EXPLORING THE ROLE OF INFRALIMBIC CORTEX INHIBITORY CIRCUITS IN CONTEXT-DEPENDENT EXTINCTION AND RENEWAL OF FEAR

Dissertation

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ABSTRACT

Exposure therapy is an effective treatment for posttraumatic stress disorder (PTSD). However, many patients experienced relapse of fear after treatment. Pavlovian fear conditioning and extinction are effective models to study the return of fear. During fear conditioning, an auditory conditioned stimulus (CS) is paired with an electric footshock (i.e., the unconditioned stimulus, US); after conditioning, the CS evoked a conditional fear response. Presenting the CS numerous times without the US causes an extinction of fear to the CS; however, the loss of fear to the CS is context-specific. That is, extinguished fear returns or "renews" outside of the extinction context. The hippocampus, the medial prefrontal cortex (mPFC) and the amygdala are thought to be essential for context-dependent extinction retrieval and fear renewal. However, how the mPFC, especially the infralimbic cortex (IL), regulates extinction memory is not clear. To clarify this question, I first used retrograde tracing techniques with immediate early gene expression to examine the activity of the mPFC-projecting neurons in the ventral hippocampus (VH) during extinction retrieval and fear renewal. Secondly, I pharmacologically manipulated $GABA_A$ receptors in the IL during either extinction retrieval or fear renewal. Lastly, I examined the activity of the interneurons in IL in extinction memory retrieval and examined their contribution to memory retrieval using cell- and circuit-specific DREADD methods. The results showed that VH projections to the prelimbic cortex (PL) and IL were both engaged by fear renewal. This pattern of results suggested that VH inhibits IL via feed-forward inhibition, a finding that was

confirmed by pharmacological manipulation of $GABA_A$ receptors in IL. Specifically, $GABA_A$ receptor agonists interfered with extinction retrieval in the extinction context, whereas $GABA_A$ receptor antagonists reduced fear renewal in a different context. However, GAD-Fos immunohistochemistry did not reveal preferential recruitment of IL interneurons during renewal. Finally, the inactivation of putative IL interneurons or activation IL-> BLA pathway did not alter fear renewal. Together, these results suggested the regulating role of IL inhibitory circuits in the context-dependent memory of extinction. Future studies will be done to understand how subtypes of IL local interneurons and the GABA receptors subtypes modulate memory of extinction.

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CHAPTER I

INTRODUCTION

Overview

Post-traumatic stress disorder (PTSD) can occur after encountering a dangerous situation, such as combat or assault. Although PTSD does not always develop after a traumatic experience, a large percentage of men and women are affected, particularly among veterans. For affected individuals, the major treatments include psychotherapy, pharmacotherapy or a combination of the two. Within psychotherapy, exposure-based treatments have received the most attention, because they target and dampen memories and anxiety associated with the traumatic event. When trauma survivors repeatedly imagine or re-experience the cues that trigger fear in a safe environment, the association between the trauma-related cues and fear is reduced or eliminated. Re-exposure of the cues can be produced by talking about them in details, returning to the site of the trauma, virtue reality, imagination, and so on. In clinical studies, exposure-based treatments such as prolonged exposure (PE) therapy are reported as effective (Bryant et al. 2003; Foa et al. 1999, 2005; Resick et al. 2002).

Since the 1980s, Pavlovian fear conditioning and extinction, forms of learning essential to establishing and suppressing fear memories, have been important models to study the development and treatment of PTSD (Pitman 1988). Fear conditioning follows the principles of Pavlovian classic conditioning. It is named after Pavlov and is based on his

famous “conditioned reflexes” experiments (Pavlov 1927). During fear conditioning, an aversive stimulus (unconditioned stimulus, US) is preceded by a neutral cue (conditioned stimulus, CS) (Davis 1992, Fendt & Fanselow 1999, LeDoux 2000, Maren 2001). A few trials of such paired training will induce a learned fear response to the CS (conditioned response, CR). Repeated exposure to the CS without presenting the US after conditioning can reduce the CR, a process referred to as fear extinction. Animal and human models of fear extinction have provided invaluable insights into exposure-based treatments (Maren 2011, Milad & Quirk 2012).

Unfortunately, extinction memories are fragile. In clinical studies, extinguished fear memories frequently relapse. In the existing literature, the relapse rate ranges from 19% to 62% in patients undergoing exposure therapy (Boschen et al. 2009, Craske & Mystkowski 2006, Kindt et al. 2009, Vervliet et al. 2013). In animal models, the return of extinguished fear may appear in three different ways: spontaneous recovery, fear renewal and reinstatement. Spontaneous recovery refers to the return of fear response simply by the passage of time. Fear renewal describes the return of fear to an extinguished CS in a context other than the one in which extinction occurs. Fear also returns when animals are simply exposed to the aversive US again after extinction, a phenomenon called reinstatement (Bouton & Moody 2004, Rescorla 1988). All of these phenomena suggest that extinction does not erase fear memory, and that the suppression of fear after extinction is context-dependent.

Contextual Control of Extinction Memory

Numerous animal and human studies have investigated the contextual regulation of fear after extinction. Fear renewal occurs when an extinguished CS is encountered outside the extinction context. In the laboratory, this is modeled using multiple contexts to condition, extinguish, and test fear responses to the extinguished CS. For example, in an "ABA" paradigm, fear conditioning is administered in context A (a place defined by a unique set of features), in which an auditory CS is paired with a US. Fear to the CS is next extinguished in a different, unique context (context B), in which the CS is delivered without the US until the fear response returns to baseline. After extinction, when the extinguished CS is presented in context B, conditional fear is low. However, if that CS is presented in context A (or any other context besides context B) fear to the CS renews. In other words, the expression of extinguished fear is context-dependent—conditional responding is low in the extinction context and high everywhere else.

Fear renewal can also be obtained in "AAB" or "ABC" paradigms, in which animals are conditioned in context A and the CS is extinguished in context A or B, and tested to the extinguished CS in context B or C (Bouton 2000, Bouton & Bolles 1979). Of course, the contexts mentioned above are physical contexts, with the presence of visual, auditory and olfactory cues (Maren et al. 2013). In addition to physical contexts, contexts can include interoceptive context defined by drug or emotional states, as well as temporal (time of day) or trial (trial spacing) contexts (Bouton et al. 2006). Human studies have also shown that context-dependent fear renewal occurs after fear conditioning and

extinction (Milad et al. 2005a). In contrast, PTSD patients demonstrate poor contextual regulation of fear in both inside and outside the extinction context (Garfinkel et al. 2014). In summary, like all memories, memory of extinction includes the phases of acquisition, consolidation and retrieval. Animals and humans demonstrate poor retrieval of extinction because of spontaneous recovery, renewal, reinstatement and pathology as shown in Figure 1.1 (adapted from Quirk & Mueller 2008).

Relapse of fear suggests that fear extinction is not the unlearning or erasure of conditioned fear (i.e., the CS-US association), but a form of new learning (i.e., CS-'no US' association) that inhibits the expression of learned fear. It is hypothesized that context functions to define the meaning of CS during these different phases of learning and memory retrieval. During extinction retrieval, the extinction context signals that the CS is safe, and outside of the extinction context, the CS is interpreted as dangerous (Bouton & King 1986, Bouton 1993, Holland 1992). For the "ABA" paradigm, context A and context B set the occasion for CS-US and CS-'no US' memories, respectively. And in "AAB" or "ABC" procedures, the CS has never been experiences in the retrieval context, and therefore the generalization of fear to the CS supports fear renewal. For this reason, "AAB" and "ABC" renewal is typically weaker than "ABA" renewal (Bouton & King 1983). Therefore, understanding the neural mechanism of context processing and how the encoded information is relayed to other parts of fear-related circuit is essential to understanding the regulation of conditioned fear after extinction (e.g. Fanselow 2000, & Quirk 2012, Myers & Davis 2006, Orsini & Maren 2012, Quirk & Mueller 2008).

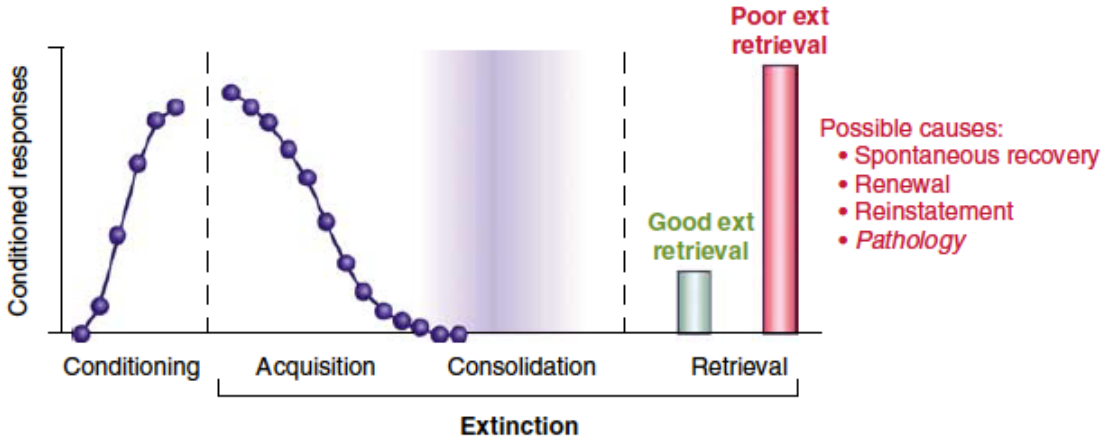

Figure 1.1 Phases of extinction learning. After conditioning and during extinction is the phase of acquisition of extinction. The long period of time after extinction is the time needed for consolidation of extinction. Good retrieval of extinction is presented by low fear response and poor extinction retrieval shows high fear response. High fear response to the extinguished CS is possibly caused by spontaneous recovery, renewal, reinstatement and pathology. Image from (Quirk & Mueller 2008).

Contextual Regulation of Extinction Memory and the Hippocampus

The hippocampus (HIP) has a critical role in learning and memory processes, particularly spatial and episodic memory (Burgess et al. 2002; Eichenbaum 2000, 2001; Moser et al. 2008, Squire & Zola 1996). The hippocampus integrates multiple sensory modalities during memory encoding (Fanselow 1990, Hirsh 1974, Nadel & Willner 1980) and contextual representations are encoded by the hippocampus to become associated with other events (Anagnostaras et al. 1999, Fanselow 2000, Kim & Fanselow 1992, Maren & Holt 2000, Phillips & LeDoux 1992, Rudy & O'reilly 1999, Rudy & O'Reilly 2001). This is accomplished with multimodal neurons in the HIP that respond to a variety of stimuli. One example is the firing of hippocampal "place" cells, which respond when an animal is located in a particular region of a maze. This firing can be

altered by exposure to different contexts, including changes in olfactory and visual stimuli (Smith & Mizumori 2006).

Several studies have defined the role of the hippocampus in the contextual regulation of extinction retrieval and fear renewal (Ji & Maren 2007). In an early study, permanent lesions of the entire HIP (Frohardt et al. 2000) or dorsal hippocampus (dHP; Wilson et al. 1995) before conditioning did not affect fear renewal to the extinguished CS after extinction when rats were placed back to their conditioning context (ABA renewal). However, later work showed that electrolytic lesions of the dorsal hippocampus (dHP) before conditioning or extinction disrupted renewal without affecting context discrimination (AAB and ABA renewal; Ji & Maren 2005). Also, reversible inactivation of the dorsal HIP with muscimol, a gamma-aminobutyric acid ($GABA_A$) receptor agonist, prior to retrieval testing, eliminated fear renewal outside of the conditioning and extinction contexts (AAB or ABC renewal), but not during ABA renewal (Corcoran & Maren 2001, 2004; Zelikowsky et al. 2012). Inactivation of the ventral hippocampus (VH) with muscimol also disrupts fear renewal (ABB renewal; Hobin et al. 2006).

Quantification of immediate early genes (IEGs) expressed in neurons has been used as an index of behaviorally relevant neural activity. One of the widely used IEGs for fear-related activity is c-Fos. Interestingly, studies showed different patterns of c-Fos expression after memory retrieval. For example, some have observed more c-Fos expressing neurons in CA1 and dentate gyrus after extinction retrieval than after fear

renewal (Knapska & Maren 2009). However, other studies observed no difference in CA1, but higher numbers of Fos-positive neurons in ventral subiculum after fear renewal, and an overall higher number in VH (Jin & Maren 2015a, Orsini et al. 2011).

Contextual Regulation of Extinction Memory and the Ventromedial Prefrontal Cortex

Considerable evidence indicates that the medial prefrontal cortex (mPFC) contributes to fear conditioning and extinction, but the precise function of prelimbic (PL) and infralimbic (IL) cortices in these processes is not clear (Giustino & Maren 2015) Although some have reported that mPFC lesions affect the fear response to a CS (but not a context) (Morgan et al. 1993), another group has reported that more selective lesions of the mPFC do not affect the acquisition, expression or retrieval of extinction (Garcia et al. 2006). Lesions of vmPFC targeting the IL impair the consolidation of extinction, resulting in extinction retrieval deficits 24 hours after extinction (Quirk et al. 2000).

The IL may also have a role in the expression of extinction. For instance, electrophysiological studies have revealed a correlation between IL activity and extinction-related freezing behavior (Barrett et al. 2003, Herry & Garcia 2002, Milad & Quirk 2002). The intrinsic responsiveness of IL neurons decreases and increases during conditioning and extinction, respectively (Santini et al. 2008, Sotres-Bayon & Quirk 2010). Excitability of IL neurons is increased during extinction consolidation and returns to baseline in animals that exhibit spontaneous recovery (Cruz et al. 2014). In addition,

after extinction, tone responses of IL neurons have a negative correlation with the freezing level during test. Pairing electrical stimulation of IL with a tone CS during extinction facilitates extinction recall and IL stimulation mimics extinction training experience in anesthetized rats (Milad & Quirk 2002, Milad et al. 2004).

Manipulation of vmPFC activity pharmacologically during extinction or extinction retrieval has a variety of effects on fear expression (Courtin et al. 2013, Giustino & Maren 2015). Pre-extinction inactivation of the vmPFC using the sodium channel blocker tetrodotoxin impairs extinction recall the following day (Sierra-Mercado et al. 2006). On the other hand, activation of IL neurons enhances extinction memory (Mueller et al. 2008). Inactivation of IL (but not PL) by infusing $GABA_A$ receptor agonist muscimol impairs extinction and fear retrieval (Laurent & Westbrook 2009; but Akirav et al. 2006). Muscimol infusion to PL prior to extinction reduces fear expression during extinction, but does not affect extinction learning or retrieval, whereas muscimol infusion to IL prior to extinction impaired extinction acquisition and retrieval the following day (Sierra-Mercado et al. 2011). Muscimol/baclofen infusions into IL prior to extinction recall also elevates freezing (Sangha et al. 2014). On the other hand, Chang & Maren 2011 have previously shown that infusion of the $GABA_A$ receptor antagonist, picrotoxin, into IL prior to immediate extinction facilitates re-extinction on the following day. Picrotoxin infusion into IL of extinction-deficit mice prior to extinction dampens freezing during early extinction and extinction retrieval (Fitzgerald et al. 2014b).

Optogenetic studies indicate that IL inactivation during extinction reduces within-session fear expression or enhances extinction recall (Bukalo et al. 2015, Do-Monte et al. 2015). In a recent study (Do-Monte et al. 2015), photoactivation of IL principal neurons during extinction reduces freezing during the entire session of extinction and retrieval of extinction on the following day. Photoactivation of those neurons during extinction retrieval facilitated retrieval as well. Interestingly, photoinactivaton of IL principal neurons did not affect extinction but facilitated retrieval. However, photoinactivation of those neurons during retrieval did not impair retrieval, which was confirmed using muscimol infusions (Do-Monte et al. 2015). Chemogenetic studies have also contributed to our understanding of the role of vmPFC in extinction memory (Laurent et al. 2016). Lastly, studies examining Fos immunohistochemistry have observed reciprocal patterns of c-fos expression in PL and IL during fear renewal and the expression of extinction, respectively (Knapska & Maren 2009, Orsini et al. 2011). This is strong evidence for the role of vmPFC in context-dependent extinction retrieval and fear renewal.

Contextual Regulation of Extinction Memory and VH-vmPFC-Amygdala Connectivity

The amygdala is known to encode memory of both fear conditioning and extinction (e.g. Barad et al. 2006, Davis 1992, Pape & Pare 2010, Pare et al. 2004). Numerous studies have shown that local microcircuits in the amygdala (Duvarci & Pare 2014, Ehrlich et al. 2009, Herry et al. 2008, Wolff et al. 2014) interact with the mPFC (Herry & Johansen 2014, Likhtik et al. 2005, Senn et al. 2014) in these learning and retrieval processes.

Anatomically, the amygdala forms reciprocal projections with PL and IL, which each receive projections from the VH; moreover, there are reciprocal projections between the VH and amygdala (Hoover & Vertes 2007, Vertes 2004). Functionally, the basolateral amygdala (BLA) forms reciprocal projections with mPFC that regulate fear conditioning and extinction (Likhtik et al. 2005, Senn et al. 2014). The reciprocal projections between BLA and HP also switch between fear learning and extinction (Herry et al. 2008). Projections within all of these networks are regulated by inhibitory interneurons (Herry & Johansen 2014, Sotres-Bayon et al. 2012).

Considerable evidence indicates that context-dependent extinction retrieval and fear renewal are mediated by the mPFC-amygdala pathway. High fear expression during fear renewal involves excitatory projections from the PL to excitatory neurons in the lateral nucleus (LA) of the amygdala, which in turn activates the medial division of central nucleus (CeM) via the basolateral and basomedial nuclei (McDonald et al. 1996, McDonald 1998, Tye et al. 2011, Vertes 2004). In the extinction context, the suppression of fear expression involves the activation of an IL-amygdala circuit, in which IL sends excitatory projections to a heterogeneous GABAergic cell group termed the intercalated cells (ITCs), which inhibit CeM both directly and indirectly via lateral division of central nucleus (CeL) (Berretta et al. 2005, Likhtik et al. 2005, Royer et al. 1999, Royer & Paré 2002, Tye et al. 2011). The critical role of ITCs in fear suppression has recently been challenged (Cassell & Wright 1986, Gutman et al. 2012, Pinard et al. 2012, Strobel et al. 2015), nonetheless it is clear that inhibitory process play a critical role. It has also been

argued that IL-BLA projections are only involved in extinction learning (Bukalo et al. 2015), but other evidence supports a role in both learning and retrieving extinction memories. Recently, VH projections to the central nucleus of the amygdala (CEA), which then projects to PAG and NST, is also involved in fear renewal (Xu et al. 2016).

Many other studies have demonstrated an interaction between the VH, mPFC and amygdala during context-dependent extinction retrieval and fear renewal. High c-fos expression has been observed in PL, LA and CeM during renewal. On the other hand, IL, ITC and CeL showed higher activity when fear is suppressed in the extinction context (Knapska & Maren 2009). Expression of Arc, a dendritically localized IEG, shows context-dependent expression in the IL and BLA: it is elevated in the IL during fear suppression in the extinction context and in the BLA during renewal of extinguished fear (Orsini et al. 2013). Transgenic rats that express a Venus reporter in Fos-active neurons have revealed that BLA neurons active during fear renewal are preferentially innervated by the VH and PL, whereas BLA neurons active during fear suppression are preferentially innervated by the IL (Knapska et al. 2012). In humans, bilateral PFC and HP BOLD activity is decreased, whereas ventromedial/orbitofrontal cortex activity is increased during learning and recall of conditioned fear (Lissek et al. 2016).

