NEURAL CIRCUITS UNDERLYING CONTEXT-DEPENDENT FEAR MEMORY RETRIEVAL

A Dissertation

by

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ABSTRACT

Exposure therapy for stress- and anxiety-related disorders is very effective, but fear return after treatment is not uncommon. During extinction, repeated exposure to a conditioned stimulus (CS) gradually decreases the probability and magnitude of the conditioned fear response (CR). However, extinction does not eliminate the fear memory; rather, it generates a new extinction memory that competes with the fear memory for control of behavior. Importantly, the extinction memory is highly context-dependent insofar as it is only expressed in the extinction context. That is, if the CS is encountered outside of the extinction context, the conditioned fear response returns or ‘‘renews’’. The renewal of extinguished fear is a considerable challenge for maintaining long-lasting fear suppression after exposure therapy. The hippocampus, the medial prefrontal cortex (mPFC) and the amygdala are thought to play essential roles for context-dependent memory retrieval after extinction, but the circuit mechanism is not clear. To explore the neural circuits underlying contextual regulation of fear memory retrieval, we first used functional tracing approach to examine the activity of prelimbic prefrontal cortex (PL)- and basal amygdala (BA)-projecting neurons in the ventral hippocampus (vHPC) during extinction retrieval and fear renewal. We then used circuit-specific chemogenetic approach to examine the role of infralimbic prefrontal cortex (IL)-projecting vHPC neurons in contextual retrieval. Finally, we used pharmacological and chemogenetic approaches to test the role of the nucleus reuniens (RE) in context-dependent fear memory retrieval. The results showed that vHPC inputs to both the PL

and BA are activated during fear renewal, with PL- and BA-dual projecting neurons showing the greatest level of activation. Moreover, we showed that IL-projecting vHPC neurons are required for fear renewal by inducing feedforward inhibition within the IL. Lastly, we demonstrated that the RE is required for extinction retrieval, and the prefrontal modulation on the RE activity is essential in this process. Together, these results provided circuit mechanisms underlying context-dependent fear memory retrieval.

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CHAPTER I

INTRODUCTION

Learning to detect potential threats and organize appropriate defensive behavior while inhibiting fear when threats are absent are highly adaptive functions associated with emotional regulation (Ohman and Mineka, 2001; Mineka and Ohman, 2002). However, in humans, this form of learning can sometimes lead to pathological fear that can cause stress- and trauma-related disorders, such as post-traumatic stress disorder (PTSD) (Rosen and Schulkin, 1998; Bouton et al., 2001). Inhibition of pathological fear is crucial given that fear memories are rapidly acquired and broadly generalized across different contexts. Moreover, cognitive behavioral therapies, such as exposure therapy, used to reduce fear and anxiety often produce transient fear reduction that is often limited to the context in which the therapy was administered (Maren et al., 2013). For these reasons, understanding neural circuits and mechanisms of fear acquisition, extinction and relapse is essential for future therapeutic interventions.

Most of what we understand about learned fear comes from studies using the Pavlovian fear conditioning paradigm (Maren, 2001). In this paradigm, an initially neutral conditioned stimulus (CS), usually a tone, is paired with an unconditioned stimulus

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(US), usually a mild but aversive footshock. Following fear conditioning, presentation of the CS alone generates various physiological (changes in heart rate, blood pressure, etc.) and behavioral (freezing, startle, etc.) conditioned responses (CR) (Fanselow, 1994). In research using rodents as a model animal, freezing behavior is usually used as an index of fear responses. Fear acquisition involves a gradual increase in the expression of the CR after CS–US presentations during conditioning. Fear memories are consolidated over time, and their retrieval can be induced and measured by presenting the CS alone in either the original conditioning context or in a novel context. Fear conditioning involves the acquisition of a CR as a result of CS–US pairings, whereas extinction refers to the reduction of CR when the CS is no longer paired with the US. Thus, during fear conditioning, animals learn that a neutral CS predicts the occurrence of an aversive US; however, if the CS is presented alone without footshock, animals then learn that the CS previously associated with aversive US no longer predicts danger. The latter process is called extinction whereby repeated presentations of the CS alone lead to a gradual reduction of conditioned fear responses to the CS (Pavlov, 1927; Dunsmoor et al., 2015). The extinction memory is labile because extinguished CRs can return under a number of circumstances including the passage of time (spontaneous recovery); encountering an extinguished CS outside of the extinction context (renewal); exposure to a novel stimulus (external disinhibition) or a noxious stimulus (reinstatement) (Bouton and Bolles, 1979; Bouton, 1993; Rescorla, 2004; Goode and Maren, 2014). Therefore, these "relapse" phenomena suggest that extinction learning does not erase the original CS-US association; instead, it leads to the formation of a new inhibitory memory that coexists

and competes with the original fear memory (Milad and Quirk, 2012). Pavlovian fear conditioning and extinction procedures are powerful methods for studying the neuronal substrates of emotional learning and memory processing. Indeed, decades of studies using this model have revealed that there is a distributed neuronal network involved in acquisition, extinction and contextual fear memory retrieval. The neuronal network consists of, but is not limited to, the amygdala, medial prefrontal cortex (mPFC) and hippocampus (HPC).

The amygdala is known as a key structure for fear learning and memory (Duvarci and Pare, 2014). The mPFC and HPC have been suggested as important structures for regulating the expression, extinction and retrieval of fear memories (Maren, 2011; Maren et al., 2013; Giustino and Maren, 2015; Jin and Maren, 2015b; Tovote et al., 2015). A significant amount of research has been devoted to studying the mechanisms within these regions underlying the acquisition, extinction and retrieval of the fear memories. However, more recently, the focus of this research has moved to examining these brain structures as part of a neuronal network mediating fear memories. Therefore, the final goal is to understand how individual components of the distributed fear network interact with each other to generate appropriate behavioral responses. In this section, the focus will be on neurobiological substrates underlying fear conditioning and extinction.

