFC inhibits these behaviors. Sadly, this dual role of the LA-mPFC circuit is manifested in the high comorbidity between PTSD and drug addiction. A recent study demonstrated the people addicted to cigarettes are twice as likely to develop PTSD after a trauma compared to controls (Koenen et al., 2005), suggesting that an imbalance between amygdala and mPFC that led to nicotine dependency also led to PTSD.

Further understanding of the interaction between the amygdala and the prefrontal cortex would generally contribute to the understanding of psychological disorders, like addiction and PTSD. However, the prevalence of these disorders is an indication that we have created a world very different from the one that shaped the evolution of our species. For example, people with emotional deficits due to brain lesions or substance abuse performed better than normal controls in a stock-market investment simulation (Shiv et al., 2005). The primacy of monetary success in the social hierarchy of Western countries is therefore selecting for people who have flattened affect. Understanding the neurobiology of psychological disorders is certainly a worthwhile endeavor, but shaping a future world that does not breed mental illness should be of equal or greater importance.

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