Consistent with these observations, functional disconnection of the VH-PL or VH-BLA projections with asymmetric lesions reduced fear renewal (Orsini et al. 2011). Moreover, functional tracing of these projections with the retrograde cholera toxin B tracer

combined with Fos immunohistochemistry reveals that VH and PL neurons projecting to the BLA are activated by fear renewal, whereas and IL neurons projecting to the BLA is activated by extinction retrieval (Orsini et al. 2011). Interestingly, a small population of VH neurons that projects to both PL and BLA are preferentially engaged by fear renewal comparing to extinction retrieval (Jin & Maren 2015a). Collectively, these data suggest that VH projections to the PL and the amygdala mediate the renewal of fear to an extinguished CS, whereas fear inhibition is mediated by IL projections to the amygdala.

The Role of Inhibitory Circuits in the Contextual Regulation of Extinction Memory

Within these hippocampal-prefrontal-amygdaloid circuits, it is clear that GABAergic interneurons play an essential role in the regulation of conditioned fear and extinction (Tovote et al. 2015). For example, early work has shown that GABAergic transmission is involved in the encoding and retrieval of contextual fear memories. For example, β-carbolines are inverse agonists of the of $GABA_A$ receptors and reduce GABAergic inhibition. Systemic injection of the β-carboline FG7142 during extinction or recall greatly increases fear during extinction retrieval, but does not alter renewal (File & Baldwin 1987). In addition, systemic administration of allosteric modulators of $GABA_A$ receptors, such as lorazepam, that potentiate GABAergic transmission leads to impaired extinction learning and recall.

Most GABAergic interneurons in cortical areas form local circuits with pyramidal neurons and with each other. They regulate firing with feedforward and feedback

inhibition (Isaacson & Scanziani 2011). The role of mPFC interneurons in fear memory is still unclear. An early study (Baeg et al. 2001) of extracellular single-unit recording in behaving rats showed a transient increase in firing rate in response to CS following learning. Ablation of NMDA receptors on prefrontal parvalbumin-containing interneurons impaired cued and contextual fear conditioning (Carlen et al. 2012).

There are three major non-overlapping types of genetically defined GABAergic interneurons (INs) in cortical areas, parvalbumin-expressing interneurons (PV+), somatostatin expressing interneurons (SOM+) and serotonin receptor type 3a (Htr3a) expressing interneurons. Novel optogenetic and chemogenetic approaches make it possible to target genetically defined subpopulations of interneurons. The interaction between each subtype of INs is summarized in (Karnani et al. 2016). In cortical areas, PV+ INs synapse with pyramidal neurons (PNs) at somata. SOM+ INs form synapses with distal dendrites of PNs and PV+ and SOM+ INs also form inhibitory synapses with each other. In cortical areas, PV+ INs strongly inhibit each other, but not other INs; SOM+ INs, on the contrary, do not inhibit each other but strongly inhibits other INs (Harris & Shepherd 2015, Pfeffer et al. 2013, Xu et al. 2013). In subcortical regions however, these relationships are reversed. In BLA, for example, conditional stimuli drive PV+ INs to inhibit SOM+ INs, thereby disinhibiting PNs to enhance auditory responses and the development of cue–shock associations (Wolff et al. 2014). SOM+ INs also regulate freezing but not “flight” response in CEA (Fadok et al. 2017). Also in VH, PV+ and SOM+ INs differentially regulate PNs. In CA1, inhibition by SOM+ INs is a major

form of regulation (Lovett-Barron et al. 2012). However, it is not known how PV+ and SOM+ INs regulate PNs in IL during fear renewal.

Attempts to Erase Fear: Reverse the Clock

Extinction training in adult animals does not erase learned fear, but results in a new association between the CS and a "safe" outcome. However, the inhibitory extinction memory is labile and changes in temporal or spatial context result in the spontaneous recovery and renewal of extinguished fear, respectively. In contrast, juvenile rats do not show either spontaneous recovery or fear renewal (Gogolla et al. 2009, Kim & Richardson 2007, Pattwell et al. 2012). In other words, it appears that, unlike in adults, extinction may in fact erase fear in juvenile rats. Inspired by the work on critical periods of plasticity in the visual cortex (Huang et al. 1999), Herry's group hypothesized that perineuronal nets (PNNs) around PV interneurons in the BLA after the critical period contributes to the maintenance of fear memory in adults (Gogolla et al. 2009). To test this possibility, they infused ChABC to remove PNNs in the amygdala in order to return the BLA to developmental state similar to that found in the juvenile critical period. When PNNs were removed before fear conditioning and extinction training, adult animals showed no spontaneous recovery or renewal; however removal of PNNs after fear conditioning, but before extinction training, failed to reduce fear responses during test, indicating the role of PV interneuron plasticity in maintaining the fear memory (Gogolla et al. 2009). Interestingly, interfering with PNNs alone does not erase the fear response without extinction training (Gogolla et al. 2009, Karpova et al. 2011). In

addition, global knockout of Nogo Receptor 1 (NgR1), a receptor that forms in adulthood, or local knockout of NgR1 in the BLA or the IL before fear conditioning significantly reduces spontaneous recovery and fear renewal after extinction, as well as increased parvalbumin expression in the IL after extinction without NgR1 (Bhagat et al. 2016). Implantation of immature interneurons into the amygdala converted it into a juvenile state and facilitated fear erasure (Yang et al. 2016).

Conclusion

In summary, the memory of extinction is labile and fear memories can relapse outside of the extinction context. VH, vmPFC and the amygdala are all involved in context-dependent extinction retrieval and fear renewal. Within each region, the inhibitory circuits are essential to regulate neural activity and behavior output. Understanding the neural substrates of fear relapse will provide invaluable information for the treatment of PTSD, in the targeting of drugs and behavioral cognitive therapy (Fitzgerald et al. 2014a, Griebel & Holmes 2013, Maren & Holmes 2016, Milad & Quirk 2012).

Hypotheses and Proposal

Exposure therapy attempts to reduce learned fear and is the primary behavioral intervention for PTSD. Reports have shown that extinguished fear is susceptible to relapse when the fearful stimulus is encountered outside of the clinic. This form of relapse, termed fear renewal, demonstrates the important role of context in retaining the newly formed extinction memory (low-fear). Numerous studies have confirmed that an

amygdala-mPFC-hippocampal circuit mediates these phenomena. Importantly, recent studies indicate that inhibitory interneurons in IL might play a crucial role in expression of fear extinction and renewal. The goal of this study is to understand the neural substrate of context-dependent extinction retrieval and fear renewal, especially the involvement of IL interneurons and the connectivity from VH to IL to BLA. These experiments will utilize optogenetic and chemogenetic approaches in a Pavlovian fear conditioning and extinction rat model to uncover the specific contribution of IL interneurons to contextual control of fear renewal. The results of this study have the potential to enhance the efficacy of treatment parameters in PTSD therapeutic intervention. From the reviewed literature, the hypothesis of this dissertation is that VH sends encoded contextual information to BLA through PL and IL, and IL gates context-dependent extinction retrieval and fear renewal.

Specific Aim 1: How does the VH->mPFC circuit regulate context-dependent extinction retrieval and fear renewal? VH projections to the mPFC are critical for fear renewal, but it is not known how different populations of VH neurons projecting to IL and PL are regulated during the retrieval or renewal of extinguished fear. In this aim I will use functional anatomical tracing methods to examine Fos activity in VH neurons projecting to IL, PL, or both area during the retrieval of extinguished fear memories. I hypothesize that the VH neurons projecting to the PL will be engaged during the renewal of fear outside the extinction context, whereas those projecting to the IL will be engaged during the suppression of fear in the extinction context.

Specific Aim 2: How does the GABAergic system in IL regulate context-dependent extinction retrieval and fear renewal? Anatomically, VH projects to both principal neurons and interneurons in the mPFC. This raises the possibility that feedforward inhibition driven by VH inputs ultimately inhibits IL principal neurons and leads to fear renewal. As a first step in testing this hypothesis, I will manipulate GABAergic transmission in the IL to explore the contribution of inhibitory synaptic transmission to the contextual control of fear. I hypothesize that activating $GABA_A$ receptors will inhibit IL activity and disrupt extinction retrieval, whereas antagonism of $GABA_A$ receptors will activate the IL and reduce fear renewal.

Specific Aim 3: Exploring the role of IL interneurons and IL->BLA pathway in fear renewal. Many studies have shown that the IL is involved in extinction retrieval. I hypothesize that GABAergic inhibition of principal neurons (mediated by VH afferents) suppresses extinction recall, resulting in fear renewal. To test this hypothesis, I use Fos immunohistochemistry in to explore the contribution of inhibitory interneurons in the mPFC to extinction retrieval and fear renewal. In addition, I will use interneuron-specific Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively activate or inhibit GABAergic interneurons in IL to define their role in renewal. Lastly, the role of mPFC-to-BLA projections in the regulation of extinction memory will be explored. BLA projecting neurons will be specifically activated or inhibited using chemogenetic approach to examine their effect on fear behavior.

Anterograde tracing will be combined with immunohistochemistry to observe the neural activity of BLA interneurons innervated by IL during renewal.

CHAPTER II

RENEWAL OF EXTINGUISHED FEAR ACTIVATES VENTRAL HIPPOCAMPAL NEURONS PROJECTING TO THE PRELIMBIC AND INFRALIMBIC CORTICES IN RATS[*]

Overview

Anatomical disconnection of the ventral hippocampus (VH) and medial prefrontal cortex (mPFC) impairs the renewal of extinguished fear in rats. Here we examined whether subpopulations of neurons in the VH that project to the mPFC, including the prelimbic cortex (PL) and infralimbic cortex (IL), are selectively or differentially engaged by the renewal of fear to an extinguished auditory conditioned stimulus (CS). Rats were ipsilaterally injected with two distinct fluorescent retrograde tracers into the IL and PL and then underwent fear conditioning, extinction and retrieval in distinct contexts. Ventral hippocampal neurons were found to project to both IL and PL, and a small number of neurons projected to both regions. Fos expression was similarly elevated in each subpopulation of mPFC-projecting neuron in animals tested outside the extinction context relative to those tested in the extinction context or home controls. Interestingly, this pattern of results is not consistent with circuit models suggesting a differential role for VH projections to PL and IL in the bidirectional regulation of fear expression after

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extinction. Rather, these data suggest that projections from the VH to both PL and IL are uniquely involved in fear renewal, but not the suppression of fear after extinction. VH neurons may drive fear renewal by fostering fear expression by exciting PL while limiting fear suppression by inhibiting IL.

Introduction

In recent years, considerable effort has been focused on understanding the neural mechanisms of extinction due to its essential role in clinical interventions, such as exposure therapy. During extinction, repeated presentations of the conditioned stimulus (CS) gradually decrease the conditioned fear response (CR), including freezing behavior. As a result, CS no longer produces fear responses at the end of extinction learning. However, substantial evidence suggests that extinction does not erase the fear memory; rather, it generates a new inhibitory memory that competes with original fear memory. Importantly, the extinction memory is highly context-dependent insofar as it is only expressed in the context where extinction occurred. If animals encounter an extinguished CS outside of the extinction context, fear returns or relapses and this phenomenon is called "renewal" (Bouton & Bolles 1979). Fear renewal is a major challenge to therapeutic interventions for trauma and stress-related disorders, including post-traumatic stress disorder (PTSD) (Goode & Maren 2014, Vervliet et al. 2013).

Considerable work has revealed that the hippocampus plays a crucial role in the context-dependence of fear memories after extinction (Fanselow 2010, Gershman et al. 2010,

Komorowski et al. 2009, Maren et al. 2013, Redish et al. 2007). For example, pharmacological inactivation of the hippocampus impairs the renewal of fear indexed both behaviorally and neurally (Corcoran & Maren 2001, 2004; Hobin et al. 2006, Ji & Maren 2008). Moreover, the medial prefrontal cortex (mPFC) is also critically involved in contextual regulation of fear memory after extinction (Giustino & Maren 2015, Jin & Maren 2015a). For instance, disconnection of ventral hippocampal (VH) projections to the mPFC impairs fear renewal (Orsini et al. 2011) and functional tracing studies indicates that VH-mPFC projections are involved in fear expression after extinction (Jin & Maren 2015b, Knapska et al. 2012, Orsini et al. 2011). Interestingly, neuroanatomical studies indicate that the VH projects to both the prelimbic region (PL) and infralimbic region (IL) of the mPFC (Hoover & Vertes 2007). PL is believed to play an important role in the expression of fear memory (Corcoran & Quirk 2007, Sierra-Mercado et al. 2011), whereas IL is preferentially involved in the suppression of conditioned fear responses after extinction (Milad & Quirk 2012, Zelikowsky et al. 2013). Moreover, neurons in the PL and IL exhibit reciprocal patterns of c-Fos expression during the renewal and suppression of fear, respectively (Knapska & Maren 2009) . Given the fact that the VH has a robust projection to both IL and PL (Hoover & Vertes 2007), we sought to determine whether VH neurons projecting to these regions are differentially involved in the suppression or renewal of fear to an extinguished CS, respectively. To answer this question, we used fluorescent retrograde tracing together with c-Fos immunohistochemistry to examine the neuronal activity in PL- and IL-projecting VH neurons during memory retrieval. Our results indicate that mPFC projecting neurons in

the VH are engaged by the renewal, but not suppression, of extinguished fear and that this effect was similar in IL- and PL-projecting populations.

Materials and Methods

Subjects. Eighteen Long-Evans male adult rats (200-224g, Blue-Spruce) were obtained from Harlan (Indianapolis, IN). The rats were individually housed on a 14/10 h light/dark cycle and had access food and water *ad libitum.* Rats were handled for 5 days before the experiment. All experimental procedures were approved by the Texas A&M University Animal Care and Use Committee.

Behavioral apparatus. All behavioral experiments were carried out in eight identical observation chambers (30 × 24 × 21 cm; MED-Associates, St. Albans, VT). Each observation chamber was constructed of a Plexiglas ceiling and rear wall, two aluminum sidewalls, a Plexiglas door. The floor of each chamber consisted of 19 stainless steel grids wired to a shock source and a solid-state grid scrambler (MED-Associates) to deliver the footshock unconditioned stimulus (US). The auditory conditioned stimulus (CS) was delivered by a speaker mounted outside of the grating in one sidewall of the chamber. A 15-W house light was fixed on the opposite sidewall and a ventilation fan was installed in each chamber. Each chamber was placed in a sound-attenuating cabinet. Three contexts were generated by the manipulation of the combination of sensory stimuli. In Context A, 1% acetic acid was used to wipe the ceiling, sidewalls, rear wall, door and grids of each chamber. The house lights and the fans were turned on. Cabinet

doors were left open. White light was on in the behavior room. Rats were transported in white transport boxes. In Context B, the chamber was wiped with 1% ammonium hydroxide. House lights, fans and computer monitor were turned off and cabinet doors were closed. Red room light was turned on in the behavior room. Black transport boxes were used for rat transportation. For Context C, the odor was generated by 70% ethanol. House lights and fans were on. Room light was white and cupboard doors were open. Black Plexiglas floors were placed on the grids. Wood chip bedding was added to white buckets for rat transportation. In all the contexts, a stainless-steel pan fill with a thin layer of the respective odor of the contexts was inserted under the grid of each chamber.

Each chamber was seated on a load-cell platform that recorded chamber displacement in response to each rat's motor activity; load-cell activity was digitized and acquired with Threshold Activity software (MED-Associates). Before the experiment, all load-cell amplifiers were calibrated to a fixed chamber displacement. Load-cell amplifier output (-10 to +10 V) from each chamber was digitized (5 Hz) and transformed to a value ranging from 0 to 100. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10) for at least 1 sec.

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 induction of anesthesia, rats were placed on stereotaxic apparatus (David Kopf Instruments) and 27-

gauge injectors were lowered into PL [anteroposterior (AP), +2.9 mm; mediolateral (ML), ±0.45 mm; dorsoventral (DV), -3.3 mm, from dura] and IL (AP, +2.8mm, ML, ±2.8 mm, DV, -4.1mm from dura, with 30° angle on the coronal plain toward the midline). Each injector was connected to polyethylene tubing, which was attached to a Hamilton syringe (10 µl) placed on an infusion pump. Alexa Fluor-594 conjugated cholera toxin B (CTb) (Life Technology) was infused into the PL and Alexa Fluor-488 conjugated CTb was infused into the ipsilateral IL at a rate of 0.1 µl/min for 5 minutes (0.5 µl each; 5 µg/µl). The injectors remained in the brain for 15 minutes before removal. Rats were placed back to their home cages for post-operative recovery for one week.

Behavioral procedures. Eighteen rats were randomly assigned to three groups: SAME (n=6), DIFF (n=5) and HOME (n=7). We used a three-context renewal procedure (Orsini et al., 2011) ("ABC") for DIFF, in which rats were conditioned in context A, extinguished in context B, and tested in context C. SAME rats were conditioned in context A, extinguished and tested in context C ("ACC"). HOME rats were conditioned in context A, extinguished in either context B or context C, and remained in their home cages during the test of other groups.

After recovery from surgery, rats were conditioned in context A, in which five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) trials were delivered. After 24 hours, rats were extinguished in either context B or C, where they received 45 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs) for two consecutive days. Before the extinction session,

rats were exposed to the alternative context (i.e., they were exposed to context C if they were extinguished in context B) to ensure that the test contexts were equally familiar for all of the rats. The following day, all the rats underwent test in context C, where they received 5 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs).

Immunohistochemistry. Ninety minutes after the first tone of the retrieval test, rats were euthanized by overdose of sodium pentobarbital (0.5 ml) and were transcardially perfused with ice-cold 0.01 M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). Brains were fixed in 4% paraformaldehyde over night at 4°C then placed in 30% sucrose solution at 4°C until sunken. Coronal brain sections (30 μm) were collected on a cryostat at -20°C. Sections containing VH were collected every 210 μm.

Immunohistochemistry was performed on free-floating sections. Brain sections were washed three times in 1 × Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.4) for 30 min each. The sections were then incubated in 10% normal donkey serum (NDS) in TBST for 2 h at room temperature followed by two washes in TBST for 5 min each. Then the tissue was incubated in primary antibody in TBST with 3% NDS (goat anti-c-Fos antibody at 1:2000; sc-52-G, Santa Cruz Biotechnology) for 48 h at 4 °C. The sections were washed three times in TBST for 10 min each, and incubated in secondary antibody in TBST with 3% NDS (biotinylated donkey anti-goat antibody at 1:200; sc-2042, Santa Cruz Biotechnology) for 2 h at room temperature. The tissue was washed three times in TBST for 10 min each and then incubated in streptavidin conjugated

AlexaFluor 350 in TBST with 3% NDS (Streptavidin-AF350 at 1:500; s-11249, Life Technology) for 1 h at room temperature. The tissue was then rinsed three times in TBS for 10 min each and mounted onto subbed slides in 0.9% saline and cover slipped with Fluoromount (Sigma-Aldrich).

