NEURAL CIRCUITRY UNDERLYING CONTEXTUAL REGULATION OF FEAR AFTER EXTINCTION

by

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CHAPTER I

INTRODUCTION

Each day, animals are faced with a multitude of situations that require the assessment of risk and organization of defensive behaviors to contend with imminent or future threats. Failure to do so might result in harm or even death. Fear is an emotion that is central to the organization of defensive behaviors against threat, and therefore has an essential role in survival. Indeed, both innate and learned fears are vital for motivating defensive behaviors that allow for successful coping in risky situations (Mineka and Ohman, 2002; Ohman and Mineka, 2001). For example, in a natural setting, when animals encounter a predator, they will flee, freeze or attempt to threaten their opponent (Bolles, 1970). Additionally, organisms use subtle predictive cues, such as noises or odors, to determine if danger is imminent and respond preemptively, which may enhance their chances of survival.

In the laboratory setting, Pavlovian fear conditioning has become the quintessential method of investigating aversive learning and memory (Maren, 2001). In this paradigm, an innocuous stimulus (conditioned stimulus, CS) is repeatedly presented with a noxious stimulus, such as a footshock (unconditioned stimulus, US). After several pairings, the CS alone predicts the US and engenders a state of fear, indicated by freezing and increased heart rate, among other reactions.

Though psychologists historically thought of fear conditioning in terms of conditioned reflexes, it is now regarded as a compilation of hierarchical associations that informs the organism about its world (Rescorla, 1988). That is, not only is the CS-US association learned, but also the relation of this aversive event to other stimuli and their structure in the environment. Thus, when an individual undergoes a traumatic event, a complex representation of the experience is formed that may persist indefinitely. These memories are typically adaptive, allowing individuals to cope with future threats. Unfortunately, in some cases, dysfunction in the fear system produces inappropriate and exaggerated fears that lead to psychopathology.

Indeed, disorders of fear and anxiety, including specific phobias and post-traumatic stress-disorder (PTSD), are largely due to and maintained by pathological fear memories. Based on recent statistics, nearly 82% of Americans will encounter a traumatic event in their life (Sledjeski et al., 2008). The National Institute of Mental Health reports that 3.5% of American adults are diagnosed with PTSD and of those, only 49% receive treatment (National Institute of Mental Health, 2011). Due to the prevalence of the disorder, research in the last several decades has focused on understanding the neural bases of fear memory formation with the aim of developing appropriate clinical interventions. One challenge that clinicians face is that fear memories endure over long periods of time and can generalize across contexts (Bouton, 1988; Rasmusson and Charney, 1997). Moreover, common behavioral therapies, including exposure therapy, tend to produce transient fear reduction that is often bound to the context in which the therapy was administered. This is also the case for extinction, a commonly used laboratory procedure in which

a CS is repeatedly presented in the absence of the US, resulting in a decrease in fear. Therefore, the resilience of fear memories and the fragility of extinction memories make successful treatment of disorders such as PTSD a challenge. However, in recent years, there have been great strides in understanding the neurobiological underpinnings of both fear and extinction memory formation. These advances offer a foundation upon which novel therapeutic interventions for fear and anxiety might be built.

Neurobiology of Aversive Learning

The amygdala

The search for the locus of emotion began in the 1920's with Walter Cannon and Phillip Bard implicating the hypothalamus and its projections in the mediation of emotional behavior. Later in 1937, James Papez extended this emotional circuit to include more medial temporal lobe structures. Specifically, he injected rabies virus into the hippocampus of a cat and observed its course throughout the brain. Based on these results, he described the emotional circuit as emanating from the hippocampus, traveling through the mammillary bodies, anterior thalamus, and anterior cingulate cortex (Papez, 1937). Paul MacLean revised this circuit to include the prefrontal cortex and amygdala and labeled it the "visceral brain" or more commonly, the "limbic system" (MacLean, 1949). The most convincing evidence for the amygdala's role in emotion, particularly fear, came from the seminal work by Kluver and Bucy (1937). They found that the bilateral removal of the medial temporal lobes in rhesus monkeys resulted in abnormal emotional behavior (Kluver

and Bucy, 1937). Before the temporal lobectomy, the monkeys were fearful and withdrew from their human handlers; after the surgical procedure, however, the monkeys no longer feared human beings and did not display anger or aggression. Importantly, they also showed avid interest in exploring objects in the environment, regardless if they posed a threat. Because Kluver and Bucy's lesions included many brain structures such as the hippocampus, amygdala, and temporal neocortex, Weiskrantz (1956) reexamined lesions restricted to the amygdala and observed the same pattern of behavior, especially the loss of fear. These behavioral phenomena would later be replicated many times in various species (Fonberg, 1972; Goddard, 1964) and would come to be known as "Kluver-Bucy" syndrome. Along with reports that amygdaloid seizures result in fear-like behavior (Depaulis et al., 1997), these early studies provide irrefutable evidence that the amygdala is crucial for attributing emotional significance to situations and regulating fear behavior.

The role of the amygdala in fear learning and memory was first demonstrated in the laboratory using instrumental conditioning paradigms, such as avoidance learning. For example, bilateral lesions of the amygdala decrease an animal's preference of a nonshocked chamber over one in which a shock was delivered (Brady et al., 1954; Robinson, 1963). Similarly, the amygdala has been implicated in mediating conditioned emotional responses, including conditioned suppression. For instance, Kellicutt and Schwartzbaum (1963) trained rats with amygdala lesions to bar-press for food and then fear conditioned them. They found that rats with amygdala lesions took longer to suppress bar-pressing in response to the CS (Kellicutt and Schwartzbaum, 1963). Several years later, Goldstein (1965)

reported that amygdala lesions resulted in deficits in the acquisition and retention of fear responses, as measured by the latency to jump out of a compartment in which a tone and shock were paired. Blanchard and Blanchard (1972) followed up these studies by showing that restricted amygdala lesions impaired the acquisition of contextual fear conditioning, in which animals learn to associate the shock with the surrounding environment. These seminal studies provided the foundation from which the neural circuit underlying fear learning and memory formation has been built.

With regard to fear conditioning, the amygdala is typically described as having two different functional subdivisions: the basolateral complex of the amygdala (BLA) and the central nucleus of the amygdala (CeA; Figure 1.1; Maren, 2003; Pitkanen et al., 1997). The BLA itself contains the lateral nucleus (LA), the basolateral nucleus (BL) and the basomedial nucleus (BM; Davis et al., 1994; Krettek and Price, 1978). Together, the BL and BM are known as the basal amygdala (BA). These nuclei themselves can be further divided into separate regions. The LA, located in the dorsal most part of the amygdala between the external capsule and CeA, is divided into the dorsolateral, mediolateral and ventrolateral regions (Pitkanen et al., 1997). The BL is situated below the LA and is made up of the magnocellular, intermediate and parvicellular regions. Lastly, the BM, also known as the accessory basal nucleus, lies ventral to the BL and also consists of the magnocellular, intermediate and parvicellular regions. The LA is the largest nucleus within the BLA, yet contains small tightly packed neurons with average soma diameters of approximately 10-15 μm. In comparison, the BL contains the largest

neurons of the BLA: the average soma diameter of BL neurons is approximately 15-20 μm. The size of the neurons in the BL ranges from large in the anterior most part of the BL to small in the more posterior section. The BM, like the LA, also consists of smaller neurons (Davis et al., 1994; Krettek and Price, 1978; Sah et al., 2003).

As a whole, the morphology of the BLA is similar to that of the cortex with the exception that BLA neurons are largely organized in a random fashion (but see Samson and Pare, 2006 for the organization of excitatory versus inhibitory neurons) rather than in layers as seen in the cortex. The population of neurons within the BLA is a heterogeneous mix insofar as there are two different types of neurons: pyramidal neurons and interneurons. Pyramidal neurons (class I) make up approximately 80% of the BLA and are large, spine-dense, and contain glutamate. These neurons form synapses on many other BLA neurons, in addition to forming most of the extrinsic connections to areas outside the BLA (i.e. CeA, hippocampus). The remaining 20% of neurons within the BLA consist of GABAergic interneurons (class II), which mostly form local circuits within the BLA. In comparison to pyramidal neurons, interneurons are small, stellate and spine-sparse. There are many different types of interneurons in the BLA, which are differentiated by unique protein expression signatures, similar to interneurons in the cortex (Bienvenu et al., 2012; Davis et al., 1994; McDonald, 1982b; Pape and Pare, 2010; Pitkanen et al., 1997; Sah et al., 2003; Swanson and Petrovich, 1998).

The CeA consists of the lateral central amygdala (CeL), the medial central amygdala (CeM) and the capsular region of the central amygdala (CeC). In general, CeA is primarily made up of GABAergic interneurons, which have been likened to

neurons in the dorsal and ventral striatopallidal region of the brain. However, there are slight differences between the cells in the CeL and the CeM. The CeL contains medium-sized spine-dense neurons that branch prolifically. Neurons in the CeM have larger soma than the CeL, yet do not contain many dendritic spines and branch sparsely. As a whole, CeA neurons express a variety of peptides, such as enkephalin, neurotensin and corticotropin-releasing hormone. CeA neurons project extensively to extrinsic structures, such as the hypothalamus and periacqueductal gray (Davis et al., 1994; McDonald, 1982a, 1985; Pape and Pare, 2010; Pitkanen et al., 1997; Sah et al., 2003; Swanson and Petrovich, 1998).

In addition, recent work reveals that local inhibitory networks exist within the CeA that regulate the overall activity in this region (Ciocchi et al., 2010; Haubensak et al., 2010). For example, Haubensak et al. (2010) have provided evidence that there are two distinct populations of neurons within the CeL based on the presence or absence of protein kinase C-δ (PKC-δ). Not only did they find that PKC-δ-positive and PKC-δ-negative cells make inhibitory connections with one another, they also observed that PKC-δ-positive cells had monosynaptic connections with CeM neurons. Interestingly, Haubensak et al. (2010) found that these separate populations of CeL cells map onto behaviorally responsive cells in vivo. For instance, when PKC-δ-positive cells in the CeL were silenced, firing activity in CeL-Off cells, or neurons that exhibit a strong inhibitory response to a CS, was suppressed. However, CeL neurons that typically display an excitatory response to a CS (CeL-On cells) were not affected by the inhibition of PKC-δ-positive cells in the CeL, suggesting that CeL-Off neurons may be PKC-δ-positive cells. Together, these

studies are the first to demonstrate that although the CeA does have important extrinsic projections, inhibitory local circuits also exist both within the CeL and between the CeL and CeM that may regulate its overall activity.

In addition to intrinsic interneurons within the BLA and CeA, there are also clusters of GABAergic neurons located at the interface of the BLA and CeA. These so-called intercalated cell masses (ITC) have recently garnered interest as a cellular substrate for gating information flow between the BLA and CeA. There are three main groups of ITC clusters located in fiber bundles in and around the amygdala (Millhouse, 1986; Sah et al., 2003). The lateral cluster is situated within the external capsule on the outside of the BLA (ITC-L). The intermediate cluster sits amidst the fibers between the BLA and CeA and consists of a dorsal cell mass located near the upper edge of CeL (ITCd) and a more ventral cell mass that is situated near the lower corner of the CeM (ITCv). Lastly, there is a large main ITC cluster located medially to the BA and ventral to the CeA (ITC-M). There are two types of neurons found within the ITC clusters, both of which have been compared to striatal neurons. The first kind consists of medium spiny neurons that synapse on neurons within the lateral, basal and central nuclei (Millhouse, 1986). The second group has a large soma and a mixture of spiny and aspiny dendrites, which travel in parallel to the BLA and CeA. This latter group, though, may not be GABAergic as it stains positive for acetylcholine rather than GABA. As a whole, the ITC local network is oriented in a dorsal to ventral direction in the rat (Amir et al., 2011; Busti et al., 2011). That is, GABAergic inhibition is always directed ventrally, which allows for an ideal mechanism for the BLA to control activity in the CeA.

In the last three decades, investigators have made great strides in uncovering the extrinsic connections of the amygdala underlying fear conditioning. Considerable work has demonstrated that the lateral nucleus of the amygdala (LA) is the primary sensory interface of the amygdala (Figure 1.1). Work by LeDoux and colleagues has established that the medial geniculate nucleus (MGN) directly relays auditory information to the LA during fear conditioning (Doron and Ledoux, 1999; LeDoux et al., 1990; LeDoux et al., 1986; LeDoux et al., 1985; LeDoux et al., 1984). As such, disrupting communication between the MGN and the amygdala results in deficits in the acquisition of fear (Iwata et al., 1986). Importantly, it has also been shown that after stimulation of the MGN, LA neurons are highly responsive to auditory stimuli (Bordi and LeDoux, 1992) and display increases in neuronal firing (Clugnet et al., 1990) as well as long-term potentiation, a cellular mechanism thought to mediate learning and memory (Clugnet and LeDoux, 1990). Auditory information is also transmitted in parallel indirectly from the MGN to the LA via the auditory cortex (Brunzell and Kim, 2001; Romanski and LeDoux, 1992). Contextual stimuli, which themselves predict aversive USs, are processed by the hippocampus (Fanselow and Poulos, 2005) and mainly sent from the ventral subiculum and ventral CA1 to the BA (Canteras and Swanson, 1992; Maren and Fanselow, 1995; Pitkanen et al., 2000).

Information about footshock unconditioned stimuli (USs) is relayed to the LA from thalamic and cortical regions. However, the exact pathways that convey US information are still not clear. Some have suggested that the posterior intralaminar nucleus of the thalamus (PIN) and the insular cortex are responsible for relaying US

information to the amygdala (Shi and Davis, 1999; Shi and Cassell, 1998). Indeed, combined lesions of the PIN and the insular cortex prevent the acquisition of fear-potentiated startle, another aversive learning paradigm (Shi and Davis, 1999). Additionally, pairing a CS with stimulation of the PIN as the US resulted in reliable conditioned responses (Cruikshank et al., 1992). However, others have found that combined lesions of the PIN and the insular cortex have no effect on fear conditioning (Brunzell and Kim, 2001; Lanuza et al., 2004). Rather, it is suggested that the PIN and insular cortex are part of a larger network of structures that process and convey US information to the amygdala (Brunzell and Kim, 2001; Lanuza et al., 2004). For example, nociceptive information can also be transmitted to the amygdala from the parabrachial nucleus and the spinal cord (Bernard et al., 1993; Lanuza et al., 2008).

As expected, LA neurons respond to both auditory and somatic stimuli (Figure 1.1; Romanski et al., 1993), suggesting a convergence of CS and US information on the same neurons within the LA. Indeed, a recent cellular imaging technique has allowed for the visualization of converging CS and US inputs within the amygdala. Cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH) capitalizes on the expression profile of the mRNA of the immediate early gene activity-regulated cytoskeletal-associated protein (*Arc*/also termed Arg3.1; Guzowski and Worley, 2001). Immediate early genes are activated rapidly in response to cellular stimuli and can result in the transcription and translation of proteins that may contribute to synaptic plasticity (Chaudhuri, 1997). *Arc* is observed within the nucleus up to 5 minutes after an animal has undergone a

behavioral task and within the cytoplasm up to 25-30 minutes after the experience (Guzowski et al., 2005). Using this technique, Barot et al. (2008) showed that amygdala neurons that respond to the CS are also activated by the US in a conditioned taste aversion paradigm. Similar results were obtained using a contextual fear conditioning paradigm (Barot et al., 2009). Rats were placed into a conditioning chamber and received one footshock 26 minutes later. This paradigm allowed for the presentation of the CS (context) and US (footshock) to be divided into 2 different experiences that could be visualized separately. Indeed, during contextual fear conditioning, there were neurons within the BLA that exhibited both cytoplasmic (due to the CS) and nuclear (due to the US) staining. This was not observed in any of the control groups (immediate shock, latent inhibition and no shock). This strongly suggests that afferents to the amygdala that carry CS and US information converge on the same population of neurons.

To generate a fear response, information must flow from the BLA to the CeA, which is thought to be the output center of the amygdala (Figure 1.1). The LA transmits CS-US information to the CeA via two routes (Krettek and Price, 1978; Pape and Pare, 2010; Pare and Duvarci, 2012; Pare and Smith, 1998). In a direct connection with the CeA, the LA sends unidirectional excitatory projections to the CeL. Subsequently, the CeL sends inhibitory projections to the CeM. Because the CeM has many connections to regions involved in fear responses, such as the periacqueductal gray (freezing) and the paraventricular nucleus of the hypothalamus (glucocorticoid release), the CeM is considered to be the ultimate output area of the amygdala (Davis and Whalen, 2001; Fendt and Fanselow, 1999;

Maren, 2001). The LA also projects to the BA; the BA, in turn, innervates neurons with the CeM. Additionally, BA neurons make connections with ITC cells before synapsing on CeM neurons (Pare and Smith, 1993, 1998; Royer et al., 1999). As these GABAergic ITC cells generate feed-forward inhibition in the CeA, it is thought that they gate the information flow between the BLA and CeA (Royer et al., 1999, 2000). As a result of multiple inhibitory inputs onto the CeM, conditioned fear responses are thus generated by the disinhibition of CeM neurons via the ITC and CeL neurons (Ehrlich et al., 2009). Evidence for the latter comes from recent work indicating that CeL-Off neurons can project to and inhibit CeM inhibitory output neurons, resulting in their net disinhibition (Ciocchi et al., 2010; Haubensak, et al., 2010).

It has been established that the BLA is the site of CS-US association insofar as lesions or reversible inactivation disrupt the acquisition of conditioned fear (Goosens and Maren, 2001; Koo et al., 2004; LeDoux et al., 1990; Maren et al., 1996a), as well as other indices of conditional fear behavior, such as fear-potentiated startle (Campeau and Davis, 1995). In fact, these deficits can be seen from one month (Maren et al., 1996a; Lee et al., 1996) up to one year (Gale et al., 2004) after training. Support from electrophysiological studies confirms the BLA's role in associative plasticity in fear memory formation. During aversive learning, neurons within the BLA exhibit enhanced responding to the CS (Maren et al., 1991; Quirk et al., 1997; Quirk et al., 1995; Repa et al., 2001; Rogan et al., 1997). These changes are associative in nature, and can be dissociated from fear states and behavioral fear responses that are consequences of fear conditioning (Goosens et al.,

2003). Importantly, the synaptic plasticity underlying aversive learning also occurs in the LA (Blair et al., 2001), as it receives converging inputs from the cortex and thalamus. Consistent with this notion, changes in the LA precede the actual behavioral changes in the animal (Repa et al., 2001) as well as tone-induced firing in other brain areas, such as the auditory cortex (Quirk et al., 1997). Disruption of LA activity does not, however, affect plasticity observed in the thalamus during fear conditioning, ruling out the possibility that plasticity in the LA is a reflection of changes in afferent structures (Schafe et al., 2005).

Evidence has also emerged that suggests that the CeA, rather than being a passive relay station to fear generating structures, is involved in fear memory formation. For example, temporary inactivation of the CeA (Wilensky et al., 2006; Ciocchi et al., 2010) or CeL alone (Ciocchi et al., 2010) prior to fear conditioning disrupts the acquisition of fear learning. Additionally, rats with BLA lesions undergoing overtraining are able to acquire conditional freezing (Ponnusamy et al., 2007; Maren, 1999a), although this is short-lived (Poulos et al., 2009). Both the bed nucleus of the stria terminalis (BNST; Poulos et al., 2010) and the CEA (Zimmerman et al. 2007) have been suggested to mediate overtrained fear in rats with BLA lesions. However, unlike the BNST, the CEA is required for both contextual and auditory CS memories, suggesting that it is ultimately responsible for mediating fear in the absence of the BLA (Zimmerman and Maren, 2010). Thus, it is conceivable that CS-US information is processed in the CeA in parallel to the LA, or that BLA-CeA projections themselves are the essential site of plasticity in fear conditioning (Maren, 2008). Indeed, auditory information can reach the CeA from the posterior

thalamic nucleus (LeDoux et al., 1985; Linke et al., 2000; Turner and Herkenham, 1991). This nucleus receives auditory input from areas of the inferior colliculus and the dorsal nucleus of the lateral lemniscus. Consistent with this possibility, thalamic stimulation results in changes in synaptic efficacy in the CeM that are independent of LA input to the CeA (Samson and Pare, 2005). In addition, US information is relayed to the CeA via the spinal cord and the parabrachial complex of the pons (Lanuza et al., 2004). This evidence suggests that amygdala is endowed with multiple routes by which fearful information can be processed and retained indefinitely.

The hippocampus

It is well known that during fear conditioning, contextual cues also become associated with the aversive stimulus. The hippocampus is responsible for assembling a contextual representation of the conditioning environment and transmitting it to the amygdala (Fanselow and Poulos, 2005; Maren, 2001). Some of the earliest investigations of the role of the hippocampus in aversive learning began by assessing the effects of electrolytic lesions of the dorsal hippocampus (DH) on contextual fear conditioning. Electrolytic lesions yielded major deficits in contextual fear (Kim and Fanselow, 1992; Phillips and LeDoux, 1992), resembling the amnesic effects seen in humans with damage to the medial temporal lobe (Scoville and Milner, 1957). The effects of DH lesions are also time-dependent insofar as the observed deficit diminishes across time (Anagnostaras et al., 1999; Kim and Fanselow, 1992). For example, Kim and Fanselow (1992) only observed contextual fear deficits in rats that received DH lesions one day after training. However, rats

retained the fear memories if they had had surgery 28 days after training. Similarly, in a within-subjects study, rats trained 50 days prior to surgery displayed intact remote context fear memory, but impaired memory for training that occurred 1 day prior to surgery (Anagnostaras et al., 1999). This effect has also been replicated with neurotoxic lesions, which spare fibers of passage (Maren et al., 1997). This accumulated evidence suggests that the hippocampus is important for the initial acquisition and storage of the contextual memory, but over time, the memory is transferred elsewhere and rendered hippocampus-independent (Frankland et al., 2004; but see Sutherland and Lehmann, 2011; Sutherland et al., 2010). However, in contrast, other groups have proposed that the hippocampus may in fact have a more permanent role in the storage of contextual fear memories (Lehmann et al., 2007; Sutherland et al., 2008; Sutherland and Lehmann, 2011; Sutherland et al., 2010; Goshen et al., 2011). For example, Goshen et al. (2011) have recently used optogenetics to demonstrate that the inhibition of hippocampal area CA1 disrupts the retrieval of fear memories when assessed 9 or 12 weeks after training. Importantly, they showed that this was effect was only obtained when optogenetic induced inhibition was limited to the duration of the test; when it was extended to include the 30 minutes prior to the test session, Goshen et al. (2011) did not observe a deficit in memory recall. This indicates that the hippocampus may have an enduring role in remote context memory, and other brain structures can rapidly compensate for its loss under some conditions.

Interestingly, many groups have reported that when neurotoxic lesions of the DH are made prior to training, there are no observable deficits in contextual fear

(Cho et al., 1999; Gisquet-Verrier et al., 1999; Maren et al., 1997; Richmond et al., 1999, but see Selden et al., 1991). Based on both the pre- and post-training lesion data, it has been theorized that an organism can acquire fear using a hippocampal-dependent configural strategy or a hippocampal-independent elemental strategy (Maren et al., 1997; Rudy and O'Reilly, 1999; Rudy et al., 2002; Maren and Holt, 2004; Biedenkapp and Rudy, 2009; Zelikowsky et al, 2011; Fanselow, 2010). For the configural strategy, an organism assembles the various elements of the context, such as odors, tactile information, and visual stimuli, into one configuration that is represented as the context and subsequently associated with the aversive US. Conversely, elemental learning consists of associating one specific salient feature of the environment with the US. With an intact hippocampus, organisms use a configural strategy in which they assemble the many sensory elements of the conditioning situation into a coherent contextual representation. Evidence for this comes from the observation that if an animal is placed into a chamber and immediately shocked, the rats do not learn the association between the context and shock (Fanselow, 1986; Fanselow, 1990). If the rats undergo pre-exposure to the context prior to the shock, this deficit is alleviated, suggesting that the hippocampus requires a certain amount of time to form a representation of the environment (Matus-Amat et al., 2004). Thus, rats with post-training lesions are still able to use a configural strategy during contextual fear learning; however, after the hippocampus is ablated, they are no longer able to retrieve that memory. In contrast, when the DH is lesioned prior to training, rats are unable to use a configural strategy and thus must employ another strategy in which to learn about the aversive situation. It has

been suggested that rats use an elemental strategy in the absence of a hippocampus, resulting in the successful acquisition and retention of contextual fear memories. These findings imply that the hippocampus can interfere with or inhibit other non-hippocampal systems that typically employ an elemental strategy during fear acquisition. A recent study by Sparks et al. (2011) has provided evidence for this notion of hippocampal overshadowing of non-hippocampal systems. They demonstrated that while rats were able to successfully acquire contextual fear memories with an inactivated hippocampus, they were impaired during the test session when hippocampal activity was restored. This suggests that under normal conditions, the hippocampus interferes with other systems during learning and retrieval; when offline, non-hippocampal systems are released from this control and can mediate contextual learning.

Though many groups have demonstrated that pre-training lesions or inactivation of the hippocampus have no deleterious effect on subsequent learning, Wiltgen et al. (2006) have found deficits under some conditions. This group found that rats with pre-training lesions of the DH show impairments in contextual fear conditioning after one training trial. Increasing the number of training trials alleviated this impairment. Importantly, it was also shown that increasing the time between placement in the training chamber and the delivery of the shock strengthened contextual conditioning in lesioned animals. Because this is characteristic of a configural strategy, it may be that organisms without a hippocampus are still able to form configural representations of the environment and thus learn about the context, albeit at a slower rate. Taken together,

accumulated data over the last decade demonstrate that the hippocampus is important for context fear learning, but in its absence, it is possible for other neural structures to compensate for this loss.