Neurobiology of fear conditioning and extinction

The amygdala

The amygdala is a critical component of the neural circuitry underlying fear learning. It is comprised of a collection of nuclei that form two distinct subsystems important for fear conditioning (LeDoux, 1995; Maren, 2001). The first subsystem of the amygdala, the basolateral complex (BLA), is comprised of the lateral (LA), basolateral (BA), and basomedial (BM) nuclei. The BLA consists of glutamatergic principal neurons (~80%) and inhibitory interneurons (~20%), and is the primary sensory interface of the amygdala (McDonald, 1992; Spampanato et al., 2011). The second subsystem of the amygdala, the central nucleus (CeA), can be divided into medial (CeM) and lateral (CeL) divisions, which constitutes the amygdala's interface to fear response systems in the brainstem. Central amygdala neurons are primarily GABAergic. An interconnected sheath of GABAergic neurons, the intercalated cells (ITC), is also found interposed between the BLA and CeA, providing an important source of inhibition (Ehrlich et al., 2009).

Within the amygdala, the LA receives multisensory information from the thalamus, cortical areas and brainstem regions (McDonald, 1998), and then projects to glutamatergic BA neurons and GABAergic ITCs (Ehrlich et al., 2009). It has been well defined that the CeM is the major output area in the amygdala. It projects to regions such as the periaqueductal gray (PAG) and lateral hypothalamus (LH) which produce behavioral and physiological responses to emotionally relevant events (Pape and Pare, 2010). After auditory fear conditioning, CeM neurons show sustained elevation in firing

rates during CS presentation (Ciocchi et al., 2010; Duvarci et al., 2011). In line with this, optogenetic activation of CeM neurons elicits freezing behavior, whereas inactivation impairs expression of conditioned freezing (Ciocchi et al., 2010). Therefore, amygdala microcircuitry seems to receive and integrate complex information to produce behavioral responses, such as freezing, and is critical for emotional processing especially for interpretation of emotionally relevant stimuli or the association of emotional relevance to otherwise neutral stimuli.

The LA has been the focus of fear conditioning studies since it has access to information regarding the sensory cues used in auditory fear conditioning. For example, the LA receives auditory information from the posterior intralaminar nuclei of the thalamus (PIN), the medial sector of the medial geniculate nucleus (MGm) and also from the ventral geniculate nucleus (MGv) via temporal auditory cortex (TE) (LeDoux et al., 1990b; LeDoux et al., 1990a). The same posterior thalamic and cortical regions that relay auditory information to LA also receive spinothalamic input and may therefore send convergent auditory CS and somatosensory US inputs to the LA (Shi and Davis, 1999). Indeed, lesion and inactivation of the LA block acquisition of conditioned freezing (LeDoux et al., 1990a; Muller et al., 1997; Nader et al., 2001), and LA neurons also develop and maintain excitatory neural responses to the auditory cue that has been paired with a footshock (Quirk et al., 1995; Collins and Pare, 2000; Repa et al., 2001; Goosens et al., 2003). Interestingly, CS and US information can converge onto single LA neurons, and the convergence of synaptic inputs about the CS and US leads to the

potentiation of synapses conveying CS information to LA through a Hebbian plasticity mechanism (Romanski et al., 1993; Uwano et al., 1995; LeDoux, 2000). Recently, Johansen and colleagues showed that optogenetic activation of pyramidal neurons in the LA combined with simultaneous tone presentation is sufficient to produce fear responses to the tone (Johansen et al., 2010). Furthermore, Nabavi and colleagues showed that activation of axonal terminals in LA from the auditory thalamus (the medial geniculate nucleus) and the auditory cortex (AC) can substitute for a tone CS when paired with footshock, thereby generating conditioned freezing and synaptic potentiation (Nabavi et al., 2014). These studies provide evidence that the LA is the critical site of synaptic plasticity for acquisition of fear memory.

Despite the fact that up to 70% of the LA neurons receive necessary sensory input, relatively few LA neurons (~20%) show increased CS responsiveness after fear conditioning (Han et al., 2007). This has led to the proposal that there is competition between LA neurons for encoding sensory input and further generating CS-evoked potentiation. Studies suggest that LA neurons with higher intrinsic excitability are preferentially recruited in this process (Han et al., 2007; Han et al., 2009). In parallel, LA neurons with increased CREB (cAMP response element-binding protein) are also preferentially recruited in the memory trace (Han et al., 2007; Zhou et al., 2009). Recently, Yiu and colleagues revisited these two findings and showed that increased expression of CREB in LA neurons immediately before training causes high intrinsic excitability, which then leads the neurons to successful recruitment in the memory trace

(Yiu et al., 2014). These studies demonstrate that a certain population of LA neurons is preferentially recruited during fear memory encoding although the majority receive necessary sensory inputs.

Because the LA is the primary input structure and the CeM is the interface to fear output systems in brainstem, convergence of the sensory information in the LA needs to reach brainstem-projecting CeM neurons in order for the conditioned fear to be expressed. However, the LA does not project directly to the CeM; instead, the LA can reach the CeM indirectly via either glutamatergic neurons in the BA or GABAergic neurons in the CeL (Pitkanen et al., 1997; Duvarci and Pare, 2014). LA neurons project strongly to the BA, and therefore sensory information entering the LA is relayed to the BA. Similar to LA neurons, BA neurons also exhibit increased CS-evoked responses during fear learning (Herry et al., 2008; Amano et al., 2011). Moreover, rats with post-conditioning BA lesions were found to have disrupted conditioned fear responses (Anglada-Figueroa and Quirk, 2005). This suggests that the BA is required for relaying the CS-evoked LA responses. In addition to the BA, the CeL also provides an important route for the LA to relay sensory information to the CeM (Duvarci and Pare, 2014). Studies suggest that the CeA is not just an output structure, but in fact is necessary for both acquisition and expression of conditioned fear. For example, NMDA receptor blockade or protein synthesis inhibition in CeA reduces the acquisition of fear learning (Goosens et al., 2003; Wilensky et al., 2006; Zimmerman et al., 2007). Further supporting this idea, a recent series of studies suggest that fear learning produces synaptic potentiation of BA

and LA inputs to neurons in the CeL (Li et al., 2013; Watabe et al., 2013).