Image analysis. Three images for the VH (-5.6, -6.3 and -6.8 mm posterior to bregma) were taken for the quantification. All images were taken at 20 × magnification with an Olympus BX53 microscope. Single-, double- and triple-labeled neurons for each fluorophore were counted. Counts for each image was averaged and standardized to counts/mm^2. For the analysis of Fos expression in PFC-projecting neurons, the number of double- or triple-labeled neurons was normalized to the total number of CTb-positive neurons in each animal. This allowed animals with different degrees of CTb transport and labeling to be compared to one another.

Data analysis. All data were analyzed with analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio. All data are presented as means ± SEM. One rat failed to extinguish and another two rats were excluded from the neuronal and behavioral analyses due to lack of tracer transport. Hence, the final group sizes were SAME (n=5), DIFF (n=5), and HOME (n=5).

Results

Representative CTb injection sites in the IL and PL are shown in Figure 2.1A along with a schematic illustration of the injection sites in Figure 2.1C. IL- and PL-projecting neurons in VH were labeled with different AlexaFluor-CTb conjugates and c-Fos was visualized with AlexaFluor 350 (Figure 2.1B). IL- and PL-projecting neurons were distributed throughout the ventral hippocampal formation, including hippocampal area CA1 and the ventral subiculum.

Fear conditioning resulted in robust increases in freezing behavior, and this did not differ between the groups (not shown). During extinction training, rats in each group exhibited high levels of freezing to the CS at the beginning of the extinction and similar reductions in conditioned freezing both within and between the two extinction sessions (Figure 2.2, left panel). This impression was confirmed by a significant main effect of extinction block [$F(3, 56)=45.52$, $p<0.0001$] without a main effect of group or a group × block interaction ($Fs<1.7$). During the retrieval test, rats exhibited low levels freezing when the CS was presented in the extinction context (SAME), whereas rats tested outside of the extinction context (DIFF) showed fear renewal [Figure 2.2, right panel; group × block interaction $F(1, 8)= 3.66$, $p<0.01$]. Importantly, differential freezing among the SAME and DIFF groups was not attributable to physical differences in the test contexts because all testing was conducted in an identical context with the same CS.

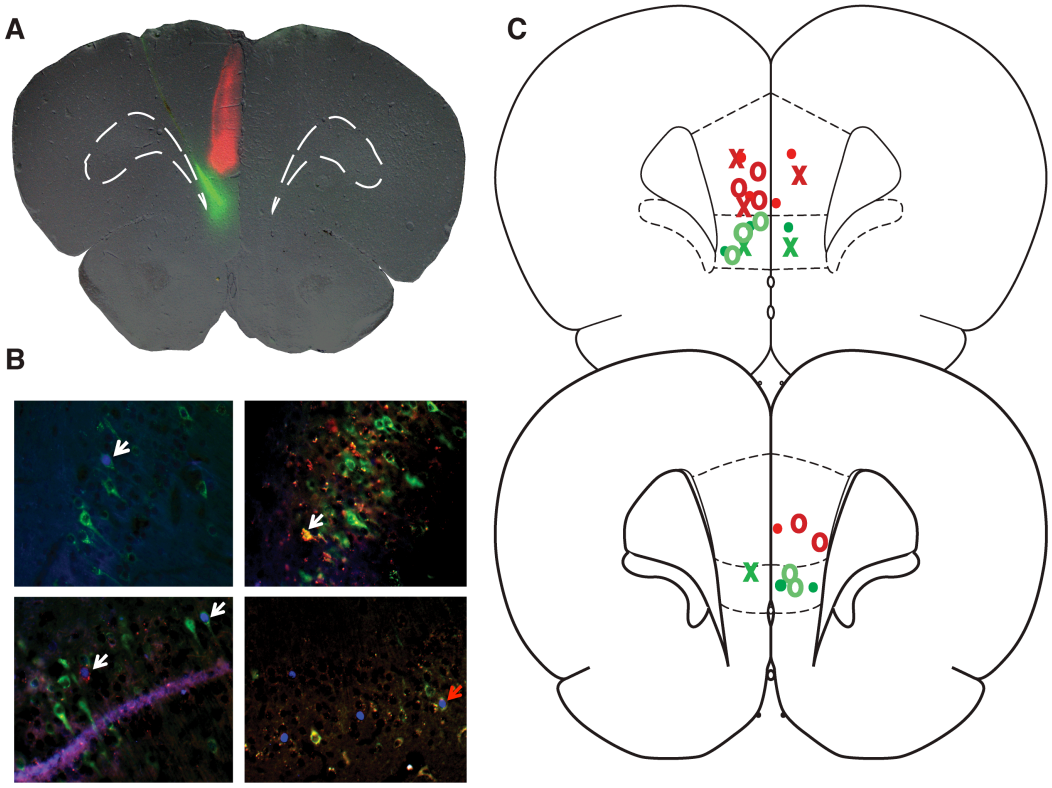

Figure 2.1 Histology. *A.* AlexaFluor conjugated cholera toxin B (CTb) infusion sites within the PL and IL. *B.* Representative coronal sections at the level of the VH showing PL- and IL-projecting neurons labeled by the different tracers. *Top left*: HOME, CA1; *Bottom left*: SAME, CA1; *Top right*, SAME, ventral subiculum; *Bottom right*: DIFF, ventral subiculum. *C.* Schematic illustration of the CTb injection sites in the mPFC. Red: AlexaFluor 594-CTb injected in PL; Green: AlexaFluor 488-CTb injected in IL; crosses: injection sites of HOME rats; circles: injection sites of IT rats; dots: injection sites of DIFF rats. PL-projecting neurons are red, IL-projecting neurons are green, and Fos-positive neurons are blue. White arrows: double-labeled neurons; red arrow: triple-labeled neuron.

Ninety minutes after retrieval testing, the rats were perfused with paraformaldehyde and their brains were extracted. IL- and PL-projecting neurons in VH were labeled with different AlexaFluor-CTb conjugates and c-Fos was visualized with AlexaFluor 350 (see Figure 2.1B). As shown in Figure 2.3A, CTb injections into the IL labeled significantly more VH neurons than injections into the PL; a small number of neurons projected to both areas [Figure 2.3A; main effect of cell type, $F(2, 14) = 19.62$, $p<0.0001$]. Post-hoc comparisons confirmed that greater numbers of VH neurons projected to IL relative to PL, both of which differed from dual-projecting neurons ($p < 0.05$). Dual-projecting neurons accounted for roughly 3~4% of the total labeled neurons in the ventral hippocampus.

We next examined c-Fos expression in the VH, independent of projection target, as an index of neuronal activity in HOME, SAME or DIFF group. As shown in Figure 2.3B, the number of c-Fos expressing neurons in the three groups differed [main effect of group, $F(2, 12) = 7.35$, $p < 0.01$]. Post-hoc comparisons revealed that both SAME and DIFF rats exhibited greater level of c-Fos expression than rats in the HOME control ($p < 0.05$; $p < 0.01$), but did not differ from each other. This confirms previous reports showing that presentation of an extinguished CS increases Fos expression in the VH independent of the context in which it is presented (Jin & Maren 2015b, Knapska & Maren 2009, Orsini et al. 2011).

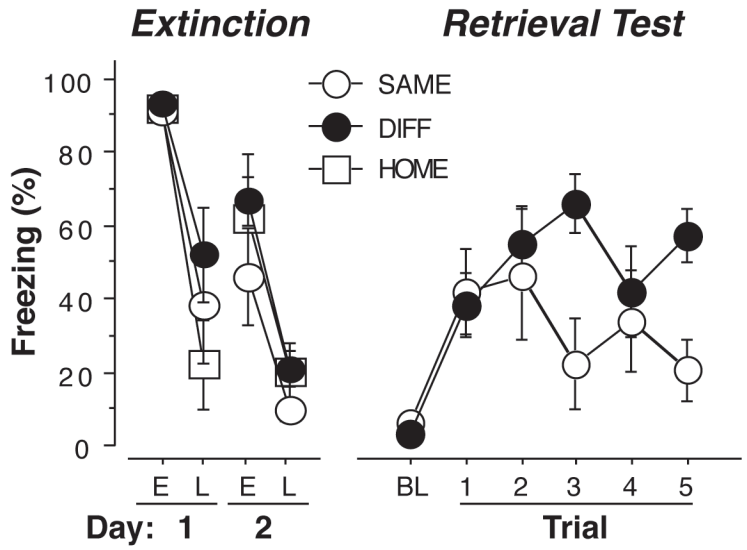

Figure 2.2 Conditioned freezing behavior. *Left:* mean percentage of freezing during the extinction sessions. Freezing was averaged across the early extinction period (E, first five trials) as well as during late extinction trials (L, last five trials). *Right:* mean percentage of freezing during the test session, which consisted of five tone-alone presentations after a baseline (BL) period.

Of course, of critical interest is the nature of retrieval-induced Fos expression in VH neurons targeting the PL or IL (or both). To this end, we examined the proportion of Fos-positive neurons among CTb-labeled neurons in the VH. As shown in Figure 2.3C, a greater proportion of PFC projectors in the VH expressed Fos in the DIFF condition relative to animals in the HOME or SAME conditions. This impression was confirmed in a two-way ANOVA with factors of group (SAME, DIFF or HOME) and cell-type (IL-, PL-, or dual-projecting), which revealed only a significant main effect of group [main effect of group, $F(2,12) = 33.7$, $p < 0.0001$]. Post-hoc comparisons ($p < 0.05$) indicated that DIFF rats exhibited a greater proportion of c-Fos-positive CTb-labeled neurons than

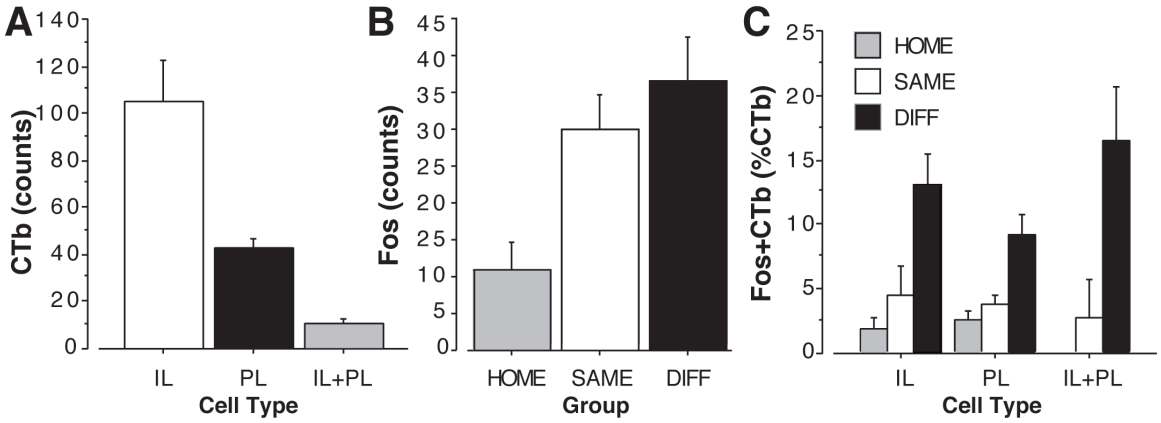

Figure 2.3 Quantification of CTb labeling and Fos expression in neurons in the VH after fear renewal. *A*. Mean cell counts for CTb-positive neurons in VH. Neurons in VH projected to the infralimbic cortex (IL, empty), prelimbic cortex (PL, black), or both areas (Dual, gray). *B*. Mean cell counts for Fos-positive neurons in VH among animals tested outside the extinction context (DIFF), inside the extinction context (SAME), or untested animals (HOME). C. Mean percentage of Fos-positive projection neurons (IL, PL, or dual-projecting) in the VH of rats in each of the three behavioral groups; counts were normalized to the total number of CTb neurons in each animal.

rats in the SAME and HOME groups, which did not differ from one another. These results indicate that renewal of fear to an extinguished CS similarly increases Fos expression in VH neurons projecting to IL, PL, or both regions. Interestingly, there was a highly significant correlation between the percentage of freezing on the retrieval test and the number of Fos-positive projection neurons (aggregated across PL, IL, and dual-projecting populations) in the VH (Figure 2.4; Pearson r = 0.789 $p < 0.01$). This replicates a previously reported correlation between retrieval-induced Fos expression and freezing behavior after extinction (Jin & Maren 2015b). Collectively, these data suggest that the ventral hippocampus plays a key role in fear renewal through its projections to the medial prefrontal cortex.

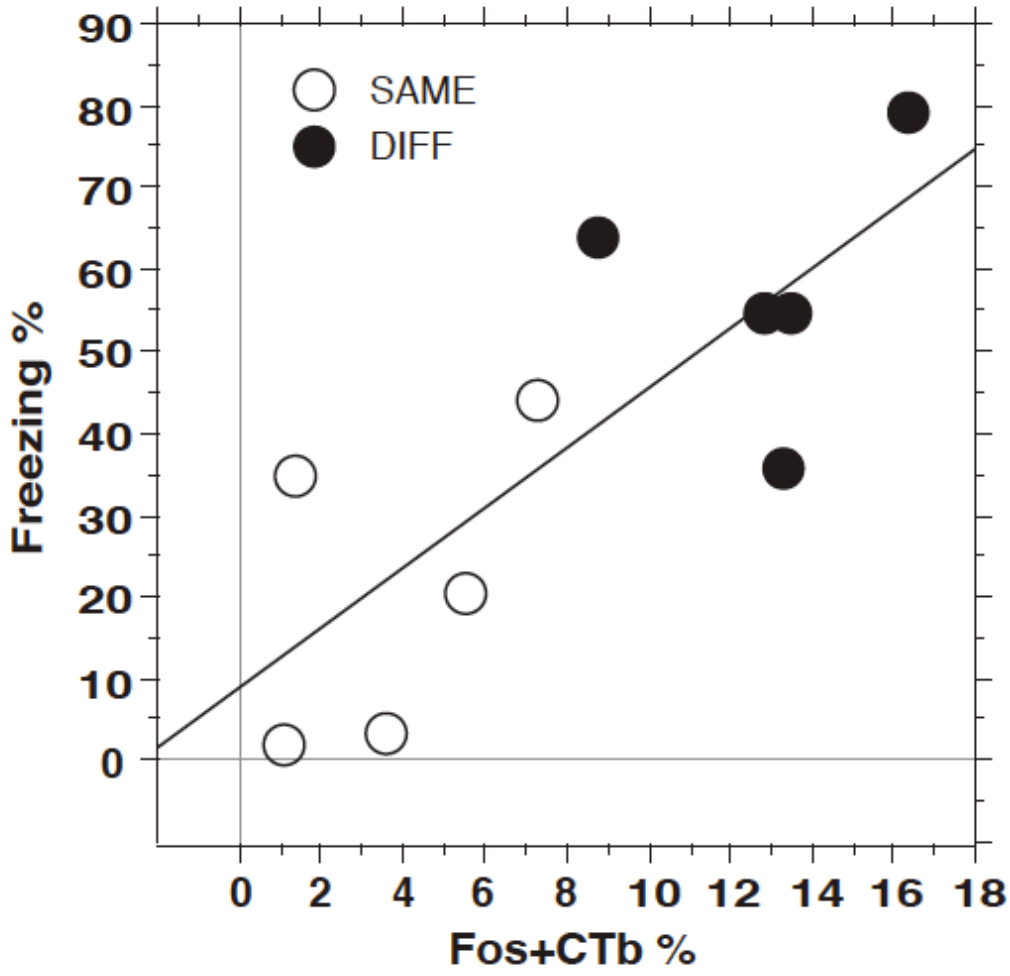

Figure 2.4 Correlation between average freezing behavior during the retrieval test among rats in SAME and DIFF and the average percentage of Fos-positive CTb-labeled cells in the VH.

Discussion

Consistent with previous work, the present study reveals that the ventral hippocampus sends direct projections to both the prelimbic and infralimbic divisions of the medial prefrontal cortex (Hoover & Vertes 2007). Interestingly, in the present study VH neurons projecting to the IL outnumbered those projecting to the PL, an observation that has not previously been reported. This might reflect the different distribution of VH efferents along the rostral-caudal extent of the mPFC, although it is possible that there was differential CTb uptake in the two areas. We also found a small number of double-labeled neurons in the VH, suggests that some VH neurons project to both the IL and PL.

As we have previously reported (Jin & Maren 2015b, Knapska & Maren 2009, Orsini et al. 2011) , ventral hippocampal Fos expression was increased after the presentation of an extinguished CS in either inside or outside the extinction context. In a previous study, we found that this pattern of retrieval-induced Fos expression was most pronounced in hippocampal area CA1, whereas ventral subicular Fos expression is selectively induced in the renewal context (Jin and Maren, 2015a). It has been suggested that VH neurons projecting to PL and IL might have different roles in the renewal and suppression, respectively, of extinguished fear (Maren 2011, Maren et al. 2013). However, we now show that both IL- and PL-projecting neurons in the VH exhibit similar increases in c-Fos expression during both the renewal of fear outside of the extinction context (DIFF) as well as the suppression of fear in the extinction context (SAME). Indeed, IL- and PL-

projecting VH neurons were preferentially activated in rats in the DIFF condition, suggesting that hippocampal-prefrontal projections have a selective role in increasing the expression of fear (e.g., during renewal) (Adhikari et al. 2010).

The observation that PL- projecting VH neurons are preferentially engaged during the renewal of extinguished fear corroborates previous reports. In this way, the hippocampus is positioned to drive fear expression through either direct projections to the amygdala (Herry et al. 2008, Knapska et al. 2012, Orsini et al. 2011, Orsini & Maren 2012) or indirectly via the PL (Corcoran & Quirk 2007, Sierra-Mercado et al. 2011). However, a surprising outcome was that VH neurons projecting to the IL were also preferentially activated after the renewal of fear outside the extinction context. Given that the IL is involved in the suppression of conditioned fear (Burgos-Robles et al. 2009, Quirk & Mueller 2008, Sierra-Mercado et al. 2011), the present results suggest that renewal-related increases in VH neurons projecting to IL might activate an inhibitory microcircuit within IL to attenuate fear inhibition (Lovett-Barron et al. 2012).

One possibility is that feed-forward inhibition generated by VH projections in IL (Gabbott et al. 2002) ultimately dampens neuronal activity in the IL thereby limiting fear suppression and permitting fear relapse. Indeed, if extinction applies an inhibitory "brake" to the expression of conditioned fear, then circumstances that result in a return of fear (such as renewal) must release the brake; VH-mediated inhibition of IL may be involved in this process. Consistent with this, previous work has revealed that electrical

stimulation of the VH produces substantial feed-forward inhibition in the mPFC (Tierney et al. 2004) and VH-mediated inhibition of mPFC can influence the expression of fear after extinction (Sotres-Bayon et al. 2012). Therefore, we propose that projections from VH to IL oppose the expression of extinction via feed-forward inhibition by GABAergic interneurons in IL. This proposed feed-forward inhibition model could potentially explain why fear renewal is associated with the activation of IL-projecting neurons in the VH activity and inhibition of neuronal activity in the IL (Knapska & Maren 2009, Orsini et al. 2011). An interaction between PL and IL during fear renewal might also contribute to the higher activity in IL-projecting neurons (Zelikowsky et al. 2013). Ultimately, the relatively stronger projection of the VH to the IL dictates that feed-forward inhibition of the IL may be greater than that in the PL, thereby yielding a net increase in fear expression when PFC-projecting neurons in the VH are engaged.