Importantly, many laboratories have also shown that the ventral hippocampus is necessary for the acquisition of both auditory and contextual fear. In contrast to the DH, the VH has robust and reciprocal connections with the amygdala (Canteras and Swanson, 1992). Thus, it is perfectly situated to regulate activity in this structure during aversive learning. Many have reported that both electrolytic (Biedenkapp and Rudy, 2009; Maren, 1999b; Trivedi and Coover, 2004) and neurotoxic (Bannerman et al., 2003; Maren, 1999b; Richmond et al., 1999) lesions impair conditioning to both contextual and auditory cues. Infusions of glutamate receptor antagonists (Zhang et al., 2001), GABA agonists (Bast et al., 2001; Esclassan et al., 2009; Maren and Holt, 2004) or sodium channel blockers (Bast et al., 2001) also prevent the acquisition of context or auditory fear. Because lesions or inactivation of the DH typically do not affect fear to explicit CSs, some believe that the dorsal and ventral hippocampus mediate different aspects of learning and more generally, cognition. Specifically, the DH is involved in the spatial and contextual aspects of learning and may transmit this information through the VH to the amygdala, where the CS-US association occurs. The VH, on the other hand, is particularly important for processing and transmitting discrete emotional stimuli to the amygdala. Consistent with the notion that the DH and VH may subserve different aspects of learning and cognition, Dong et al. (2009) found that the dorsal CA1 area of the hippocampus (CA1d) and ventral CA1 area of the hippocampus

(CA1v) display clear regional-specificity with regard to the expression of certain genes. For example, gene markers in the CA1d correlate highly with those found in the cortical and subcortical structures innervated by the CA1d that are involved in spatial processing and navigation. On the other hand, the CA1v shares gene expression patterns with other areas of the brain that receive projections from the CA1v and that have been shown to mediate endocrine and emotional responses. Thus, it appears that rather than being a homogenous structure, the hippocampus can in fact be parceled into distinct subregions, each with their own gene expression patterns. As a result, each hippocampal subfield mediates different aspects of behavior (Fanselow and Dong, 2010).

Extinction of Conditioned Fear

Though a great deal of information has been learned about the neural bases of fear conditioning, the mechanisms underlying fear extinction are not as well characterized. Given that extinction has significant implications for clinical interventions in treating anxiety disorders, phobias and PTSD, recent research has been devoted to developing a framework for understanding extinction processes. Indeed, extinction has strong parallels to exposure therapy, a common cognitive behavioral therapeutic technique, in which the patient receives extensive exposure to the fear-evoking stimulus. Repeated exposure of the aversive stimulus results in a gradual decrease in fear behavior. Because of the similarity between extinction and exposure therapy, it seems prudent to investigate the mechanisms underlying

extinction so as to improve methods used to treat patients with pathogenic fear disorders.

Behavioral features and theories of extinction

Ivan Pavlov was the first to document the extinction process with appetitive conditioned stimuli (Pavlov, 1927). In his hallmark experiment, Pavlov paired the sound of a metronome (CS) with the delivery of meat powder (US). Initially, the meat powder alone elicited a salivary response (unconditioned response, UR), but upon several CS-US pairings, the metronome alone resulted in a salivary response (CR). Importantly, the repeated presentation of the CS in the absence of the US led to a gradual decrement in the magnitude of the CR. Similarly, repeated CS presentations without an aversive US result in the extinction of the fear response. Though seemingly simple, extinction is actually a complicated phenomenon. In this section, several core behavioral properties of extinction will be described, followed by some of the more prominent theories of extinction learning.

To begin, it is important to appreciate that extinction is not the same as forgetting (Myers and Davis, 2007). Forgetting implies that there is a decrease in the fear response due to the passage of time. In fact, fear memories are resistant to forgetting as they can persist for over a year (Gale et al., 2004). However, extinction specifically refers to a decrement in behavior due to the presentation of the CS without the US.

An important property of extinction is its context-dependence. An illustrative example of this is the renewal effect, which refers to the return of conditioned fear when the CS is presented in a context different from that in which

extinction occurred (Bouton, 2004; Bouton and Bolles, 1985). This effect has been observed even after massive extinction training consisting of up to 160 CS-alone presentations (Bouton and Swartzentruber, 1989; Denniston and Miller, 2003; Gunther et al., 1998). Importantly, renewal is not due to excitatory or inhibitory contextual conditioning occurring during fear conditioning and extinction, respectively. Rather, the context comes to modulate or "set the occasion" for CS-US and CS-'no US' associations (Bouton, 1993). As such, after extinction, the CS has two possible meanings: 1) the CS predicts the US and 2) the CS predicts the absence of the US. The context in which the CS is presented ultimately determines which association is retrieved and thereby determines whether fear is expressed or not. For example, if an extinguished CS is presented in the context in which extinction occurred, fear is suppressed. Conversely, if the CS it presented in an ambiguous or novel context, fear to the CS will return or renew. Thus, fear behavior after extinction depends specifically on the environment in which the cue is presented.

Extinction is not permanent loss of conditional responding. That is, learned fear responses are quick to return if there is a delay between extinction and retention testing. This property of extinction is termed spontaneous recovery (Rescorla, 2004). It is typically reported that the longer the extinction-to-test interval, the more robust the recovery is (Quirk, 2002). Similar to renewal, spontaneous recovery can also be explained by contextual modulation. Rather than a change in spatial cues, the passage of time creates a change in "temporal context" (Bouton, 1993). Interestingly, if there is both a physical and temporal change, there

is even more of an enhanced recovery than that observed with each context shift alone (Rosas, 1998).

Lastly, extinguished fear responses can be reinstated with unsignaled presentations of the US (Bouton, 1993). Specifically, if the US is delivered after extinction, there will be a recovery of conditioned responding when the CS is subsequently presented. There are two important points to demonstrate that reinstatement is a context-specific. First, reinstatement will only occur if the CS test after extinction occurs in the context in which the US was presented. Second, reinstatement does not occur if an unsignaled US is presented after fear conditioning and prior to any extinction training. This suggests that reinstatement of fear is due to contextual conditioning between the US and the context in which it is presented; this contextual conditioning triggers a fear response upon the presentation of the extinguished CS. Thus, the return of fear during reinstatement is the result of the summation of a weak context-US association and the residual excitatory associative properties of the extinguished CS.

Given the various behavioral properties of extinction, several different theories of what is learned during extinction have been posited. Robert Rescorla and Alan Wagner's model of associative learning describes extinction as a loss of associative strength to a CS that had accrued during conditioning; that is, extinction is a form of unlearning (Delamater, 2004; Miller et al., 1995; Rescorla and Wagner, 1972). Key to their associative learning model, Rescorla and Wagner claim that extinction learning is determined by US expectancy. By this view, CS-alone presentations lead to a surprising absence of the US, resulting in a decrease in the

associative strength of the CS. There are various empirical reports that support the idea that extinction occurs because of a violation of US expectancy (Holtzman-Assif et al.; Huh et al., 2009; McNally and Westbrook, 2003). For example, Huh et al. (2009) reported that there was increased phosphorylated extracellular signal-regulated kinase (pERK), a signature of cellular changes during learning, in the hippocampus when an expected footshock was not delivered. Furthermore, increasing US expectancy was associated with faster extinction and increases in pERK in the hippocampus. Huh et al. (2009) claimed that ERK signaling during extinction was specific to coding prediction error, as there was no ERK activation during habituation, or continual reinforcement. Others have also reported that opioid signaling within the periacqueductal gray (PAG) also mediates negative prediction errors during extinction (McNally et al., 2004; McNally and Westbrook, 2003; Quirk and Mueller, 2008) whereas dopamine in the nucleus accumbens is important for regulating prediction error during extinction (Holtzman-Assif et al., 2010)

However, the Rescorla-Wagner model cannot account for the recovery of conditional responding observed during renewal, reinstatement and spontaneous recovery. As such, others have proposed that extinction is actually a form of new learning, a notion that is supported by the observed properties of extinction (Bouton, 2004; Pearce and Hall, 1980; Konorski, 1967) as well as the fact that fear can be rapidly re-acquired after extinction (Bouton, 2004). By this view, a new inhibitory association between the CS and US is formed (a CS-"no US" association) during extinction that co-exists and competes with the original CS-US memory

(Konorski, 1967; Bouton, 1993; Myers and Davis, 2007). After extinction, the net sum of each association is zero; subsequent contextual cues, whether temporal, interoceptive or spatial in nature, gate which association is retrieved and ultimately expressed.

Other theorists have suggested that extinction is a form of non-associative learning. For example, extinction has been likened to the process of habituation, in which there is a decrease in responding to a stimulus when the stimulus is repeatedly presented over a long period of time (McSweeney and Swindell, 2002; Storsve et al., 2010). By this view, an organism may initially attend to the fact that the US no longer follows the CS, but once it is familiar with CS-alone presentations, it will ignore the CS (Kamprath and Wotjak, 2004; McSweeney and Swindell, 2002; Pearce and Hall, 1980). Interestingly, Rescorla and Heth (1975) argued that during CS extinction, habituation to the US also occurs. In other words, with initial CS presentations, the US representation is reactivated as it is a fundamental part of the CS-US association, but over the course of extinction, the US representation is devalued (Rescorla and Heth, 1975; Storsve et al., 2010). Though habituation and extinction have some features in common (McSweeney and Swindell, 2002), habituation does not account for the context-dependence of extinction.

Ultimately, it seems that multiple processes contribute to the acquisition of extinction. Both new inhibitory learning and loss of associative strength together might explain extinction learning. For example, it has been shown in numerous studies that the age at which extinction occurs critically determines whether new learning occurs during extinction (Kim and Richardson, 2007b, 2010). Kim et al.

(2007b) extinguished rats on either postnatal day 17 (P17) or postnatal day 24 (P24). Both age groups demonstrated low levels of fear at the end of extinction, but, remarkably, only the P24 rats renewed their fear the following day (Kim and Richardson, 2007b). In addition to renewal, it was also shown that P17 rats do not exhibit reinstatement and spontaneous recovery (Gogolla et al., 2009; Kim and Richardson, 2007a). This suggests that extinction erases the original CS-US association in preweanling rats.

What mediates the transition from "unlearning" to "new learning" during extinction? It has been suggested that the participation of several neural structures during extinction is limited in P17 rats. In adult rats, extinction is mediated by a distributed network of neural structures that includes the hippocampus, amygdala and prefrontal cortex (PFC). In P17 rats, however, only the amygdala seems to be required for the extinction of conditioned fear (Kim et al., 2009; Kim and Richardson, 2008). This is consistent with developmental literature showing that the hippocampus and the PFC are delayed in their full maturation (Van Eden and Uylings, 1985a, b; Wilson, 1984). As such, the unlearning or erasure that occurs during extinction in the P17 rats may be due to their relatively immature neural organization. Another contributing factor to the development from "unlearning" to "new learning" may be the involvement of perineuronal nets (PNNs), an extracellular matrix consisting of chondroitin sulfate proteoglycans (CSPGs). PNNs have been found to be involved in the induction of ocular dominance in the visual cortex during a critical period of development. Along these lines, Gogolla et al. (2009) hypothesized that PNNs in the amygdala may enable plasticity during

development that ultimately prevents the erasure of the original fear memory typically observed in young animals. They found that the time course for the development of PNNs and the persistence of fear memories after extinction were positively correlated (Gogolla et al., 2009). More importantly, intracranial infusions of a compound that disrupts CSPGs into the BLA in adult mice prevented renewal and spontaneous recovery after extinction. This evidence, along with other developmental literature, suggests that the dissociable extinction mechanisms in young and adult rats are due to distinct neural and cellular developmental processes.

Others have also shown that the interval between fear conditioning and extinction is a critical determinant of extinction learning. Maren and Chang (2006) demonstrated that if extinction occurs 15 minutes after fear conditioning (immediate extinction), there is no long-term retention of the extinction memory. That is, there is a within session decrease in fear during extinction, but when tested 48 hours later, there is recovery of the fear response (Maren and Chang, 2006, but see Myers et al., 2006). In a follow-up study, Chang and Maren (2009) showed that if rats were tested 15 minutes after immediate extinction, rats do show suppression of fear behavior, but it does not last (Chang and Maren, 2009). This short-term fear suppression, however, is context-independent insofar as rats that received immediate extinction did not renew their fear when tested 15 minutes after extinction in a different context. Based on their findings, they concluded that rather than learning a CS-"no US" association during immediate extinction, rats were using nonassociative mechanisms during extinction. Specifically, animals were

habituating to the CS, independent of the context in which it was presented. Like extinction, habituation can display spontaneous recovery (McSweeney and Swindell, 2002), which is consistent with the fact that rats exhibited high levels of fear during the retention test. Unlike extinction, however, short-term habiutation does not seem to be context-dependent. These results are especially informative when considering therapeutic treatments for anxiety disorders as it suggests that early interventions may actually exacerbate the recovery of traumatic memories.

Neurobiology

Similar to fear conditioning, extinction is not mediated by one specific brain region. Rather, extinction depends on plasticity within a distributed neural network. The amygdala, PFC and hippocampus have all been implicated in the acquisition, consolidation and retrieval of extinction of conditioned fear (Quirk and Mueller, 2008). Specifically, the amygdala is thought to be the site of acquisition and storage of the extinction memory; the PFC, specifically the infralimbic area (IL), is thought to mediate the consolidation of extinction. Finally, the hippocampus plays a role in the context-dependent expression of extinction. In this section, the focus will be on how the circuit-level interactions between these brain regions mediate extinction.

There are robust reciprocal connections between the amygdala and the hippocampus (Canteras and Swanson, 1992; Pitkanen et al., 2000). Specifically, projections from the hippocampus to the amygdala arise in the ventral subiculum/ventral CA1 region of the hippocampus and traverse through the ventral angular bundle (VAB). Projections from the ventral subiculum terminate heavily in

the LA, BM, BA and CeM of the amygdala. The ventral CA1 however primarily projects only to the BA. Projections from the amygdala to the hippocampus mostly arise in the BA of the amygdala and terminate in the ventral subiculum, CA1, CA2 and CA3 subfields of the hippocampus. Given the heavy connections between these two areas, it is possible for both the amygdala and hippocampus to communicate with one another. This communication seems to be especially important for the context-specific retrieval of extinction. Indeed, it has been shown that the hippocampus regulates context-specific firing within the amygdala after extinction (Maren and Hobin, 2007). That is, infusions of muscimol into the dorsal hippocampus block the increase in LA firing typically observed during the renewal of fear (Maren and Hobin, 2007; Hobin et al., 2003). Furthermore, it has been shown that the ventral hippocampus projects onto neurons within the amygdala that are selectively active during renewal (Herry et al., 2008). It is possible that communication between these two structures also is involved in extinction learning, given that hippocampal inactivation impairs the acquisition of extinction (Corocoran et al., 2005). It is important to point out that these effects are different than those observed with pre-training hippocampal lesions (Frohardt et al, 2000; Zelikowsky et al., 2011) as they do not interfere with the acquisition of extinction. This is because other neural structures are able to compensate for the loss of the hippocampus during conditioning and thus encode the memory using an elemental strategy (but see Wiltgen et al., 2006). If the hippocampus is intact during conditioning, however, any manipulation thereafter will cause impairments in fear expression and extinction, as the source of the configural representation of the context is gone.

Like the amygdala, the prefrontal cortex is a major target of hippocampal projections (Jay and Witter, 1991; Vertes, 2004; Cenquizca and Swanson, 2007; but see Swanson, 1981). The PFC is comprised of various subregions, but the ones that have been specifically implicated in extinction are the prelimbic (PL) and infralimbic areas located in the ventromedial PFC. Both the PL and IL receive input from the ventral CA1 and ventral subiculum (Hoover and Vertes, 2007). Low-frequency electrical stimulation (LFS) of the dorsal hippocampus attenuates extinction-related long-term potentiation within the PFC (Farinelli et al., 2006) and impairs extinction recall. In contrast, high-frequency stimulation of the dorsal hippocampus restores extinction-related potentiation within the PFC and, importantly, facilitates the recall of extinction. Similarly, others have shown that the stimulation of the ventral hippocampus results in similar LTP-like changes in the PFC as those observed after extinction (Hugues et al., 2006). Moreover, this prefrontal plasticity can be blocked by infusions of a mitogen-activated protein kinase (MAPK; a protein kinase known to be important in the cellular cascades underlying synaptic plasticity) inhibitor. Interestingly, a more recent study suggests that hippocampal input to the IL is a major source of brain-derived neurotrophic factor, a growth factor that is necessary for successful suppression of conditioned fear (Peters et al., 2010). Taken together, this evidence suggests that hippocampal projections to the PFC elicit synaptic changes that may be responsible for the consolidation of extinction.

Additionally, there are strong reciprocal connections between the PFC and the amygdala. Anatomical studies show that IL projects to the BM, CeA and ITC, whereas the PL sends robust projections to the BLA and CeA (McDonald et al., 1996;

Vertes, 2004). Interactions between the prefrontal cortex and amygdala have received the most attention with regard to extinction. Specifically, it is thought that the mPFC influences CeA activity, which can result in the suppression of fear. However, there are various theories as to how this actually occurs. *In vivo* work in anesthetized rats has shown that the stimulation of either the IL or PL results in the inhibition of BLA through the activation of BLA inhibitory interneurons (Grace and Rosenkranz, 2002; Rosenkranz and Grace, 2001). In fact, it has been demonstrated that PFC stimulation in anesthetized rats suppressed LA activity in response to the presentation of a previous conditioned stimulus (Rosenkranz et al., 2003). This suggests that during extinction, the excitability of BLA projections neurons is reduced via local inhibitory circuits. This subsequently results in a decrease in CeA activity and fear behavior.

It has also been posited that the mPFC, specifically the IL, regulates amygdala activity through its projections to the ITC cells. For example, Quirk et al. (2003) were the first to show that stimulation of the PFC reduced CeA responsiveness to BLA excitatory input. However, this decrease in CeA activity is not thought to be due to PFC synapses on BLA interneurons, as Rosenkranz and Grace (2001) proposed. Specifically, others have shown that PFC stimulation excites BLA neurons (Likhtik et al., 2005) and that the PFC projects onto BA neurons selectively active during extinction (Herry et al., 2008). This indicates that, downstream of the BLA, there is an active gating mechanism that inhibits CeA activity during extinction (Likhtik et al., 2005; Quirk et al., 2003). The ITC cells have emerged as likely candidates because they are GABAergic, project to the CeA and receive

glutamatergic input from both the IL and BLA (Royer et al., 1999; Royer and Pare, 2002). Consistent with this, chemical stimulation of the IL with picrotoxin results in an increase in c-fos in ITC cells (Berretta et al., 2005) while selective lesions of ITC cells impair the expression of extinction (Likhtik et al., 2008). In a recent study, Amano et al. (2010) examined how the IL modulates BLA input onto ITC cells during extinction. Twenty-four hours after extinction, rats were sacrificed and coronal slices of their amygdala were prepared (Amano et al., 2010). Whole-cell recordings were made from CeM neurons during BLA stimulation. They found that in rats that were extinguished, there was greater synaptic inhibition in the CeM. In addition, they reported that ITC cells of extinguished rats were significantly more responsive than ITC cells of control groups (animals that were only conditioned or received unpaired CS and US presentations). This enhancement of ITC responsiveness was due to an increase in BLA neurotransmitter release and an alteration in the phosphorylation level of ionotropic glutamate receptors on ITC cells. Lastly, they demonstrated that the increased BLA-ITC efficacy is dependent upon the IL. This provides strong evidence that suppression of fear during extinction is mediated to a large extent by the regulation of ITC cells by IL glutamatergic activity.

Contextual modulation of the expression of extinction

As described above, retrieval of an extinction memory is under the control of contextual cues. For example, the expression of extinction will only occur if the extinguished CS is presented in the context in which extinction occurred. However, if the CS is presented in a different context, fear to the CS will renew (Bouton and

Bolles, 1979). Similarly, fear to a CS will become reinstated only if it is presented in the context in which an unsignaled US occurred after extinction (Bouton, 2004). In the last decade, considerable research has focused on the neurobiology underlying the contextual modulation of behavior after extinction (Maren and Quirk, 2004; Maren, 2005, 2011). It is widely accepted that, similar to the acquisition of extinction, the context-specific expression of extinction is mediated by a distributed network, including the hippocampus, prefrontal cortex and amygdala. In this section, I will review the extant literature pertaining to the contextual modulation of fear after extinction.

Neurobiology

Because the hippocampus has been shown to be important for contextual processing and discrimination (Fanselow, 2000), it has been hypothesized that this region may be responsible for contextually modulating fear behavior after extinction. Early studies show that pre-conditioning lesions of the fornix (Wilson et al., 1995) or the hippocampus (Frohardt et al., 2000) only abolish reinstatement (Frohardt et al., 2000) and spontaneous recovery (Wilson et al., 1995), but do not have any disrupting effects on renewal (Frohardt et al., 2000; Wilson et al., 1995). However, the results from these early studies do not preclude the hippocampus from being involved in the renewal of fear: because lesions were performed prior to any behavioral sessions, it is possible that other brain structures compensated for the loss of the hippocampus. To avoid this methodological problem, we have used inactivation techniques in our laboratory to temporarily inhibit the hippocampus only during the test session. With this procedure, we have demonstrated that

reversible inactivation of the dorsal (Corcoran et al., 2005; Corcoran and Maren, 2001, 2004) or ventral hippocampus (Hobin et al., 2006) with muscimol, a GABA-A agonist, prior to the retention test eliminated renewal, as evidenced by low levels of fear when the CS presentation occurred outside the extinction context. More recently, Knapska and Maren (2009) have shown that c-fos expression is elevated in the ventral CA1 and dentate gyrus of the hippocampus during both renewal and extinction memory retrieval. Together with the inactivation data, this suggests that the hippocampus is involved in disambiguating the meaning of CS using contextual cues.

In a further attempt to explore the role of the hippocampus in the renewal of fear, Zelikowsky et al. (2011) varied the time in which rats received DH lesions and assessed the effects of the lesions on renewal of fear. Similar to previous work (Frohardt et al., 2000), rats that received pre-training lesions of the DH renewed their fear to the extinguished CS. In contrast, rats that received post-extinction lesions failed to renew their fear. This suggests that if the hippocampus participates in the acquisition and extinction of fear, it is necessary for the renewal of fear. However, if rats are trained and extinguished without a hippocampus, they are still able to display renewal, which is presumably mediated by another compensatory brain structure. This pattern of results is similar to that seen with the effects of pre-training versus post-training lesions on the expression of non-extinguished fear. Interestingly, Zelikowsky et al. (2011) also showed that rats could only renew their fear in the absence of a hippocampus if the tone duration during extinction training and test matched. When lesioned rats were extinguished with a discrete tone, but

were tested with a continuous tone, renewal was severely impaired. Based on these findings, Zelikowsky and colleagues (2011) claim that without a hippocampus, rats become more sensitive to temporal changes in the CS. In intact rats, however, the hippocampus allows the animal to generalize across temporal differences in CSs to guide conditional responding.

The prefrontal cortex has also been implicated in regulating fear behavior after extinction. For example, the PL is thought to be involved in renewal of fear. Support for this comes from studies showing that the PL is involved in the expression of conditioned fear (Blum et al., 2006; Corcoran and Quirk, 2007). Furthermore, stimulation of the PL results in freezing behavior (Vidal-Gonzalez et al., 2006) and elicits firing within the BA (Likhtik et al., 2005). Burgos-Robles et al. (2009) have recently shown that PL firing patterns parallel freezing behavior during fear conditioning and extinction. That is, there is sustained firing within the PL during fear conditioning, which gradually decreases during extinction (Burgos-Robles et al., 2009). Finally, renewal of fear is associated with elevated levels of c-fos expression within the PL (Knapska and Maren, 2009). In contrast, the IL is thought to mediate the retrieval of extinction. In early studies, it was shown that lesions of the medial prefrontal cortex that specifically included the IL prevented retention of the extinction memory (Lebron et al., 2004; Quirk et al., 2000, but see Garcia et al., 2006). Furthermore, inactivation of the IL prior to an extinction retrieval test results in high levels of freezing, indicating the IL is necessary for the retrieval of the extinction memory (Sierra-Mercado et al., 2006). Using electrophysiological methods, it has been shown that the IL selectively responds to

extinguished CSs, which is associated with low levels of fear (Herry and Garcia, 2002; Milad and Quirk, 2002, but see Chang et al., 2010). Interestingly, the recall of extinction is accompanied by an increase in neuronal bursting in the IL, which is thought to increase the chance of the IL activating inhibitory cells within the amygdala to gate fear expression (Chang et al., 2010; Santini et al., 2008). Additionally, if the IL is stimulated in conjunction with a non-extinguished CS, rats display low levels of freezing, analogous to a post-extinction state (Milad and Quirk, 2002; Milad et al., 2004). Finally, Knapska and Maren (2009) observed a significant increase in c-fos expression within the IL during the retrieval of extinction as compared to the renewal of fear. Taken together, it seems that the prelimbic and infralimbic areas work in an opposing manner: activity in the prelimbic results in fear perseveration whereas activity in the IL promotes successful suppression of fear.

Given that the amygdala is important for the acquisition of extinction (Falls et al., 1992), it is no surprise that it has been shown to be important for the context-dependent expression of extinction. In an elegantly designed experiment, Hobin and Maren (2003) examined the role of the LA in the context-dependent expression of extinguished fear. Rats were trained with two different CSs (CS1 and CS2), which were then extinguished in different contexts the following day. After implanting a recording electrode in the LA, rats underwent retention tests in which each CS was presented within its extinction context (consistent, CONS) as well as in the context in which the other CS was extinguished (inconsistent, INCONS). During the CONS test, rats exhibited low levels of fear; in contrast, rats displayed significantly more

fear during the INCONS test. Remarkably, spike firing within the LA was highest when the CS was presented outside the extinction context. This indicates that after extinction, the LA represents CSs that are ambiguous with respect to their associative meaning. Interestingly, it was further shown that the inactivation of the dorsal hippocampus eliminates this context-dependent firing pattern in the LA (Maren and Hobin, 2007). This finding suggests that contextual information processed by the hippocampus converges on both inhibitory and excitatory associations within the amygdala; this integration results in either the expression of extinction or renewal, respectively.