Interestingly, different subpopulations of neurons in the amygdala are affected differently by extinction training. Firstly, within the LA, dorsal LA neurons exhibit a rapid reduction of CS-evoked responses, whereas ventral LA neurons maintain CS responses (Repa et al., 2001). Extinction training also changes CS responsiveness in the BA, and BA inactivation seems to impair extinction. For instance, infusing NMDA receptor antagonists or $GABA_A$ receptor agonists into the BA prior to extinction training results in extinction impairments (Falls et al., 1992; Chhatwal et al., 2005; Laurent et al., 2008; Makkar et al., 2010; Sierra-Mercado et al., 2011). Furthermore, extinction learning induces expression of the immediate early gene (IEG) c-Fos in the BA, while the expression decreases in animals showing impaired extinction learning (Herry and Mons, 2004; Muigg et al., 2008). Similar to LA neurons, different subpopulations of BA neurons show different responses to extinction training (Herry et al., 2008). For example, single-unit recordings indicate that there are two types of neurons in the BA depending on their responses to CS during fear memory acquisition and extinction. “Fear neurons” develop excitatory CS responses as a result of fear conditioning but lose them after extinction training, whereas “extinction neurons” only become CS responsive following extinction training but not fear conditioning (Herry et al., 2008). The existence of neurons with different responsiveness to the CS within both the LA and BA suggests that different neural circuits, consisting of different populations of neurons, may coexist and compete with each other in different behavioral states.

The medial prefrontal cortex

Early studies have shown both anatomical and functional differences between two important structures within the mPFC, the prelimbic (PL) and infralimbic (IL) prefrontal cortex, in fear memory acquisition and extinction. Anatomically, the PL has connections with sensorimotor and association neocortical areas, while the IL has rather extensive connections with the amygdala and temporal, limbic association cortices (Heidbreder and Groenewegen, 2003). In addition, the IL strongly projects to limbic structures such as the septum and hypothalamus, while the projections from PL to these regions are rather limited. It has become clear following decades of studies that both the PL and IL play important roles in fear learning and memory. Interestingly, studies indicate that the PL and IL exert distinct, sometimes opposing, roles on fear expression and extinction (Giustino and Maren, 2015; Gourley and Taylor, 2016). In particular, it has been shown that the PL is critically involved in generating conditioned fear responses as a 'go' structure whereas the IL is a key brain region for extinction learning as well as fear suppression after extinction as a 'stop' structure (Quirk et al., 2006; Myers and Davis, 2007; Quirk and Mueller, 2008; Herry et al., 2010; Giustino and Maren, 2015; Gourley and Taylor, 2016). In this section, the dichotomy of PL-go/IL-stop will be discussed in terms of the fear conditioning and extinction paradigms.

A series of studies showed that inactivation of the PL eliminates the expression of conditioned fear to both contextual and auditory cues (Corcoran and Quirk, 2007; Laurent and Westbrook, 2008; Sierra-Mercado et al., 2011), without affecting

acquisition of fear memory (Corcoran and Quirk, 2007; Laurent and Westbrook, 2008), expression of innate fears (Corcoran and Quirk, 2007) or acquisition of extinction memory (Laurent and Westbrook, 2008; Sierra-Mercado et al., 2011; Kim et al., 2016). These studies suggest that the PL is necessary for fear expression, but not for the plasticity required during fear conditioning or extinction. In line with this idea, microstimulation of the PL potentiates the expression of conditioned fear (Vidal-Gonzalez et al., 2006). Interestingly, PL neurons have sustained activity to aversive CSs during fear recall, and indeed, the PL is the first structure to exhibit conditioned responses that model fear responses. That is to say, PL neurons exhibit a sustained increase in activity that mirrors the time course of freezing (Burgos-Robles et al., 2009). Indeed, inactivation of PL interneurons disinhibits PL projections to the amygdala thereby enhancing the expression of conditioned fear (Courtin et al., 2014). Recent studies further showed that PL-amygdala projections constitute a key brain circuit for retrieving newly formed fear memory (Do-Monte et al., 2015), and inhibitory-excitatory balance in the BLA-projecting PL neurons is decreased after fear conditioning (Arruda-Carvalho and Clem, 2014). Furthermore, rats that fail to successfully retrieve extinction memory show increased activity in the PL (Burgos-Robles et al., 2009; Knapska and Maren, 2009), and administration of propranolol or cannabidiol, which has been shown to reduce fear expression, directly into the PL also reduces fear expression (Rodriguez-Romaguera et al., 2009; Lemos et al., 2010). These studies suggest that the PL is important in generating and maintaining conditioned fear responses.