In sum, the present results reveal that the presentation of extinguished CSs induces Fos in ventral hippocampal neurons projecting to the medial prefrontal cortex. Importantly, neurons targeting the prelimbic and infralimbic cortices did not differ in their propensity to exhibit renewal-related Fos expression. However, both the substantially greater projection of the VH to IL and the potent feed-forward inhibition in this circuit suggests that the dominant effect of VH activation is an inhibition of IL output. The inhibition of infralimbic output may permit fear renewal by releasing the amygdala from the IL-mediated inhibition that normally contributes to the suppression of fear after extinction.

Ultimately, suppressing the activity of inhibitory interneurons in the infralimbic cortex may be a novel strategy for fostering the expression of extinction memories and preventing fear relapse.

CHAPTER III

GABAA RECEPTORS IN THE INFRALIMBIC CORTEX REGULATE BOTH THE EXPRESSION OF EXTINCTION AND RENEWAL OF FEAR IN RATS

Overview

There is considerable interest in the role of the infralimbic (IL) region of the medial prefrontal cortex in the regulation of conditioned fear. We have previously shown that infusion of the $GABA_A$ receptor antagonist, picrotoxin, into IL impairs the expression of freezing to an auditory conditioned stimulus (CS) (Chang and Maren, 2011). This suggests that GABAergic inhibition in IL is involved in fear regulation and may have a critical role in the regulation of extinguished fear. To examine this issue, we conducted two experiments in which rats received either muscimol (Experiment 1) or picrotoxin (Experiment 2) infusions into the IL prior to an extinction recall test or fear renewal test, respectively; freezing served as the index of conditional fear. Infusions of muscimol into the IL impaired the expression of extinction (Exp. 1) and resulted in a relapse of conditioned freezing, whereas infusions of picrotoxin into the IL (Exp. 2) yielded low levels of conditioned freezing and prevented fear renewal. These data suggest that $GABA_A$ receptors in IL bidirectionally regulate the expression of fear after extinction. Importantly, the IL is required for the retrieval of fear and safety memories after extinction.

Introduction

Extinction of conditioned fear memory is labile. After extinction, a change of context, a reminder unconditional stimulus (US), or simply the passage of time will cause fear to the extinguished CS to return (Bouton 2000, 2002; Goode & Maren 2014, Hermans et al. 2006, Maren et al. 2013, Vervliet et al. 2013). The return of fear caused by the change of context is termed "fear renewal".

Many studies have suggested that infralimbic cortex (IL) regulates the acquisition, consolidation and context-dependent retrieval of extinction, as well as fear renewal (Milad & Quirk 2012, Quirk & Mueller 2008, Tovote et al. 2015). One focus of interest is on GABAergic transmission in IL. GABA receptors are ubiquitous in IL (Bowery et al. 1987) and previous pharmacological studies have shown that the acquisition, consolidation or retrieval of extinction can be influenced by infusion of GABA receptor modulators into the IL. Prior to extinction, infusion of muscimol, a $GABA_A$ receptor agonist, into IL interferes extinction learning (Sierra-Mercado et al. 2011). IL infusion of $GABA_A$ receptor antagonist picrotoxin into extinction-deficit mice prior to extinction rescues early extinction (Fitzgerald et al. 2014b). Muscimol infusion into IL prior to re-extinction also increases freezing during re-extinction of contextual fear (Laurent & Westbrook 2009).

Pre-extinction muscimol infusions into the IL interfere with extinction to an auditory CS or context (Laurent & Westbrook 2009, Sierra-Mercado et al. 2011). Picrotoxin

infusions into the IL have also been reported to facilitate extinction under some conditions (Chang & Maren 2011, Fitzgerald et al. 2014b). However, the timing of drug infusions in these studies does not allow one to differentiate the effects of the drugs on acquisition versus consolidation. Post-extinction muscimol infusion increases freezing during extinction retrieval the following day, indicating a specific role for IL in consolidation of extinction (Laurent & Westbrook 2009). However, interestingly, in another study, muscimol infusion to IL prior to extinction facilitated acquisition of extinction (Akirav et al. 2006). This result is the opposite of previous findings, although histology revealed infusion sites to be both IL and some ventral area of the adjacent prelimbic cortex (PL) (Akirav et al. 2006). After contextual conditioning and extinction, muscimol infusion into IL interferes extinction retrieval (Laurent & Westbrook 2009). Cocktail of muscimol with $GABA_B$ receptor agonist baclofen infusion to IL prior to extinction recall test also elevates freezing level (Sangha et al. 2014). However, IL infusion of muscimol alone did not alter extinction retrieval to an extinguished CS during a short test (Do-Monte et al. 2015).

Clearly, GABAergic inhibition in IL appears to have a critical role in the regulation of extinguished fear. In previous studies, $GABA_A$ receptor agonists or antagonists was infused at different time points in order to examine the role of IL in extinction acquisition, consolidation and retrieval. Therefore, to clarify the role of muscimol on context-dependent extinction retrieval to the extinguished CS, and picrotoxin on fear renewal, we conducted two experiments in which rats received either muscimol or

picrotoxin infusions into the IL prior to extinction retrieval test (Exp.1) or fear renewal test (Exp.2), respectively. Infusions of muscimol into the IL impairs the expression of extinction and results in a relapse of conditioned freezing, whereas infusion of picrotoxin into the IL yields low levels of conditioned freezing and prevents fear renewal. Thus, $GABA_A$ receptors in IL bi-directionally regulate fear behavior after extinction.

Materials and Methods

Subjects. Forty-seven adult male rats (200-224g, Long-Evans Blue-Spruce) were obtained from Envigo (Indianapolis, IN). The rats were individually housed on a 14/10 h light/dark cycle and had access food and water *ad libitum.* Rats were handled for 5 days before the experiment. All experimental procedures were approved by the Texas A&M University Animal Care and Use Committee.

Behavioral apparatus. All behavioral experiments were carried out in eight identical observation chambers (30 × 24 × 21 cm; MED-Associates, St. Albans, VT). Each observation chamber was constructed of a Plexiglas ceiling and rear wall, two aluminum sidewalls, a Plexiglas door. The floor of each chamber consisted of 19 stainless steel grids wired to a shock source and a solid-state grid scrambler (MED-Associates) to deliver the footshock unconditioned stimulus (US). The auditory conditioned stimulus (CS) was delivered by a speaker mounted outside of the grating in one sidewall of the chamber. A 15-W house light was fixed on the opposite sidewall and a ventilation fan was installed in each chamber. Each chamber was placed in a sound-attenuating cabinet.

Three contexts were generated by the manipulation of the combination of sensory stimuli. In Context A, 1% acetic acid was used to wipe the ceiling, sidewalls, rear wall, door and grids of each chamber. The house lights and the fans were turned on. Cabinet doors were left open. White light was on in the behavior room. Rats were transported in white transport boxes. In Context B, the chamber was wiped with 1% ammonium hydroxide. House lights, fans and computer monitor were turned off and cabinet doors were closed. Red room light was turned on in the behavior room. Black transport boxes were used for rat transportation. For Context C, the odor was generated by 70% ethanol. House lights and fans were on. Room light was white and cupboard doors were open. Black Plexiglas floors were placed on the grids. Wood chip bedding was added to white buckets for rat transportation. In all the contexts, a stainless-steel pan fill with a thin layer of the respective odor of the contexts was inserted under the grid of each chamber.

Each chamber was seated on a load-cell platform that recorded chamber displacement in response to each rat's motor activity; load-cell activity was digitized and acquired with Threshold Activity software (MED-Associates). Before the experiment, all load-cell amplifiers were calibrated to a fixed chamber displacement. Load-cell amplifier output (-10 to +10 V) from each chamber was digitized (5 Hz) and transformed to a value ranging from 0 to 100. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10) for at least 1 sec.

Surgical procedures. Rats were anesthetized with isoflurane (5% for induction; ~2% during surgery), and were placed on stereotaxic apparatus (David Kopf Instruments). A single 8mm steel guide cannula unilaterally lowered into midline targeting both IL cortices (Sierra-Mercado et al. 2006; +2.65ap, -1.0ml, -4.1dv from dura, angled 11° toward the midline in the coronal plane). Cannulas were secured with jeweler's screws and dental cement. Stainless steel obturators (30 gauge, 9 mm; Small Parts) were placed in each guide cannula and were changed twice prior to behavioral tests. Rats were placed back to their home cages for post-operative recovery for one week.

Behavioral procedures. In Experiment 1, thirty-one rats were randomly assigned to four groups in a 2x2 factorial design: ACC-MUS (n=8), ACC-VEH (n=8), CCC-MUS (n=7) and CCC-VEH (n=8). Rats were conditioned in either context A or C and both extinguished and tested in context C ("ACC" or "CCC"). This design was arranged to determine whether the contribution of IL to extinction retrieval is greater for procedures in which conditioning and extinction occur in different contexts (ACC) versus in the same context. In Experiment 2, 16 rats were randomly assigned to two groups: PIC (n=8) and VEH (n=8). We used a three-context procedure (Orsini et al., 2011) ("ABC") for fear renewal, in which rats were conditioned in context A, extinguished in context B, and tested in context C.

After recovery from surgery, rats were conditioned in context A or context C, in which five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) trials were delivered.

After 24 hours, rats were extinguished in either context B or C, where they received 45 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs). Before the extinction session for fear renewal experiment, rats were exposed context C to ensure that the test context were familiar for the rats. The following day, all the rats underwent test in context C, where they received 45 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs). Extinction retrieval test was delivered immediately after muscimol or vehicle infusion (0.2µl 1mg/ml Muscimol in 0.9% sterile saline or 0.2 µl 0.9% sterile saline at the rate of 0.1 µl/min). Renewal test was delivered immediately after picrotoxin or vehicle infusion (100ng picrotoxin in 0.5 µl 0.9% sterile saline or 0.5 µl 0.9% sterile saline at the rate of 0.1 µl/min).

Histology. After the tests, rats were euthanized by overdose of sodium pentobarbital (0.5 ml) and were transcardially perfused with ice-cold 0.9% saline (pH 7.4) followed by 4% formalin in 0.1M PBS (pH 7.4). Brains were fixed in 4% formalin over night at 4°C then placed in 30% sucrose solution at 4°C until sunken. Coronal brain sections (30 µm) were collected on a cryostat at -20°C. Sections containing IL were collected every 60 µm, mount with PBS solution onto gelatin-coated glass slides. Thionin staining was performed on slide. Stained sections were imaged on a Leica microscope (MZ FLIII) for cannula placement.

Data analysis. All data were analyzed with analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio. All data are presented as means ±

SEM. In experiment 1, two rats did not have patent cannula; hence, the final group sizes were ACC-MUS (n=7), ACC-VEH (n=8), CCC-MUS (n=7), CCC-VEH (n=7).

Results

Experiment 1. Muscimol in IL impairs extinction retrieval, despite conditioning context difference. In order to explore whether IL activity regulates fear expression during extinction retrieval through $GABA_A$ receptors, we infused $GABA_A$ agonist muscimol to inactivate IL prior to retrieval test. Experimental schemes and histology are shown in Figure 3.1. A single cannula was placed targeting the midline IL with a small angle on the coronal plane. Extinction retrieval was tested once a day for two consecutive days. Each animal was infused with either muscimol or vehicle the first day, and switched to vehicle or muscimol the next day. Muscimol caused high fear expression during retrieval in both test sessions [Figure 3.2. Test 1: Main effect of two-way ANOVA, $F(1, 27)=35.355$, $p<0.0001$; Test 2: $F(1,27)=5.087$, $p<0.05$]. Within-subject analysis revealed that muscimol infusion increased average freezing level of the entire test session [Figure 3.2, $F(1,28)=43.456$ $p<0.0001$]. Interestingly, in both test sessions, the effect of muscimol had an onset later than the first five trials (Figure 3.2). It is possible that all animals showed high fear expression because of spontaneous recovery, which was evident during the first five trials of every extinction session (Figure 3.2, early extinction). That may have created a ceiling effect of the vehicle animals.

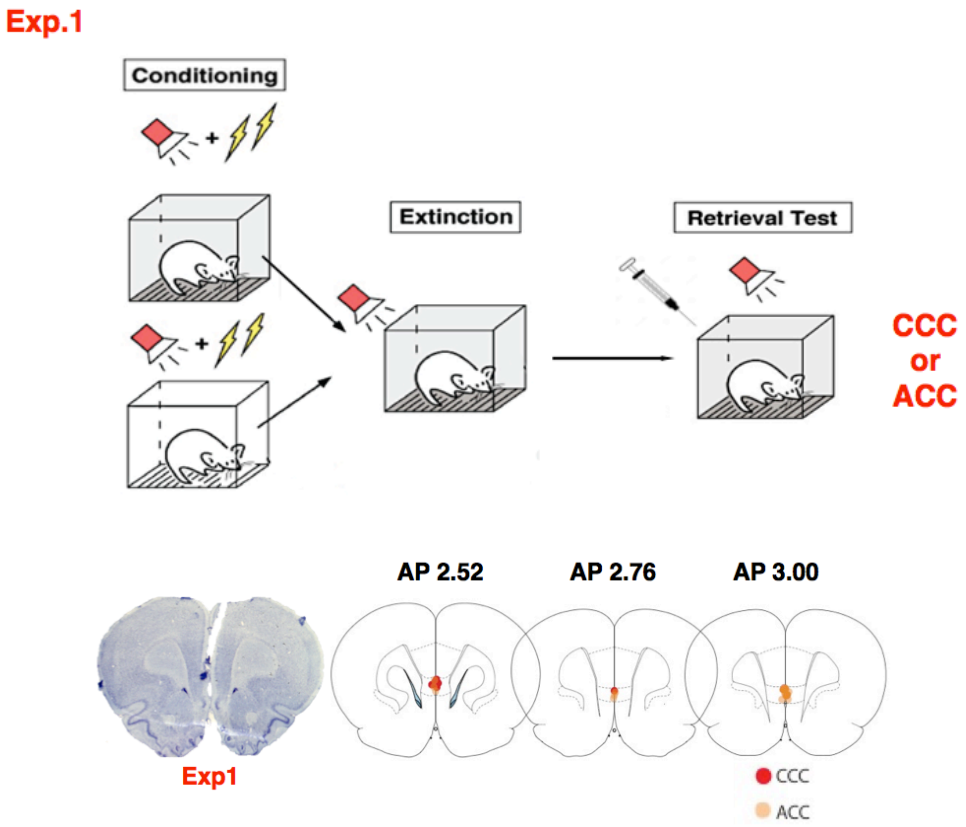

Figure 3.1 Experiment scheme and histology of Experiment 1. Top: experimental scheme. All animals were submitted in fear conditioning, extinction and extinction retrieval test. Extinction and retrieval occurred either in the same or a different context (context C) than the conditioning context (context A or C). Muscimol or vehicle was infused immediately prior to test. Bottom: examples of midline cannula implantation and the map of all cannula tip locations at the midline IL. Red dots: placement of CCC rats; orange dots: placement if ACC rats.

Animals in this experiment were trained in either context A or C (Figure 3.1). Those trained in context C showed higher freezing than those trained in context A [Figure 3.3; $F(1,27)=9.59$ $p<0.05$]. Fear response to the CS was extinguished in context C. During the first few tones of the first extinction, animals with either training background recalled fear training, then animals trained in context A showed faster extinction than

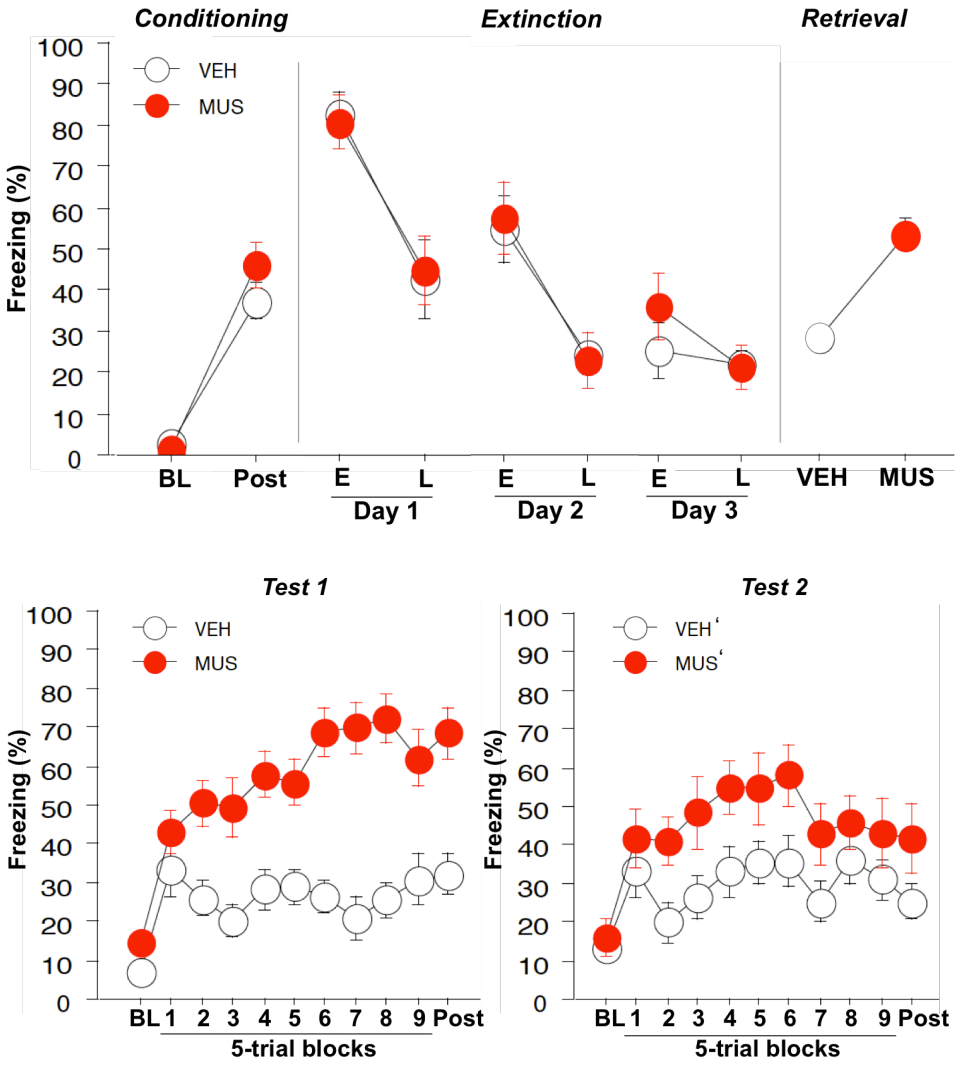

Figure 3.2 Muscimol effect on extinction retrieval. *Top*: fear conditioning, extinction and average freezing of within-subject retrieval test. After three days of extinction, freezing of both groups returned to the baseline. E: early extinction; L: late extinction. *Bottom*: extinction retrieval test on Day 1 and Day 2. All animals were given 45 trials in each test session. Animals receive either vehicle or muscimol alternatively between sessions. Note that the onset of muscimol effect was later than the first 5-trial block in both sessions.

those train in context C, indicated by lower freezing in all three extinction sessions. (Figure 3.3, $p<0.01$). By the end of the third extinction, both groups returned to baseline freezing. Interestingly, when analyzing extinction retrieval separately by training group, muscimol in IL elevated freezing behavior in both groups non-differentially (Figure 3.3; $p=0.24$). During the first five trials of the first test, all animals showed fear relapse. However, in Test 2, animals trained in context A that were infused with vehicle showed a trend of lower fear during the first five trials comparing to the other three groups ($p=0.0504$ comparing to CCC-VEH). The results indicate that muscimol infusion in IL prior to extinction retrieval increases fear behavior despite training backgrounds.