In a more recent study, Herry et al. (2008) discovered that within the BA, there are two separate populations of neurons that are selectively active during extinction ("extinction" neurons) and renewal ("fear" neurons). To first identify these populations, these authors used a discriminative fear conditioning protocol in which CS+ was paired with a footshock whereas CS- was presented alone. "Fear" neurons in the BA showed an increase in firing to the CS+ during and after conditioning; extinction of the CS+ eliminated firing within these BA neurons. Importantly, "fear" neurons did not respond to the CS-. During extinction of the CS+, however, there was an increase in firing in the "extinction" neurons that was absent during and after fear conditioning. To further demonstrate that "fear" and "extinction" neurons were selectively recruited during fear behavior and extinction, respectively, mice underwent a discriminative extinction paradigm in which two different CSs (CS1 and CS2) were fear-conditioned, but only CS1 was extinguished. "Fear" neurons only responded to the non-extinguished CS2 whereas the

"extinction" neurons specifically fired in response to the extinguished CS2. Remarkably, during extinction, "extinction" neurons began to increase their firing pattern before the activity of the "fear" neurons disappeared, clearly demonstrating that within the BA, neuronal activity can be switched between active and inactive states very quickly. Extending these findings, the authors went on to show that activity in "fear" neurons reemerges after extinction if the CS is presented outside the extinction context. Similarly, when mice are tested for extinction memory retrieval, "extinction" neurons are selectively engaged. Interestingly, it was shown that these segregated populations of neurons have distinctly different connections with the hippocampus and prefrontal cortex. Using orthodromic and antidromic stimulation of BA afferents and efferents, respectively, Herry et al. (2008) showed that "fear" neurons receive input from the ventral hippocampus and project to the prefrontal cortex. "Extinction" neurons, on the other hand, were reciprocally connected with the prefrontal cortex only. The notion that neurons within the BA are active during both the expression of extinction and renewal is consistent with findings from Knapska and Maren (2009) in which they observe high levels of c-fos expression in the BA during both renewal and extinction recall. Thus, this evidence suggests that the BA is required for the context-dependent expression of extinction, possibly to allow for discrimination between CSs with different meanings. Interestingly, there is indirect evidence that indicates that there may also be similar populations of neurons within the LA. For example, Repa et al. (2001) describe LA neurons that are extinction resistant whereas Hobin et al. (2003) report that approximately 25% of LA neurons selectively fire within the extinction context,

suggesting the presence of "fear" and "extinction" neurons within the LA, respectively. Whether these populations of neurons truly exist within the LA, however, has not been proven and thus, requires further scrutiny.

Specific Aims and Hypotheses

The main focus of this dissertation is to explore how contextual information comes to modulate behavioral responses after extinction. Though there seems to be concrete evidence as to the individual roles of the hippocampus, amygdala and prefrontal cortex in the context-dependent expression of fear, it is less clear how this contextual modulation occurs. It is currently appreciated that the hippocampus is positioned in such a way to influence amygdala activity, as it has connections with both the amygdala and the prefrontal cortex (Canteras and Swanson, 1992; Pitkanen et al., 2000; Figure 2; Vertes, 2004). As such, we hypothesized that hippocampus modulates amygdala activity through its direct connections to the BA and through its indirect pathway via the prefrontal cortex. In Chapter 2, we first explored this hypothesis by measuring neuronal activation, as assessed by c-fos, in cells in the prefrontal cortex and VH that project to the BA during context-dependent retrieval of fear. We found that BA-projecting neurons in both the PL and VH are engaged during the renewal of fear, whereas BA-projecting neurons within the IL are active during the recall of extinction.

Though Chapter 2 reveals crucial anatomical information as to the neural circuit involved in renewal, it does not unequivocally identify how contextual information is relayed from the hippocampus to the amygdala. Thus, in Chapter 3,

we examined the necessity of each of the pathways from the VH to the BA through the use of asymmetrical lesions of the VH and BA or the VH and PL. Interestingly, we found that disconnection of either pathway resulted in the elimination of renewal. Together with the results of Chapter 2, these findings indicate that convergent input from the PL and VH within the BA is required for the contextual expression of fear after extinction.

Herry and colleagues (2008) have recently reported that within the BA, there exists two distinct populations of neurons: those that are engaged during fear conditioning and renewal ("fear" neurons) and those that are engaged during the presentation of an extinguished CS ("extinction" neurons). This seems to suggest that discrimination between a fearful and extinguished CS occurs at least at the level of the BA. However, it is unclear whether this pattern extends to the prefrontal cortex and VH. As such, in Chapter 4, we used cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH) to examine whether segregated populations of neurons in the prefrontal cortex, hippocampus and amygdala were engaged during renewal and extinction recall. As previously reported with c-fos (Knapska and Maren, 2009), we observed that the expression of *Arc,* another immediate early gene, was contextually regulated after extinction. In addition, we found that within the BA, there were segregated populations of neurons devoted to renewal or extinction recall, as has been previously shown (Herry et al., 2008). Importantly, we noticed that the increase in functional activity of these cell assemblies seemed to be a result of extinction training. Unlike the BA, the PL contained neurons that responded to the CS regardless of extinction training

and the context in which the CS was presented. Lastly, we observed that within the VH, there was a heterogeneous population of neurons that were engaged during renewal, extinction or both. As a whole, these findings suggest that the extinguished CS is represented differentially within the circuit. Furthermore, they indicate that through the convergence of VH and PL input in the BA during extinction, discrete cell assemblies emerge in the amygdala that respond to the CS in a context-dependent manner.

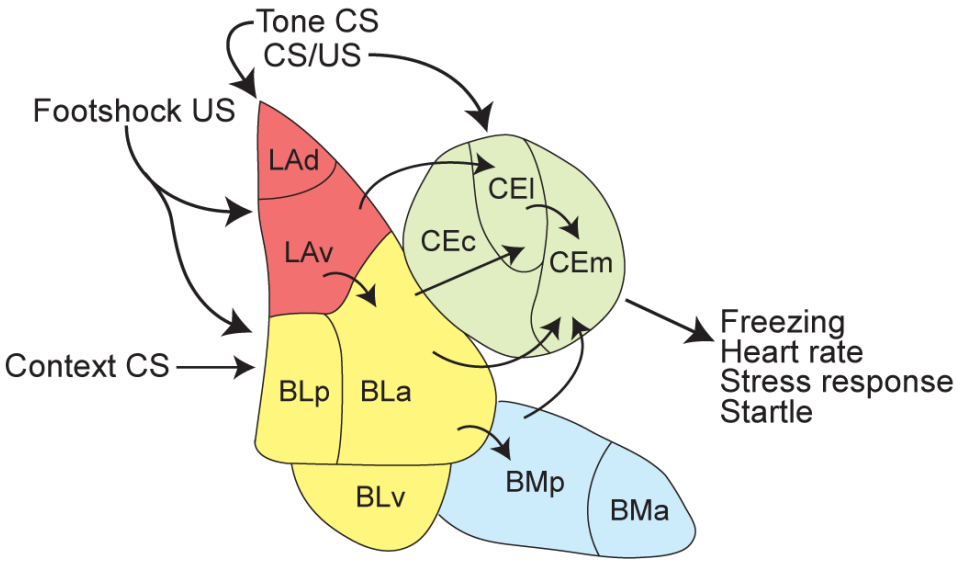

Figure 1.1. Amygdala anatomy and connectivity during fear conditioning in rats. The amygdala consists of several nuclei that are integral to the acquisition and retention of fear memories. Tone conditioned stimulus (CS) and footshock unconditioned stimulus (US) information converges within the lateral amygdala [LA; dorsal lateral amygdala (LAd) and ventral lateral amygdala (LAv)], as well as the basolateral amygdala [BL; posterior basolateral amygdala (BLp), anterior basolateral amygdala (BLa) and ventral basolateral amygdala (BLv)]. Additionally, CS and US information are processed in parallel within the central amygdala [CeA; centrolateral amygdala (CEl) and centromedial amygdala (CEm)]. Lastly, contextual conditioned stimulus information is transmitted to the basal amygdala. From the BL, information is relayed to the CeM, thought to be the output of the amygdala, either through the CeL or by coursing through the basomedial amygdala [BM; posterior basomedial amgydala (BMp) and anterior basomedial amygdala (BMa)]. The CeM projects to downstream structures, such as the periaqueductal gray, that produce various fear responses, including freezing and increases in heart rate, among others.

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CHAPTER II

HIPPOCAMPAL AND PREFRONTAL PROJECTIONS TO THE BASAL AMYGDALA ARE ENGAGED DURING THE CONTEXT-SPECIFIC EXPRESSION OF EXTINCTION IN RATS

In recent years, considerable interest has emerged in the extinction of learned fear insofar as it is central to several clinical interventions, including exposure therapy. During extinction, a previously conditioned stimulus (CS) is repeatedly presented without the unconditioned stimulus (US). This results in a gradual decrease in learned fear responses, such as freezing behavior (Maren, 2001). However, extinction does not erase the original fear memory; rather, it yields a new inhibitory memory that reduces fear to the CS (Quirk and Mueller, 2008). Which memory is retrieved depends on the retrieval context; fear to an extinguished CS is suppressed in the extinction context but "renews" when it is presented outside the extinction context (Bouton and Bolles, 1979). The renewal of extinguished fear presents obvious challenges for the efficacy of behavioral interventions for fear and anxiety disorders.

Recently, substantial progress has been made in understanding the neural mechanisms for the context-dependence of extinction (Maren, 2005; Maren, 2011) This work has revealed that the hippocampus, a structure critical for context processing (Fanselow, 2000), plays an important role in the contextual modulation of fear after extinction. For example, pharmacological inactivation of either the

dorsal (DH; Corcoran and Maren, 2001) or ventral hippocampus (VH; Hobin et al., 2006) in rats eliminates the renewal of fear to an extinguished CS outside the extinction context. Interestingly, hippocampal inactivation also eliminates the contextual modulation of CS-evoked spike firing in the amygdala after extinction (Hobin et al., 2003; Maren and Hobin, 2007), suggesting that hippocampo-amygdala projections (Canteras and Swanson, 1992; Pitkanen et al., 2000) mediate the context-dependence of extinction. Because the VH is the primary source of contextual information to the amygdala (Pitkanen et al., 2000), it is conceivable that this direct projection is necessary for the renewal of fear. Indeed, neurons within the basal amygdala (BA) that are active during renewal receive direct projections from the VH (Herry et al., 2008).

Another route by which the VH can influence BA activity is via the prelimbic cortex (PL). The VH has robust projections to the PL (Vertes, 2006), which in turn has reciprocal connections with the BA (McDonald et al., 1996; Vertes, 2004). Prelimbic lesions or inactivation impair fear expression (Blum et al., 2006; Corcoran and Quirk, 2007) and microstimulation of the PL results in both freezing behavior (Vidal-Gonzalez et al., 2006) and increases in BA firing (Likhtik et al., 2005). Additionally, PL activity during fear conditioning correlates with the expression of freezing (Burgos-Robles et al., 2009). Lastly, we have found increased c-fos expression in the PL after the renewal of fear (Knapska and Maren, 2009). Hence, it is possible that the hippocampus contributes to the context-dependence of extinction through either direct or indirect projections to the BA.

To explore this question, we used functional retrograde tracing to determine whether BA-projecting neurons in the VH and PL are differentially activated (as indexed by c-fos expression) during renewal of fear after extinction. Collectively, our results indicate that input from the PL and VH to the BA is required for the renewal of fear after extinction.

General Methods

Subjects

Subjects were male Long-Evans rats (220-224 g; Blue Spruce), obtained from a commercial supplier (Harlan Sprague-Dawley, Indianapolis, IN). Animals were individually housed in clear plastic hanging cages and were kept on a 14:10 light: dark cycle and had free access to food and water. Rats were handled 15-20 sec/day for five days before the start of the experiment so as to acclimate the animals to the experimenter. All experimental procedures were carried out in accordance with the protocols approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Behavioral apparatus

All behavioral sessions were carried out in eight identical observation chambers (30 X 24 X 21 cm; Med-Associates, St. Albans, VT), each located in an individual sound-attenuating cabinet. The observation chambers were constructed of two aluminum sidewalls and a Plexiglas ceiling, back and door. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) used for delivery of the footshock unconditioned stimulus (US). The rods were wired to a shock source

and a solid-state shock scrambler (Med-Associates, St. Albans, VT). To deliver the acoustic CS, a speaker was mounted on one wall of each chamber. Additionally, ventilation fans and house lights were installed in each chamber to allow for the manipulation of contexts during training, extinction, exposure and testing. We used a three-context ("ABC") renewal procedure that allows both fear and c-fos expression evoked by an extinguished CS to be assessed independent of background fear to the context (Corcoran and Maren, 2001; see procedure below). For Context A (conditioning context), house lights and room lights were on, ventilation fans (65 db) were turned on, cabinet doors were left open and the chambers were cleaned with 1% acetic acid. For Context B (extinction and test context), house lights and ventilation fans were turned off, the cabinet doors were closed and the chambers were cleaned with 1% ammonium hydroxide. Additionally, the room was illuminated by fluorescent red lights. For Context C (test context), house lights were on, ventilation fans were off, the room was illuminated with fluorescent red light and cabinet doors were left open. Black Plexiglas floors were placed on the grid of each chamber and chambers were cleaned with 10% ethanol. In each context, stainless steel pans were filled with a thin layer of the context's respective odor and inserted below the grid floor.

During the behavioral sessions, motor activity was measured by recording the displacement of each chamber by a load cell platform located below each chamber. Prior to the experiment, all load cell amplifiers were calibrated to a fixed chamber displacement and the output of each amplifier was set to a gain that optimally detected freezing behavior (vernier dial = 8; somatomotor immobility,

except that required for breathing). Load-cell amplifier output (-10 to +10V) was then digitized (5 Hz) and acquired online with Threshold Activity software (Med-Associates, St. Albans, VT). Absolute values of load-cell voltages were multiplied by 10, which resulted in an activity score that ranged from 0 to 100. A bout of freezing was scored if at least five contiguous load-cell values fell below the freezing threshold. In other words, activity had to be below threshold for at least 1 sec to be scored as freezing behavior. This method of assessing freezing behavior correlates highly with time sampling of behavior by trained observers (Maren, 1998).

Behavioral procedures

Thirty-six rats were randomly assigned to groups that were to be tested either within the extinction context (SAME) or outside the extinction context (DIFF), and those that were left in the home cages during the test session (HOME). We used a three-context ("ABC") renewal procedure in which rats were conditioned in context A, extinguished in context B or C, and then tested in context C (extinction and test contexts were counterbalanced). This yielded groups tested in the extinction context (SAME, ACC) or in another, familiar context that had not hosted extinction (DIFF, ABC). This renewal design is critical because it allows the assessment of fear and c-fos expression to an extinguished CS independent of fear to the context in which the CS is tested (i.e., rats are never tested in the conditioning context as they are in a typical ABA design). Moreover, all rats were tested identically and in the same physical contexts so that any differences in behavior and c-fos expression could be attributed to the meaning of the CS in that context, and not the CS or context itself.

One week prior to all behavioral sessions, rats received unilateral intracranial injections of cholera toxin subunit b (CTb; List Biological #104) into the BA. The side of the injection was counterbalanced across all behavioral groups. After allowing for one week of recovery, rats underwent fear conditioning in context A. Conditioning consisted of five tone (CS; 10 sec, 85 db, 2 kHz)-footshock (US; 1.0 mA, 2.0 sec) pairings with 60 sec interstimulus intervals (ISIs). The chamber position of each animal was counterbalanced across each experimental group and training squad. Twenty-four hours later, animals underwent extinction in context B or C (counterbalanced across groups) where they received 45 tone-alone (10 sec) presentations with 30 sec ISIs. Prior to this session, the rats were exposed to the alternate context (i.e. they were exposed to context B if they were extinguished in context C) to ensure that the test contexts were equally familiar for all of the rats. Twenty-four hours after extinction, the rats were returned to the observation chambers (Context C) for a test session consisting of five tone-alone (10 sec) presentations with 30 sec ISIs. Rats were sacrificed ninety minutes after the first tone presentation to assess c-fos expression induced by the test session. In all behavioral sessions, freezing was used as an index of fear.

Surgical procedures

Rats were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) and given atropine sulfate (0.4 mg/kg, i.p.). After being placed into the stereotaxic apparatus (David Kopf instruments, Tujunga, CA), the scalp was incised and retracted. The head was leveled to ensure that lambda and bregma were in the same horizontal plane. One small burr hole was drilled to allow for a 30-gauge

injector (Small Parts, Inc) to be lowered into the basal amygdala (AP: -3.1; ML: ± 4.6-5.1; DV: -8.1 from dura). The injector was attached to polyethylene tubing, which was connected to Hamilton syringes (10 μl) located on an infusion pump. Cholera toxin subunit b (List Biological, #104) was unilaterally infused at a rate of 0.1 μl/minute for 1 minute (0.1 μl total volume). The injector remained in the basal amygdala for another five minutes to allow for the diffusion of CTb. The hemisphere of CTb injection was counterbalanced across all behavioral groups (DIFF, SAME, HOME). The rats recovered on a heating pad before returning to their home cages and were allowed one week for post-operative recovery.

Immunohistochemistry

Ninety minutes after the first tone presentation, rats were overdosed with pentobarbital and transcardially perfused with ice-cold 0.1 M phosphate-buffered solution (PBS; pH 7.4) and 4% paraformaldehyde (PFA; pH 7.4). Brains were extracted and placed into 4% PFA for one hour and then transferred into a 30% sucrose/0.1M PBS solution. Brains were mounted and cut (30 μm) on a cryostat maintained at -20° Celsius. Coronal sections were collected every 150 μm and placed in 0.1 M PBS with 0.2% sodium azide. Additional sections were mounted on subbed slides with 70% ethanol for Nissl staining.

Immunohistochemistry was performed on free-floating sections. Sections were first washed two times in 0.1M PBS for ten minutes, followed by a third wash in 0.1M PBS with 0.3% Triton-X-100 for ten minutes. Tissue was then incubated at room temperature in 10% normal donkey serum (NDS) and 0.3% Triton-X-100 in 0.1M PBS for 30 minutes. Sections were then immediately transferred into the

primary antibody solution for a 48-hour incubation period [goat anti-c-fos at 1:1000 (Santa Cruz, sc-52-G); rabbit anti-cholera toxin subunit b at 1:3000 (Sigma, C3062); in 0.1M PBS with 10% NDS and 0.3% Triton-X-100) at 4° Celsius. Forty-eight hours later, tissue was washed three times in 0.3% Triton-X-100 in 0.1M PBS for ten minutes and then incubated in 10% NDS for 30 minutes. Sections were then incubated in the secondary antibodies [biotinylated donkey anti-goat at 1:200 (Santa Cruz, sc-2042); donkey anti-rabbit conjugated to Alexa Fluor 488 at 1:500 (Invitrogen, A-21206); in 0.3% Triton-X-100 and 10% NDS in 0.1M PBS) for 2 hours at room temperature. After being rinsed in 0.1 M PBS with 0.3% Triton-X-100, tissue was incubated in streptavidin conjugated to Alexa Fluor 594 (1:200 in 0.3% Triton-X-100 and 10% NDS in 0.1 M PBS; Invitrogen, S11223) for 1 hour at room temperature. To aid in the fluorescent detection, Signal Enhancer (3 drops per 8 ml solution; Invitrogen) was added to both the secondary antibody and streptavidin solution. After a final wash in 0.1 M PBS, tissue was mounted onto subbed slides in 0.9% saline and coverslipped with Vectashield (Vector Laboratories, H-1200).

Image analysis

We quantified the number of CTb- and c-fos-positive nuclei in the prelimbic cortex (PL), infralimbic cortex (IL), perirhinal cortex (PRh), auditory cortex (AC) and ventral hippocampus (VH; consisting of the ventral subiculum and ventral CA1 subfield). Multiple images were captured for each region, consistent with other published immunohistochemical reports (Herry and Mons, 2004; Berretta et al., 2005; Kim et al., 2009). Specifically, for the prelimbic region, four images were captured (two sets bilaterally at + 3.7 and +2.7 millimeters anterior to bregma). For

the infralimbic region, four images were captured (bilaterally at +3.2 and +2.7 millimeters anterior to bregma). Three images for the perirhinal cortex (-3.0, -3.6 and -4.0 millimeters posterior to bregma), auditory cortex (-3.6, -4.0, and -4.6 millimeters posterior to bregma) and ventral hippocampus (-5.6, -6.3 and -6.8 millimeters posterior to bregma) were taken on the side of the CTb injection. To verify the borders of cortical areas and the hippocampal layers, adjacent thionin-stained sections were used. All images were taken at 20X magnification (443 x 331 μm; 0.15 mm^2) with a Leica DM6000 B microscope (Wetzlar, Germany) outfitted with filters for different excitation/emission wavelengths (Knapska and Maren, 2009; Hamlin et al, 2009). For each region, the number of c-fos-positive, CTb-positive and double-labeled neurons were counted using an image analysis software program (ImageJ). Importantly, double-labeled neurons were readily observable as the resulting stain was visible in a center-surround manner for c-fos and CTb, respectively (Marchant et al., 2009; Leman et al., 2000). Counts for each image of the brain region were averaged across the number of images taken and group differences in absolute cell counts were analyzed with an ANOVA and Fisher's protected least significant difference (PLSD) post hoc tests. Results are represented as means (±*SEM*).

Behavioral data analysis

Freezing behavior was measured continuously during all of the behavioral sessions, including the pre-CS "baseline" period as well as during tone presentations and interstimulus intervals. Freezing was then analyzed and reported for each trial, which consisted of a CS presentation and the subsequent interstimulus interval.

For each period, the percentage of total observations in which freezing occurred was calculated. The percentage of freezing values were submitted to analysis of variance (ANOVA) and Fisher's PLSD post hoc tests were performed after significant omnibus *F*-ratios were obtained. All data are represented as means (±*SEM*).

Results

Freezing behavior during conditioning (context A), extinction (contexts B or C) and retrieval testing (context C) is displayed in Figure 2.1. Freezing behavior during conditioning and extinction was typical and did not differ between the groups [*F*s < 1]. As expected, conditional freezing differed markedly between the groups during the retrieval test (Figure 2.1). Rats tested outside the extinction context (DIFF) exhibited renewal of conditional freezing to the extinguished CS, whereas those tested within the extinction context (SAME) displayed low levels of fear. This impression was confirmed in an ANOVA performed on the CS-elicited freezing across the test trials [main effect of group: F (1, 23) = 24.3, $p < 0.01$]. Importantly, renewal of fear was not due to contextual fear because the levels of pre-CS freezing in the two contexts were not statistically different [DIFF = 19.3 ± 6.4%; SAME = 10.9 ± 2.9 %; F (1, 23) = 1.9, p = 0.2]. Moreover, differential freezing among the SAME and DIFF groups was not due to physical differences in the test contexts; all testing was conducted in identical contexts with the same CS.

After retrieval testing, rats were sacrificed to assess c-fos expression in BA afferents. A representative CTb injection site in the BA is shown in Figure 2.2A, along with a schematic illustration of maximal and minimal infusions (Figure 2.2C).

Only rats for which CTb labeling was confined to the BA were included in the analysis. Four rats were excluded because their CTb injections were not confined to BA. Another twelve rats were excluded because the CTb infusions were too small, resulting in weak staining. The final group sizes were: DIFF (n=9), SAME (n = 16), and HOME (n = 11).

Retrieval testing induced c-fos expression in the PL, IL, and VH, and many c-fos positive nuclei were co-localized with CTb-positive cells (Figure 2.3). The absolute counts of CTb- and c-fos positive nuclei as well as the percentage of double-labeled [(double-labeled cells/total CTb cells) X 100] neurons for the PL, IL and VH are shown in Figures 2.4A-C. Two-way ANOVAs with a within-subjects factor of brain region and between-subjects factor of group were performed for each dependent variable. The extent of CTb labeling in the groups did not differ in any of the brain regions [Figure 2.4A; *p*s > 0.05], although there was a main effect of brain region [F (4, 132) = 18.6, $p < 0.001$]. Post-hoc comparisons revealed that there were significantly more CTb-positive neurons in the IL than the PL ($p < 0.001$) and the VH ($p < 0.01$). Additionally, the AC had significantly less CTb-positive neurons than all other brain regions ($p < 0.001$) whereas the PRh had significantly more CTb-positive neurons than all other brain regions ($p < 0.01$), with the exception of the IL. This pattern of retrograde labeling is generally consistent with the distribution and density of projections from these regions to the BA (Romanski and LeDoux, 1993; Pitkanen et al., 2000; Vertes, 2004).

As we have previously reported, either the renewal or suppression of fear was associated with different patterns of c-fos expression in the PL, IL, and VH

(Figure 2.4B). This was confirmed by significant main effects of group [(F (2, 33) = 17. 5, $p < 0.001$] and brain region [F (4, 132) = 54.0, $p < 0.001$], and a significant group by region interaction [F (8, 132) = 8.3, $p < 0.001$] in the ANOVA. Within the PL, post-hoc comparisons revealed that rats in both the SAME and DIFF groups exhibited significant increases in c-fos expression relative to rats in the HOME group ($p < 0.001$). In the IL, c-fos expression was greater in the SAME group relative to both the DIFF and HOME groups ($p < 0.05$); c-fos expression was not significantly different between the DIFF and HOME groups ($p > 0.05$). In the VH, rats in the DIFF group exhibited significantly more c-fos expression than rats in the HOME group ($p < 0.05$). There were no significant group differences for c-fos expression in the AC and PRh ($p > 0.05$). This suggests that the PL, IL, and VH are differentially engaged by the renewal or suppression of fear to an extinguished CS.

Of considerable interest is the proportion of retrogradely labeled neurons in each area that express c-fos during retrieval testing (Figure 2.4C). We found significant group differences in the number of double-labeled neurons in the PL, IL, and VH. In particular, the VH and PL exhibited significantly more double-labeled neurons in the DIFF condition relative to the SAME and HOME conditions, whereas the number of double-labeled cells in the IL was greatest in the SAME condition. These impressions were confirmed in the ANOVA by significant main effects of group [F (2, 32) = 9.4, $p < 0.001$] and brain region [F (4, 128) = 22.2, $p < 0.001$] as well as a group by brain region interaction [F (8, 128) = 8.1, $p < 0.001$). Within the PL, post-hoc comparisons revealed that there were significantly more double-labeled neurons in the DIFF group than the SAME ($p < 0.001$) and the HOME group

($p < 0.001$). Within the IL, however, there were significantly more double-labeled neurons in the SAME group relative to the DIFF ($p < 0.05$) and HOME groups ($p < 0.001$). Additionally, there were more double-labeled neurons in the IL in the DIFF group than the HOME group ($p < 0.05$). In the VH, there were significantly more double-labeled neurons in the DIFF group relative to SAME ($p < 0.01$) and HOME ($p < 0.005$) groups. Within the VH, the SAME and HOME groups were not significantly different from one another, and there were no significant group differences in the AC or PRh. These data reveal that BA-projecting neurons in the VH and PL are preferentially engaged during fear renewal, whereas those in IL are engaged by the suppression of fear during the expression of extinction. Importantly, these patterns of c-fos expression are related not to the specific sensory properties of the CS or the test context (both of which are the same for the SAME and DIFF groups), but rather the meaning of the CS in a particular context.