One of the earliest observations regarding the neural mechanisms of fear extinction was that lesions in the mPFC impair extinction of conditioned fear (Morgan et al., 1993). Using pharmacological and electrophysiological tools, it has been identified that the IL plays a major role in acquisition and consolidation of extinction memory. In particular, Quirk and colleagues showed that rats with IL lesions have difficulty recalling extinction memory 24 hours after acquisition (Quirk et al., 2000). Recording studies show elevated tone responses of IL neurons during extinction training (Chang et al., 2010; Holmes et al., 2012), and increased intrinsic excitability of the IL neurons after extinction training (Santini et al., 2012). Extinction learning also induces plasticity (Sepulveda-Orengo et al., 2013) and IEG expression in the IL (Knapska and Maren, 2009). Additionally, acquisition of extinction is impaired by inactivation of IL neurons during extinction training (Sierra-Mercado et al., 2006; Burgos-Robles et al., 2007; Fontanez-Nuin et al., 2011; Sierra-Mercado et al., 2011; Do-Monte et al., 2015; Kim et al., 2016). In contrast, electrical stimulation or optogenetic activation of the IL during extinction learning strengthens extinction memory (Milad and Quirk, 2002; Quirk et al., 2003; Vidal-Gonzalez et al., 2006; Maroun et al., 2012; Do-Monte et al., 2015). Indeed, electrical stimulation of the IL decreases the excitability of brainstem-projecting CeM neurons, and reduces the responsiveness of CeM output neurons to BLA stimulation, and then eventually decreases the expression of conditioned fear (Royer and Pare, 2002; Quirk et al., 2003). Neuroanatomical studies support the idea that the IL projects to the GABAergic ITC cells (McDonald et al., 1996; Hoover and Vertes, 2007), and the ITC cells in turn project to the CeM and are responsible for feed-forward inhibition of CeM

output neurons (Pare and Smith, 1993; McDonald et al., 1996; Royer et al., 1999). Because CeM output neurons receive only sparse projections directly from the IL (McDonald et al., 1996), it has been suggested that IL inhibits CeM output indirectly via projections of the IL to the ITCs. Indeed, Amano and colleagues showed that extinction-induced potentiation of tone responses in IL neurons caused feed-forward inhibition in the CeM, thereby suppressing fear responses (Amano et al., 2010). Consistent with this idea, it has been shown that stimulation of the IL increased c-Fos expression in amygdala ITC cells, and lesion of the ITC cells disrupted expression of fear extinction (Berretta et al., 2005; Likhtik et al., 2008). ITC cells also exhibit NMDA-mediated plasticity, suggesting that they may participate in long-term storage of extinction (Royer and Pare, 2002; Muigg et al., 2008).

In addition to this, the IL also plays a critical role in expression of the extinction memory (Milad and Quirk, 2002; Quirk et al., 2006; Knapska et al., 2012; Kim et al., 2016). Specifically, it has been demonstrated that CS-evoked activity in IL neurons is increased during retrieval of extinction memory *in vivo* (Milad and Quirk, 2002). This observation suggests that IL activity is correlated with extinction retrieval such that its activation by the CS might mediate the expression of fear extinction. However, there are also some studies that have failed to demonstrate the role of the IL in extinction retrieval (Garcia et al., 2006; Sierra-Mercado et al., 2006; Do-Monte et al., 2015). For example, Do-Monte and colleagues re-visited the role of the IL in fear extinction using optogenetic techniques. Consistent with previous findings, they showed that IL activity during

extinction training is necessary for the encoding of extinction memory. However, they showed that IL activity does not appear to be necessary for retrieval of extinction memory. More recently, Kim and colleagues (2015) conducted similar experiments using optogenetics and showed that IL is able to bidirectionally control extinction retrieval. In their study, increased IL activity at the time of retrieval led to better extinction recall, and decreased IL activity resulted in the opposite effect. One major difference between these two studies is that Kim and colleagues targeted both glutamatergic and GABAergic neurons in the IL while Do-Monte and colleagues only targeted glutamatergic neurons during optogenetic manipulation. This suggests that inhibitory interneurons within IL might also play a crucial role in extinction memory retrieval by interacting with local excitatory neurons (Do-Monte et al., 2015; Kim et al., 2016).

In summary, the fact that animals show normal auditory fear conditioning in the absence of cortical areas might suggest that the amygdala and its subcortical connections are sufficient for fear learning. However, as described above, PL and IL are required for fear expression and extinction under normal circumstances. This suggests that the mPFC is able to process the information that the amygdala does not have access to, including the emotional information associated with a stimulus, context, time, internal state, and cognitive-mnemonic information (Sotres-Bayon and Quirk, 2010). Medial prefrontal cortical neurons integrate convergent signals from other brain areas and then drive fear responses via projections to the amygdala once the overall fear signal exceeds a certain

threshold. In the same way, integration of diverse types of information in IL may be needed to determine whether or not to express extinction to a given stimulus in a given context.

The hippocampus

During fear conditioning, rats acquire not only the CS-US association, but also the environmental information where the CS-US association took place. Whereas information regarding the CS and US appears to reach the amygdala via direct projections from primary sensory areas, information concerning contextual cues requires the HPC. Prior work has suggested that the HPC is responsible for assembling contextual representations and transmitting these representations to the amygdala (Maren, 2001; Fanselow and Poulos, 2005). Importantly, different subregions of the HPC are functionally distinct. For instance, the dorsal hippocampus (dHPC), which corresponds to the posterior hippocampus in primates, is primarily involved in spatial representations, whereas the ventral hippocampus (vHPC) is part of the temporal lobe associated with motivation and emotion (Fanselow and Dong, 2010). Pavlovian fear conditioning requires the integration of both spatial and nonspatial (emotional) information, so it is reasonable that both the dHPC and vHPC are importantly involved in the process.