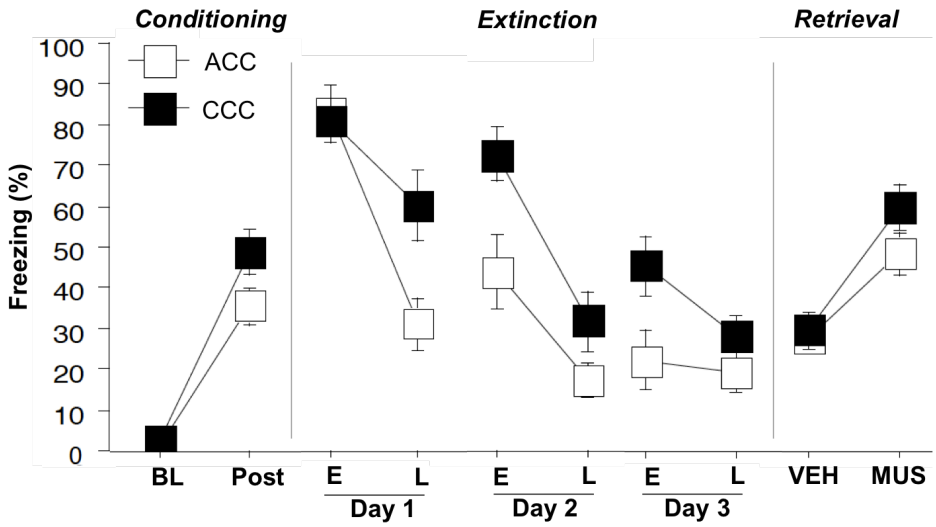

Figure 3.3 Contextual effect on conditioning, extinction and extinction retrieval. Animals were submitted to fear conditioning in either context A or context C. Extinction was administrated in context C in three consecutive days. E: early extinction; L: late extinction. Extinction retrieval was administrated in context C for two consecutive days. Average freezing of both days of test under vehicle and muscimol is shown.

Experiment 2. Picrotoxin in IL dampens fear renewal. Muscimol is $GABA_A$ receptor agonist. IL inactivation induced higher fear expression during extinction retrieval test. To further confirm that IL causally regulates fear behavior through GABA system, we activated IL and tested fear renewal. Fear renewal was induced using an "ABC renewal paradigm". Animals were conditioned in context A. The CS was extinguished in context B. Renewal was tested in context C, a third context (Figure 3.4). Prior to renewal test, we infused $GABA_A$ receptor antagonist picrotoxin into the midline IL (Figure 3.4). Controlled animals showed returned fear response to the extinguished CS. Animals infused with picrotoxin showed lower freezing behavior comparing the control animal [Figure 3.5; $F(1, 14) = 7.569$; $p<0.05$]. Lower fear expression had an onset as early as the first tone and lasted throughout all the 45 trials.

Discussion

In this study, we showed that $GABA_A$ receptors in IL bi-directionally regulate fear expression during extinction retrieval and fear renewal. Despite distinct training contexts, IL infusion of $GABA_A$ receptor agonist muscimol reversibly increases freezing behavior in response to the extinguished CS during the extinction retrieval test. On the other hand, IL infusion of $GABA_A$ receptor antagonist picrotonxin reduces fear renewal.

Muscimol's impairment of extinction retrieval is in accordance with previous studies (Sangha et al. 2014), in which a mixture of $GABA_A$ and $GABA_B$ receptor agonists was infused into IL prior to extinction retrieval test and increased freezing. This study

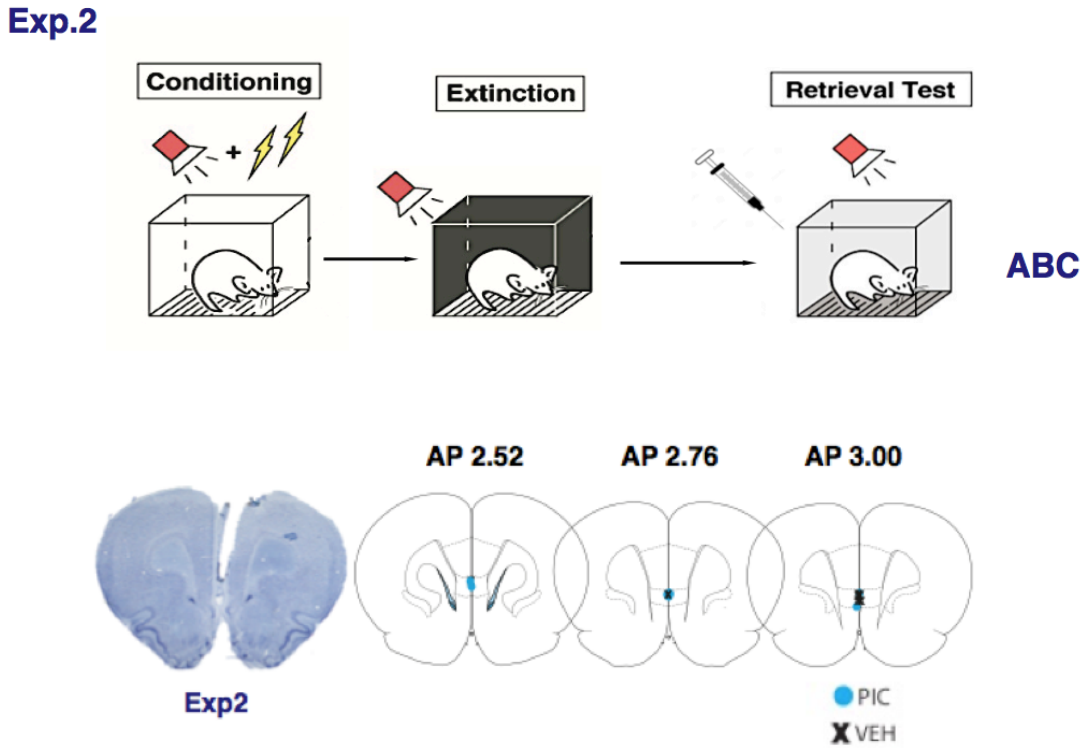

Figure 3.4 Experimental scheme and histology of Experiment 2. *Top*: scheme of Experiment 2. Fear conditioning, extinction and extinction retrieval test was in three distinctive contexts (context A, B and C). Picrotoxin or vehicle was infused prior to extinction retrieval test. *Bottom*: examples of midline cannula implantation and the map of all cannula tip locations at the midline IL. Blue dots: picrotoxin infusion sites. Black X: vehicle infusion sites.

suggested that $GABA_A$ receptor agonist alone is sufficient to alter freezing behavior, given the fact that the quantity of $GABA_A$ binding sites outnumbers $GABA_B$ binding sites in the prefrontal cortex (Bowery et al. 1987). Our data also confirm a previous study in which IL inactivation by sodium channel blocker tetrodotoxin impaired extinction retrieval (Sierra-Mercado et al. 2006). Interestingly, we have found that the impairment of extinction retrieval after muscimol infusions occurs is not manifest in the earliest test trials, but requires several trials to develop. This suggests that spontaneous

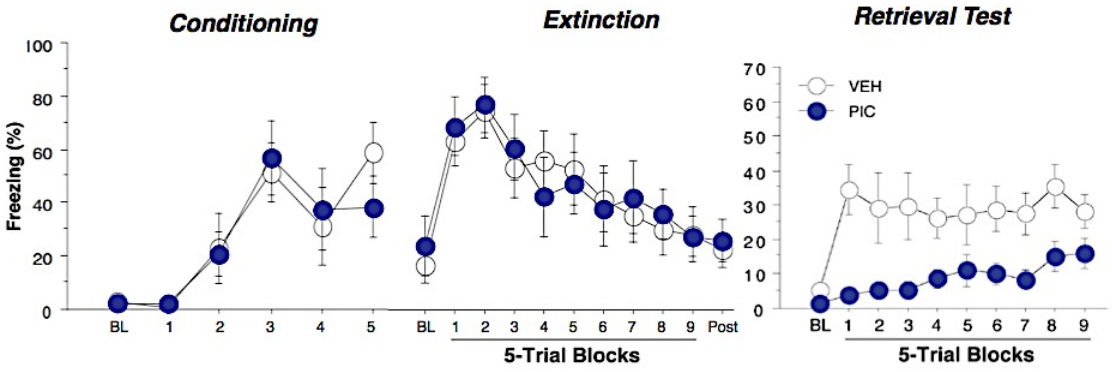

Figure 3.5 Picrotoxin effect on fear renewal. *Left*: freezing behavior of all animals during conditioning and extinction. *Right*: freezing behavior during the extinction retrieval test in a third context immediately after infusion of vehicle or picrotoxin.

recovery in the early test trials may limit IL contribution to performance and thereby the effect of muscimol on extinction retrieval. Evidence of such hypothesis is in the average of the first five tone responses on the second test day, during which vehicle animals conditioned in context A show low freezing, although their muscimol background during Test 1 may need to be considered. That is, those animals were tested for 45 trials infused with muscimol during Test 1 and with vehicle during Test 2, and Test 1 could be considered as another full extinction. In that case, it is inconclusive whether muscimol actually facilitated extinction or not.

It has recently been reported that optical silencing of IL principal neurons during extinction retrieval did not impair retrieval, which was confirmed by muscimol infusion test (Do-Monte et al. 2015). On the surface, the result of the current study is contradictory to the earlier report. However, closer scrutiny proved otherwise. Do-Monte

et al. 2015 tested extinction retrieval over four trials. During the first four trials of the current study, muscimol infusion into IL did not increase freezing comparing to the animals infused with vehicle. The onset of muscimol effect on freezing is after the first first five trials. Moreover, muscimol in IL activates all $GABA_A$ receptors in all cell types, not only principal neurons. Indeed, in another study, photoinactivation of both IL cell types after extinction using virus with hSyn promoter impairs extinction retrieval (Kim et al. 2016). Therefore, the current result is consistent with the previous findings and extends the time course of muscimol effect.

The results indicate that muscimol in IL impairs extinction retrieval independent of how the extinction procedure was conducted. In the "ACC" paradigm, extinction is conducted outside of the conditioning context which therefore signals "safe" CS-'no-US' information (Bouton et al. 2006, Maren et al. 2013). On the other hand, in "CCC" paradigm, the extinction (and test) context is the same as the conditioning context, which produces ambiguity--the context has been both "dangerous" and "safe" (Bouton 1988, 2002; Bouton et al. 2006). That may explain the slight fear relapse of the control group during the first five trials in Test 2. However, after three session of extinction, the strength of CS-'no-US' meaning of context C is stronger than the CS-US meaning. Inactivation of IL with muscimol seems to push the ambiguous signal to the more "dangerous" end. Indeed, IL receives direct projection from the hippocampus that encodes contextual information (Hobin et al. 2006, Hoover & Vertes 2007, Ji & Maren 2005). In this case, "safe" and "ambiguous" signals from the hippocampus to IL are

disrupted by the activation of inhibitory $GABA_A$ signaling. In turn, downstream of the circuit, inactivated IL fails to inhibit the amygdaloid nuclei from fear expression (Ehrlich et al. 2009; Likhtik et al. 2005, 2008; Quirk & Mueller 2008, Vertes 2004).

Experiment 2 suggested that activation of IL by inhibiting $GABA_A$ receptor disrupts context-dependent fear renewal. Previously, studies have shown that picrotoxin infusion into IL before extinction reduces conditioned fear response in extinction, and facilitates the later re-extinction of fear (Chang & Maren 2011, Thompson et al. 2010). Here I show that activation of IL inhibits fear expression in the renewal context. In comparison, infusion of picrotoxin before extinction attenuates fear expression immediately. During the test on the following day, fear expression start up high and decreases within session. After extinction, infusion prior to renewal test reduces freezing in the entire test session comparing to the animals infused with vehicle. In a context out of the extinction context, inhibition of $GABA_A$ receptors inhibits the contextual information encoded by the hippocampus and relayed to IL, thus disinhibits the inhibition of fear expression by IL.

To summarize, as shown in Figure 3.6, $GABA_A$ signaling in IL bi-directionally regulates context-dependent fear expression. After acquisition and consolidation of extinction, in a "safe" context, IL relays context information from hippocampus to the downstream ITC and BLA inhibitory neurons, and suppresses fear expression, showing retrieval of extinction. In an ambiguous test context in the "CCC" and "ABC" paradigm, $GABA_A$ receptors put a brake on the IL principal neurons, and integrate the strength of the

meaning of the context as to CS-US versus CS-no-US. There are muscimol and picrotoxin binding sites at all types of neurons. Therefore, further study to exam the role of specific interneurons will provide more information about how IL regulates conditioned fear memory after extinction and specific pharmacological targets in the ventromedial prefrontal cortex in human for fear relapse after exposure therapy.

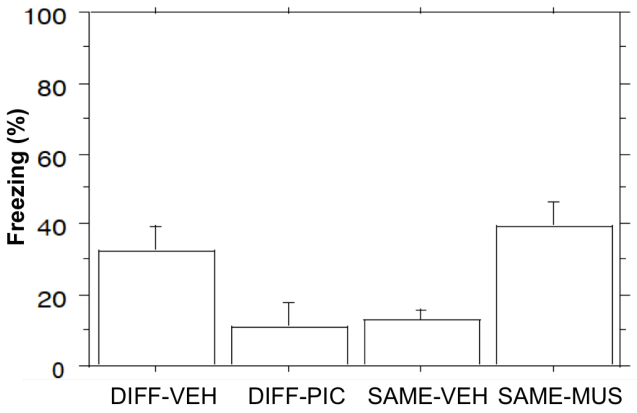

Figure 3.6 Summary of fear expression during extinction retrieval and fear renewal though $GABA_A$ receptors in IL. Inhibition of $GABA_A$ signaling reduces freezing in the renewal context to the level of extinction retrieval whereas activation of $GABA_A$ receptor signaling increases freezing in the extinction context to the level of fear renewal.

CHAPTER IV

EXPLORING THE ROLE OF INFRALIMBIC INTERNEURONS IN RETRIEVAL OF EXTINCTION MEMORY AND FEAR RENEWAL

Overview

By the end of Chapter III, it was suggested that $GABA_A$ receptors in IL bi-directionally context-dependent extinction memory. In this chapter, we sought to explore how the activity of IL interneurons regulated conditioned fear after extinction. First, we examined the neuronal activity during extinction retrieval and fear renewal. Then we used DREADD technology to inactivate IL interneuron or activate IL->BLA projection neurons in order to confirm whether it affected fear renewal. The results showed no activity difference of IL interneurons. Also, DREADD inactivation of IL interneurons or activation of IL->BLA neurons did not alter fear renewal. It seems like there is no role of IL interneurons or the downstream pathway to BLA in fear regulation during extinction retrieval and fear renewal. However, technical factors should be taken into consideration to make any conclusion.

Introduction

Memory of traumatic experiences is critical for survival. Fear memories enable the discrimination of safe or dangerous situations and motivate "fight or flight" decisions. However, failure to control fear memory leads to disorders such as posttraumatic stress disorder (PTSD) or specific phobias. Exposure therapy is the most effective method to

treat PTSD. However, after exposure therapy, fear relapse frequently occurs. In animal studies, Pavlovian fear conditioning is an important model to study learning, extinction and the relapse of fear. During Pavlovian conditioning, a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US). After a few trials of training, the animals acquired a conditioned response (CR) to the CS. Exposure to the CS along without US extinguishes the CR to the CS, a process called fear extinction (LeDoux 2000, Maren 2001). Extinction learning has the phases of acquisition, consolidation and retrieval. Extinction retrieval occurs in the context where extinction acquisition occurs. Unfortunately, after extinction, fear responses to the extinguished CS can return under a number of conditions; fear relapse that occurs outside of the extinction context is called "fear renewal" (Bouton 1993, Bouton & Bolles 1979, Maren 2011).

Immediate early gene (IEG) expression is a common tool to examine neural activity related to learning and memory (Davis et al. 2003, Plath et al. 2006). Previously, we have shown that the infralimbic cortex (IL) is differentially activated by extinction retrieval and fear renewal. Greater numbers of IL neurons were activated during extinction retrieval than fear renewal, which negatively correlated with freezing behavior (the index of fear memory) (Knapska & Maren 2009, Orsini et al. 2011). However, quantification of Fos-positive neurons in these studies did not consider the specific cell types in which Fos was expressed. In the cerebral cortex, 80 - 90% of the neurons are principal cells and 10 -20% are interneurons; processing of information depends on the

interaction of the two broad categories of neurons (DeFelipe & Fariñas 1992, Gabbott et al. 2005). Neurons in the prefrontal cortex are also defined by their connectivity within specific layers. For example, IL neurons projecting to the amygdala in IL arise primarily from in layers II and V (DeFelipe & Fariñas 1992, Gabbott et al. 2005, Pinto & Sesack 2008, Vertes 2004). Therefore, quantification of Fos-positive neurons by cell types and layers is necessary to understand how IL local circuits regulate context-dependent fear.

In addition to measuring neural activity during behavior, activation or inhibition of IL using electrical lesion or pharmacological methods reveal its function in fear expression after extinction (Bentefour et al. 2016, Chang & Maren 2011, Farrell et al. 2010, Fitzgerald et al. 2015, Garcia et al. 2006, Laurent & Westbrook 2009, Milad et al. 2004, Milad & Quirk 2002, Mueller et al. 2008, Sierra-Mercado et al. 2011, Thompson et al. 2010, Vidal-Gonzalez et al. 2006, Vollmer et al. 2016). However, these studies were not able to parse the contribution of specific cell types to behavior. Fortunately, optogenetics and chemogenetics enables cell type-specific manipulations of neuronal circuits (Johansen et al. 2012, Roth 2016, Tye & Deisseroth 2012). Recent studies indicate that optical manipulation of IL principal neurons produces bidirectional effects on extinction learning (i.e., inhibition impairs while excitation enhances extinction learning). Interestingly, silencing IL principal neurons during extinction retrieval did not impair retrieval during the four-trial test, but inhibiting both interneurons and principal cells did impair renewal (Do-Monte et al. 2015, Kim et al. 2016). Therefore, in this study, we sought to specifically silence IL interneurons prior to fear renewal test using transgenic

and virally transduced hM4Di DREADD (Designer Receptor Exclusively Activated by Designer Drug) whose expression was driven by a novel a inhibitory interneuron-specific GAD65 promoter.