Interestingly, the proportion of double-labeled neurons across the PL, IL, and VH within a retrieval condition was significantly different. Within the DIFF condition, post-hoc comparisons showed that the PL had the highest proportion of double-labeled neurons relative to all of the other brain regions ($p < 0.05$). Hence, although BA-projecting neurons in the VH exhibited greater c-fos expression in the DIFF relative to SAME conditions, the proportion of BA-projecting neurons exhibiting c-fos was much lower than that in the PL. In the SAME condition, however, the IL exhibited the highest proportion of double-labeled cells relative to the other groups ($p < 0.05$). Together, these results indicate that a greater number of BA-projecting neurons in the PL are engaged during renewal than BA-projecting

neurons in the VH. Conversely, a greater number of BA-projecting neurons in the IL are engaged during the retrieval of extinction relative to the PL and VH.

General Discussion

The present data reveal that both projections from the VH and PL to the BA are engaged during the renewal of fear after extinction. Importantly, we found that significantly more BA-projecting neurons in the PL and VH exhibited c-fos expression during the renewal of fear as compared to the recall of extinction. Conversely, more BA-projecting neurons within the IL exhibited c-fos expression during the recall of extinction than during renewal. These results reveal that the contextual regulation of extinguished fear memories requires a distributed neural network involving the VH, PL, and BA.

These results replicate and extend recent work from our laboratory that has shown that c-fos expression in the prefrontal cortex and hippocampus is regulated by the context in which an extinguished CS is retrieved (Knapska and Maren, 2009). In both studies, for example, we found that c-fos expression in the IL was significantly greater during extinction recall than during renewal. The present results extend this work by demonstrating that BA-projecting neurons within the IL are strongly recruited during extinction recall. Interestingly, we also found that BA-projecting neurons in the IL are engaged during renewal of fear, albeit to a lesser extent than those in the PL. Although IL activation in the renewal context was weak, it suggests that IL neurons that project to the BA may nonetheless have some role in promoting fear expression during renewal. Collectively, these data support the

view that the IL has an important role in the retrieval of extinction memories (Milad and Quirk, 2002; Sierra-Mercado et al., 2006).

The present results also confirm our previous findings showing c-fos expression in the PL and VH during renewal of fear to an extinguished CS (Knapska and Maren, 2009). However, unlike Knapska and Maren (2009), we observed an increase in c-fos expression within the PL during both the renewal of fear and during the expression of extinction (at levels similar to that in the IL). One potential reason for this discrepancy may be the differences in renewal paradigms used in each study; we used a three-context renewal design (ABC), whereas Knapska and Maren (2009) used a two-context design (AAB). In addition, different immunohistochemical detection methods for c-fos were used in each study. Regardless of these differences, however, both studies clearly demonstrate that neurons within PL and VH are engaged during fear renewal.

Of course, it is possible that the retrograde labeling we observed in BA afferents is due to CTb spread into neighboring amygdala regions. For example, CTb labeling in the IL could be a result of CTb diffusion into the adjacent intercalated cell masses (ITC), clusters of GABAergic interneurons that receive glutamatergic projections from the IL (Berretta et al., 2005). We believe that this possibility is unlikely, however, insofar as we were careful to only include subjects for which we had focal BA CTb injections. In addition, the afferent pathways we chose to investigate have very specific connections with the BA. For example, the BA is the only region of the amygdala that receives robust input from the ventral CA1 area of the hippocampus (Pitkanen et al., 2000). Given that cholera toxin is a selective

monosynaptic retrograde tracer (Bruce and Grofova, 1992; Conte et al., 2009), we are confident that the CTb labeling we have observed reflects specific afferent projections of the BA.

Although considerable data indicate an important role for the hippocampus in the renewal of extinguished fear (Corcoran and Maren, 2001; Corcoran et al., 2005; Hobin et al., 2006; Maren, 2011), we now show for the first time that the PL has an important role in this process. We found significantly greater numbers of BA-projecting neurons in the PL relative to the VH during the renewal of fear. The involvement of the PL in renewal is consistent with the emerging view that it has an important role in the expression of conditioned fear. For example, previous work has shown that CS-evoked neuronal activity in the PL parallels freezing behavior during conditioning and extinction (Burgos-Robles et al., 2009) and inactivation of the PL disrupts the expression of learned fear (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2010). It should be noted that the PL is not required for fear expression because PL inactivation spares unconditioned freezing (Corcoran and Quirk, 2007). Moreover, we have found that the expression of fear to a non-extinguished CS does not induce c-fos in PL neurons (Knapska and Maren, 2009). These data suggest that the PL, like the hippocampus, has an important role in contextual memory retrieval rather than in fear expression per se.

As a whole, these results provide valuable insight into how the hippocampus and prefrontal cortex communicate with the amygdala during contextual retrieval of fear. During renewal, it appears that the VH and PL actively project to the BA whereas during the recall of extinction, IL-BA communication is required. The latter

finding is consistent with previous data showing that the prefrontal cortex synapses on "extinction" neurons in the BA (Herry et al., 2008). That the VH projects to the BA during renewal of fear also aligns well with the findings of Herry et al. (2008), who show that the VH monosynaptically projects to "fear" neurons in the BA. Interestingly, these authors also show that "fear" neurons do not receive any input from the prefrontal cortex; rather, a large proportion of these neurons project back to the cortex. There are several possible reasons for these discrepancies. First, Herry et al. (2008) used mice whereas in our study, we used rats. Secondly, the two studies differ in how communication between these structures was identified. Whereas Herry and colleagues (2008) used orthodromic activation paired with electrophysiological recordings, we used tract tracing in conjunction with c-fos expression. These methodological differences could easily account for the disparities observed with respect to the prefrontal cortex's projections to the BA during renewal. It is also important to note that our findings do not preclude the fact that there may be projections from the BA to the prefrontal cortex during renewal, as well as extinction (as seen by Herry et al., 2008). Our retrograde tracing did not allow us to assess this projection pattern; the use of anterograde tracing may help confirm these findings.

In conclusion, our results provide new insight into the neural circuitry involved in the contextual regulation of fear memories after extinction. Specifically, we found that BA-projecting neurons in both the PL and VH are preferentially active during the renewal of fear to an extinguished CS. Because fear renewal poses a challenge to patients and clinicians, it is critical to understand how dysfunction in

hippocampo-prefrontal-amygdaloid connectivity underlies psychopathology. Insights into the neural mechanisms of fear and extinction promise to facilitate the development of more effective therapeutic interventions for individuals suffering from fear and anxiety disorders, such as post-traumatic stress disorder.

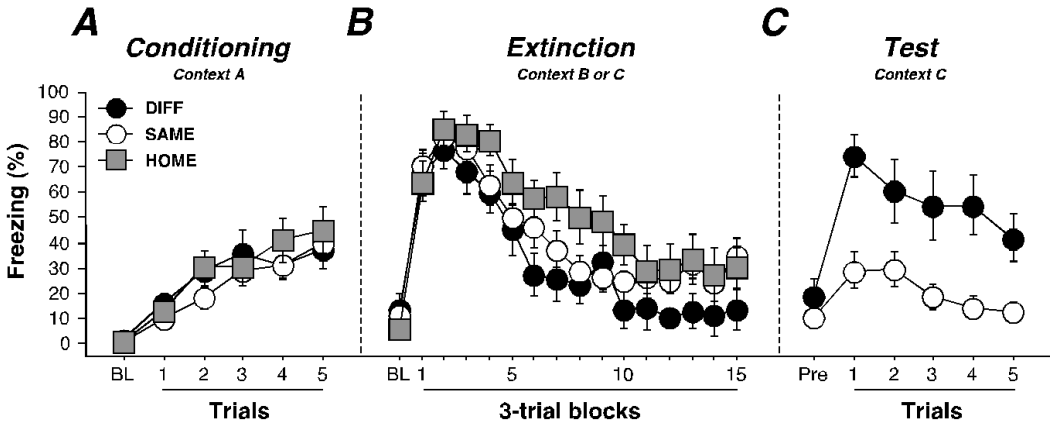

Figure 2.1. Conditioned freezing behavior in rats previously infused with CTb. *(A)* Mean percentage of freezing (±SEM) during fear conditioning, which consisted of a 3 min baseline period followed by 5 tone-shock pairings. Freezing was averaged across the pre-CS baseline (Pre) as well as during each of the five conditioning trials; each trial consisted of the average of freezing during each CS presentation and the subsequent interstimulus interval. *(B)* Mean percentage of freezing (±SEM) during the 45-tone alone extinction session. Freezing was averaged across the baseline period (Pre) as well as during the 45 extinction trials; as with conditioning, each trial consisted of the average of freezing during each CS presentation and subsequent ISI (data were binned into 15 blocks of 3-trial averages). *(C)* Mean percentage of freezing (±SEM) during the test session that consisted of 5 tone-alone presentations with 30 sec ISIs. Freezing was measured during the baseline (Pre) period and during the five trials, each of which consisted of a CS presentation and the subsequent ISI. Data are shown for rats that were tested outside the extinction context (DIFF; black circles), tested within the extinction context (SAME; open circles) or not tested at all (HOME; gray squares).

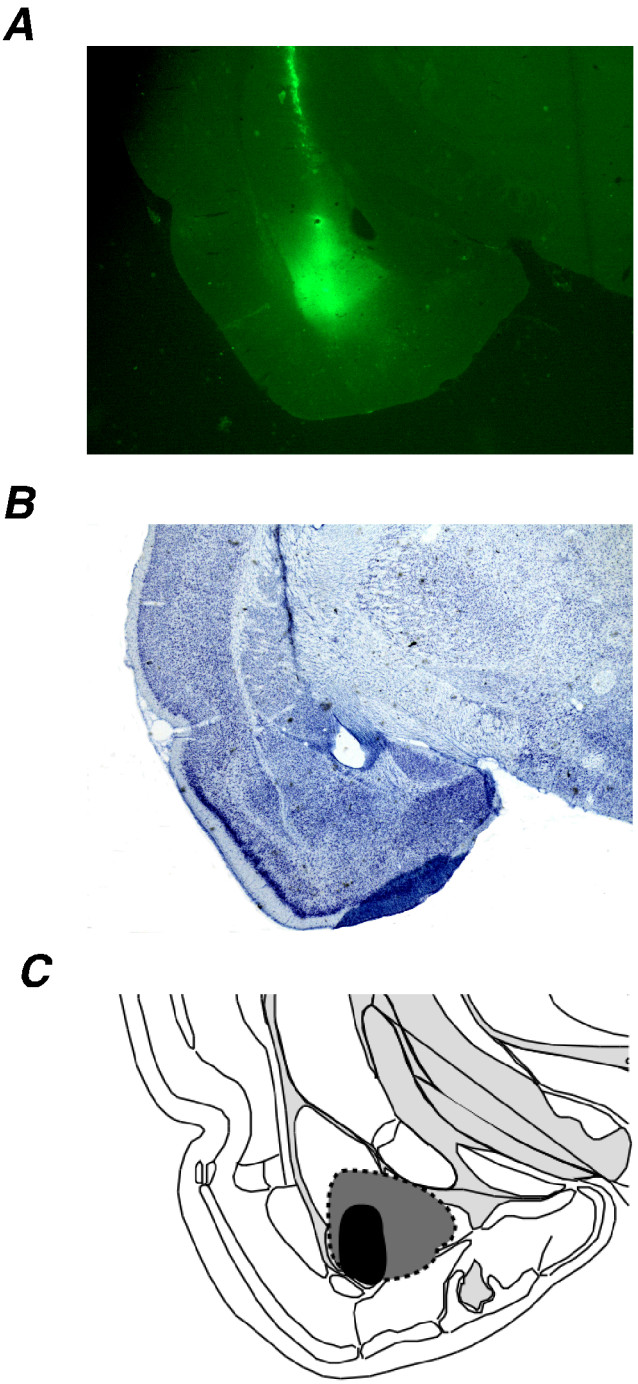

Figure 2.2. Illustrations of the CTb injection site and CTb spread within the basal amygdala (BA). *(A)* A representative CTb stained coronal section displaying the site of the CTb injection *(B)* Adjacent thionin-sections were used to ensure that CTb spread did not extend beyond the boundaries of the BA *(C)* Schematic of CTb spread within each behavioral group; black indicates rats with maximal CTb spread and gray represents rats with the smallest injections of CTb. The cartoon image was adapted from Swanson (2004).

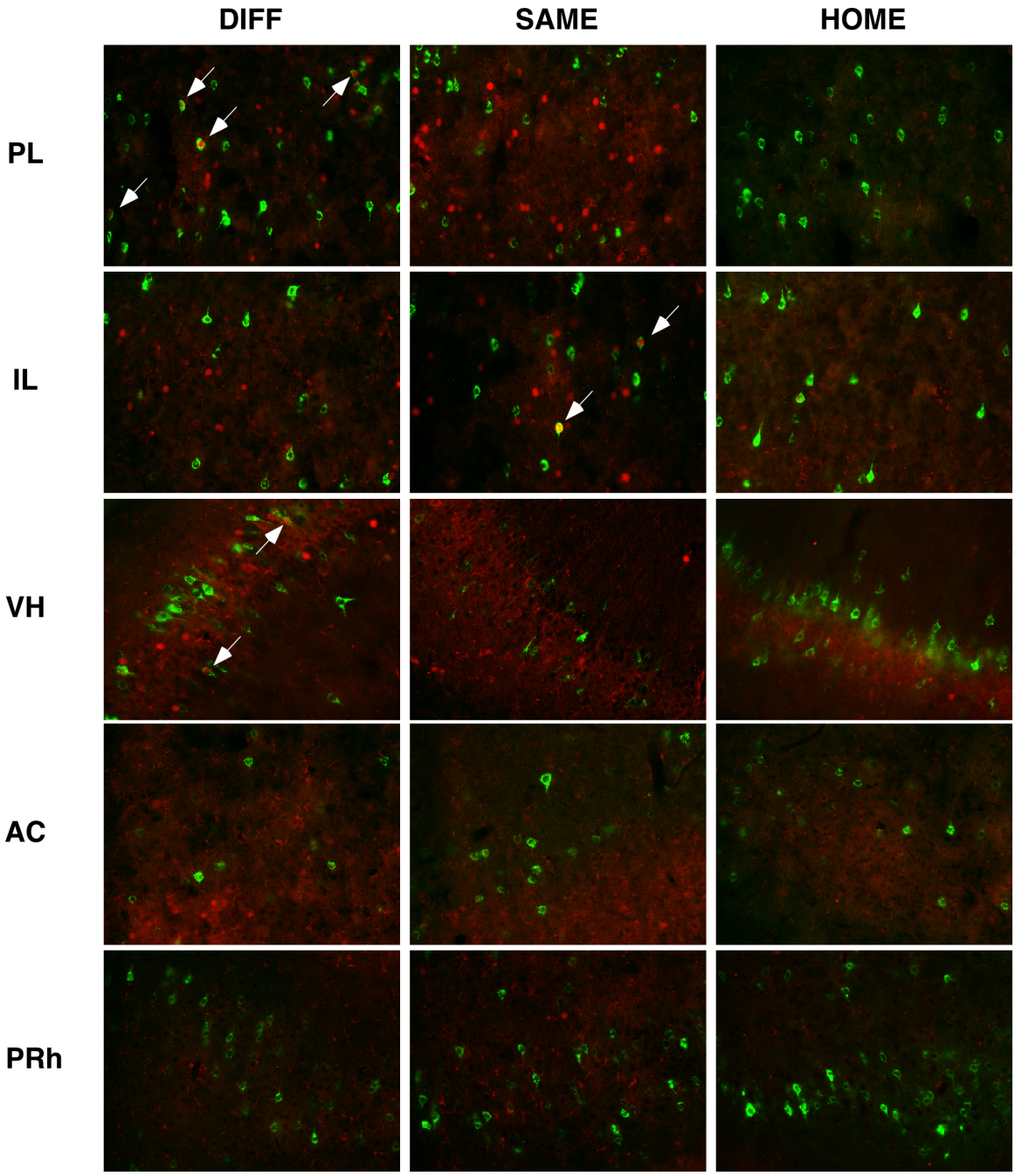

Figure 2.3. Photomicrographs of representative double-labeled neurons in the prelimbic area (PL), infralimbic area (IL), ventral hippocampus (VH), auditory cortex (AC) and perirhinal cortex (PRh) for each behavioral group (DIFF, SAME, HOME). White arrows indicate double-labeled neurons; CTb-positive neurons are stained green and c-fos-positive neurons are stained red.

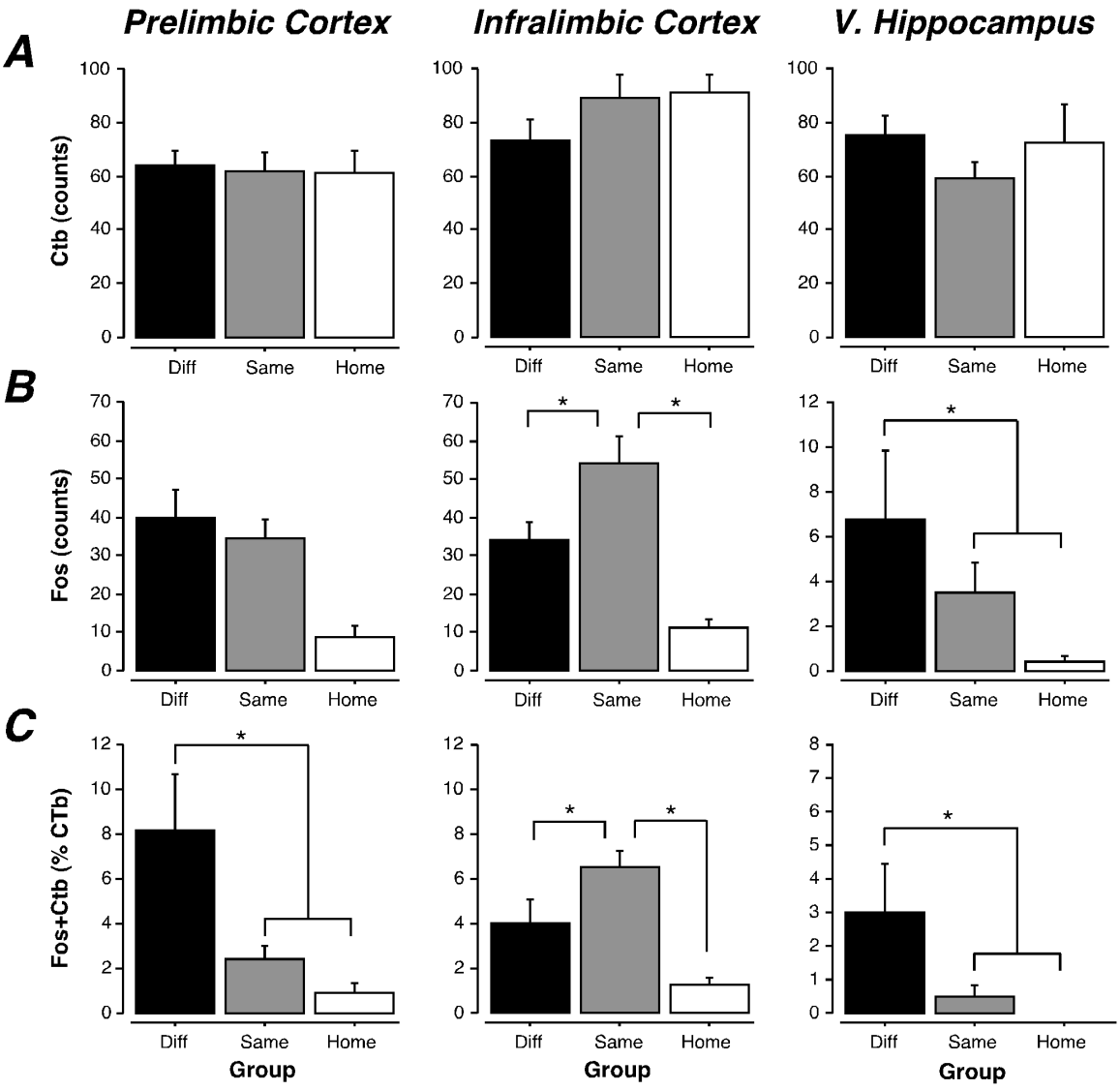

Figure 2.4. Quantification of CTb-positive, c-fos positive and double-labeled neurons in the prelimbic area (PL), infralimbic area (IL) and ventral hippocampus (VH). *(A)* Mean (±SEM) cell counts for CTb-positive neurons in the PL, IL and VH *(B)* Mean (±SEM) cell counts for c-fos positive neurons in the PL, IL and VH *(C)* Mean (±SEM) percentage of double-labeled neurons [(counts of CTB + c-fos-positive divided by counts of CTB alone)X100] in the PL, IL and VH.

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CHAPTER III

BOTH THE DIRECT AND INDIRECT PATHWAYS FROM THE VENTRAL HIPPOCAMPUS TO THE BASAL AMYGDALA ARE REQUIRED FOR THE RENEWAL OF FEAR IN RATS

In the last decade, research has heavily concentrated on exploring the neural basis of traumatic fear, with a specific focus on its reappearance after extinction. Similar to exposure therapy commonly used by clinicians, extinction is a phenomenon in which the fear-invoking stimulus is repeatedly presented alone, resulting in a gradual reduction in fear (Maren, 2001). This is commonly studied in the laboratory using a Pavlovian fear conditioning paradigm. After an innocuous conditioned stimulus (CS; tone) is presented with an aversive unconditioned stimulus (US; footshock), the CS is repeatedly presented alone during extinction. Importantly, extinction results in the formation of a new memory that competes with the original memory (Quirk and Mueller, 2008). What dictates the retrieval of each memory is the context in which the CS is presented. For instance, the presentation of the CS outside the extinction context results in the renewal of fear responses (Bouton and Bolles, 1979). Renewal not only demonstrates the context-specificity of the expression of extinction, but also the persistence of fear, which poses obvious challenges for those hoping to promote permanent suppression of traumatic fear.

Considerable progress has been made in identifying some of the structures involved in contextual modulation of fear after extinction (Maren, 2005; Maren, 2011). Known to be essential for forming contextual representations (Fanselow, 2000), the hippocampus has been shown to play an important role in the expression of fear after extinction. For example, pharmacological inactivation of either the dorsal (Corcoran et al., 2005; Corcoran and Maren, 2001, 2004) or ventral (Hobin et al., 2006) hippocampus with muscimol, a GABA-A agonist, prevents the renewal of fear. Additionally, Zelikowsky et al. (2011) reported that post-extinction lesions of the dorsal hippocampus impaired renewal, as evidenced by low levels of fear. Though it seems that the hippocampus has an unequivocal role in renewal, it is less clear how contextual information from the hippocampus is relayed to the amygdala. Anatomical evidence shows that hippocampal projections to the basal amygdala (BA) arise from the ventral hippocampus (VH; ventral CA1 and ventral subiculum regions; Canteras and Swanson, 1992; Pitkanen et al., 2000). Consistent with this, Herry and colleagues (2008) demonstrated that BA neurons that are selectively active during renewal ("fear" neurons) receive projections from the VH. More recently, using tracing techniques, we have shown BA-projecting neurons in the VH are engaged during the renewal of fear (Orsini et al., 2011). Together, these recent findings suggest a potential route in which contextual information is relayed to the BA during renewal.

In addition to this direct projection from the VH to the BA, contextual information can also be transmitted indirectly to the BA from the VH through the prelimbic (PL) area of the prefrontal cortex. Indeed, the VH directly projects to the

PL (Vertes, 2006), which has reciprocal connections with the BA (McDonald et al., 1996; Vertes, 2004). Importantly, we have previously shown that BA-projecting neurons in the PL are engaged during the renewal of fear (Orsini et al., 2011). This evidence is consistent with other data indicating a role of the PL in the expression of fear. For instance, the inactivation of the PL disrupts the expression of learned fear (Corcoran and Quirk, 2007; Blum et al., 2006) whereas its stimulation results in freezing behavior (Vidal-Gonzalez et al., 2006) and elicits firing in the BA (Likhtik et al., 2005). Others have demonstrated that PL neuronal firing parallels freezing behavior during fear conditioning and extinction (Burgos-Robles et al., 2009). Lastly, Knapska and Maren (2009) observed elevated levels of c-fos expression in the PL during renewal of fear. As a whole, it seems that the PL is an ideal intermediary structure in which contextual information from the VH can be routed to the BA during renewal.

Though our laboratory has demonstrated that BA-projecting neurons in the PL and VH are engaged during renewal, it is unclear whether both the direct and indirect pathways are required for contextual expression of fear after extinction. It is conceivable that only the direct pathway is necessary since it has been previously shown that hippocampal inactivation eliminates context-specific firing of amygdala neurons (Maren and Hobin, 2007). Alternatively, it is also possible that both pathways need to be intact in order for renewal to occur. To examine the necessity of each pathway, we used asymmetric lesions to disconnect the VH from either the BA and PL (Olton et al., 1982) after extinction of conditioned fear. Interestingly, we found that the elimination of either pathway disrupted renewal, indicating that both

routes of communication are required for the contextual expression of fear after extinction.

General Methods

Subjects

Subjects were male Long-Evans rats (220-224 g; Blue Spruce), obtained from a commercial supplier (Harlan Sprague-Dawley, Indianapolis, IN). Animals were individually housed in clear plastic hanging cages and were kept on a 14:10 light: dark cycle and had free access to food and water. Rats were handled 15-20 sec/day for five days before the start of the experiment so as to acclimate the animals to the experimenter. All experimental procedures were carried out in accordance with the protocols approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Behavioral apparatus

All behavioral sessions were carried out in eight identical observation chambers (30 X 24 X 21 cm; Med-Associates, St. Albans, VT), each located in an individual sound-attenuating cabinet. The observation chambers were constructed of two aluminum sidewalls and a Plexiglas ceiling, back and door. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) used for delivery of the footshock unconditioned stimulus (US). The rods were wired to a shock source and a solid-state shock scrambler (Med-Associates, St. Albans, VT). To deliver the acoustic CS, a speaker was mounted on one wall of each chamber. Additionally, ventilation fans and house lights were installed in each chamber to allow for the

manipulation of contexts during training, extinction, exposure and testing. For Context A (conditioning context), house lights and room lights were on, ventilation fans (65 db) were turned on, cabinet doors were left open and the chambers were cleaned with 1% acetic acid. For Context B (extinction and test context), house lights and ventilation fans were turned off, the cabinet doors were closed and the chambers were cleaned with 1% ammonium hydroxide. Additionally, the room was illuminated by fluorescent red lights. For Context C (extinction and test context), house lights were on, ventilation fans were off, the room was illuminated with fluorescent red light and cabinet doors were left open. Black Plexiglas floors were placed on the grid of each chamber and chambers were cleaned with 10% ethanol. In each context, stainless steel pans were filled with a thin layer of the context's respective odor and inserted below the grid floor.