The first evidence for a role of the HPC in contextual fear conditioning came from studies showing that lesions of the dHPC prevented both the acquisition and expression

of contextual fear (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Post-training fiber-sparing neurotoxic lesion of the dHPC also disrupted the expression of contextual fear (Maren et al., 1997), suggesting that the dHPC is important for the initial acquisition and storage of contextual memory. Interestingly, the effect of dHPC lesion is time-dependent. For example, Kim and Fanselow (1992) observed contextual fear deficits in rats that received dHPC lesions 1 day after training but not 28 days after training (Kim and Fanselow, 1992). Similarly, rats trained 50 days prior to surgery displayed intact remote contextual fear memory, but showed impaired memory if the training occurred 1 day prior to surgery (Anagnostaras et al., 1999). These studies suggest that the dHPC is crucial for initial acquisition and storage of the contextual memory, but over time, the memory is transferred elsewhere and becomes hippocampus-independent (Frankland et al., 1998; Sutherland et al., 2010; Sutherland and Lehmann, 2011). However, later studies using more precise temporal control in HPC inactivation suggest that this might not be the case. Goshen and colleagues showed that precise real-time inactivation of the HPC abolished retrieval of context fear memory even after a long period of time; but if the inactivation extended to typical time course of pharmacological inhibition, context memory became HPC-independent (Goshen et al., 2011). This suggests that HPC is indeed responsible for long-term contextual memory retrieval but is capable of adaptively shifting its function to other brain areas when it's not available for a longer period of time. Further, Wilgen and colleagues demonstrated that retrieval of detailed memories still require the HPC but become HPC-independent when the details are lost (Wiltgen et al., 2010). The result suggests that it is not the ages of memory, but is the

detail of the memory that determines the hippocampal involvement. Together, these studies challenged the time-dependent role of the HPC in memory retrieval.

Interestingly, later studies showed that axon-sparing pre-training lesion of the dHPC does not lead to contextual fear conditioning deficits (Maren et al., 1997; Frankland et al., 1998; Cho et al., 1999; Richmond et al., 1999). Therefore, these pre- and post-training lesion studies together indicate that the dHPC is the primary brain structure for contextual encoding and storage when it is intact, but other brain regions compensate for its function when it is damaged. Subsequent studies have suggested that animals with an intact dHPC use a configural strategy to encode the context such that a unified representation of the cues associated with the context is formed in the dHPC (Rudy and O'Reilly, 1999). Once acquired in this manner, the contextual fear memory is sensitive to post-training hippocampal lesions. However, rats without a functional dHPC use an alternative hippocampus-independent strategy to form the contextual fear memory by associating the context directly to the elements, which is less efficient than forming a configural representation (Young et al., 1994; Maren, 2001; Matus-Amat et al., 2004; Maren et al., 2013). Interestingly, memory acquired by a hippocampaus-independent strategy cannot be retrieved during later retrieval test, if the hippocampal activity is restored at the time of the test (Sparks et al., 2011). This indicates that the hippocampus and other brain regions that can compensate for its function interact with each other during contextual encoding and retrieval.

Importantly, contextual representations are acquired during a period of exploration in the conditioning chamber before the delivery of footshock. During exploration, animals acquire sensory and spatial information, and are able to associate the information into a unified contextual representation (Fanselow, 1986, 1990). A lack of exploration prior to the footshock, which occurs when footshock is presented immediately after placing the rats into the conditioning chambers, results in little or no freezing at test in the conditioning context (Fanselow, 1986). The deficit caused by immediate shock can be eliminated by context pre-exposure in the to-be-conditioned context (Fanselow, 1990). NMDA antagonists or protein synthesis inhibitors microinfused into the dHPC prior to the context pre-exposure can disrupt this context pre-exposure facilitation effect (Barrientos et al., 2002; Stote and Fanselow, 2004). Interestingly, dHPC lesions seem to spare fear conditioning to auditory cues (Kim and Fanselow, 1992; Phillips and LeDoux, 1994; Anagnostaras et al., 1999), suggesting that the dHPC is not directly involved in forming CS-US association in auditory fear conditioning. Collectively, the evidence indicates that the dHPC is preferentially involved in contextual encoding in a way that assembles elements into a single unified representation.

Compared to the dHPC, the role of the vHPC in fear conditioning is more complicated. In contrast to the dHPC, vHPC lesions do not results in deficits in tasks that require spatial memory such as Morris water maze or radial arm maze (Moser et al., 1995; Pothuizen et al., 2004). However, in studies that involve emotional responses, the vHPC, but not dHPC, seems to play a crucial role (Kjelstrup et al., 2002). Ferbinteanu and

colleagues showed that rats with dHPC lesions exhibit decreased performance in returning to the previously rewarded arm in the conditioned place preference test but rats with vHPC lesions show increased performance in the same test (Ferbinteanu and McDonald, 2001). The opposing effects suggest that dHPC lesion interfered with spatial memory processing, whereas vHPC lesion spared spatial memory but influenced the affective aspect of the memory associated with the food reward. Similarly, vHPC manipulations in fear conditioning tasks impair fear memories to both context and auditory cues, but the effects are less straightforward than dHPC manipulations. Deficits caused by either lesions or inactivation of the vHPC are more pronounced and general compared to that of the dHPC (Fanselow and Dong, 2010). Similar to the dHPC manipulation, NMDA antagonists infused into the vHPC block the acquisition of context fear but not tone fear (Zhang et al., 2001; Quinn et al., 2002). However, lesions or muscimol infusion in the vHPC blocks tone fear and produces less consistent effects on context fear (Maren and Holt, 2004; Hunsaker and Kesner, 2008). The discrepancy could be partially explained by the fact that the vHPC and amygdala have strong reciprocal projections (Hoover and Vertes, 2007). The interconnecting projections enable the vHPC to modulate emotional responses mediated by the amygdala (Henke, 1990). Furthermore, the vHPC might act as a relay structure between the dHPC and amygdala that translates cognitive and spatial knowledge into motivation and action critical for survival (Bast, 2007; Dong et al., 2009).

Interestingly, inactivation of the dHPC prior to extinction attenuates extinction

acquisition and disrupts the contextual encoding (Corcoran et al 2005), indicating that the HPC also plays a role in encoding extinction memory (Radulovic and Tronson, 2010). Moreover, a series of other studies indicate that the HPC is a key structure for context-dependent memory retrieval after extinction. For example, inactivation of either the dorsal or ventral HPC prior to retrieval test disrupts fear renewal but not extinction retrieval (Corcoran and Maren, 2001, 2004; Hobin et al., 2006). Importantly, neuronal responses in the amygdala associated with fear renewal were abolished by inactivation of the dHPC, suggesting that the HPC is regulating contextual retrieval by interacting with the amygdala. Given the important role of the HPC in contextual retrieval after extinction, its role within the neuronal circuit underlying contextual memory retrieval will be discussed further.