The inhibition of fear by the IL may involve projections to intercalated cells (ITCs) in the amygdala that gates central nucleus of amygdala (CeA) (Berretta et al. 2005, Likhtik et al. 2005, Royer et al. 1999, Royer & Paré 2002). However, recent studies have challenged the nature of IL projections to the ITC (Cassell & Wright 1986, Gutman et al. 2012, Pinard et al. 2012, Strobel et al. 2015). An alternative is that monosynaptic IL projection to the basolateral amygdala (BLA) regulates behavioral output. Indeed, IL and BLA showed reciprocal pattern of Fos expression after context-dependent fear behavior after extinction (Knapska & Maren 2009, Orsini et al. 2011). Moreover, studies have shown that IL projection to ITCs occurs through monosynaptic projections to BLA (Cho et al. 12, Knapska et al. 2012, Orsini et al. 2011, Strobel et al. 2015). Previously, photostimulation or photoinhibition of IL-to-BLA pathway did not change extinction retrieval (Bukalo et al. 2015). In this study, we sought to activate IL-to-BLA pathway using hM3D DREADD technology and explore its role in context-dependent fear renewal to an extinguished auditory CS.

Materials and Methods

Subjects. Figty-six Long-Evans male adult rats (200-224g, Blue-Spruce) were obtained from Envigo (Indianapolis, IN). The rats were individually housed on a 14/10 h

light/dark cycle and had access food and water *ad libitum.* Rats were handled for 5 days before the experiment. All experimental procedures were approved by the Texas A&M University Animal Care and Use Committee.

Behavioral apparatus. All behavioral experiments were carried out in eight identical observation chambers (30 × 24 × 21 cm; MED-Associates, St. Albans, VT). Each observation chamber was constructed of a Plexiglas ceiling and rear wall, two aluminum sidewalls, a Plexiglas door. The floor of each chamber consisted of 19 stainless steel grids wired to a shock source and a solid-state grid scrambler (MED-Associates) to deliver the footshock unconditioned stimulus (US). The auditory conditioned stimulus (CS) was delivered by a speaker mounted outside of the grating in one sidewall of the chamber. A 15-W house light was fixed on the opposite sidewall and a ventilation fan was installed in each chamber. Each chamber was placed in a sound-attenuating cabinet. Three contexts were generated by the manipulation of the combination of sensory stimuli. For Experiment 1, in Context A, 1% acetic acid was used to wipe the ceiling, sidewalls, rear wall, door and grids of each chamber. The house lights and the fans were turned on. Cabinet doors were left open. White light was on in the behavior room. Rats were transported in white transport boxes. In Context B, the chamber was wiped with 1% ammonium hydroxide. House lights, fans and computer monitor were turned off and cabinet doors were closed. Red room light was turned on in the behavior room. Black transport boxes were used for rat transportation. For Context C, the odor was generated by 70% ethanol. House lights and fans were on. Room lights were white and cabinet

doors were open. Black Plexiglas floors were placed on the grids. Wood chip bedding was added to white buckets for rat transportation. For Experiments 2&3, in Context A, 1% ammonium hydroxide was used to wipe the ceiling, sidewalls, rear wall, door and grids of each chamber. The house lights and the fans were turned on. Cabinet doors were left open. White light was on in the behavior room. Rats were transported in white transport boxes. In Context B, the chamber was wiped with 3% acetic acid. House lights, fans and computer monitor were turned off and cabinet doors were closed. Red room light was turned on in the behavior room. In all the contexts, a stainless steel pan fill with a thin layer of the respective odor of the contexts was inserted under the grid of each chamber.

Each chamber was seated on a load-cell platform that recorded chamber displacement in response to each rat's motor activity; load-cell activity was digitized and acquired with Threshold Activity software (MED-Associates). Before the experiment, all load-cell amplifiers were calibrated to a fixed chamber displacement. Load-cell amplifier output (-10 to +10 V) from each chamber was digitized (5 Hz) and transformed to a value ranging from 0 to 100. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10) for at least 1 sec.

Surgical procedures. Rats were anesthetized with isoflurane (5% for induction; ~2% during surgery), and were placed on stereotaxic apparatus (David Kopf Instruments). For

Experiment 2, after induction of anesthesia, rats were placed on stereotaxic apparatus (David Kopf Instruments) and pulled glass injectors were lowered into IL (AP, +2.68mm, ML, ±3.1 mm, DV, -4.9mm from dura, with 30° angle on the coronal plain toward the midline). Cocktail of interneuron-specific inhibitory DREADD virus with "trace virus" was injected into IL bilaterally (AAV8-Gad65-hM4D(Gi)-Flag: 1.14E+13 GC/ml, 0.45ul/hemisphere; AAV-CMV-GFP: 7.8E+12 GC/ml, 0.05ul/hemisphere. Virus was obtained from Ploski Lab in University of Texas at Dallas). Injections were controlled by Nanoject (Drummond) at a rate of 0.23nl/s pulse and 3 pulses/minute bilaterally. The injectors remained in the brain for 10 minutes before removal. Rats were placed back to their home cages for post-operative recovery for two week. For Experiment 3, after induction of anesthesia, rats were placed on stereotaxic apparatus (David Kopf Instruments) and 30-gauge injectors were lowered into IL (AP, +2.68mm, ML, ±3.1 mm, DV, -4.9mm from dura, with 30° angle on the coronal plain toward the midline) and BLA (AP, -2.8mm, ML, ±5.0 mm, DV, -8.55 mm from Bregma). Each injector was connected to polyethylene tubing, which was attached to a Hamilton syringe (10 μl) placed on an infusion pump. AAV5-Cre-GFP virus was infused bilaterally in BLA (2.5E+12 pp/mL, 1.8 μl /hemisphere, UNC Vector Core) and rAAV8-hSyn-DIO-hM3Dq-mCherry DREADD virus was infused bilaterally in IL (5.9E+12 molecules/ml, 1.8 μl /hemisphere, UNC Vector Core) at a rate of 0.2 μl /minute. The injectors remained in the brain for 10 minutes before removal. Rats were placed back to their home cages for post-operative recovery for six week.

Behavioral procedures. In Experiment 1, we used a three-context procedure ("ABC") for fear renewal, in which rats were conditioned in Context A, extinguished in Context B, and tested in Context C. For extinction retrieval, rats were conditioned in Context A, extinguished and tested in Context C ("ACC"). HOME rats were conditioned in Context A and extinguished in Context C. In Experiments 2 and 3, we used a two-context procedure ("ABA") for fear renewal, in which rats were conditioned in Context A, extinguished in Context B, and tested back in Context A. In order to reduce contextual fear, animals were exposed to context A for the same amount of time as extinction, before each extinction session.

After handling for 5 days, rats were conditioned in context A, in which five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) trials were delivered. After 24 hours, rats were extinguished in either context B, where they received 45 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs). Reminder shock is 0.5 mA, 2 s. For Experiment 1, the following day, all the rats underwent test in context C, where they received 5 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs). For Experiments 2, the following day, rats received IP injection of clozapine-N-oxide (CNO; 3mg/kg; 1ml/kg; 2.5% DMSO) or vehicle (2.5% DMSO) 30 minutes before tested in Context A. Two test sessions were administered in two consecutive days. Animals were injected with CNO or vehicle alternatively. For Experiments 3, rats received IP injection of CNO (3mg/kg; 1ml/kg; 2.5% DMSO) or vehicle (2.5% DMSO) 30 minutes before tested in Context A.

Immunohistochemistry. Ninety minutes after the first tone of the retrieval test, rats were euthanized by overdose of sodium pentobarbital (0.5 ml) and were transcardially perfused with ice-cold 0.01 M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). Brains were fixed in 4% paraformaldehyde over night at 4°C then placed in 30% sucrose solution at 4°C until sunken. Coronal brain sections (30 μm) were collected on a cryostat at -20°C. Sections containing mPFC were collected every 30 μm.

Immunohistochemistry was performed on free-floating sections. Brain sections were washed three times in 1 × Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.4) for 10 min each. The sections were then incubated in 10% normal donkey serum (NDS) in TBST for 2 h at room temperature followed by two washes in TBST for 5 min each. Then the tissue was incubated in primary antibody in TBST with 3% NDS (goat anti-c-Fos antibody at 1:1000, Millipore; mouse anti-GAD67 antibody at 1:1000, Millipore) for 48 h at 4 °C. The sections were washed three times in TBST for 10 min each, and incubated in secondary antibody in TBST with 3% NDS (donkey anti-goat Alexa Fluor 488 at 1:200, Life Technology; donkey anti-mouse Alexa Fluor 594 at 1:200, Life Technology) for 2 h at room temperature. The tissue was then rinsed three times in TBS for 10 min each and mounted onto subbed slides in 0.9% saline and cover slipped with Fluoromount (Sigma-Aldrich).

Image analysis. Three images for the mPFC (+3.2, +2.7 and +2.3mm anterior to bregma) were taken for the quantification. All images were taken at 10 × magnification

with a Zeiss Imager M2 microscope. Single- and double-labeled neurons for each fluorophore in the PL and IL were counted. Counts for each image was averaged and standardized to counts/mm^2. The percentage of c-Fos expression in GAD67+ neurons and the percentage of GAD67 expression in c-Fos+ neurons are used to compare between groups. For layer-specific analysis, Layer II and Layer V are recognized based on the description: Layer II is the thin layer with high concentration of small granular neurons next to Layer I where no neuron can be observed. Layer III contains small neurons as well with lower concentration, next to Layer V with high concentration of larger neurons.

Data analysis. All data were analyzed with analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio. All data are presented as means ± SEM. Four rats were excluded because of lack of viral expression.

Results

Experiment 1. GAD67 and c-Fos analysis of the PL and IL after extinction retrieval or fear renewal. We have previously found that IL bidirectionally regulates fear expression during extinction retrieval and fear renewal through GABA signaling. To test whether local interneurons modulate PL and IL principal neurons in fear expression, we quantified GAD67-positive interneurons and Fos-positive neurons during the retrieval and renewal of extinquished fear. Animals showed clear fear renewal and extinction

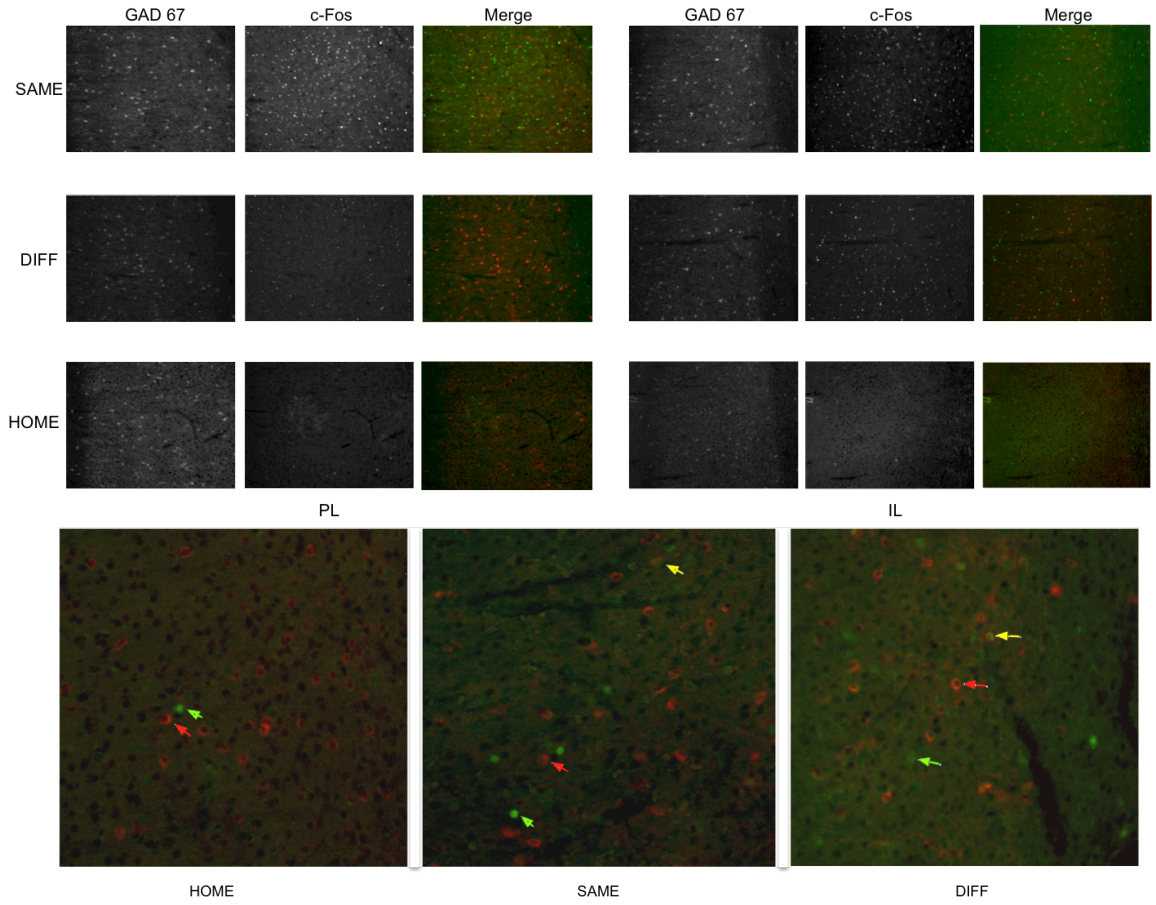

Figure 4.1 Example of immunohistochemistry. *Top*: Animals were perfused ninety minutes after the first tone of the extinction retrieval or fear renewal test. GAD67+ and Fos+ neurons in PL and IL was quantified. *Bottom panel*: Merged images in IL of HOME, SAME and DIFF animals, showing labeled cells. Red channel and arrow: GAD67-positive neurons. Green channel and arrow: Fos-positive neurons. Yellow arrow: double-labeled neurons with GAD67 labeling the perineuronal region and Fos labeling the nucleus.

retrieval were selected for analysis. Immunohistochemistry is shown in Figure 4.1. Ninety minutes after the first CS presentation during the retrieval test, the animals were perfused. Quantification of neurons is shown in Figure 4.2. In PL, GAD67 expression was elevated in the DIFF group relative to the HOME condition (Fisher's PLSD, $p<0.05$). In IL, more GAD67 neurons were observed in both DIFF and SAME comparing to HOME ($p<0.05$), but no differences were observed between the DIFF and SAME conditions. In PL, more robust c-Fos expression was observed during fear renewal than extinction retrieval ($p<0.05$), and both of these groups exhibited more Fos expression than animals in the HOME condition ($p<0.0001$). Interestingly, double-labeled neurons in DIFF outnumbered those in SAME as well ($p<0.05$).

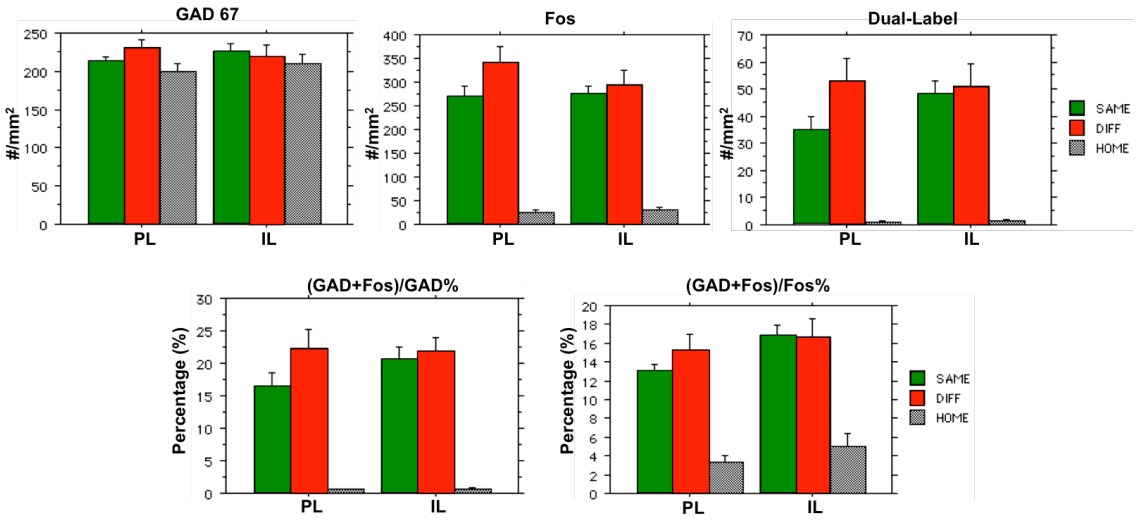

Figure 4.2 Quantification of activated interneurons in PL and IL during extinction retrieval and fear renewal. *Top*: cell counts of GAD67+ neurons, Fos+ neurons and dual-labeled neurons. *Bottom*: analysis of the percentage of dual-labeled neurons within all the GAD67+ or the Fos+ neurons. (SAME: n=10; DIFF: n=6; HOME: n=8)

In order to examine the cell type of the activated neurons, we measured the percentage of dual-labeled neurons among all Fos+ neurons. Also, in order to examine the activity of interneurons in both behavioral tests, we measured the percentage of dual- labeled neurons among all GAD67 neurons. In PL, the percentage of activated neurons within all the GAD67 neurons did not differ between groups. Also, among the activated neurons, the percentage of GAD67 neurons is consistent between groups. In IL, Fos+ and double-labeled neurons were consistent between SAME and DIFF groups.

Previously, we showed that Fos expression was more robust during extinction retrieval than fear renewal in IL. However, the localization of Fos activity to specific cortical layers in IL was not examined. Here, we analyzed layer II and layer V of IL, the major source of projections to the amygdala (Figure 4.3). In this analysis, SAME and DIFF animals showed the same level of expression of GAD67, Fos and there were no differences in the number of double-labeled neurons. There was a trend towards a greater percentage of dual- labeled neurons in the PL during fear renewal, but this was not statistically reliable (ANOVA, p=0.1037).