During the behavioral sessions motor activity was measured by recording the displacement of each chamber by a load cell platform located below each chamber. Prior to the experiment, all load cell amplifiers were calibrated to a fixed chamber displacement and the output of each amplifier was set to a gain that optimally detected freezing behavior (vernier dial = 8; somatomotor immobility, except that required for breathing). Load-cell amplifier output (-10 to +10V) was then digitized (5 Hz) and acquired online with Threshold Activity software (Med-Associates, St. Albans, VT). Absolute values of load-cell voltages were multiplied by 10, which resulted in an activity score that ranged from 0 to 100. A bout of freezing was scored if at least five contiguous load-cell values fell below the freezing threshold. In other words, activity had to be below threshold for at least 1 sec to be

scored as freezing behavior. This method of assessing freezing behavior correlates highly with time sampling of behavior by trained observers (Maren, 1998).

Behavioral procedures

One hundred and twenty-five rats were randomly assigned to groups that would receive contralateral (C) lesions, ipsilateral (I) lesions or sham (SH) surgeries in the VH-BA or VH-PL circuits after extinction training. Additionally, these groups were further divided into rats that would be tested outside the extinction context (DIFF) or within the extinction context (SAME). Prior to surgery, rats underwent fear conditioning, which consisted of five tone (CS; 10 sec, 85 db, 2 kHz)-footshock (US; 1.0 mA, 2.0 sec) pairings with 60 sec interstimulus intervals (ISIs). The chamber position of each animal was counterbalanced across each experimental group and training squad. Twenty-four hours later, animals underwent extinction in context B or C (counterbalanced across groups) where they received 45 tone-alone (10 sec) presentations with 30 sec ISIs. Prior to this session, rats were exposed to the alternate context (i.e. they were exposed to context B if they were extinguished in context C) to ensure that the test contexts were equally familiar for all of the rats. Twenty-four to 96 hours after extinction, rats underwent surgery. After one week of recovery, the rats were placed back into either Context B or Context C for the test session [45 tone alone (10 sec) presentations with a 30 sec ISI]. In this experiment, the extinction and test contexts were counterbalanced across all groups. Renewal was assessed by measuring freezing during the first 5 trials of the test session in the DIFF context relative to that in the SAME context.

To assess whether the disconnections produced nonspecific impairments in freezing behavior, we ran an additional experiment with another cohort of fifty-nine rats. The rats were conditioned, as described above, but they did not receive extinction training. Rather, they were merely exposed to either Context B or Context C (no CS presentations) for the same duration as the extinction session in the foregoing experiment. Twenty-four hours later, rats received either contralateral or ipsilateral lesions placed in the VH and BA or VH and PL or sham surgeries. After one week of post-operative recovery, the rats were tested for their retention of fear to the non-extinguished CS in either Context B or C. As before, the extinction and test contexts were counterbalanced across lesion groups.

Surgical procedures

Rats were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) and given atropine sulfate (0.4 mg/kg, i.p.). After being placed into the stereotaxic apparatus (David Kopf instruments, Tujunga, CA), the scalp was incised and retracted. The head was leveled to ensure that lambda and bregma were in the same horizontal plane. To disconnect the VH and BA or VH and PL, unilateral electrolytic lesions were placed in contralateral hemispheres; ipsilateral lesions served as a control (Figure 3.1). We chose to use electrolytic lesions because we were able to produce much more focal damage to the BA and PL than we obtained with excitotoxic lesions. For the VH-BA disconnections, insulated insect pins (size 00; except for 1 mm at the tip; Fine Science Tools) were placed in the VH (AP: -6.7, --6.3, -5.8; ML: +/- 5.6, 5.4, 5.2; DV: -5.6, -5.8, -6 from dura) and the BA (AP: -2.85, -3.6; ML: +/- 5; DV: -9, -9.1 from skull). For the VH-PL disconnections, the electrodes

were placed in the PL (AP: +3.6, + 2.7; ML: ± 0.5; DV: -3.7 from skull) and VH (AP: -6.7, -6.3, -5.8; ML: ± 5.6, 5.4, 5.2; DV: -5.6, -5.8, -6 from dura). Lesions were produced by passing anodal current (0.5 mA, 8 sec) across the insect pins. Lesion placement in the left or right hemispheres was counterbalanced. The incision was closed with stainless steel wound clips, treated with antibiotic ointment, and the animals were administered an analgesic (carprofen, 5 mg/kg; i.p.). The rats recovered on a heating pad before returning to their home cages and were allowed one week for post-operative recovery.

Histology

After behavioral testing, rats were overdosed with pentobarbital and perfused across the heart with 0.9% saline followed by 10% formalin. Brains were extracted and post-fixed in 10% formalin for 2 days at which time brains were transferred into a solution of 30% sucrose in 10% formalin. Brains were sectioned (45 μm) on a cryostat maintained at -20° Celsius. Tissue was wet mounted on subbed slides with 70% ethanol and subsequently stained with 0.25% thionin to verify lesion placement and extent.

Behavioral data analysis

Freezing behavior was measured continuously during all of the behavioral sessions, including the pre-CS "baseline" period as well as during tone presentations and interstimulus intervals. Freezing was then analyzed and reported for each trial, which consisted of a CS presentation and the subsequent interstimulus interval. For each period, the percentage of total observations in which freezing occurred was calculated. The percentage of freezing values were submitted to analysis of

variance (ANOVA) and Fisher's PLSD post hoc tests were performed after significant omnibus *F*-ratios were obtained. All data are represented as means (±*SEM*).

Results

To determine the role of direct and indirect projections of the VH to the BA, we disconnected the VH and BA or VH and PL with asymmetric unilateral lesions in each structure (Figure 3.1). Control rats received the same lesions, but they were localized in the same hemisphere leaving connections between the VA, PL, and BA intact in the opposite hemisphere. Representative photomicrographs of the lesions are shown in Figure 3.2. We used strict criteria when analyzing the histology in order to ensure that only animals with focal lesions in the targeted brain regions were included in the analysis. For the BA, we excluded animals for which the lesions extended dorsally into the lateral amygdala. Similarly, subjects were excluded from the analysis if lesions of the PL encroached either upon the cingulate cortex dorsally or the infralimbic cortex ventrally. Finally, we required that ventral hippocampal lesions were localized to the ventral subiculum and ventral CA1 area; if damage to the entorhinal cortex or dentate gyrus was apparent, the subject was excluded from the analyses. In all cases, lesions had to encompass at least two-thirds of the intended target to be considered a "hit" and included in the statistical analyses. Based on these criteria, we excluded sixty-eight rats, which left a total of 125 animals in the analysis. The final group sizes were: SH-Same (n = 20); SH-Diff (n = 22); I-HPL-Same (n = 8); I-HPL-Diff (n = 6); I-HBA-Same (n = 14); I-HBA-Diff (n = 14); C-HPL-Same (n = 8); C-HPL-Diff (n = 8); C-HBA-Same (n = 12); C-HBA-Diff

(n=13). It is important to note that the relatively large number of rats in the sham groups is a consequence of the exclusion of many rats with lesions that did not meet our histological criteria for inclusion in the analysis.

Freezing behavior during the conditioning, extinction and test session is displayed in Figure 3.3. Prior to surgery, there were no differences in freezing behavior during the conditioning and extinction sessions [*p*s > 0.1]. During the test session, pre-CS freezing in the SAME and DIFF conditions for each group did not differ from one another (F (1, 115) = 2.4, p = 0.1), nor did pre-CS freezing in any of groups differ from one another [F (4, 115) = 0.9, p = 0.4; data not shown]. We therefore normalized the average CS-elicited freezing (freezing during the CS and interstimulus intervals averaged across each test trial) to the pre-CS baseline for each rat. As shown in Figure 3.3C, the groups exhibited marked differences in the degree of fear renewal during the test session. This was indicated by a main effect of lesion [F (4, 115) = 5.2, p < 0.001] and significant interaction of lesion and test context in the ANOVA [F (4, 115) = 2.9, p < 0.05]. Post-hoc comparisons revealed that both sham animals and rats with ipsilateral lesions (I-HPL and I-HBA) renewed their fear to the CS outside the extinction context (*ps* < 0.05). However, rats with contralateral lesions (C-HPL and C-HBA) failed to exhibit renewal (*ps* > 0.05). Hence, either VH-BA or VH-PL disconnections eliminated the contextual retrieval of fear after extinction.

Although control rats with ipsilateral lesions exhibited normal renewal, it is possible that the deficit in rats with contralateral disconnections was due to a non-specific impairment in fear expression to the auditory CS rather than a deficit in

renewal *per se*. To examine this possibility, we conducted an additional experiment examining the consequences of ipsilateral or contralateral lesions in the VH-BA and VH-PL circuits on the expression of conditioned fear to an auditory CS that was not extinguished. The behavioral paradigm was identical to the previous experiment, except that animals did not hear auditory CSs during the post-conditioning session in context B (i.e., there was no extinction training). Twenty-one rats were excluded from the analysis because their lesions did not meet our histological criteria; this left a total of fifty-nine rats in the analyses. The final group sizes were: SH (n = 15); I-HPL (n = 8); I-HBA (n = 14); C-HPL (n = 7); C-HBA (n = 15). Freezing behavior during fear conditioning and the context exposure session was not different between any of the groups (*p*s > 0.05). As shown in Figure 3.4, the test session reveals that neither VH-BA nor VH-PL disconnections affected the expression of freezing behavior during the retention test [F (4, 54) = 1.8, p > 0.1]. Nonetheless, there was a non-significant trend for reduced freezing in all of the rats with lesions, regardless of whether they were placed ipsilaterally or contralaterally in the VH-BA and VH-PL circuits. However, this general suppression of fear in rats with ipsilateral or contralateral lesions does not account for the selective deficit in renewal in rats with contralateral lesions in the previous experiment. Therefore, deficits in fear renewal in rats with contralateral disconnections in the previous experiment are not merely due to deficits in the expression of conditional freezing.

General Discussion

The present study focused on dissecting the relative importance of two separate pathways from the VH to the BA. Using asymmetric lesions to disconnect the VH from the BA or the PL, we found that both routes of communication are required for renewal of fear. These impairments were specific to renewal insofar as the disconnections had no observable effect on the expression of fear in general. These findings provide important insight into how contextual information reaches the amygdala and comes to modulate fear behavior after extinction.

That the direct projection from the VH to the BA is required for renewal aligns well with previous work demonstrating the importance of this connectivity in fear behavior. For instance, Maren and Fanselow (1995) found that the stimulation of the ventral angular bundle, the fiber tract that carries information from the VH to the BA, resulted in long-term potentiation, a putative synaptic correlate of learning, in the amygdala. More recently, Gonzalez-Pardo and colleagues (2012) reported interregional correlations of metabolic activity between the ventral hippocampus and basal amygdala during contextual fear conditioning, which is suggestive of communication between the two structures. Others have shown that "fear" neurons receive projections from the ventral hippocampus. By orthodromically activating the ventral hippocampus while recording in the BA, Herry et al. (2008) demonstrated that the ventral hippocampus synapses upon neurons within the BA that are selectively active during renewal. Consistent with this, our laboratory has also shown that BA-projecting neurons in the VH are engaged during renewal (Orsini et al., 2011). Given these previous findings, it is no surprise that the

disconnection of this direct pathway interferes with contextual modulation of fear behavior.

In addition to the direct pathway, it appears that the communication between the VH and BA also requires the PL. Disconnection of the VH and PL eliminated renewal of fear, resulting in levels of fear no different than that observed during extinction recall. These results are consistent with extant literature insofar as the PL has previously been shown to be involved in the expression of fear (Corcoran and Quirk, 2007; Blum et al., 2006; Sierra-Mercardo, et al., 2010) as well as renewal of fear (Knapska and Maren, 2009; Orsini et al., 2011). However, our findings further extend the role of the PL in renewal as being a conduit for contextual information between the VH and BA. Given that our asymmetric lesions prevent the VH from communicating with the PL, it is conceivable that this manipulation deprived the BA of contextual information arriving from the PL. Consistent with this, Stevenson (2011) also found that interrupting communication between the PL and posterior portion of the BA disrupts contextual fear expression. Moreover, we have recently reported that BA-projecting neurons in the PL are selectively engaged during renewal (Orsini et al., 2011). By this view, the indirect pathway via the PL is a necessary route in which contextual information from the VH reaches the BA to modulate fear behavior after extinction.

Because both the VH and PL are important routes by which contextual information reaches the BA to regulate fear output, we propose that the convergence of hippocampal and prefrontal input in the BA is essential for the contextual regulation of extinction memory (Figure 3.5A). In support of this model,

interrupting hippocampal input to either the BA or PL eliminates the renewal of fear. In the former case, VH and BA disconnections spare BA-PL interconnection in one hemisphere, but isolate this circuit from hippocampal input. In the latter case, VH and PL disconnections spare VH-BA connections in one hemisphere, but isolate this circuit from prelimbic input. By this view, convergent excitatory input from the VH and PL may be required to overcome inhibitory networks in the amygdala that suppress fear responses after extinction. Extinction related inhibition might come from either local inhibitory interneurons in BA (Chhatwal et al., 2005) or through IL-gated inhibitory networks in ITC clusters (Pare et al., 2004; Amano et al., 2010). Although neurons in the BA that respond to CSs during fear renewal receive hippocampal input, it is not clear that they also receive convergent prelimbic input (Herry et al., 2008). However, it has been reported that single VH neurons project to both PL and BA (Ishikawa and Nakamura, 2006). This raises the possibility that VH neurons projecting to PL and BA form a common anatomical hub to regulate excitability in both the PL and BA, as well as encouraging coherence in PL-BA activity (Seidenbecher et al., 2003; Pape et al., 2005; Adhikari et al., 2010). Nonetheless, our data do not address whether single BA neurons receive convergent VH and PL input, or whether VH and PL projections that converge on the BA do so on different populations of BA neurons. Dual anterograde tracing of VH and PL projections to the amygdala, as well as simultaneous neural recordings in this network, will be important steps to assess this anatomical question as well as the validity of this model.

Another possibility is that the convergence of VH and BA information in the PL, rather than VH-PL convergence in BA, is required for fear renewal (Figure 3.5B). It is well known, for example, that the BA has robust projections to both the medial prefrontal cortex and hippocampus (Pitkanen et al., 2000; Hoover and Vertes, 2007) and that BA inactivation impairs fear renewal (Herry et al., 2008). It is also apparent from our circuit model (Figure 3.5) that VH-BA and VH-PL disconnections eliminate not only VH and PL convergence in BA, but also the convergence of VH and BA input in PL. Consistent with the possibility that BA and VH convergence in PL is important for fear renewal, Herry et al. (2008) report that neurons in the BA that are active during fear renewal project to the medial prefrontal cortex. Indeed, PL neurons receive convergent hippocampal and amygdala input (Ishikawa and Nakamura, 2003). Hence, this alternative model predicts that BA inactivation, which impairs fear renewal (Herry et al., 2008), would also eliminate renewal-induced c-fos signals in the PL. Experiments to test this hypothesis are underway.

In summary, we found that disruption of communication between the VH and BA either directly or indirectly impairs the contextual expression of fear after extinction. We propose that our manipulations deprived the amygdala of the contextual information necessary to determine the appropriate behavioral response. These findings provide valuable insight into how hippocampal-prefrontal-amygdaloid dysfunctions may contribute to fear and anxiety psychopathologies. Given that fear persists across environmental shifts, combating such psychopathologies poses a major challenge to both neuroscientists and clinicians. Further work probing the neural network, both at the systems and

cellular level, underlying contextual modulation of fear behavior after extinction will be beneficial in developing better therapeutic interventions for victims of fear and anxiety disorders.

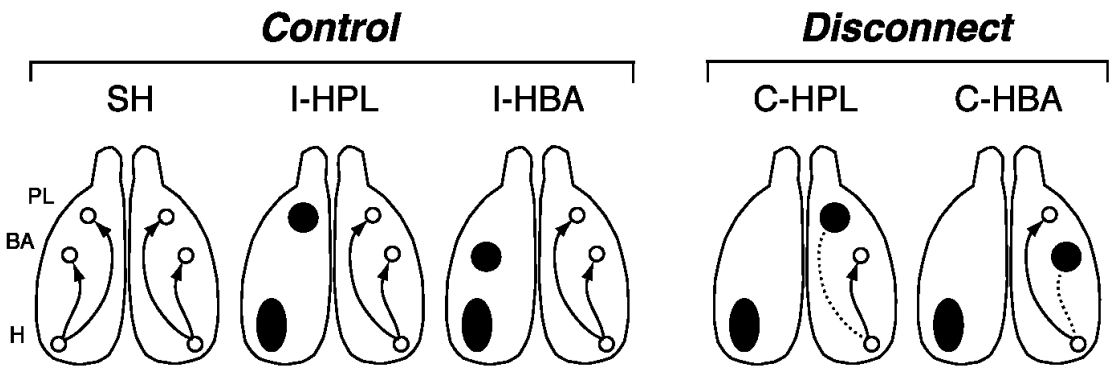

Figure 3.1. A schematic of the projections between the hippocampus (H), BA and PL in control rats and rats in which the projections were disconnected. In intact brains (SH), there are ipsilateral projections (arrows) from the VH (H) to both the BA and PL. Controls rats with ipsilateral lesions of either the VH and PL (I-PL) or the VH and BA (I-BA) maintain connectivity between the hippocampus, amygdala and PL in the intact hemisphere. However, contralateral lesions of the VH and PL (C-HPL) or VH and BA (C-HBA) disconnect the hippocampus (dashed lines) from the PL and BA, respectively.

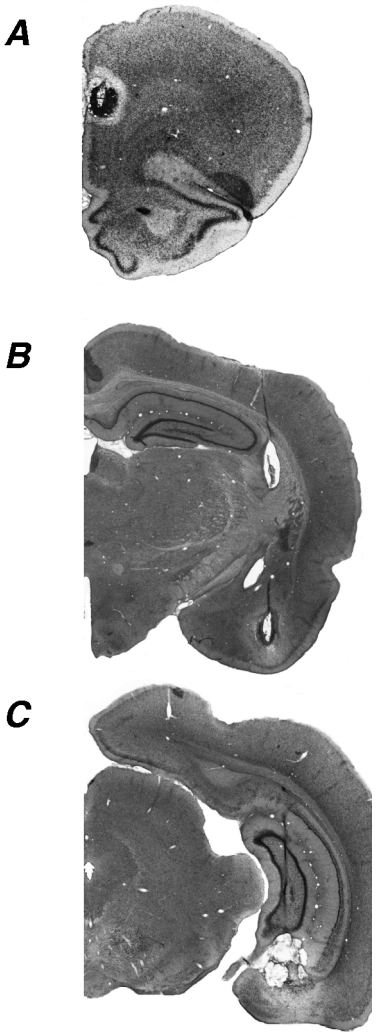

Figure 3.2. Photomicrographs of thionin-stained coronal sections showing representative lesions. *(A)* Representative image of an electrolytic lesion in the prelimbic cortex. *(B)* Representative image of an electrolytic lesion in the basal amygdala. *(C)* Representative image of an electrolytic lesion in the ventral hippocampus.

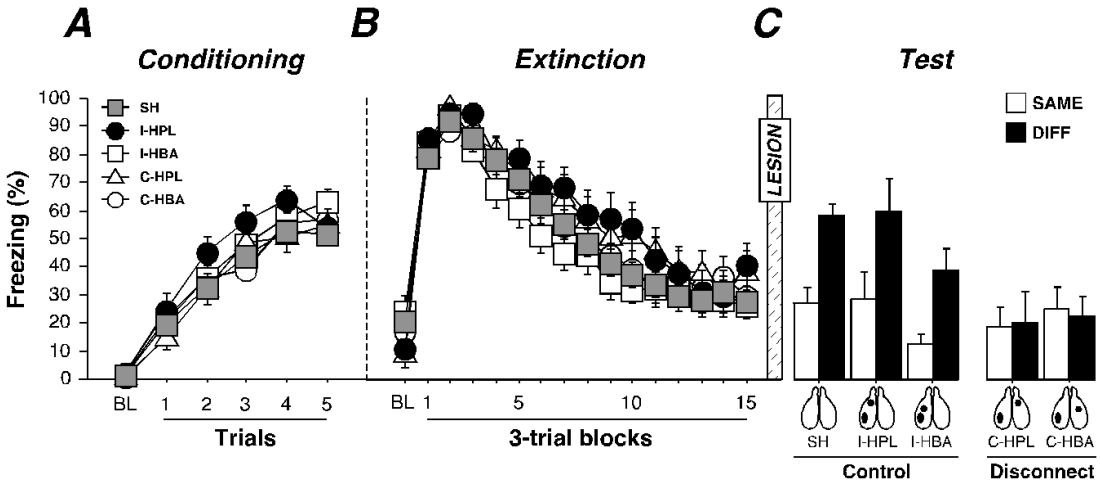

Figure 3.3. Conditioned freezing in rats that received post-extinction ventral hippocampal (VH), prelimbic (PL) or basal amygdala (BA) lesions. *(A)* Mean percentage of freezing (±SEM) during fear conditioning that consisted of a 3 min baseline period (Pre) followed by 5 tone-shock presentations. Freezing was averaged across the pre-CS baseline (Pre) as well as during each of the five conditioning trials; each trial consisted of the average of freezing during each CS presentation and subsequent ISI. *(B)* Mean percentage of freezing (±SEM) during the 45 tone-alone extinction session. As with conditioning, freezing was averaged across the 3 min baseline (Pre) period as well as during the 45 trials, each of which consisted of a CS presentation and the subsequent ISI [data were binned into 15 blocks of 3-trial averages]. *(C)* Mean percentage of freezing (±SEM) during the first 5 minutes of the test session. Freezing was measured during the 3 min baseline (Pre) period as well as during the first five trials. Data are shown for rats that received sham surgeries (SH; gray squares), rats that received ipsilateral lesions of the VH and PL (I-HPL; black circles), ipsilateral lesions of the VH and BA (I-HBA; open squares), contralateral lesions of the VH and PL (C-HPL; open triangles) or contralateral lesions of the VH and BA (C-HBA; open circles).

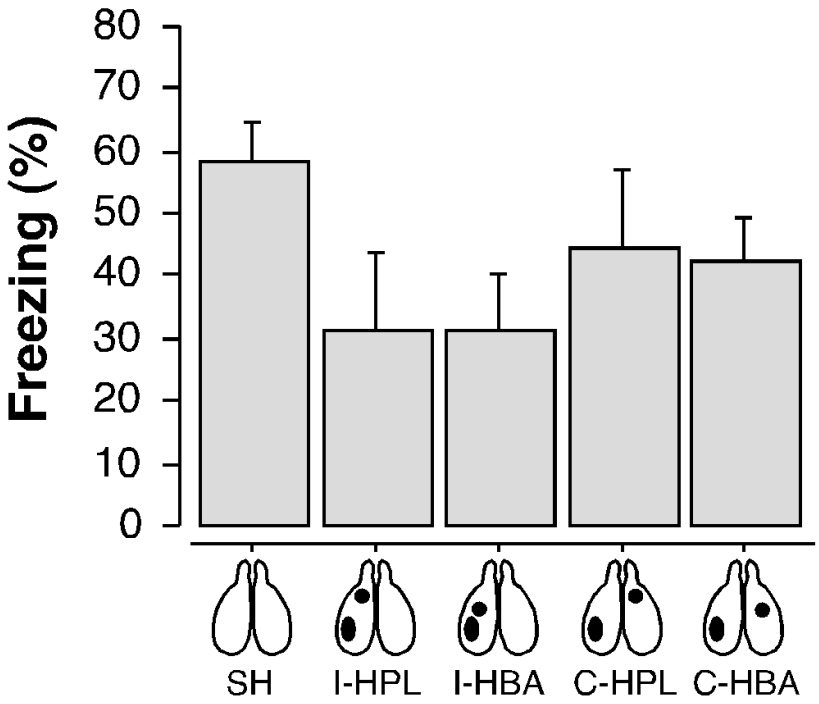

Figure 3.4. Conditioned freezing in rats that received ventral hippocampal (VH), prelimbic (PL) or basal amygdala (BA) lesions after fear conditioning. During the test session, freezing was averaged across the Pre period as well as during the 45 trials, each of which consisted of a CS presentation and the subsequent ISI. The data are represented by the mean percentage of freezing (±SEM) during the first 5 minutes of the test session.

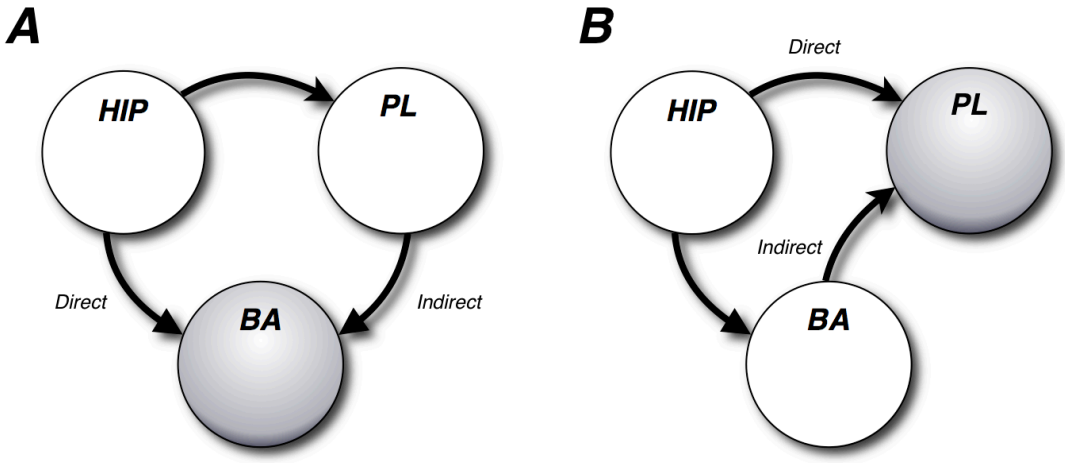

Figure 3.5. Circuit model of hippocampal-prefrontal-amygdaloid interactions in the renewal of fear. *(A)* In this scenario, both direct and indirect projections of the VH to the BA are required for the renewal of fear after extinction. Disconnection of either the direct or indirect pathways deprives the BA of convergent input from the VH and PL. *(B)* Another possibility is that convergence of direct and indirect projections from the VH to the PL mediate the renewal of fear. Indeed, disconnection of either the VH-PL or VH-BA projection prevents convergence of VH and BA input in the PL.