Neurobiology of contextual fear memory retrieval

The contextual information encoded during an experience supports the later retrieval of that information, a phenomenon known as encoding specificity (Tulving and Thomson, 1973) and contextual retrieval (Hirsh, 1974). That is, the content of what is remembered about a particular event is often critically dependent on where that memory is retrieved. Deficits in contextual retrieval are associated with memory impairments accompanying a variety of neural insults including age-related dementia, traumatic brain injury, stroke, and neurodegenerative disease (Maren et al., 2013). As such, understanding the neural circuits mediating contextual retrieval is essential for targeting interventions to alleviate memory disorders and associated cognitive impairments.

Decades of research in both humans and animals have revealed that the HPC and mPFC are essential for the encoding and retrieval of memories (Kennedy and Shapiro, 2004; Hasselmo and Eichenbaum, 2005; Diana et al., 2010; Preston and Eichenbaum, 2013). Indeed, prior work suggests that communication between these brain areas is essential for episodic memory processes (Simons et al., 2002; Preston and Eichenbaum, 2013). Anatomically, neurons in the HPC have a robust projection to the mPFC, including the IL and PL in rats (Swanson and Kohler, 1986; Jay and Witter, 1991; Thierry et al., 2000; Varela et al., 2014). In primates, there are projections that originate from hippocampal CA1 and terminate in the orbital and medial frontal cortices (areas 11, 13, 14c, 25 and 32) (Zhong et al., 2006). These PFC connectivity patterns seem to be similar in humans and monkeys; for example, both humans and monkeys have fimbria/fornix fibers (which originate from the hippocampus and subiculum) terminating in the medial orbital PFC (Cavada et al., 2000; Croxson et al., 2005). For these reasons, models of memory retrieval have largely focused on the influence of contextual representations encoded in the HPC on retrieval processes guided by the mPFC (Maren and Holt, 2000; Hasselmo and Eichenbaum, 2005; Ranganath, 2010). Yet emerging evidence suggests that the mPFC itself may be critical for directing the retrieval of context-appropriate memories in the HPC (Navawongse and Eichenbaum, 2013; Preston and Eichenbaum, 2013). This suggests that indirect projections from the mPFC to HPC may be involved in contextual memory retrieval (Davoodi et al., 2009; Davoodi et al., 2011; Hembrook et al., 2012; Xu and Sudhof, 2013). Moreover, abnormal interactions between the HPC and mPFC are associated with decreased mnemonic ability as well as disrupted emotional control,

which are major symptoms of psychiatric disorders such as schizophrenia, depression, specific phobia, and PTSD (Sigurdsson et al., 2010; Godsil et al., 2013; Maren et al., 2013). This section will focus on the anatomy, physiology and functional role of the HPC-mPFC pathway in relation to memory and emotion in an effort to understand how dysfunction in this network contributes to contextual control of fear memory.

It has long been appreciated that there are both direct monosynaptic projections, as well as indirect polysynaptic projections between the HPC and the mPFC (Hoover and Vertes, 2007). In rats, injections of retrograde tracers into different areas of the mPFC robustly label neurons in the vHPC and subiculum (Jay et al., 1989; Hoover and Vertes, 2007). In addition, injections of the anterograde tracer, Phaseolus vulgaris-leucoagglutinin (PHA-L), into the HPC reveal direct projections to the mPFC (Jay and Witter, 1991). Hippocampal projections to the mPFC originate primarily in ventral CA1 and ventral subiculum; there are no projections to the mPFC from the dHPC or dentate gyrus. Hippocampal projections course dorsally and rostrally through the fimbria/fornix, and then continue in a rostro-ventral direction through the septum and the nucleus accumbens (NAcc), to reach the IL, PL, medial orbital cortex, and anterior cingulate cortex (Jay and Witter, 1991; Cenquizca and Swanson, 2007). Afferents from CA1 and the subiculum are observed throughout the entire rostro-caudal extent of the mPFC, with only sparse projections to the medial orbital cortex.

Indirect pathways from the HPC to mPFC include projections through the NAcc and

ventral tegmental area (VTA), amygdala, entorhinal cortex (EC), and midline thalamus (Maren, 2011; Russo and Nestler, 2013; Wolff et al., 2015) (Figure 1-1). These complex multi-synaptic pathways from both subcortical and cortical areas are critically involved in higher cognitive functions that are related to several major psychiatric disorders. For example, the mPFC projects to the thalamic nucleus reuniens (RE), which in turn has dense projections to the HPC (Varela et al., 2014). Importantly, these projections are bidirectional, which provides another route for the HPC to influence the mPFC. Interestingly, it has been shown that single RE neurons send collaterals to both the HPC and mPFC (Hoover and Vertes, 2012; Varela et al., 2014), which places the RE in a key position to relay information between the mPFC and HPC and to coordinate their functions (Davoodi et al., 2009; Davoodi et al., 2011; Hembrook et al., 2012; Hoover and Vertes, 2012; Xu and Sudhof, 2013; Varela et al., 2014; Griffin, 2015; Ito et al., 2015). Interestingly, the CA1 and subiculum send direct projections back to the mPFC, allowing a functional loop that enables interactions between cortical and subcortical areas during memory encoding and retrieval (Preston and Eichenbaum, 2013).