Experiment 2. Inactivation of putative interneurons in IL did not reduce fear renewal. Here we attempt to specifically inhibit GABAerigic interneurons using an hM4D DREADD virus with a GAD65 promoter. As shown in Figure 4.4, DREADD expression was localized to the IL and a small portion of ventral PL. After two days of

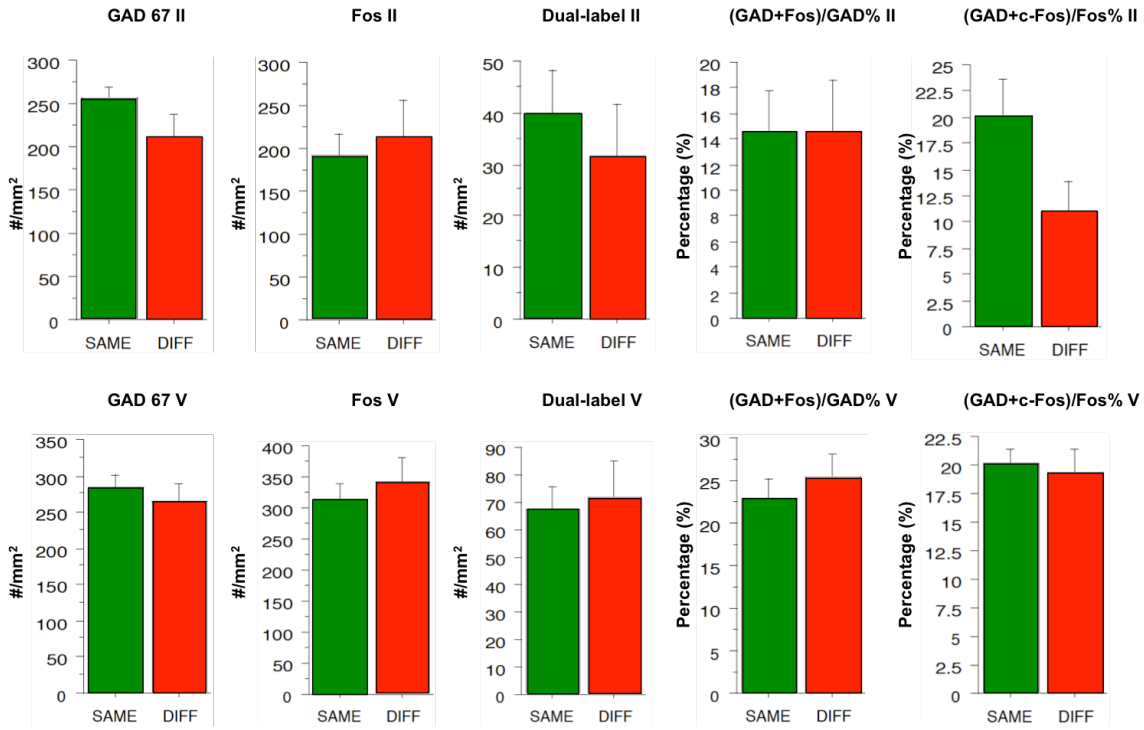

Figure 4.3 Normalized quantification of interneuron activity of Layer II and Layer V of IL during extinction retrieval and renewal test. The number of Fos expressing neurons represents neural activity. GAD67 marks interneurons. The percentage of dual-labeled neurons among all Fos+ and GAD67+ neurons is analyzed in each layer.

extinction in context B, animals were injected with CNO or vehicle, alternatively prior to each of the two renewal test sessions. CNO infusion did not reduce the renewal of freezing to the extinguished CS comparing to the control group (within-subject; Figure 4.4). However, analysis of each test revealed an effect of test order. In animals that received CNO during the first test, CNO infusion slightly reduced fear expression, although this effect was not significant (Figure 4.4; p=0.3364). However, animals receiving CNO during the second test, exhibited significantly higher levels of freezing [Figure 4.4; $F(1,12)=9.062$; $p<0.05$]. Although CNO did not reduce fear renewal overall,

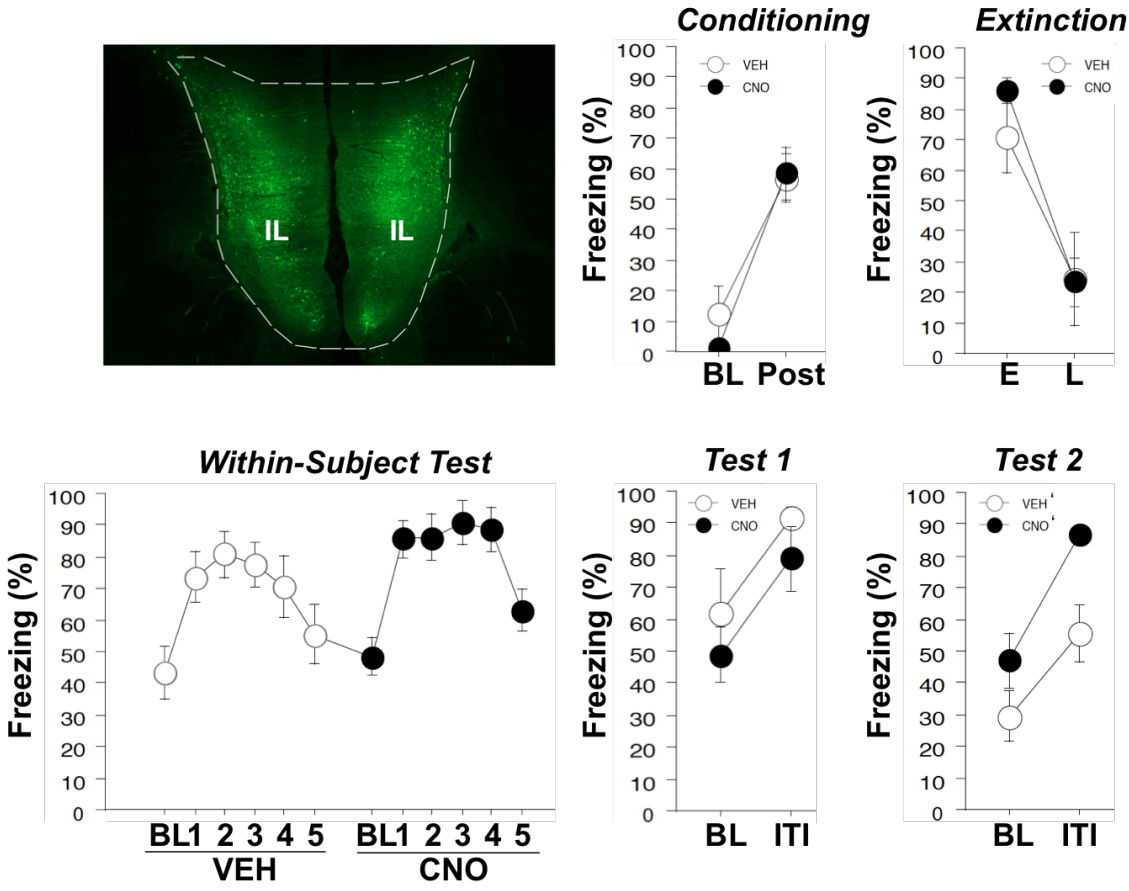

Figure 4.4 Effect of inactivation of putative IL interneurons on fear renewal. *Top left*: histology. DREADD virus infected bilateral IL and a small portion of PL. *Top middle and right*: fear conditioning in context A and extinction in context B. *Bottom left*: within-subject analysis of CNO effect. Inactivation of putative IL interneurons by CNO injection did not reduce fear renewal in context A [n=14; $F(5,65)=0.624$; $p=0.6819$]. *Bottom middle and right*: separate view of fear renewal Test 1 and Test 2. Animals were injected with vehicle or CNO alternatively prior to the two test sessions. CNO slightly reduced fear expression, but not significant [VEH: n=6; CNO: n=8; $F(1,12)=1.425$; $p=0.2557$]. During Test 2, CNO injection increased fear renewal comparing to the VEH group (VEH': n=8; CNO'; n=6; $F(1,12)=6.661$; $p<0.05$).

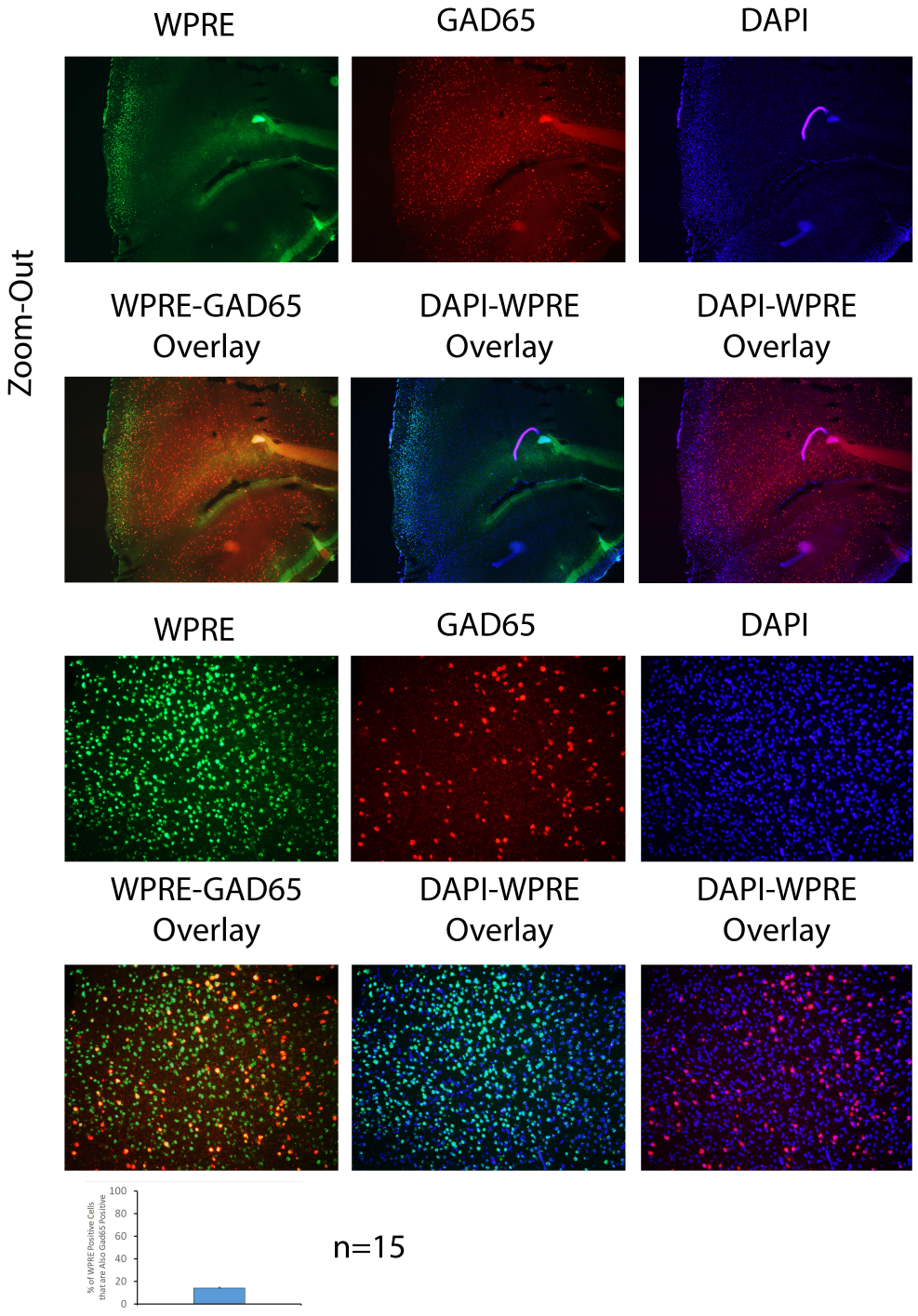

Figure 4.5 *In situ* test of the colocalization of expression of DREADD virus with a GAD65 promoter and GAD65. Viral expression is detected with WPRE. All neurons are marked with DAPI. Interneurons are marked with GAD65. Only 14% of all infected neurons are GAD65- positive.

it apparently has an influence on conditional freezing under some conditions. Importantly, an immunohistochemical analysis revealed that GAD65-DREADD virus did not exclusively express in GAD65 neurons. Only 14% of all infected neurons are GAD65+ neurons (Figure 4.5). This indicates that our manipulation was not specific to interneurons.

Experiment 3. Activation of IL-BLA pathway did not reduce fear renewal. Recent studies indicate that BLA is a gateway of fear expression, which forms feedforward inhibition circuit to CeA through ITCs (Strobel et al. 2015) and reduce fear expression. Previously, photostimulation and inhibition of IL-BLA pathway did not alter extinction retrieval (Bukalo et al. 2015). Here we sought to determine whether activation of this pathway reduce freezing behavior in renewal context. To specifically target IL-BLA pathway, we infused a retrograde virus constructed with Cre recombinase into BLA, and Cre-dependent excitatory hM3D DREADD virus into IL (Figure 4.6). Viral expression was detected in IL (Figure 4.6). After conditioning in context A and extinction in context B (Figure 4.6), animals were injected with CNO in order to activate the BLA- projecting neurons in IL. Activation of this population of neurons did not alter fear expression.

Discussion

In the present experiments, we attempted to explore the neural activity of interneurons in the infralimbic cortex (IL) during extinction retrieval and fear renewal, measured by Fos

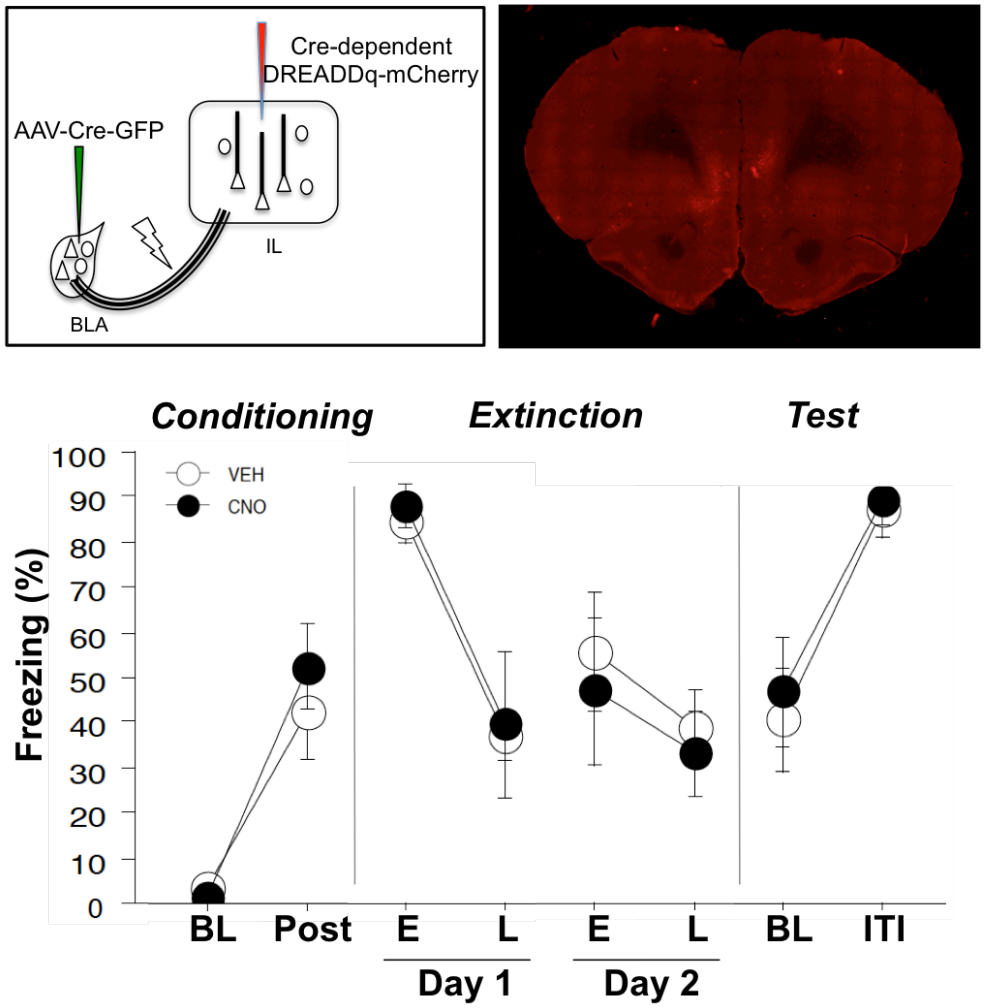

Figure 4.6 Stimulation of IL-to-BLA pathway did not reduce fear renewal. *Top left*: virus with cre-recombinase was infused to BLA bilaterally, and cre-dependent excitatory DREADD virus in IL. *Top right*: cre-dependent expression of DREADD in IL neurons. *Bottom*: animals were trained in context A and the conditioned CS was extinguished in context B. Prior to fear renewal test, animals were injected with vehicle or CNO. Fear renewal was tested in context back in context A. DREADD inactivation of IL-to-BLA pathway did not reduce fear renewal (VEH: n=6; CNO: n=6; p=0.7482).

expression. IL neuronal activity is at the same level during extinction retrieval and fear renewal, which did not replicate previous studies (Knapska & Maren 2009, Orsini et al. 2011). This may be due to the use of different methods for Fos immunohistochemistry and differences in how the brain sections and regions were quantified. We also observed substantial differences in the labeling of interneurons using antibodies from different suppliers, which introduced considerable variability in the outcomes (not shown). Lastly, individual differences within each experiment may have contributed to the results as well. Indeed, contradictory reports about the function of IL in expression of extinction have been discussed (Giustino & Maren 2015). Fos expression within interneurons was not influenced by the test context and there were no differences between cortical layers in Fos or GAD expression.

Consistent with previous reports (Knapska et al. 2012, Orsini et al. 2011), we found more Fos expressing neurons during fear renewal than extinction retrieval in PL. Interestingly, more dual-labeled neurons were found after fear renewal in the PL, although the percentage of interneurons among all activated neurons did not differ between groups. This suggests that CS presentations in the renewal context induced more neural activity in PL principal neurons and interneurons without altering the excitatory/inhibitory balance compared to the extinction context.

In the second experiment, CNO activation of inhibitory DREADDs in IL interneurons failed to reduce fear renewal. However, we the inhibitory DREADD virus was not

specifically expressed in GAD65 interneurons; in fact only 14% of infected neurons were GAD+ (Figure 4.6). Thus CNO activation of DREADDs inhibited primarily principal neurons in the IL. Consistent with this, we observed that IL inhibition increased freezing in at least one test, which would be expected if the inhibitory influence of the IL was removed. Consistent with this, it was previously shown that photostimulation of IL neurons infected by opto-virus with hSyn promoter during extinction retrieval reduced freezing during retrieval test, and silencing the neurons during retrieval impaired retrieval (Kim et al. 2016).

In the third experiment, we did no observe an influence of driving IL neurons projecting to the BLA on fear renewal. This is consistent with a recent report that photoactivation or inhibition of IL-BLA pathway does not alter extinction retrieval (Bukalo et al. 2015). In the same study, photoactivation of IL-BLA pathway during partial extinction enhanced extinction retrieval and photoinhibition during full extinction impaired retrieval (Bukalo et al. 2015). These results suggest a role for IL projections to BLA in extinction encoding, but not retrieval. In the current study, in the fear renewal context, activation of IL-BLA pathway did not reduce freezing as we hypothesized. Combining the two studies, it is suggested that after the formation of extinction memory, this pathway is not causally involved in the expression of fear in both the extinction context and renewal context. Indeed, synaptic study suggested that mPFC (IL and a part of PL) -to- BLA pathway was involved in extinction learning, but IL-ITC pathway was involved in retaining extinction (Cho et al. 2013). More specifically, however, IL-LA pathway

was activated during extinction retrieval, but not fear renewal (Knapska et al. 2012). In terms of the alternative pattern of c-fos expression in IL and BLA in extinction retrieval and fear renewal (Knapska & Maren 2009, Orsini et al. 2011), it is possible that some heterosynaptic connections are involved. Especially, extinction retrieval and fear renewal is context dependent, to which the hippocampal input is essential (Jin & Maren 2015a, Orsini et al. 2011).