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CHAPTER IV

CONTEXT-DEPENDENT NEURONAL ENSEMBLES IN THE AMYGDALA, PREFRONTAL CORTEX AND HIPPOCAMPUS AFTER FEAR EXTINCTION IN RATS

Over the last decade, the extinction of conditioned fear has received considerable interest as it has direct parallels with commonly used cognitive-behavioral treatments, such as exposure therapy. In the laboratory, extinction is modeled using a paradigm in which a conditioned stimulus (CS; tone) previously paired with an unconditioned stimulus (US; footshock) is repeatedly presented alone (Maren, 2001). This results in a decrease in fear behavior, often measured as freezing. Importantly, extinction is considered new learning that yields a distinct memory, separate from the original fear memory (Maren, 2011). Unique to extinction, its expression is context-dependent. That is, suppression of fear is only observed within the extinction context; fear will renew if the CS is presented in a different context (Bouton and Bolles, 1979; Bouton, 2004). The ability of fear to readily reappear after a contextual shift unequivocally demonstrates the fragility of extinction memories. Moreover, it is clear that contextual cues strongly influence the interpretation of the fearful stimulus, leading to specific behavioral consequences.

The contextual modulation of fear after extinction is thought to involve a tripartite circuit, involving the hippocampus, prefrontal cortex and amygdala

(Maren, 2005; Quirk and Mueller, 2008; Herry et al., 2010; Orsini and Maren, 2012). Individually, each structure has been shown to be involved in the context-dependent retrieval of extinguished fear. For instance, inactivation of the ventral hippocampus (VH; Hobin et al., 2006) or the basal amygdala (BA; Herry et al., 2008) eliminates renewal of fear. Similarly, others have demonstrated specific roles for the prelimbic (PL) and infralimbic (IL) cortices of the prefrontal cortex in renewal and extinction recall, respectively (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Knapska and Maren, 2009). However, the interaction between these brain structures is what drives the contextual regulation of fear after extinction (Herry et al., 2008; Orsini et al., 2011; Orsini and Maren, 2012). It is theorized that the hippocampus contextually controls the circuit such that during renewal, it promotes amygdala activity and during extinction, it suppresses amygdala activity, presumably through its connections with the prefrontal cortex. Consistent with this model, we have shown that the inactivation of the hippocampus disrupts context-dependent firing in the amygdala (Maren and Hobin, 2007). Furthermore, the elimination of hippocampal input to the BA, either directly or indirectly via the PL, disrupts renewal of fear (Orsini et al., 2011). With respect to the expression of extinction, we have found that BA-projecting neurons in the IL are selectively engaged during the recall of extinction (Orsini et al., 2011). Others have also shown that impaired extinction is possibly related to aberrant prefrontal-amygdala interactions (Herry and Mons, 2004; Muigg et al., 2008), strongly suggesting that communication between the prefrontal cortex and amygdala is required for the suppression of fear after extinction. Together, these findings advocate a circuit

model in which structures upstream of the BA contextually sculpt its activity and subsequently bias fear behavior.

Though the circuitry underlying contextual modulation of fear is fairly well understood, it is less clear how cell assemblies within these key brain areas orchestrate the selection of behavior (i.e. fear or extinction expression) to an extinguished CS. Neurophysiological recordings in the BA suggest that there are two separate populations of neurons that are engaged during either the renewal or suppression of fear (Herry et al., 2008). These "fear" and "extinction" neurons are randomly distributed within the BA and are monosynaptically connected with the VH and prefrontal cortex, respectively. Interestingly, there are also neurons in the BA that are resistant to extinction (Amano et al., 2011). These studies suggest that contexts may select unique populations of neurons in the BA to regulate the expression of fear to extinguished CSs. It is not known, however, whether there is a similar segregation of cell assemblies in the prefrontal cortex and hippocampus. By examining the cellular distribution of the immediate early gene *Arc,* Guzowski and colleagues (1999) demonstrated that neuronal ensembles in the hippocampus are differentially activated when a rat explores two disparate environments, suggesting that this type of segregation does exist in the hippocampus. Similarly, others have demonstrated that the dorsal hippocampus contains non-overlapping cell assemblies that are recruited during contextual fear conditioning and extinction training (Tronson et al., 2009). In our own laboratory, we have shown that neurons in the PL (Orsini et al., 2011) and the VH (Knapska and Maren, 2009) are recruited during both the renewal and suppression of fear, but it is not clear whether it is the

same or different neurons responding to the CS in each case. Moreover, even in the amygdala, there has been no systematic anatomical examination of the context-dependent regulation of neuronal activity.

To address these questions, the present study uses cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH), a method that allows one to visualize neuronal activation to two different behavioral experiences (Guzowski and Worley, 2001). Here, we will explore the cellular distribution of the mRNA of the immediate early gene (IEG) *Arc* to index neuronal activity in the prefrontal cortex, hippocampus and amygdala in response to presentation of an extinguished CS in two different contexts (the extinction context and another context). This will allow us to address whether the same CS recruits distinct neuronal assemblies depending on the context in which it is presented, and whether the hippocampus, prefrontal cortex and amygdala exhibit similar patterns of context-dependent activity. Ultimately, these results will provide important information about how the brain processes and organizes emotionally salient information to select appropriate behavioral responses.

General Methods

Subjects

Experimental subjects were male Long-Evans rats (200-224 g; Blue Spruce) obtained from a commercial supplier (Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed individually in clear plastic hanging cages and were maintained on a 14:10 light:dark cycle with access to food and water *ad libitum*. Prior to the

start of the experiment, rats were handled 15-20 sec/day for five continuous days so as to acclimate the animals to the experimenter. All experimental procedures were carried out in accordance with the protocols approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Behavioral Apparatus

All behavioral sessions occurred in eight identical observation chambers (30 X 24 X 21 cm; Med-Associates, St. Albans, VT), constructed of a Plexiglas ceiling, back and door and two aluminum sidewalls. The floor of each observation chamber consisted of 19 stainless steel rods (4 mm in diameter) by which the footshock US was delivered. The rods of the floor were wired to a shock source and a solid-state shock scrambler (Med-Associates, St. Albans, VT). Within each observation chamber, a speaker was mounted on one sidewall to deliver the acoustic CS. Lastly, each chamber contained a house light and ventilation fans that could be manipulated to create distinct contexts in the experimental. Importantly, each observation chamber was situated within a sound-attenuating cabinet.

A three-context ("ABC/ACB") renewal design was used in this experiment. For Context A (fear conditioning context), room lights, house lights and ventilation fans (65 db) were turned on and the cabinet doors were left open. Each observation chamber was cleaned with 1% acetic acid. In Context B (extinction and test context), house lights and ventilations fans were turned off and the cabinet doors were closed. Chambers were cleaned with 1% ammonium hydroxide and the room was illuminated by fluorescent red lights. For Context C (extinction and test context), ventilation fans were left off, but the house lights were turned on. The

cabinet doors were left open and the room was lit by fluorescent red lights. Chambers were cleaned with 10% ethanol and black Plexiglas floors were put on top of the grids in each chamber. For each context, stainless steel plans containing a thin layer of the context's respective odor were inserted below the grid floor of each observation chamber.

In each behavioral session, motor activity was measured by recording the displacement of each chamber by a load cell platform located beneath each chamber. Before the experiment commenced, each load cell amplifier was calibrated to a fixed chamber displacement and the output of each amplifier was set to a gain that optimally detected freezing behavior (vernier dial = 8; somatomotor immobility except that necessitated for breathing). The output of each load cell amplifier (-10 to +10V) was subsequently digitized (5 Hz), resulting in one observation per rat every 200 milliseconds (300 observations per rat per minute), and acquired online using Threshold Activity software (Med-Associates, St. Albans, VT). The absolute values of the load cell voltages were multiplied by 10, yielding an activity score that ranged from 0 to 100. If at least five continuous load-cell values (or at least 1 second's worth) fell below the freezing threshold (threshold = 10), freezing was scored for that time period. This method of assessing freezing behavior has been used previously and is tightly correlated with time sampling of freezing behavior by trained observers (Maren, 1998).

Behavioral Procedures

Twelve rats were randomly assigned to two groups: those that received extinction training (EXT; n = 8) and those that did not (NO-EXT; n = 4). The

experiment consisted of a three-context renewal design whereby animals were fear conditioned in Context A, extinguished in either Context B or C and tested in Context B and C (the extinction context was counterbalanced across experimental groups). This yielded conditions in which EXT rats were tested in the extinction context (SAME; ABB/ACC) and in another context that had not hosted extinction (DIFF; ABC/ACB). Rats in the NO-EXT group were tested in both of the test contexts. The EXT group was further subdivided into rats that received the DIFF test first and the SAME test last (D/S) and rats that received the SAME test first and the DIFF test last (S/D). Similarly, NO-EXT rats were divided into rats that were tested in Context B first and tested in Context C last (B/C) and those that were tested in Context C first and Context B last (C/B).

One week after being housed, rats underwent fear conditioning, which consisted of five tone (10 sec, 85 db, 2kHz)-footshock (1.0 mA, 2.0 sec) pairings with interstimulus intervals (ISIs) of 60 seconds. The chamber position of each rat was counterbalanced across experimental group and test order (S/D, D/S, B/C, C/B). Twenty-four hours after conditioning, EXT rats underwent extinction (45 tone-alone presentations with 30 sec ISIs) in either Context B or Context C. NO-EXT rats were also placed in either Context B or Context C, but did not receive CS presentations. Prior to the extinction/no-extinction session, all animals were exposed to the alternate context. For example, rats that were extinguished in Context B were exposed to Context C beforehand. This ensured that all animals were equally familiar with all contexts involved in the experiment. Twenty-four hours after extinction, rats were returned to the observation chambers for the first of two tests.

Each test session consisted of 3 tone-alone presentations with 30 sec ISIs in either Context B or C. After the first test session, rats were returned to their home cage for 18 minutes before being tested again in the alternate context. Immediately after the last test, rats were lightly anesthetized with isofluorene and killed for brain tissue extraction. Brains were quickly extracted, flash frozen in a vial of isopentane that was immersed in dry ice and subsequently stored at -80° Celsius until sectioning. The relative timing of this design was used to parallel the expression profile of *Arc* mRNA. Under basal conditions, *Arc* expression is very low (Guzowski et al., 2005). However, upon a behavioral experience (or any type of stimulation associated with synaptic plasticity), *Arc* mRNA can be observed in the nucleus within 5 minutes and in the cytoplasm within 25 minutes. Importantly, this allows one to assess neuronal activation induced by two temporally disparate behavioral experiences (Guzowski and Worley, 2001; Guzowski et al., 2005). As such, in the present experiment, cytoplasmic staining would correspond to the first test session and nuclear staining would correspond to the last test session. In all behavioral sessions, freezing was used as the index of fear.

Fluorescent in situ hybridization (FISH)

Upon completion of the experiment, coronal sections (15 μm) were collected with a cryostat maintained at a constant temperature of -20° Celsius and arranged on electrostatic slides (Histobond). Slides were stored at -80° Celsius until FISH procedures commenced.

Digoxigenin (DIG)-labeled Arc riboprobes were generated using a commercial MAXIscript T7/T3 *in vitro* transcription kit (Ambion). After being

treated with DNase, the riboprobes were subsequently purified using Mini Quick Spin RNA Columns (Roche). Successful yield of the DIG-labeled riboprobe was confirmed by a gel electrophoresis; purity and concentration was assessed on a NanoDrop. The riboprobe was then stored at 80° Celsius until use.

For FISH procedures, slide-mounted sections were first thawed to room temperature (RT) and were then fixed in 4% buffered paraformaldehyde for 10 minutes. After a wash in a 2X saline-sodium citrate buffer (SSC), sections were treated with acetic anhydride/triethanolamine and then incubated in a 1:1 acetone/methanol mix. Following another 2X SSC wash, 200 μl of 1X pre-hybridization buffer (Sigma) was applied to each slide and coverslips were overlaid. Slides were incubated in a humid chamber for 30 min at RT after which, 150 μl of 1X hybridization buffer containing the DIG-labeled riboprobe (100 ng) was applied to each slide. Slides with overlying coverslips were incubated in a humid chamber overnight at 56° Celsius. Twenty-four hours later, slides were washed several times in 2X SSC buffer and then treated with RNase (1:1000; diluted in 2X SSC) for 30 min at 37° Celsius. Slide-mounted sections were then washed in several stringent SSC washes, two of which were 0.5X SSC at 56° Celsius. After the final SSC wash, slides were incubated in a 1% H_20_2 solution for 30 min, quenching any endogenous peroxidase activity in the tissue. After two separate 2X SSC washes, slides were introduced to a tris-buffered solution (TBS) for 5 minutes, followed by the application of 150 μl of blocking buffer [Normal Donkey Serum (NDS; JacksonImmuno) mixed with blocking reagent (Roche)] to each slide. Slides were incubated with overlying coverslips in the blocking buffer for 30 min in a humid

chamber at RT, after which 150 μl of the primary antibody solution [mouse anti-DIG conjugated to horseradish peroxidase (HRP; JacksonImmuno) at 1:300; diluted in blocking buffer without NDS] was applied to each slide and coverslips were placed on each slide. Slides were incubated in a humid chamber for 2 hours at RT and subsequently washed several times in TBS with 0.05% Tween-20 (TBS-T). To amplify the Arc signal, 100 μl of tyramide-signal amplification (TSA)-biotin conjugated solution was applied. Coverslips were overlaid, and slides were incubated in a humid chamber for 30 min at RT. Slides were subsequently washed twice in TBS-T and once in TBS. To detect Arc mRNA and stain neuronal nuclei, streptavidin conjugated to AlexaFluor 488 (1:300; Invitrogen) and Hoechst (1:500; Sigma), respectively, were diluted in TBS and 150 μl of this solution was added to each slide. Coverslips were overlaid and slides were incubated for 1.5 hr at RT. Finally, slides were washed twice in TBS-T, once in TBS and coverslipped with Vectashield mounting medium (Vector Labs; without DAPI).

Confocal microscopy and image analysis

Stained sections were imaged on an Olympus Fluoview (FV1000) confocal microscope equipped with 6 lasers (405, 458, 488, 515, 561 and 633 nm lasers). Images were collected using an Olympus 40X/1.30 oil immersion lens. Two image samples from the PL, IL, VH, BA and lateral amygdala (LA) were captured, similar to other published catFISH studies (Barot et al., 2008; Barot et al., 2009; Chung et al., 2011); each image was *z*-sectioned in 0.5 μm optical sections. Using the publicly available ImageJ software, cells were characterized as one of the following: nuclear, cytoplasmic, or nuclear/cytoplasmic. Importantly, only those cells that 1) were not

cut off on the edges of the image and 2) were present throughout the entire *z*-stack were included in the analyses. Cells were denoted as "nuclear" if they showed one or two robust foci with high levels of saturation that were restricted only to the nucleus. Neurons were denoted as "cytoplasmic" if they showed a "halo" of *Arc* staining around the nucleus and/or diffuse perinuclear staining present across multiple sections. The nuclear/cytoplasmic (N/C; double-labeled) designation was given to cells that showed both of the aforementioned properties. Cells were counted by an investigator blind to each rat's experimental condition. Cell counts were averaged across the two samples from each region of interest then divided by the area occupied by the structure in the image (211 μm by 211 μm; 45 μm^2). Group differences in cell counts were analyzed with an ANOVA and Fisher's protected least significant difference (PLSD) post hoc tests. Results are represented as means (±*SEM*).

Behavioral data analysis

During all behavioral sessions, freezing was measured during the pre-CS "baseline" periods, CS presentations and interstimulus intervals. Freezing was then analyzed and reported for each trial, which consisted of a CS presentation and its subsequent interstimulus interval. For each trial, the percentage of total observations in which freezing occurred was calculated and these values were submitted to analysis of variance (ANOVA). If a significant omnibus *F*-ratio was obtained, Fisher's PLSD post-hoc tests were performed. All data are represented as means (±*SEM*).

Results

Freezing behavior during conditioning and extinction is displayed in Figure 4.1. During the pretrial period of the training session, there was no freezing behavior prior to the first tone-shock presentation. With each subsequent tone-shock presentation, freezing gradually increased until it reached asymptote. As such, there was a significant effect of trial [F (5, 50) = 10.21, $p < 0.0001$]. However, there were no significant differences between groups [F (1, 10) = 0.11, $p > 0.05$] and no significant interaction between trials and group [F (5, 50) = 0.35, $p > 0.05$]. During extinction, both EXT and NO-EXT rats displayed low levels of fear during the pretrial period. With the first tone presentation, EXT rats exhibited high levels of freezing, which gradually decreased over the course of the session. Rats in the NO-EXT condition, however, showed very little freezing during the extinction session. There was no significant main effect of group [F (1, 10) = 0.038], but there was a significant effect of trial [F (15, 150) = 4.7, $p < 0.0001$] and a significant trial by group interaction, [F (15, 150) = 10.75, $p < 0.0001$]. Across the two test sessions (Figure 4.2), extinguished rats displayed significantly higher levels of fear when tested outside the extinction context, [F (1, 7) = 60.15, $p = 0.0001$]. Importantly, there was no effect of test order [D/S or S/D; F (1, 6) = 0.8]. In the NO-EXT condition, there were no differences between the Context B test and the Context C test, [F (1, 3) = 8.4] and no effect of test order [B/C or C/B; F (1, 2) = 0.4].

Immediately after the second retrieval test, rats were killed for brain extraction and tissue processing. Only those rats in the EXT group that had all regions of interest stained evenly throughout were included in the within-subjects

analyses (n=6). Two additional EXT rats were included in the between-subjects comparison with the NO-EXT rats (n=4). Non-extinguished rats were only included in the between-subjects comparisons as not all regions of interest were stained reliably within each brain.

Both retrieval tests induced *Arc* mRNA in the PL, IL, BA, VH and LA (Figure 4.3). Within each brain region of each rat, the number of cells activated by renewal (DIFF) or extinction recall (SAME) was counted. For instance, cells with only nuclear labeling in the S/D condition and only cytoplasmic labeling in the D/S condition were summed to yield the total number of unique neurons engaged during renewal. We call these "context-selective neurons". To assess the total cellular activation due to renewal (DIFF) or extinction (SAME), we summed the context-selective neurons for each condition with the number of double-labeled (or non-selective) cells (DBL), which were active under both retrieval conditions.

Similar to c-fos expression, *Arc* expression in the brain areas we quantified was context-dependent (Figure 4.4). This was confirmed by a significant main effect of brain region [F (4, 20) = 9.8, $p < 0.01$] and a significant interaction between brain region and condition [DIFF or SAME; F (4, 20) = 6.7, $p < 0.0001$]. Pair-wise comparisons between the SAME and DIFF conditions for each brain region revealed a pattern of *Arc* expression similar to what we have previously reported with c-fos (Knapska and Maren, 2009). For instance, we observed significantly more *Arc*-positive cells in the DIFF condition relative to the SAME condition in the PL [F (1, 5) = 10.0, $p < 0.05$]. Conversely, there were significantly more *Arc*-positive cells in the SAME condition relative to the DIFF condition in the IL [F (1, 5) = 23.0, $p < 0.01$].

Within the VH, there were no significant differences between DIFF and SAME in the number of *Arc*-positive neurons [F (1, 5) = 0.09, $p > 0.05$]. Though there appeared to be more *Arc*-positive neurons in the BA in the DIFF condition relative to the SAME condition, there was only a trend towards significance [F (1, 5) = 4.3, $p = 0.09$]. Finally, within the LA, there were significantly more *Arc*-positive neurons within the DIFF condition than the SAME condition [F (1, 5) = 6.9, $p < 0.05$]. Together, these results extend our previous findings with c-fos to another immediate early gene, *Arc*.

To further explore these results, we investigated whether there were differences in the percentages of context-selective neurons within each brain region (Figure 4.5). It is important to note that in these analyses, the DIFF and SAME conditions do not include non-selective neurons. Though there was not a main effect of condition in the BA [F (2, 10) = 2.3, $p > 0.05$], a *post-hoc* comparison revealed that there was a significant difference between the DIFF and SAME conditions ($p < 0.05$). For the VH, we observed that there was no main effect of condition [F (2, 10) = 2.2, $p > 0.05$]. In other words, out of all *Arc*-positive neurons in the VH, there were no differences in the percentage of context-selective or non-selective neurons (DBL; $p > 0.05$). Within the PL, however, there was a significant main effect of condition [F (2, 10) = 82.3, $p < 0.0001$) and *post-hoc* comparisons revealed that out of all the *Arc*-positive neurons, there were significantly more non-selective neurons than context-selective neurons ($p < 0.05$). Additionally, there was a significantly higher percentage of *Arc*-positive neurons in the DIFF condition relative to the SAME condition ($p < 0.0001$). In the IL, there was a significant main

effect of condition [F (2, 10) = 19.9, p > 0.01) such that the percentage of *Arc*-positive neurons was significantly higher in the SAME and non-selective conditions than the DIFF condition (p < 0.01). Finally, like the PL and IL, there was a significant main effect of condition [F (2, 10) = 5.0, p < 0.05] in the LA and *post-hoc* comparisons showed that out of all the *Arc*-positive neurons, there were significantly more in the DIFF and non-selective condition than in the SAME condition (p < 0.05).

The catFISH procedure allowed us to identify both 1) context-specific neurons that were engaged by CSs presented in either the extinction or renewal contexts and 2) non-selective neurons that were active in both contexts. Of particular interest is the ratio of context-selective neurons to non-selective neurons because this provides an index of the relative representation of unique, context-dependent cell assemblies in each brain area (Figure 4.6). A one-way within subjects ANOVA revealed a significant main effect of brain region [F (4, 20) = 5.5, p < 0.01] in the ratio of context-selective to non-selective neurons in EXT rats. *Post-hoc* tests revealed that there were significantly more context-selective neurons in the BA compared to all other brain regions (p < 0.05). We then used a paired-wise comparison to determine whether the BA ratio was significantly different from 1, a value indicating equal proportions of context-selective and non-selective neurons. This comparison revealed that there was only a trend towards significance (p = 0.06). For the VH, the ratio of context-selective to non-selective cells was approximately equal: there was no significant difference between the VH ratio and

1, ($p > 0.05$). In addition, *post-hoc* tests showed that the ratio of cells in the VH was not significantly different than the ratios of the PL, IL and LA ($p > 0.05$).

Of all brain regions, the PL contained the most non-selective neurons. First, the PL ratio value was significantly lower than 1 ($p < 0.05$). Secondly, in addition to being significantly different than the BA, the PL also had significantly more non-selective neurons than the IL ($p < 0.05$). Lastly, the IL and LA appeared to have slightly more context-selective than non-selective neurons; when comparing these ratio values to 1, we found that the IL had a significantly higher ratio value ($p < 0.05$) whereas the LA did not ($p > 0.05$). Comparing across brain regions, the IL had significantly more context-selective neurons than then PL ($p < 0.05$) and both the IL and LA had significantly less than the BA ($p < 0.05$). Overall, these results demonstrate that in extinguished rats, there are more neurons in the BA that are engaged separately by renewal and extinction recall whereas the PL contains a greater number of overlapping cell assemblies during context-dependent retrieval of fear.

Of particular interest is whether the increased functional activity of these cell assemblies, especially within the BA, depended on extinction. To explore this question, we compared the ratio of context-selective neurons in each brain region between non-extinguished and extinguished rats (Figure 4.7). Each brain area was analyzed separately because we did not have a sufficient number of animals in the NO-EXT condition with measurements in all of the brain areas. In general, extinction training greatly increased the number of context-selective neurons in the brain areas we quantified. This was most pronounced in the BA [$F(1, 8) = 6.5$, $p < 0.05$],

but also occurred in the PL [F (1, 10) = 10.8, $p < 0.01$] despite the fact that the majority of the neurons were not context-selective. There were no significant differences between these two behavioral groups in the LA, IL or VH. These data suggest that, within the BA, extinction training contributes to the emergence of distinct cell ensembles that are recruited in a context-dependent manner.

General Discussion

It is currently appreciated that context-dependent retrieval of fear involves communication between the hippocampus, amygdala and prefrontal cortex (Orsini and Maren, 2012). The present study extends these findings by characterizing cell assemblies engaged within these brain regions during the renewal and suppression of fear. We found that the BA contained two distinct neuronal ensembles that were selectively recruited during the renewal or suppression of fear. Importantly, the emergence of these cell ensembles depended on extinction training. Conversely, the PL contained largely overlapping populations of cells that were activated by the CS in both contexts. Consistent with previous findings, we observed that the IL and LA were heavily recruited during extinction recall and renewal, respectively, though these regions also contained neurons that were engaged in both renewal and suppression of fear. Lastly, we found that the VH contained a heterogeneous population of cells that responded during renewal, extinction recall or both.

Our laboratory has previously demonstrated neuronal activation patterns related to context-dependent expression of extinction using the IEG product, c-fos (Knapska and Maren, 2009; Orsini et al., 2011). Importantly, the present study

replicates and extends these findings to *Arc*, another IEG. All three studies show that IEG expression in the prefrontal cortex is regulated by the context in which the CS is presented. For instance, there is elevated c-fos expression (Knapska and Maren, 2009) and *Arc* (present study) in the PL during renewal whereas c-fos (Knapska and Maren, 2009; Orsini et al., 2011) and *Arc* (present study) expression is higher in the IL during the recall of extinction. We also found that *Arc* expression in the LA, like c-fos expression (Knapska and Maren, 2009), is higher during renewal than extinction recall. Finally, similar to previous observations (Knapska and Maren, 2009), the present study showed that the VH is recruited during both renewal and extinction recall. In contrast, Orsini et al. (2011) have shown higher c-fos expression in the VH during renewal than extinction recall. Given that the findings of the present study and of Knapska and Maren's (2009) report converge across different IEGs and behavioral paradigms (the latter study used an AAB/ABB renewal design), we believe that the VH is likely engaged during both renewal and extinction recall to aid in contextually disambiguating the meaning of the CS.