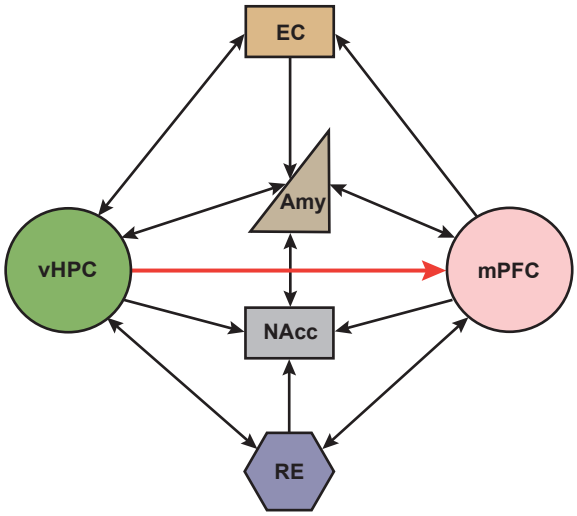

Figure 1-1 Neural circuits between the medial prefrontal cortex and hippocampus. vHPC has strong direct projections to the mPFC, but there are no direct projections from the mPFC back to the HPC. The reuniens (RE) and amygdala have reciprocal connections with both the mPFC and HPC. NAcc receives inputs from mPFC, HPC, RE and amygdala. mPFC also project to entorhinal cortex (EC) which in turn has reciprocal projections with HPC. vHPC, ventral hippocampus; EC, entorhinal cortex; Amy, amygdala; NAcc, nucleus accumbens; RE, nucleus reuniens; mPFC, medial prefrontal cortex.

The physiology of projections between the HPC and mPFC has been extensively investigated in rodents. These projections consist of excitatory glutamatergic pyramidal neurons that terminate on either principal neurons or GABAergic interneurons within the mPFC (Jay et al., 1992; Carr and Sesack, 1996; Tierney et al., 2004). Electrical stimulation in hippocampal area CA1 or the subiculum produces a monosynaptic excitatory postsynaptic potential (EPSP) followed by fast and slow inhibitory postsynaptic potentials (IPSPs); the latter are due to both feedforward (Jay et al., 1992; Tierney et al., 2004) and feedback inhibition (Degenetais et al., 2003). Excitatory responses evoked in mPFC neurons by electrical stimulation of the HPC are antagonized by CNQX but not by AP5, indicating that these responses are AMPA receptor-dependent (Jay et al., 1992). Hippocampal synapses in the mPFC exhibit activity-dependent plasticity including long-term potentiation (LTP), long-term depression (LTD), and depotentiation (Laroche et al., 1990; Jay et al., 1996; Burette et al., 1997; Takita et al., 1999; Laroche et al., 2000). These forms of plasticity are NMDA receptor-dependent and involve activation of serine/threonine kinases such as CaMKII, PKC, and PKA (Dudek and Bear, 1992; Bliss and Collingridge, 1993; Jay et al., 1995; Burette et al., 1997; Jay et al., 1998; Takita et al., 1999). Within the indirect mPFC-RE-HPC pathway, a large proportion of RE projection neurons are glutamatergic (Bokor et al., 2002). RE stimulation produces strong excitatory effects on both HPC and PFC neurons (Dolleman-Van der Weel et al., 1997; Bertram and Zhang, 1999; McKenna and Vertes, 2004), suggesting that the RE is capable of modulating synaptic plasticity in both the HPC and mPFC (Di Prisco and Vertes, 2006; Eleore et al., 2011).

When humans and animals form new memories, contextual information associated with the experience is also routinely encoded without awareness (Tulving and Thomson, 1973). Contextual information plays an important role in memory retrieval since the content of what is recalled is often critically dependent on where that memory is retrieved (Hirsh, 1974; Maren and Holt, 2000; Bouton, 2002). This contextual retrieval process allows the meaning of a cue to be understood according to the context in which it is retrieved (Maren et al., 2013). For example, encountering a lion in the wild might be a life-threatening experience to someone, but seeing the same lion kept in its cage in the zoo might be an interesting (and non-threatening) experience. Therefore, the same cue in different contexts has totally different meanings. Contextual processing is highly adaptive because it resolves ambiguity during memory retrieval (Bouton, 2002; Maren et al., 2013; Garfinkel et al., 2014). Research in both humans and animals has revealed that the HPC and mPFC are essential for contextual retrieval (Kennedy and Shapiro, 2004; Hasselmo and Eichenbaum, 2005; Diana et al., 2010; Maren et al., 2013). Humans and animals with disconnections in the HPC-mPFC network have deficits in retrieving memories that require contextual information (Schacter et al., 1984; Shimamura et al., 1990; Simons et al., 2002).

Recent work indicates that the HPC-mPFC interaction plays a critical role in regulating context-dependent fear memory retrieval after extinction (Orsini et al., 2011; Maren et al., 2013; Jin and Maren, 2015a). Disconnection of the vHPC from the mPFC impairs fear renewal after extinction (Orsini et al., 2011). Inactivation of the vHPC also

modulates the activity of both interneurons and pyramidal neurons in the PL, and influences the expression of fear behavior in extinguished rats (Sotres-Bayon et al., 2012). Moreover, vHPC neurons projecting to both the mPFC and amygdala are preferentially involved in fear renewal (Jin and Maren, 2015a), suggesting that vHPC might modulate memory retrieval by coupling activity in the mPFC and amygdala. Ultimately, the hippocampus appears to gate reciprocal mPFC-amygdala circuits involved in the expression and inhibition of fear (Herry et al., 2008; Knapska and Maren, 2009; Knapska et al., 2012). It has also been shown that the vmPFC-HPC network is involved in the context-dependent recall of extinction memories in humans as well (Kalisch et al., 2006; Milad et al., 2007). These observations support the idea that the HPC-mPFC pathway is critically involved in the context-specificity of fear memories, whereby the transmission of contextual information from the HPC to the mPFC generates context-appropriate behavioral response by interacting with the amygdala. Interestingly, the indirect HPC-mPFC interaction via the RE might be another important pathway that could regulate contextual memory retrieval. It is well-documented that damage to the midline thalamic nuclei, including the RE, results in profound amnesia in humans (Mennemeier et al., 1992). Moreover, recent work has revealed that the mPFCàREàHPC circuit is involved in maintaining the specificity of context fear memories (Xu and Sudhof, 2013). This raise the possibility that the mPFC mediates the retrieval of context-appropriate memories by interacting with the HPC, and that the RE might be a critical relay structure for this interaction.