In conclusion, we did not observe differences in the activity of IL interneurons between extinction retrieval and fear renewal, adding to the confusion of the contradictory findings (Giustino & Maren 2015). Due to the complex organization of subtypes of interneurons and their inhibition to each other (Harris & Shepherd 2015, Karnani et al. 2016), it is possible that activity of certain subtype of interneurons in IL regulates expression of extinction memory with finer temporal control (Courtin et al. 2014), which is difficult to examine with Fos expression. New technology attempting to specifically inactivate GAD65 interneurons needs improvement in order to be conclusive. Lastly, driving the IL-BLA pathway did not reduce fear renewal, which may yield to alternative pathways for the retaining of context dependent extinction memory.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary of Findings

In this dissertation, I aimed to explore the role of infralimbic cortical (IL) inhibitory circuits in context-dependent extinction retrieval and fear renewal. By the end of Chapter I, it was suggested that hippocampus (HP) is the hub for spatial processing and forming contextual representations and amygdala mediates the expression of fear responses. The medial prefrontal cortex (mPFC) is involved in higher executive function including decision-making. Anatomically, the prelimbic and infralimbic cortices of the mPFC both receive projections from the ventral hippocampus (VH), including ventral CA1 and vental subiculum. Both PL and IL send projections to the basolateral amygdala (BLA). Meanwhile, IL projects to the intercalated neurons (ITC) alongside the BLA, which in turn make inhibitory synapses in the central amygdala. The PL and IL send projections to each other. Therefore, contextual information encoded by the hippocampus can be relayed to the PL and IL, integrated, and passed onto the amygdala.

To test this hypothesis, in Chapter II, I aimed to dissect the direct pathways from VH to PL and IL. I injected retrograde tracer cholera toxin b (CTb) conjugated with two different fluorphores into PL and IL and analyzed neuronal activity in the VH projection neurons during extinction retrieval and fear renewal by quantifying c-fos expression. VH projections to IL outnumbered those to PL. This suggests that contextual modulation of

fear after extinction is mediated by the information flow from VH (including ventral CA1 and ventral subiculum) to PL and IL. Consistent with previous findings, more IL-projecting neurons were found in VH than PL- projecting neurons (Hoover & Vertes 2007). Also a few dual- projecting neurons were found. In the renewal context (context C of "ABC" paradigm), both PL-, IL- and dual- projecting neurons in VH were activated to higher percentage relative to in extinction context (context B of "ABC" paradigm). On the surface, it seemed that PL- and IL- projecting neurons did not discriminate contexts differentially. Previously, we used Fos expression to examine PL and IL activity during extinction retrieval and fear renewal. PL and IL showed opposite patterns of c-fos expression, that was, PL was more activated during fear renewal and IL was more activated during extinction retrieval. Therefore, PL- projecting neurons in VH and PL neurons have the same pattern of Fos expression, and IL- projecting neurons and IL neurons have the opposite pattern. Based on this result, I hypothesized that VH projections to IL recruit feedforward inhibition in IL, that is, VH projection neurons form synapses with both IL interneurons and principal neurons. Indeed, the hippocampus projects to both IL interneurons and principal neurons (Gabbott et al. 2002, Ishikawa & Nakamura 2003).

Therefore, in Chapter III, I infused $GABA_A$ receptor modulators into the IL prior to extinction retrieval or renewal tests to test the involvement of IL inhibitory circuits in the context-dependent regulation of fear after extinction,. Muscimol greatly interfered with extinction retrieval by elevating freezing in the extinction context and picrotoxin

dampened fear renewal. These results suggested that $GABA_A$ receptors in the IL bi-directionally regulate conditioned fear in extinction retrieval and fear renewal. Also, muscimol infusion interfered with extinction retrieval in both "ACC" and "CCC" tests to the same level despite their training backgrounds.

Those results suggest a critical role for GABAergic transmission in the regulation of extinguished fear, and imply that feedforward inhibition in hippocampal afferents might regulate fear. However, $GABA_A$ receptors are ubiquitous, not only on the principal neurons, but also in interneurons. So activation or inhibition of $GABA_A$ receptors affect both cell types. In order to test the specific role of IL interneurons in context-dependent fear regulation after extinction, in Chapter IV, I first assessed the neuronal activity in IL by labeling the interneurons with GAD67 antibody and measuring Fos expression to index neuronal activity. The overall levels of Fos expression did not differ between SAME and DIFF groups where extinction retrieval and fear renewal were tested, and the number of GAD67 neurons in the SAME groups was more than HOME controls. Extinction retrieval and fear renewal did not induce different numbers of activated interneurons labeled by both GAD67 and Fos. To assess the level of activity of the interneurons, I used the percentage of double-labeled neurons among all GAD67-labeled neurons as an indicator. Extinction retrieval and fear renewal tests did not yield different numbers of Fos+ interneurons in the mPFC and the ratio of interneurons to principle neurons differ between the two tests.

In a parallel test, in order to directly inactivate IL interneurons prior to renewal tests, we attempted to develop a DREADD virus that would selectively target inhibitory We infused inhibitory hM4D DREADD virus bilaterally into IL. The virus was constructed with a GAD65 promoter so that expression would be specifically in interneurons. Inhibitory DREADD was activated by CNO prior to renewal test. Inactivation of IL interneurons by CNO did not reduce fear renewal in the conditioning context relative to animals infused with vehicle.

Finally, I tried to examine the role of IL projections to BLA in context-dependent fear after extinction. To clearly dissect the IL-BLA pathway without affecting other populations, I used DREADD technology with the combination of a retrograde virus expressing Cre-recombinase with Cre-dependent DREADD infused in the afferent target. Specifically, I infused retrovirus expressing Cre-recombinase into the BLA, and Cre-dependent excitatory hM3D DREADD virus into IL. The virus can be taken up by axons in BLA and travel back to the cell bodies in IL. There, Cre-recombinase would activate the expression of Cre-dependent DREADD in IL. CNO injection would activate BLA- projecting neurons in IL. Immunohistochemistry revealed a fair amount of IL neurons labeled by the reporter protein constructed into Cre-dependent virus. CNO was injected prior to renewal test. However, it did not change freezing during fear renewal.

To rule out a “floor effect” in the vehicle group, I tested them again after delivered a reminder shock. The result did not change. Inhibition of the local IL interneurons

activates the IL principal neurons. In order to directly test whether the activation of the excitatory IL- to –BLA pathway reduce the fear response in the renewal test, I infused retrograde virus carrying Cre into BLA and Cre-dependent virus carrying excitatory DREADDs. Injection of CNO did not induce lower freezing during the renewal test. The results in Chapter IV indicate that the IL local interneurons do not regulate fear expression.

To summarize, VH projections to PL and IL responded more robustly to renewal context than extinction context. VH projections to IL may drive feedforward inhibition of IL principal neurons, which was supported by the behavior output of the $GABA_A$ receptor manipulation. However, Fos expression among interneurons in the mPFC did not correlate with freezing levels under any of the conditions. Chemogenetic inactivation of IL interneurons or chemogenetic activation of IL-BLA pathway did not reduce fear renewal as hypothesized.

The Role of IL Inhibitory Circuits in Gating VH-IL-BLA Information Flow During Context-Dependent Extinction Retrieval and Fear Renewal

The present results support the hypothesis that IL inhibitory circuits regulate context-dependent extinction retrieval and fear renewal. PL- and IL- projecting neurons in the VH responded to the extinguished CS more in the renewal context compared to the extinction context. However, in contrast to previous reports (Knapska & Maren 2009, Orsini et al. 2011), we did not observe difference in Fos expression in PL and IL during

the retrieval and renewal of extinguished fear. Nonetheless, the present data suggest that VH projections to the mPFC regulate fear, a finding that was supported by the bi-directional regulation of extinguished fear by $GABA_A$ receptors modulators. According to this view, during fear renewal, VH neurons engage feedforward inhibition of the IL and thereby limit IL inhibition of the amygdala, which leads to increased freezing (Maren & Holmes 2016, Orsini & Maren 2012). In other words, the IL normally puts a brake on fear expression, and VH input inhibits the IL to release this brake.

The results from Chapter III and Chapter IV appear contradictory. The robust bidirectional effect of $GABA_A$ receptor modulators infused into the mPFC was not reflected in the activity of GAD+ interneurons, nor did the chemogetic manipulation of putative GAD+ interneurons reproduce the pharmacological manipulations. One major reason for this discrepancy is the lack of specificity of the GAD-DREADD, which was expressed in both interneurons and projection neurons. In fact, only 14% of the infected neurons were GAD65+, suggesting that the majority (>85%) of the infected neurons were projection cells. Despite these technical complications, it is likely that in any event GABAergic interneurons do not account for all cortical GABA transmission. For example, cortical neurons may also receive GABA transmission from other brain regions. Evidence has shown that some cortical areas send functional long-range GABAergic projections to other brain regions (Lee et al. 2014). Also, it has been reported that cabindin+, PV+ and some pyramidal neurons in IL receive GABAergic

inputs from the basal forebrain and the ventral tegmental area (VTA) (Carr & Sesack 2000, Henny & Jones 2008).

In addition, GABA released by local interneurons may modulate GABA receptor subtypes and cell types differentially during recall of extinction. Indeed, GABA receptor subtypes mediate freezing behavior differentially. Our unpublished data has shown that antagonism of $GABA_B$ receptors does not reduce fear renewal as robustly as that of $GABA_A$ receptors (not shown in this dissertation). Also, the distribution of GABA subtypes is cell-type specific (Sieghart & Sperk 2002). For instance, $GABA_B$ receptors modulate GABA release of PFC PV+ and SOM+ neurons during various behaviors (Liu et al. 2007). Therefore, taken together, it is not surprising that the overall effect of local GABA release differs from the behavioral outcome by the manipulation of local $GABA_A$ receptors alone. Lastly, GABA transmission not only inhibits the activity of principal neurons, but also that of interneurons, which have a complex reciprocal inhibitory network (Karnani et al. 2016). For example, PV+ interneurons are strongly inhibited by themselves and SOM+ interneurons, and suppress SOM+ and VIP+ neurons (Karnani et al. 2016). These interactions can be altered by behavior (Wolff et al. 2014). As part of the VH->IL circuit, subtypes of interneurons in IL are possibly engaged in fear behavior differentially as well. It has been shown that VH projects more strongly to PV+ neurons than SOM+ neurons in IL (unpublished data). Therefore, manipulation of $GABA_A$ receptors may have different effects on different subtypes of interneurons, and these behavioral outcomes may not be replicated by shutting down the entire interneuron

population. Taking all of these nuances into consideration, it is possible that all $GABA_A$ receptors in all cell types have robust overall functions on freezing during context-dependent extinction memory, but all interneurons do not mediate the effect as a whole, as evident in Chapter IV.

In the BLA, "fear neurons" and "extinction neurons" signal conditioned fear responses and extinction (Herry et al. 2008). Anatomically, "fear neurons" project to the mPFC and "extinction neurons" reciprocally connect to mPFC (Herry et al. 2008, Senn et al. 2014). By the end of Chapter IV, it was suggested that IL->BLA pathway is not engaged in the context-dependent memory of extinction, given the evidence that activation of this pathway using DREADD did not alter fear renewal. This result is consistent with previous report with optogenetic technology (Bukalo et al. 2015). In the amygdala, IL also projects to ITCs, a pathway previously reported to be essential for inhibition of fear expression (Berretta et al. 2005, Quirk et al. 2003). This result has suggested that activation of IL->BLA pathway alone is not sufficient to activate extinction neurons in order to suppress fear expression in the renewal context. Alternatively, it is possible that the excitation of inhibitory circuit in IL in the renewal context suppresses the IL->ITCs pathway. Activation of IL->BLA pathway alone is not enough to alter the fear expression by the activation of the other excitatory pathways, including the direct input from VH to BLA (Jin & Maren 2015a, Orsini et al. 2011). Future activation of IL->ITCs pathway is necessary to test the hypothesis.

Clinical Implications

Since the 1980s, fear extinction has been a model to study the treatment of PTSD (Pitman 1988). Human fMRI studies have revealed that the functional human homolog of IL is the ventromedial prefrontal cortex (vmPFC), a brain area that is important for extinction recall in humans. Structurally, the thickness of vmPFC is positively correlated with fear extinction (Hartley et al. 2011, Milad et al. 2005b). Functionally, its activity is increased during extinction recall (Kalisch et al. 2006, Phelps et al. 2004). Milad and colleagues have also observed that the magnitude of vmPFC activation is positively correlated with the magnitude of extinction retention (Milad et al. 2007) (Milad et al. 2005a). Interestingly, patients with PTSD have normal responses during fear conditioning and within-session extinction learning, but are not able to recall extinction (Milad et al. 2009). Their impaired extinction recall is due to hypoactivity of vmPFC and hyperactivation of dorsal anterior cingulate cortex (dACC), the human homolog of PL (Milad et al. 2009).

Pharmacologically, drugs targeting the GABA-benzodiazepine system are mainly used for treatment of generalized anxiety disorder (GAD) and social anxiety disorder (SAD), but not PTSD (Griebel & Holmes 2013). However, understanding of the role of specific brain regions and cell types in extinction memory regulation is significant for potential clinical applications (Cruz et al. 2013, Dejean et al. 2015, Urban & Roth 2015). Especially, brain stimulation based treatment such as deep brain stimulation, transcranial direct current stimulation or transcranial magnetic stimulation (TMS) a complement to

pharmacological and behavioral therapies, for their advantage to target brain regions (Boggio et al. 2010, Dejean et al. 2015, Isserles et al. 2013, Koek et al. 2014, Saunders et al. 2015, Watts et al. 2012). Repetitive TMS enables excitatory or inhibitory stimulations using different frequencies (Watts et al. 2012), and the specific protocols can target PV+ GABAergic interneurons in cortical areas (Benali et al. 2011). Additionally, viral constructs of Cre-recombinase with different promoter could enable cell-type specific or activity driven expression of DREADDs or other relevant proteins (Cruz et al. 2013, Urban & Roth 2015). Therefore, targeting the inhibitory circuits in vmPFC is potentially effective in preventing relapse of fear outside of the site of PTSD treatment.

Future Directions

In this dissertation, I sought to explore the role of the IL inhibitory circuits in the contextual regulation of conditioned fear in extinction retrieval and fear renewal, especially in the VH-IL-BLA pathway. I have looked at the specific involvement of PL- and IL- projecting neurons in the VH, the role of $GABA_A$ receptors and local interneurons in the IL and the effect of activating the IL-BLA pathway.

By the end of Chapter II, it was suggested that IL local inhibitory system is a possibility to suppress IL activity during extinction retrieval, which is activation by projections from VH. Indeed, PL and IL interneurons receive projections from the CA1 (Gabbott et al. 2002, Ishikawa & Nakamura 2003). However, the activation of PL- and dual-projecting neurons in the VH during fear renewal is as the same activity pattern as the

PL, whereas IL- projecting neuronal activity has an opposite pattern comparing the IL activity during fear renewal and extinction retrieval. In order to understand the seemingly opposite overall effect of the local interneurons in the PL and IL, it is necessary to assess the ratio of the synapses on the principal neurons vs. onto the interneurons. Additionally, context-CS induced dynamic change of the synaptic strength and the timing of such change may also contribute to effect. Also, in order to directly examine the behavior effect, activation or inhibition of the projection neurons using optogenetic or chemogenetic methods during extinction retrieval and fear renewal can be used and *in vivo* electrophysiological measurements will be taken. Furthermore, on the circuit level, VH makes direct projections to IL and BLA (Hoover & Vertes 2007, Jin & Maren 2015b, Orsini et al. 2011). VH projections to PL and BLA has the same responses to the CS in extinction or renewal contexts (Jin & Maren 2015b). But it is clear whether there are dual- projection neurons to IL and BLA or what their activity patterns are to the CS in different contexts. Using the same retrograde tracing with Fos imaging techniques will clarify that.

By the end of Chapter III, the results suggested that the $GABA_A$ receptors in the IL bi-directionally regulate context-dependent conditioned fear. $GABA_A$ receptor is one type of GABA receptors, and subtypes of $GABA_A$ receptors differ in physiological properties (Farrant & Nusser 2005). It is curious how other GABA receptors affect the memory of extinction. Indeed, mixed infusion of $GABA_A$ and $GABA_B$ agonists interfered extinction retrieval, as the same effect as $GABA_A$ agonist (Sangha et al. 2014). But the effect of

$GABA_B$ receptor agonist alone is not clear. Similarly, the effect of $GABA_B$ antagonist, or the mixture of $GABA_A$ and $GABA_B$ receptor antagonists on fear renewal is not clear. Furthermore, it will in interesting to know the specific role of each subtype of GABA receptors in extinction memory.

In Chapter IV, I have examined the overall effect of GABAergic interneurons in IL with the techniques of immunohistochemistry and direct manipulations of neuronal activity of the interneurons with DREADDs. As mentioned in Chapter I, cortical interneurons are population of neurons with great diversity and they are interconnected with each other in a specific order (Harris & Shepherd 2015, Karnani et al. 2016). Because of such interconnection, other than the inhibition of principal neurons at different locations (Muller et al. 2006, 2007), they inhibit each other and the overall effect may be various in specific situations. With the development of optogenetic and chemogenetic methods, combining with transgenic models, their interconnection with each other and with principal neurons with precise spatial and temporal control has become a trend of interest in the study of fear (Tovote et al. 2015). For example, PV+ and SOM+ interneurons in BLA were inhibited during aversive footshock, but PV+ neurons disinhibit BLA neurons by inhibiting SOM+ neurons during auditory CS (Wolff et al. 2014). Also, in PL, inhibition of PV+ neuronal activity disinhibits projection neurons and synchronizes their firing by resetting local theta oscillations, leading to fear expression. Inhibition of PV+ neuronal activity disinhibits prefrontal projection neurons and synchronizes their firing by resetting local theta oscillations, leading to fear

expression (Courtin et al. 2014). Therefore, mapping out the subpopulation of interneurons in the IL and examining their activities in context-dependent fear expression after extinction is necessary. Indeed, we attempted to use some indirect method to interfere PV+ neurons by erasing perineuronal nets (PNNs), which was reported to surround PV+ neurons alone (Galtrey & Fawcett 2007, Gogolla et al. 2009). However, preliminary data showed no difference in extinction retrieval or fear renewal when PNNs in IL was erased prior to test (not reported). It may be because of low amount of PNNs in IL (Ueno et al. 2017) or the low specificity around PV+ neurons (Carstens et al. 2016). Furthermore, combining specific antibody of PV+, SOM+, or 5HT3a interneurons with Fos expression after extinction retrieval and fear renewal can provide a more accurate understanding of neuronal activity during each test. Additionally, with the availability of PV-, SOM- and VIP-Cre animals, it is possible to use Cre-dependent DREADDs or opto-virus to directly excite or inhibit subpopulations of interneurons during retrieval and renewal tests. Especially with optogenetic methods, there is more accurate temporal control of the interneurons coupled with behavior tests. This way, it will provide more accurate understanding about how the inhibitory microcircuits in IL integrate information from the hippocampus and relay to amygdala for fear regulation.

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