The present study also confirms findings of Herry et al. (2008) and Amano et al. (2011), who have shown that there are segregated populations of neurons in the BA that are engaged during renewal and extinction recall. Using neurophysiological recordings, they identified neurons that responded to a fearful CS ("fear" neurons) and neurons that were activated by an extinguished CS ("extinction" neurons). Here, we also show that after extinction, there are cells in the BA that selectively respond to renewal or extinction, with very little overlap. Moreover, we observed that out of all activated neurons, there appeared to be more neurons in the BA that

were engaged during renewal than extinction recall. Interestingly, it seems that the emergence of these cell assemblies depends on the process of extinction. We observed that there was a three-fold increase in context-selective neurons in the BA due to extinction. These results align well with the notion that extinction training yields its own memory that is different than the original fear memory (Quirk and Mueller, 2008). In our hands, the BA was transformed from having cells that responded to the CS regardless of the context in which it was presented to having distinct ensembles of neurons whose activation was regulated by contextual information. Together, this demonstrates that extinction training creates distinct neuronal ensembles in the BA that form context-dependent representations of the CS.

To determine whether similar neuronal activation patterns extended beyond the BA, we assessed the proportion of context-selective neurons in the PL, IL, VH and LA. Unlike the BA, we observed that the majority of neurons in the PL were engaged independent of where the CS was presented. In other words, the PL consisted of overlapping cell assemblies that were recruited during both renewal and extinction recall. Importantly, we observed this pattern prior to extinction as well. Though there was a slight increase in context-selective neurons after extinction, it appears that the PL predominantly contains neuronal ensembles that are generally recruited during fear behavior. Interestingly, though we observed that a large proportion of neurons in the IL were engaged during extinction recall, a majority of neurons were also engaged in both extinction recall and renewal. To date, there is no evidence that supports our finding that the prefrontal cortex

responds in a context-independent manner. In fact, that the prefrontal cortex responded to the CS independent of where it was presented stands in contrast to recent literature showing that prefrontal cortical activity is context-dependent (Hyman et al., 2012). Hyman and colleagues (2012) demonstrated through the use of electrophysiology that neuronal ensembles in the prefrontal cortex exhibit changes in activity when rats move through different environments. Specifically, they observed that different contexts elicited different and separate patterns of neuronal activity in cell assemblies in this region. Furthermore, they showed that the context in which an organism was placed can influence how behavior is encoded in cell populations in the prefrontal cortex (Hyman et al., 2012). In light of our findings, however, we believe that the prefrontal cortex is important for contextual processing insofar as it encodes global contextual representations and tracks contextual shifts. Previous work details a role for the PL and IL in the ability to flexibly shift behavioral strategies when conditions require an organism to do so, as is the case with contextual changes (Delatour and Gisquet-Verrier, 2000; Dalley et al., 2004; Gisquet-Verrier and Delatour, 2006; Ragozzino, 2007). By this view, the prefrontal cortex should be engaged regardless of where the CS was presented, as it is actively monitoring environmental shifts to determine the appropriate behavioral output. Additionally, it has been proposed that the PL may allow an organism to execute new strategies that may inhibit previously used strategies (Ragozzino, 2007). Thus, after extinction, the PL may be specifically involved in the suppression of fear within the extinction context as it is employing a new behavioral strategy to accommodate the environmental conditions.

In contrast to the prefrontal cortex, we observed that the VH contained three distinct cell assemblies: cells engaged during renewal or extinction recall and those activated during both renewal and extinction recall. This indicates the VH consists of a heterogeneous population of neurons that are differentially engaged after extinction. Importantly, these results suggest that different neuronal ensembles may represent different forms of contextual information. These findings are consistent with those of Guzowski et al. (1999), who also used catFISH to demonstrate that the hippocampus consists of three different cell populations that are engaged during the exploration of two distinct environments. Two of the cell assemblies were activated by a specific environment whereas the third population responded to both contexts (Guzowski et al., 1999). Along with the current study, these results nicely complement electrophysiological work that shows that the hippocampus consists of cell ensembles that encode spatial information for a specific location in the environment (O'Keefe and Dostrovsky, 1971; Thompson and Best, 1990; Wilson and McNaughton, 1993). Together, these "place" cells create a map of the environment. Though it was typically thought that "place" cells were only in the dorsal region of the hippocampus, one group has recently reported the existence of "place" cells within the VH (Kjelstrup et al., 2008). Thus, the activation pattern we observed during renewal and extinction may correspond to the activation of "place" cells that are responding to specific components of each environment. These neurons in the VH may deliver contextual information during extinction recall and renewal to downstream structures, such as the amygdala and prefrontal cortex.

As the LA has previously been shown to be involved in the context-dependent expression of fear (Hobin et al., 2003; Maren and Hobin, 2007; Knapska and Maren, 2009), we extended our analysis to include this region. We found that there were two distinct populations of neurons within the LA: those engaged solely by renewal and those that responded to the CS during both extinction recall and renewal. The presence of the former is consistent with previous literature showing that the LA contains neurons that respond to the CS during fear conditioning and return to baseline levels during extinction (Quirk et al., 1995; Repa et al., 2001). Furthermore, our laboratory has demonstrated that the LA exhibits context-specific firing after extinction whereby spike firing to the CS is increased during renewal relative to extinction recall (Hobin et al., 2003). The notion that a proportion of LA neurons are also engaged in extinction recall (in addition to renewal) is not as well supported by previous literature. For example, Herry et al. (2008) explored whether the LA contained populations of neurons devoted to extinction and found that unlike the BA, there was no evidence for the presence of "extinction" neurons. One possible explanation for these discrepancies is that the recall of extinction retrieves part of original fear memory, possibly due to phenomena such as spontaneous recovery; this would result in the appearance of neurons engaged during both extinction recall and renewal. Further work, possibly with optogenetic inactivation, needs to be carried out to tease apart this activation pattern.

We have recently a proposed a model by which contextual information comes to regulate fear behavior (Orsini et al., 2011). Specifically, we suggest that context-dependent retrieval of fear requires convergent input in the BA from the VH

and PL. This is evidenced by the fact that BA-projecting neurons in the VH and PL are engaged during renewal and that the disruption of communication between the VH and PL or BA impairs renewal. The present study extends these findings by providing insight into how distinct CS representations emerge in the BA after extinction. We propose that convergent input from the PL and VH in the BA during extinction causes the appearance of segregated cell assemblies devoted to extinction recall or renewal. In support of this claim, we have previously found that the disconnection of the VH and PL or VH and BA had no effect on non-extinguished fear, but severely impaired the recovery of extinguished fear (Orsini et al., 2011). Furthermore, the present study shows that whereas fear to a non-extinguished CS is represented by overlapping populations in the BA, segregated cell assemblies emerge after extinction that are selectively activated during renewal or extinction recall. Of course, these results do not indicate whether PL and VH input converge on similar neurons or how their activity actually causes these cell assemblies to emerge. Interestingly, it has been shown that the same VH neurons project to cells in both the amygdala and PL (Ishikawa and Nakamura, 2006) and that firing activity of neurons within the prefrontal cortex and amygdala can become entrained to hippocampal theta rhythm (Pape et al., 1998; Seidenbecher et al., 2003; Jensen, 2005; Pape et al., 2005; Adhikari et al., 2010; Colgin, 2011). By this view, it is conceivable that the VH serves as an anatomical hub that promotes the synchronized activity of the circuit during extinction. This entrainment could aid in sculpting the formation of discrete populations of cells in the BA that are active

during renewal or suppression of fear. Further work using physiology and tract tracing methods need to be employed to confirm this hypothesis.

In conclusion, the results of the present study provide new insight into how an extinguished CS is represented across the neural circuit known to be involved in the contextual regulation of fear after extinction. Whereas the BA mostly contained segregated cell populations engaged during renewal or extinction recall, we found that the VH consisted of a heterogeneous population of responsive neurons and the PL predominantly had overlapping cell assemblies. Additionally, we observed that the IL and LA were recruited during extinction recall and renewal, respectively, though they also contained neurons that responded to both conditions. Most importantly, we found that extinction training caused the emergence of the distinct cell assemblies seen in the BA, presumably due to PL and VH input. Understanding how these brain regions represent emotionally salient information and interact with one another to orchestrate fear behavior is especially important if we are to successfully combat fear and anxiety pathologies. In particular, the relapse of traumatic fear poses a real challenge as it demonstrates how weak extinction training is and how fragile the resulting memories are. Continuing to explore the brain basis for fear and extinction behavior will enable us to build a better model of how contextual information regulates these processes and hopefully lead to more promising therapeutic treatments.

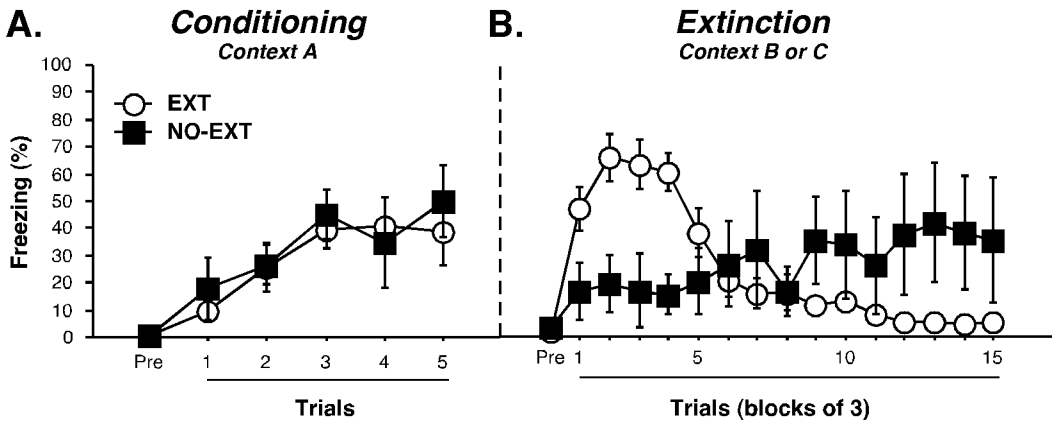

Figure 4.1. Conditioned freezing behavior during fear conditioning and extinction. Mean percentage of freezing (±SEM) during fear conditioning, which consisted of a 3 min baseline period followed by 5 tone-shock pairings. Freezing was averaged across the pre-CS baseline (Pre) as well as during each of the five conditioning trials; each trial consisted of the average of freezing during each CS presentation and the subsequent interstimulus interval. *(B)* Mean percentage of freezing (±SEM) during the 45-tone alone extinction session. Freezing was averaged across the baseline period (Pre) as well as during the 45 extinction trials; like conditioning, each trial consisted of the average of freezing during each CS presentation and subsequent ISI (data were binned into 15 blocks of 3-trial averages). Data is shown for rats that underwent extinction (EXT) and those that did not receive extinction (NO-EXT).

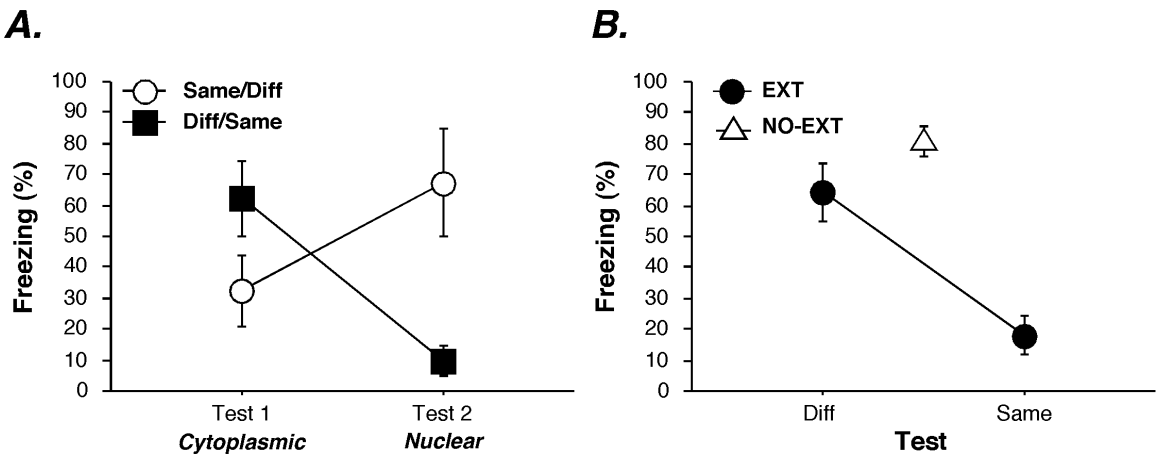

Figure 4.2. Freezing behavior in extinguished and non-extinguished rats during the test sessions. *(A)* Mean percentage of freezing (±SEM) during the two test sessions that each consisted of 3 tone-alone presentations with 30 sec ISIs. Freezing was measured during the baseline (Pre) period and during the three trials, each of which consisted of a CS presentation and the subsequent ISI. Data are shown for rats that were first tested in the extinction context, followed by the renewal context (Same/Diff) and rats that were first tested in the renewal context, followed by the extinction context. Importantly, we note that the first test corresponds with cytoplasmic staining whereas the second test corresponds to nuclear staining. *(B)* Mean percentage of freezing (±SEM) during the test sessions for extinguished (EXT) and non-extinguished (NO-EXT) rats. For extinguished rats, freezing was collapsed across Same/Diff and Diff/Same groups to yield an overall mean percentage of freezing (±SEM) for the renewal and extinction test. Similarly, freezing across the two tests was collapsed for non-extinguished rats.

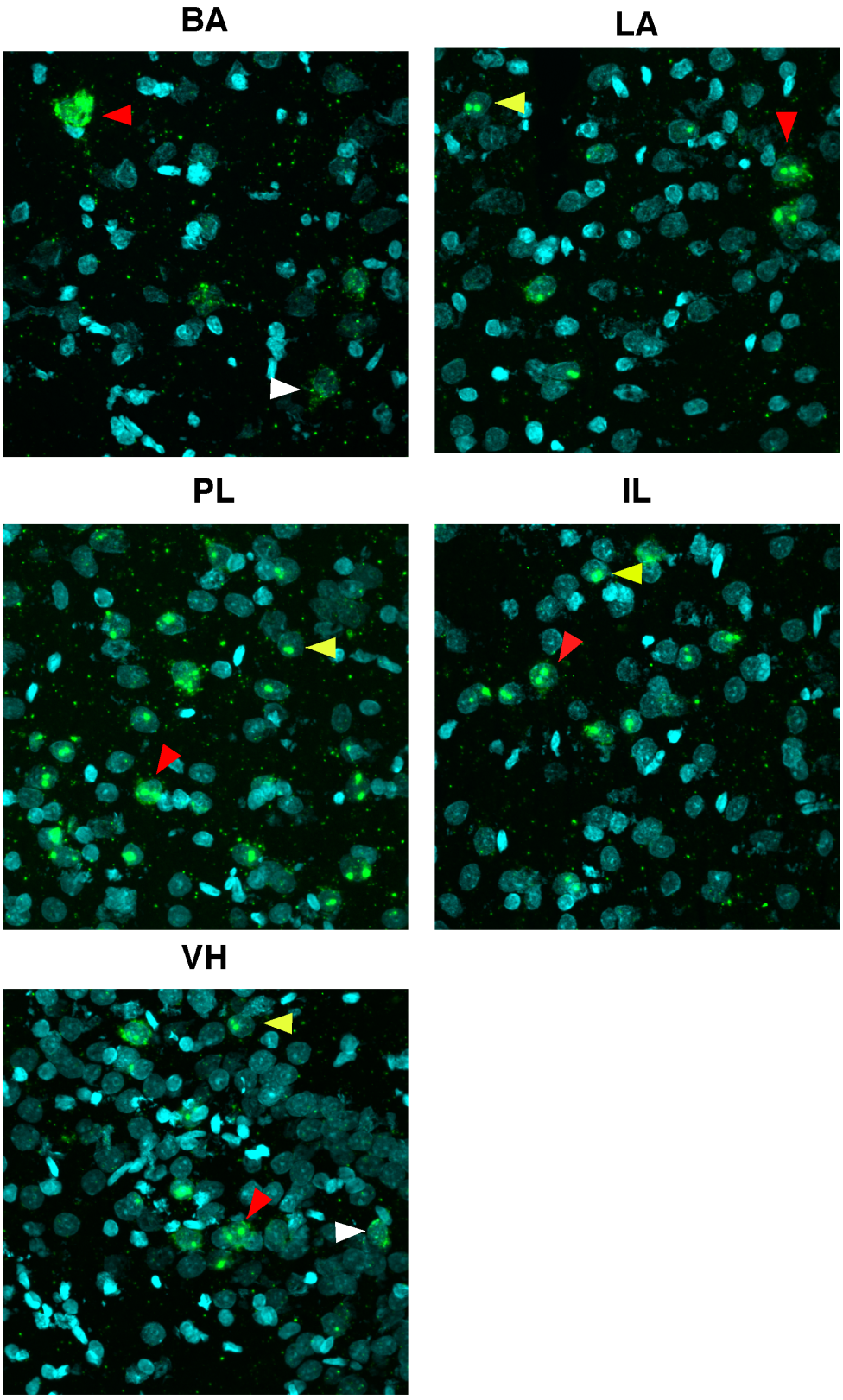

Figure 4.3. Representative confocal images for the basal amygdala (BA), ventral hippocampus (VH), prelimbic area (PL), infralimbic area (IL) and lateral amygdala

(LA) taken at 40X magnification. White arrowheads indicate cytoplasmic staining and yellow arrowheads indicate nuclear staining. Red arrowheads indicate nuclear and cytoplasmic staining (non-selective neurons).

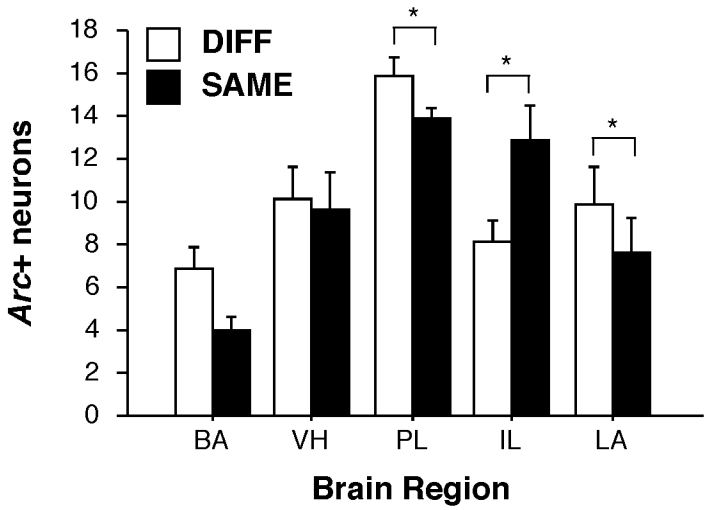

Figure 4.4. Context-dependent expression of *Arc* in the basal amygdala (BA), ventral hippocampus (VH), prelimbic cortex (PL), infralimbic cortex (IL) and lateral amygdala (LA). Cell counts for the DIFF condition were calculated by taking the sum of *Arc*-positive cells activated during renewal and those activated during both renewal and extinction recall. Cell counts for the SAME condition were obtained by adding together *Arc*-positive neurons engaged during extinction recall and those engaged during both renewal and extinction recall. DIFF and SAME are represented as the mean (±SEM) cell counts of *Arc*-positive neurons of extinguished rats for each brain region.

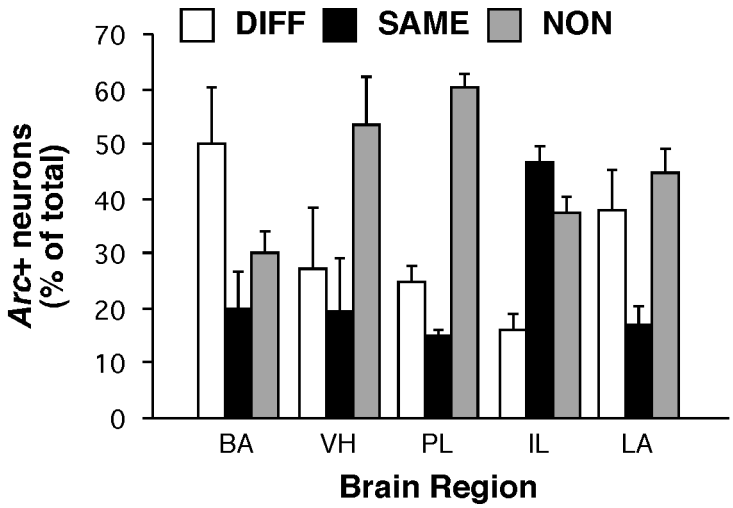

Figure 4.5. Percentage of total *Arc*-positive neurons in extinguished rats. The data are represented as mean (±SEM) percentage of *Arc*-positive neurons activated during renewal (DIFF), extinction recall (SAME) or during both renewal and extinction recall (non-selective; NON) in the basal amygdala (BA), ventral hippocampus (VH), prelimbic cortex (PL), infralimbic cortex (IL) and lateral amygdala (LA). Percentages were acquired by dividing cell counts for each condition (DIFF, SAME or NON) by the total count of *Arc*-positive neurons.

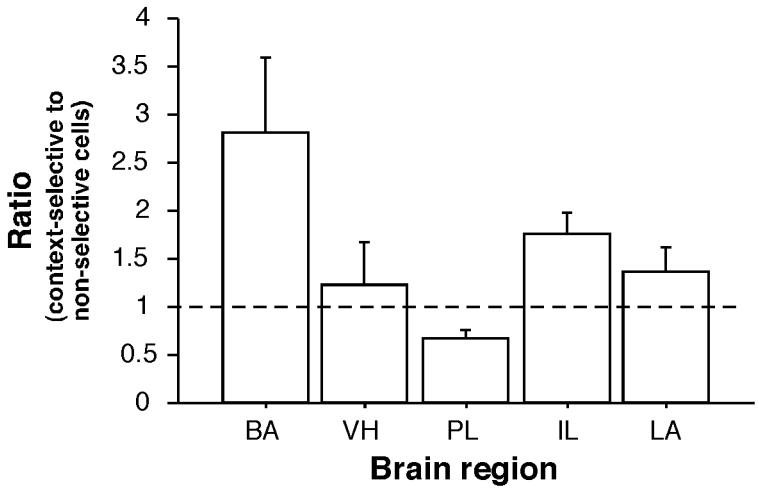

Figure 4.6. Ratio of context-selective neurons to non-selective neurons in extinguished rats. Ratios are represented as means (±SEM) for the basal amygdala (BA), ventral hippocampus (VH), prelimbic cortex (PL), infralimbic cortex (IL) and lateral amygdala (LA). The dashed line represents equal proportions of context-selective and non-selective neurons.

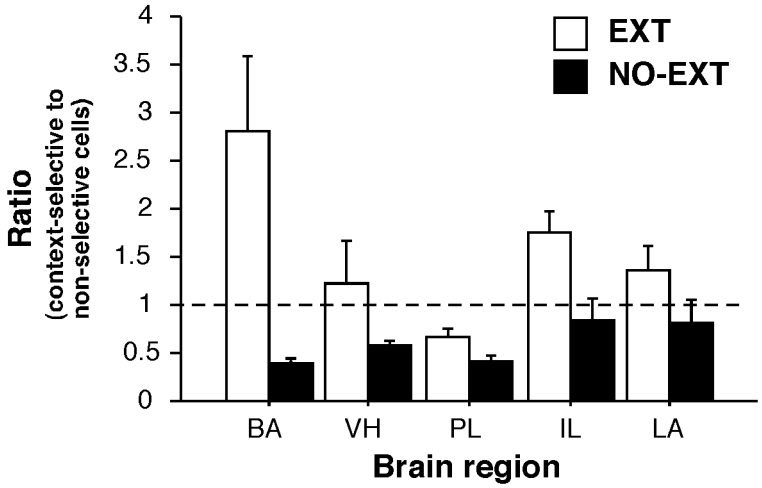

Figure 4.7. Comparison of the ratio of context-selective to non-selective neurons between extinguished (EXT) and non-extinguished rats (NO-EXT). Ratios are represented as means (±SEM) for the basal amygdala (BA), ventral hippocampus (VH), prelimbic cortex (PL), infralimbic cortex (IL) and lateral amygdala (LA). The dashed line represents equal proportions of context-selective and non-selective neurons.

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CHAPTER V

CONCLUSION

Summary of findings

At the end of Chapter 1, it was suggested that the contextual modulation of fear after extinction is mediated by a distributed neural network consisting of the hippocampus, prefrontal cortex and amygdala. Though a great deal is known about how each of these structures contributes to this behavior, the way in which these structures interact to produce an appropriate response is not as well understood. Therefore, in this dissertation, I sought to explore how these brain regions represent conditioned stimuli (CS) after extinction and how this information is conveyed across the circuit.

Given the hippocampus' role in spatial processing and forming contextual representations (Fanselow, 2000), it has been posited that it provides the amygdala with contextual information used to disambiguate the meaning of a CS after extinction. Anatomically, the ventral hippocampus (ventral CA1/ventral subiculum; VH) has direct projections to the basal amygdala (BA; Canteras and Swanson, 1992; Pitkanen et al., 2000); in addition, the VH can access the BA indirectly through the prelimbic cortex (PL) of the prefrontal cortex (PFC). Thus, contextual information can be relayed to the BA via two different pathways during the contextual retrieval of fear after extinction. In Chapter 2, I explored whether both pathways are

engaged during renewal of fear. I infused cholera toxin subunit b (CTb) into the BA to label cells in structures afferent to the BA. I then assessed neuronal activation, as measured by c-fos expression, in BA-projecting neurons in the VH and prefrontal cortex during renewal or extinction recall. As expected, I observed that BA-projecting cells in the PL and VH were selectively recruited during renewal of fear. In contrast, BA-projecting neurons in the infralimbic cortex (IL), an area of the prefrontal cortex known to be involved in the consolidation and retrieval of extinction (Milad and Quirk, 2002; Milad et al., 2004; Quirk et al., 2006), were engaged during the recall of extinction.

Though the results of Chapter 2 suggest that the PL and VH communicate with the BA during the contextual retrieval of fear, it does not elucidate whether both pathways are required for renewal or whether one intact pathway is sufficient for this behavior to occur. As such, Chapter 3 focused on examining the necessity of the direct and indirect pathway from the VH to the BA during renewal of fear. Using asymmetric electrolytic lesions, I disconnected the VH from either the BA or PL after the extinction of conditioned fear. I found that the elimination of either pathway severely disrupted renewal. Importantly, this effect was not due to the lesions interfering with general expression of fear, as the disconnections did not affect freezing in non-extinguished rats. Thus, these results suggest that both pathways need to be intact for the reappearance of fear after extinction. Along with Chapter 1, it also indicates that BA-projecting neurons in the VH and PL may converge in the BA during renewal.

Results from Chapters 2 and 3 provide evidence that the hippocampus, prefrontal cortex and basal amygdala actively communicate during the context-dependent retrieval of fear. However, these results do not reveal how cell assemblies in these regions use contextual information to organize behavioral selection. For example, it has been shown that the BA contains unique cell populations that are selectively recruited during renewal or extinction (Herry et al., 2008). It is not known whether a similar segregation of neurons exists in the prefrontal cortex and hippocampus. Therefore, in Chapter 4, I used cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH), a method that allowed me to visualize neuronal activation to two different behavioral experiences. Specifically, I examined the cellular distribution of the mRNA of the immediate early gene *Arc* in the VH, prefrontal cortex and BA during renewal and extinction recall. Consistent with previous reports (Herry et al., 2008), I found that the BA contained distinct cell ensembles that responded to the CS in a context-dependent manner. Importantly, the emergence of these neuronal populations was due to extinction training. Unlike the BA, the PL largely consisted of overlapping cell assemblies that were activated regardless of extinction training and the context in which the CS was presented. The IL also contained neurons that responded to the CS independent of the context; however, I also observed a separate population in the IL that was solely engaged during extinction recall. Within the VH, I observed three distinct cell populations: those that responded during renewal or extinction, and those that responded during both. Finally, the lateral amygdala (LA) contained neurons that were explicitly active during renewal, though there were

also neurons engaged during both renewal and extinction recall. These data demonstrate that contextual information modulates neuronal ensembles differently across the brain and that extinction has a role in the emergence of these populations.

Interactions between the VH, BA and prefrontal cortex

The individual roles of the ventral hippocampus, amygdala and prefrontal cortex in the context-dependent expression of fear are quite well understood given the plethora of data that has emerged in the last decade. For example, the inactivation of the ventral hippocampus disrupts renewal of fear (Hobin et al., 2006). Similarly, selective inactivation of the BA produces the same results (Herry et al., 2008). Within the prefrontal cortex, the PL is preferentially involved in the renewal of fear (Knapska and Maren, 2009; Orsini et al., 2011) whereas the IL has been shown to be involved in the consolidation and expression of extinction (Milad and Quirk, 2002; Quirk et al., 2006; Knapska and Maren, 2009; Orsini et al., 2011). Together, all of this evidence suggests that the contextual modulation of fear after extinction recruits all of these brain regions, presumably at the same time. To date, however, there has not been a systematic evaluation of how these particular neural structures interact and relay contextual information through the circuit.

Results from Chapters 2 and 3 now show that pathways from the VH to the amygdala, directly or indirectly, are recruited during renewal of fear. BA-projecting neurons in both the VH and PL were active during renewal; interfering with communication between the VH and BA or VH and PL eliminated renewal entirely.

Importantly, these latter findings from Chapter 3 suggest that the convergence of both pathways in the BA is necessary for renewal to occur. For example, severing the connection between the VH and PL spares VH-BA communication in one hemisphere, but completely deprives the BA of PL input. Similarly, disconnecting the VH and BA still allows communication between the VH and PL in one hemisphere, but prevents all VH input to the BA. Thus, in order for renewal to occur, both the direct and indirect pathway must be intact in order for the PL and VH to access the BA simultaneously.

Ultimately, however, these data show that the VH is required to contextually control behavior through its projections to the BA (both directly and indirectly). Interestingly, it has been shown that the same neurons in the VH project to both the PL and BA (Ishikawa and Nakamura, 2006). Along with our data, this suggests that the VH serves as an anatomical hub from which it can coordinate fear behavior through its connections with the prefrontal cortex and amygdala. Such neural organization can be accomplished through the synchronization of theta rhythms between brain areas. Theta rhythms are oscillatory patterns in local field potentials in the 4-10 Hz range that typically occur during active behavior and REM sleep (Buzsaki, 2002; Colgin, 2011). Not only have theta rhythms been observed in the hippocampus (Buzsaki, 2002), but they also occur in the prefrontal cortex (Hyman et al., 2005; Jones and Wilson, 2005b; Siapas et al., 2005) and amygdala (Pare and Gaudreau, 1996), among several other structures. This oscillatory pattern has been linked to many different neural functions (Buzsaki, 2005), most important of which is the coordination of interactions between brain regions. For example, when the

VH is engaged, firing in the prefrontal cortex becomes phase-locked to hippocampal theta rhythms (Jones and Wilson, 2005a, b). This process is thought to reflect information transfer and ultimately, the strengthening of synapses between these areas (Siapas et al., 2005). In addition, synchrony with hippocampal theta oscillations promotes the formation of cell ensembles within the interacting brain structures (Benchenane et al., 2010). Thus, it is possible that the synchronization of prefrontal and amygdala theta rhythms with hippocampal theta rhythms during extinction not only forms the network mediating contextual regulation of fear after extinction, but also allows for the emergence of neuronal ensembles within these structures, as seen in Chapter 4.

Interestingly, synchronous activity in the hippocampal-prefrontal pathway has received widespread attention with regard to its role in organizing behavior. In one of the first studies to show theta phase-locking between the prefrontal cortex and hippocampus, Siapas et al. (2005) found that the prefrontal cortex phase-locked to hippocampal theta with a 50 millisecond delay, confirming that the directionality of modulation is from the hippocampus to the prefrontal cortex. Importantly, others have similarly shown that prefrontal cortical neurons phase-lock to hippocampal theta rhythms during behavioral epochs that require spatial information to guide an organism's behavior (Jones and Wilson, 2005a, b). This suggests that this pathway may be used for the transfer of contextual information from the ventral hippocampus to the prefrontal cortex, consistent with our conclusions in Chapter 3.

More recently, one group investigated how synchronous activity between the ventral hippocampus and prefrontal cortex mediates anxiety behavior (Adhikari et

al., 2010, 2011). By recording in the VH and deep layers of the PL during the exploration of the elevated-plus maze (EPM) and the open field test, they found that these two brain regions were highly synchronized (Adhikari et al., 2010). Interestingly, they observed that both prefrontal theta frequency activity and coherence between the VH and PFC increased when the animal entered "safer" regions of these environments, but decreased when entering the "aversive" regions, indicating that the interaction between the VH and PFC may be important for regulating exploratory behavior during anxiety-inducing situations. Furthermore, Adhikari et al. (2011) found that prefrontal neurons differentially represent the "safe" (closed) and "aversive" (open) arms of the EPM. Prefrontal neurons with the strongest representations of these task-related components were those neurons that were most strongly modulated by hippocampal theta oscillations. Thus, it is conceivable that in anxiety-related situations, the VH relays contextual information to the prefrontal cortex, specifically the prelimbic region; this can be integrated with input from other brain regions, such as the mediodorsal nucleus of the thalamus, that provide more motivational/affective information (Vertes, 2006). In turn, the prelimbic cortex selects the appropriate behavioral response and guides its output through its connections with the amygdala. In our hands, this suggests that hippocampal-prefrontal synchrony is a way in which contextual information can organize and elicit appropriate behavioral responses after extinction. By this view, the disconnection of these two areas would not only interrupt VH-PL synchronized activity, but also prevent the PL from selecting the appropriate behavioral response during renewal (freezing) through its interactions with the amygdala.

Not only can the VH-PFC pathway become synchronized at the theta frequency, but it has also been shown that this occurs in the hippocampal-amygdala pathway. For example, synchronized theta activity in the hippocampus and amygdala increases after fear conditioning and is significantly pronounced upon conditioned stimulus presentation after fear conditioning (Seidenbecher et al., 2003), implicating a role for hippocampal-amygdala synchronization in long-term fear memory retrieval (Narayanan et al., 2007). More recently, it was shown that artificially induced entrainment between these brain regions resulted in the persistence of freezing during extinction (Lesting et al., 2011). These findings suggest that theta synchronization between the hippocampus and the amygdala is important for the expression of fear. Given that renewal involves fear expression, it is possible that theta synchrony between the VH and BA is involved in generating this response when tested outside the extinction context. This theory is consistent with our findings in Chapters 2 and 3 as well as those of Herry et al. (2008), which unequivocally demonstrate a role for the VH to BA pathway in the expression of fear after extinction.

Given the plethora of data detailing the importance of synchronous activity of the hippocampus with the prefrontal cortex and amygdala, it stands to reason that this may be a mechanism by which the VH can modulate BA activity. Interestingly, one important result may be the emergence of the cell assemblies in the BA that we observed in Chapter 4. It has been shown, for example, that during increases in hippocampal-prefrontal coherence, theta-modulated prefrontal neurons organize into cell assemblies whose firing phases shift to fire in synchrony with hippocampal

cell assemblies (Benchenane et al., 2010). Though this has not been shown in the pathways to the BA, it is a possible hypothesis for how these populations appear during extinction. Reverberating theta oscillations in the circuit could bring about repeated depolarizations in the amygdala, which could differentially couple specific cells with afferent structures (hippocampus or prefrontal cortex). That is, neurons solely engaged during renewal could be specifically connected with VH input during theta oscillations. As it has been shown that interneurons can also become phase-locked to hippocampal theta rhythms (Hartwich et al., 2009; Benchenane et al., 2010), another possibility is that during these oscillations, interneurons can interact with activated pyramidal neurons. This interaction could aid in sculpting the formation of different neuronal populations. Regardless of the mechanism, however, it is clear that the VH's rhythmic properties and projection patterns make the structure an ideal candidate for being able to contextually regulate fear behavior.

Amygdala microcircuitry involved in fear regulation

Converging input in the amygdala from the ventral hippocampus and prefrontal cortex clearly regulates fear behavior. However, the way in which the amygdala itself processes incoming information and subsequently organizes the appropriate behavioral output after extinction is not as well understood. In the last two years, great strides have been made in uncovering the amygdala microcircuitry involved in fear expression and extinction. Though it is not clear whether fear expression in non-extinguished animals is neurobiologically the same as fear

expression during renewal, it nonetheless provides a framework from which to understand how the amygdala can differentially represent and regulate fear states.

It appears that after extinction, neurons in both the LA and BA differ in their responsiveness to the CS. For example, neuronal firing in the dorsal portion of the LA diminishes during extinction while cells in the ventral portion of the LA remain persistently active throughout extinction (Repa et al., 2001). This latter group of LA neurons could possibly correspond with the cells in the LA that selectively responded to the CS during renewal in Chapter 4. Within the BA, there are distinct populations of projection neurons that respond to a fearful CS ("fear" neurons) or an extinguished CS ("extinction" neurons; Herry et al., 2008; Chapter 4). Recently, another group has corroborated the existence of two different cell populations in the BA (Popescu and Pare, 2011). Though they did not demonstrate the populations' specific relevance to fear states, they showed that there are two different types of projection cells in the BA that differ in their relationship to inhibitory interneurons. Specifically, the activation of approximately 15% of BA projection cells, presumably through cortical or hippocampal input, results in their activation of BA interneurons, which subsequently inhibit the remaining portion of BA projection neurons. This interaction between GABAergic neurons and glutamatergic neurons may explain how "extinction" neurons and "fear" neurons regulate each other's activity to bias a specific behavioral output. For instance, activation of "fear" neurons by ventral hippocampal input (Herry et al., 2008) could activate BA interneurons, which could then inhibit "extinction" neurons. This, of course, is only a hypothesis and thus requires further scrutiny.

Though the BA may contain unique cell populations that represent the CS differently, it ultimately is not responsible for controlling fear output. Rather, the central nucleus of the amygdala (CeA) is thought to be the interface between the BA and response-generating structures. The CeA contains GABAergic neurons and can be divided into three different subnuclei: the lateral central amygdala (CeL), the medial central amygdala (CeM) and the capsular region of the central amygdala (CeC; Sah et al., 2003). The CeL and CeM have garnered a lot of interest with respect to their role in fear behavior. The CeL is thought to be important for fear memory acquisition whereas the CeM is considered to be the output of the CeA, with a specific role in fear expression (Ciocchi et al., 2010; Duvarci et al., 2011). Interestingly, the CeL is thought to tonically inhibit the CeM (Ciocchi et al., 2010), suggesting the importance of an inhibitory interaction between the CeL and CeM in regulating behavioral output. Indeed, findings in the last two years have shown that both the CeL and CeM contain segregated inhibitory cell populations and intricate microcircuitry between these subregions and their distinct cell populations contribute to the overall fear response (Ciocchi et al., 2010; Haubensak et al., 2010; Duvarci et al., 2011; Viviani et al., 2011; Pare and Duvarci, 2012).

Ciocchi and colleagues (2010) first demonstrated that the CeL contains two distinct inhibitory cell populations that either exhibit an excitatory response to the CS (CeL-On cells) or an inhibitory response to the CS (CeL-Off cells). They found that CeL-Off cells receive inhibitory projections from CeL-On cells and that both of these cells project to and inhibit the CeM. In a parallel study, Haubensak et al. (2010) also observed two separate cell types in the CeL that were distinguished by

the presence or absence of protein kinase C-δ (PKC-δ). Importantly, cells that were positive for PKC-δ (PKC-δ+) physiologically mapped onto CeL-Off cells and that like CeL-On and CeL-Off cell interactions, PKC-δ+ cells received inhibitory input from PKC-δ- cells. Interestingly, they also found that PKC-δ+ neurons synapsed on neurons in the CeM that projected to the periaqueductal gray (PAG), a brainstem structure known to be responsible for freezing behavior (LeDoux et al., 1988). Behaviorally, Haubsenak and colleagues (2010) demonstrated that silencing PKC-δ+/CeL-Off neurons resulted in freezing behavior. Together, these studies suggest that the generation of a fear response involves the disinhibition of CeM neurons as a result of the inhibition of PKC-δ+/CeL-Off neurons by CeL-On neurons.

It has been reported that the CeM also contains different cell populations. Viviani et al. (2011) identified two distinct populations that differed with respect to their projection targets. They observed that one CeM cell assembly selectively projected to the PAG while another population projected to the dorsal vagal complex (DVC), which is responsible for the cardiovascular responses to fear stimuli (an increase in heart rate; Danielsen et al., 1989). Not only did these populations differ in their projection patterns, but they also differed in their response to the application of oxytocin, a neuropeptide that has been shown to inhibit CeM neurons through its effect on neurons in CeL (Huber et al., 2005). PAG-projecting neurons in the CeM were inhibited when oxytocin was applied to the CeA; DVC-projecting neurons were unaffected. Interestingly, when oxytocin was infused into the CeA during a context test after contextual fear conditioning, freezing to the context was reduced while the cardiovascular response remained intact. Thus, it seems that the

CeM differentially regulates fear expression through cell populations with distinct projections to the brainstem. Moreover, the activation of these assemblies is under the control of oxytocin's effect on CeL neurons. Interestingly, Haubensak et al. (2010) found that a proportion of PKC-δ+ neurons in the CeL also express oxytocin receptors. This suggests that this neuropeptide can contribute to the inhibition of PAG-projecting neurons in the CeM by acting on PKC-δ+ neurons in the CeL, resulting in the suppression of fear (as seen in extinction).

Thus, to successfully produce a behavioral response, the LA and BA must interact with the CeA, specifically the CeM. The amygdala is equipped with several ways in which this can occur. First, the LA can directly send excitatory projections to CeL neurons, which can then project to and inhibit the CeM, leading to the suppression of freezing. Consistent with this, the optogenetic activation of this projection has been shown to result in a decrease in unconditioned fear and the inhibition of CeM neurons (Tye et al., 2011). In contrast, neurons in the BA can directly access the CeM (Krettek and Price, 1978; Pare et al., 1995; Pitkanen et al., 1997). Through this excitatory projection, the activation of the CeM by the BA can contribute to the expression of fear. The LA and BA can also access the CeM via its connections with the intercalated cell masses (ITC), groups of GABAergic neurons that lie between the basolateral amygdala and CeA (Millhouse, 1986; Sah et al., 2003). There are several clusters of ITC cells in this area: a dorsal cluster closer to the top of the CeL (ITCd) and a more ventral group that is located near the bottom of the CeM (ITCv). Cells in the BA project to the ITCv cluster, which directly projects to cells in the CeM (Amir et al., 2011). In contrast, neurons in the LA project to the

ITCd cluster, which can then regulate CeM output through its connection to the CeL and/or its projection to the ITCv. Given their location and their intricate connections, the ITC cells are situated perfectly to dynamically modulate fear output. For example, activation of ITCd cells can disinhibit CeM neurons by inhibiting CeL cells, resulting in fear expression; conversely, direct activation of ITCv neurons by the BA will inhibit CeM neurons, resulting in fear suppression. Consistent with the role of the ITCv in the expression of extinction, it was recently shown that the expression of the immediate early gene *zif268* was higher in the ITCv cells than the ITCd cells during extinction recall (Busti et al., 2011).

Recently, Pare and Duvarci (2012) have taken this entire body of evidence and incorporated it into a model that attempts to explain how the amygdala processes CSs during fear or extinction recall. The results presented in this dissertation not only align well with their model, but also suggest how input from the prefrontal cortex and hippocampus may interact with amygdala microcircuitry (Figure 5.1). During the retrieval of fear, neurons in the LA respond to the CS, similar to our observations in Chapter 4. These neurons can then excite "fear" neurons in the BA, as well as ITCd cells and CeL-On neurons in the CeL. These latter projections result in the disinhibition of PAG-projecting neurons in the CeM through the inhibition of CeL-Off cells (for the freezing conditioned response). In addition, the activation of ITCd cells can then inhibit ITCv neurons, which, again, would result in the disinhibition of CeM neurons. Results from Chapters 2 and 3 suggest that in addition to LA input, "fear" neurons in the BA may receive monosynaptic convergent input from neurons in the PL and VH. As previously suggested, "fear" neurons could

then activate BA interneurons, which would in turn inhibit "extinction" neurons. To promote freezing behavior, "fear" neurons in the BA could send excitatory projections to PAG-projecting neurons in the CeM. The net activity of the circuit results in the release of the CeM from inhibition by the CeL.

Conversely, during the recall of extinction, "extinction" neurons in the BA are activated (Chapter 4), possibly by input from the IL (Chapter 2). Though we did not find any evidence for VH input to the BA during extinction, it is possible that it can contextually regulate behavior through its projection to the IL. "Extinction" neurons can suppress "fear" neuron activity through the activation of BA interneurons. Through their excitatory projections to ITCv neurons, "extinction" neurons can then inhibit PAG-projecting neurons in the CeM, leading to the suppression of fear. Consistent with this, a recent study found that extinction resulted an increase in the potentiation of BA inputs to ITC cells that directly projected to the CeM (Amano et al., 2010). Interestingly, this potentiation of BA to ITC input depended on input from IL. Finally, CeL-Off neurons can directly inhibit CeM neurons, possibly through the activation of oxytocin receptors. As a whole, this suggests that the suppression of fear observed during extinction is a result of the net inhibition of CeM.

Future Directions

Though we suggest that PL and VH both project to and converge on "fear" neurons in the BA, this remains unproven. It has been shown that the VH projects to "fear" neurons whereas the prefrontal cortex only synapses on "extinction" neurons (Herry et al., 2008). However, results from Chapter 2 show that BA-projecting

neurons in the PL are engaged during renewal, though they do not indicate whether these prefrontal neurons specifically synapse on "fear" neurons. As such, I believe that an important next step would be to test this hypothesis. This could be addressed by injecting an anterograde tracer into the PL prior to our typical renewal behavioral paradigm. Through the use of immunohistochemistry and catFISH, we could then identify the overlap of BA target neurons from the PL with "fear" and "extinction" neurons. Additionally, if another anterograde tracer were simultaneously injected into the VH, this would additionally indicate whether VH and PL projections converge on identical neurons in the BA during renewal, as suggested in Chapter 3.

In Chapter 2, we showed the BA-projecting neurons in the VH are selectively engaged during renewal of fear. However, in Chapter 4, we observed that the VH contains neurons that are activated during renewal and extinction recall. Thus, the question of how VH cells contribute to extinction recall still remains. It is possible that, like the PL, the IL receives projections from the VH during extinction; in turn, it can then project to the BA to suppress fear, as evidenced by our results in Chapter 2. To easily test this hypothesis, we could disconnect the VH from IL and assess its effect on the recall of extinction. I hypothesize that this manipulation would result in high levels of fear comparable to that observed in renewal. Presumably, this is due to the removal of the input that drives the inhibition of the CeM.

Lastly, an important issue is how the results presented in this dissertation relate to other behaviors that can be modulated by contextual information. Of considerable interest, for example, is how contextual cues can cause the relapse of

previously extinguished drug seeking (Crombag and Shaham, 2002; Crombag et al., 2008). In humans, contexts that were previously associated with drug taking can cause the relapse of drug use (Wikler, 1973; O'Brien et al., 1992). To study this behavior in the laboratory, an animal is trained to associate a cue with the delivery of a drug (or a stimulus that indicates drug availability) in one context. In a different context, the animal then undergoes extinction in which the cue is repeatedly presented without drug delivery. Finally, during the test session, the conditioned drug cue is re-introduced to the animal in the training context. Similar to fear renewal paradigms, drug seeking is "reinstated" within the training context to a level comparable to pre-extinction. It has been shown that context-induced drug relapse involves the prefrontal cortex (McLaughlin and See, 2003; Hamlin et al., 2008; Bossert et al., 2011; Bossert et al., 2012), ventral hippocampus (Vorel et al., 2001; Atkins et al., 2008; Lasseter et al., 2010) and amygdala (See et al., 2003; Fuchs et al., 2005; Hamlin et al., 2008; Hamlin et al., 2009). To date, however, there is no data that suggests how these regions interact during the contextual modulation of drug seeking. Given that the inactivation of the VH eliminates context-induced reinstatement (Lasseter et al., 2010), it is conceivable that, like the contextual regulation of fear, it modulates behavioral output through its interactions with the prefrontal cortex and amygdala. It would be interesting to take the techniques used in this dissertation and apply them to context-induced reinstatement of drug seeking. The use of catFISH, for instance, could show whether extinguished drug cues activate cell populations in a context-dependent manner. Furthermore, it would be intriguing to compare this against how the brain

represents extinguished aversive cues (Chapter 4). Because contextual cues exert such a strong influence on drug seeking behavior, probing the neural circuitry underlying reinstatement would provide invaluable insight into how the brain processes drug-related cues (both discrete and contextual) so as to better develop therapies for drug addiction.

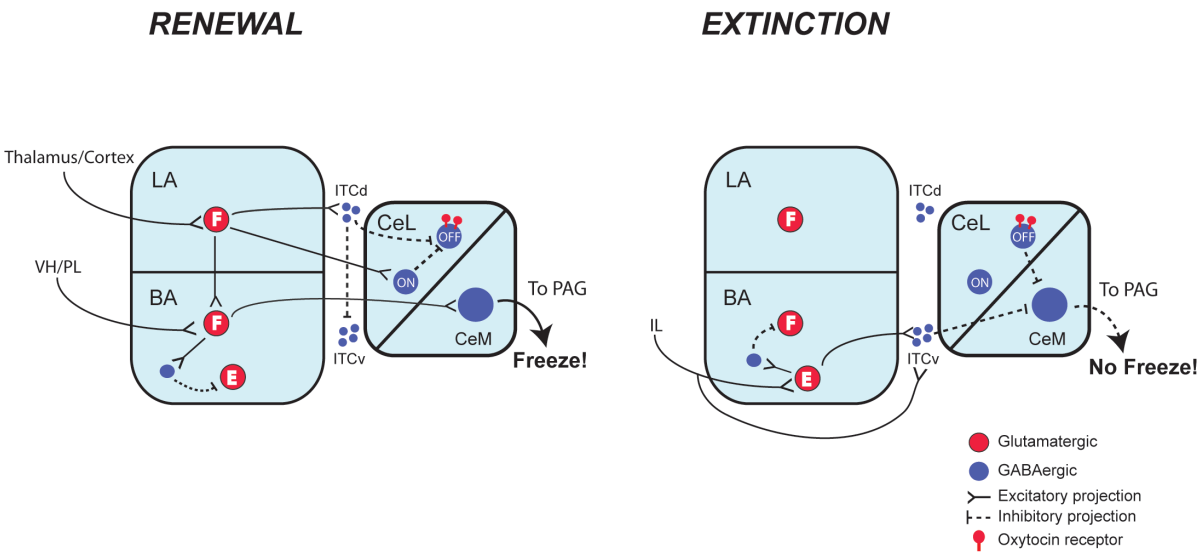

Figure 5.1. Proposed model of the contextual modulation of fear after extinction. After extinction, rats suppress their fear to the conditioned stimulus (CS) within the extinction context, but exhibit high levels of fear to the CS in other contexts. Input from the prefrontal cortex [prelimbic cortex (PL) and infralimbic (IL)] and the ventral hippocampus (VH) to the amygdala regulates extinction recall and renewal. Recent work has demonstrated that the participation of distinct neuronal assemblies within amygdala microcircuitry contributes to the selection of the appropriate behavioral response. During renewal (left panel), fear neurons (F) in the lateral amygdala (LA) and basal amygdala (BA) are activated by the thalamus/cortex and VH/PL, respectively. Fear neurons in the LA send excitatory projections to fear neurons in the BA, as well as GABAergic neurons in the dorsal region of the intercalated cell mass (ITCd) and the lateral central amygdala (CeL). Both the ITCd neurons and CeL-On neurons subsequently inhibit a separate neuronal population in the CeL (CeL-Off). Due to CeL-Off inhibition, a subset of medial central amygdala (CeM) neurons that project to the periaqueductral gray (PAG) are disinhibited, resulting in freezing behavior. Fear neurons in the BA also contribute to CeM activity as they send direct excitatory projections to this output center of the central amygdala. In addition, they interact with interneurons in the BA to suppress extinction neurons (E). During extinction recall (right panel), the IL projects onto extinction neurons, which inhibit fear cells through BA interneurons. Extinction cells project onto GABAergic neurons in the ventral region of the intercalated cell mass (ITCv); in turn, these neurons inhibit PAG-projecting neurons in the CeM. Interestingly, it has been shown that the IL also projects to ITC cells and actually potentiates BA input to these GABAergic cells. In addition to ITCv neurons, CeL-Off cells are free to inhibit PAG-projecting neurons in the CeM due to the absence of feed-forward inhibition from CeL-On and ITCd cells. The activation of oxytocin receptors on CeL-Off neurons may contribute to the inhibition of CeM neurons.

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