Specific aims and hypotheses

The main focus of the present dissertation is to explore how direct and indirect interactions between the mPFC and HPC modulate contextual fear memory retrieval. The vHPC is required for fear renewal via interactions with both the amygdala and mPFC. Within the mPFC, the PL and IL have opposing roles in expression and extinction of conditioned fear. While the PL has been implicated in fear expression as well as fear renewal after extinction, the IL is known as a key structure for extinction learning and fear suppression after extinction. The vHPC interacts with both the PL and IL via its direct projections, but what role each pathway plays in contextual memory retrieval is not clear. In addition to the hippocampal-prefrontal direct pathway, the indirect prefrontal-hippocampal pathway is also critically involved in contextual retrieval, especially of episodic memories. The mPFC is capable of guiding expression of context-appropriate memory by interacting with the HPC, but this interaction is indirect due to the lack of direct projections from the mPFC to HPC. Thus, the indirect prefrontal projections to the HPC via the RE might be one of the candidates for the modulation. The RE has reciprocal projections to both the mPFC and HPC, and it has shown that the RE is critically involved in behavioral tasks that require interactions of the mPFC and HPC. Therefore, it is highly possible that indirect prefrontal-hippocampal modulation of contextual retrieval requires RE to be a relay center. Accordingly, in Chapters II and III, I (?) explored the role of vHPC→mPFC pathways in contextual memory retrieval, and in Chapter IV, we examined the possibility that contextual memory retrieval also receives modulation from mPFC→RE→HPC indirect pathway.

The behavioral paradigm for contextual modulation of fear memory after extinction is shown in Figure 1-2.

Specifically, in Chapter II, we examined if one of two pathways, either between the vHPC and PL or vHPC and BA, was predominantly activated during renewal over the other. At the same time, we sought to determine if PL- and BLA-dual projecting vHPC neurons were also preferentially involved in fear renewal. To this end, we infused two different fluorescently labeled retrograde tracers (AlexaFluor-conjugated cholera toxin B, CTb) into the BLA and PL. We then examined whether CTb-labeled vHPC-projecting neurons exhibit distinct activity-dependent c-Fos expression. We hypothesized that vHPC-projecting neurons to the PL, BLA or both are preferentially activated during fear renewal but not during extinction retrieval. We also expected that vHPC→PL would be preferentially involved in fear renewal.

In Chapter III, we hypothesized that the ventral hippocampal projections to the IL are involved in fear renewal by suppressing inhibitory control of the IL in the extinction context. To test the hypothesis, we examined whether inhibition or activation of vHPC projections to IL prevents or promotes fear renewal. Furthermore, we examined whether GABAergic signaling within the IL is involved in context-dependent memory retrieval, and if this GABAergic modulation depends on the vHPC→IL pathway. To answer these questions, we used adeno-associated viral (AAV) vectors to express DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) in a circuit-specific

manner. Specifically, we infused CAV2-Cre into the efferent region of interest (IL), where incoming projections to the region will take up and express Cre recombinase. At the same time, we infused Cre-dependent AAVs expressing either hM4d(Gi) or hM3d(Gq) into the vHPC, inducing Cre-expressing neurons in the vHPC to express Cre-dependent DREADDs. Systemic injections of the designer ligand, clozapine-*N*-oxide (CNO) were used to inactivate or activate DREADD-expressing neurons during fear retrieval/renewal testing. We expected that inactivation of vHPC→IL circuit would decrease fear renewal whereas activation of the same circuit in safe context will cause fear relapse.

Finally, in Chapter IV, we hypothesized that in addition to the hippocampal control on the mPFC, indirect prefrontal control on the HPC, via the RE, is also crucial in contextual retrieval. To explore this possibility, we first ran a series of experiments to see if the RE is required for contextual retrieval. The experiments included muscimol inactivation of the RE during retrieval and renewal test, and detection of c-Fos expression in the RE during extinction retrieval and renewal. Then we manipulated activity of specific neural circuits interconnecting the mPFC, RE and HPC, again using a circuit-specific DREADDs approach, to interrogate their contributions in contextual memory retrieval. We expected that both mPFC→RE and RE→HPC pathways would be critical for contextual memory retrieval.

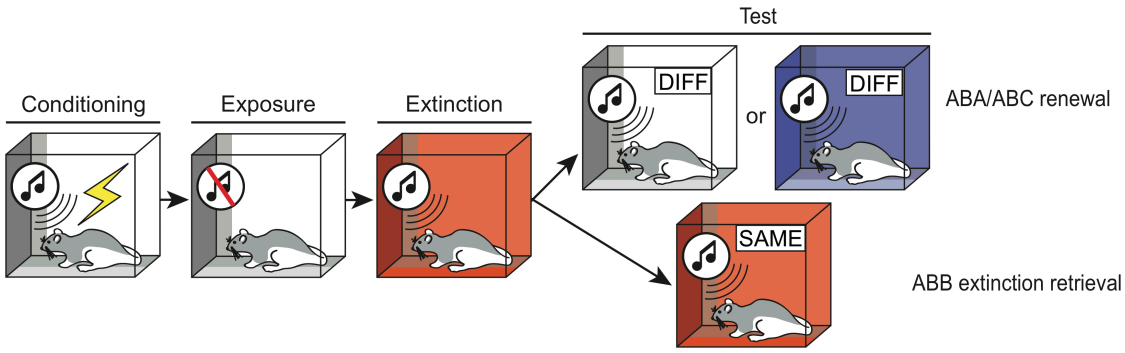

Figure 1-2 Fear renewal and extinction retrieval paradigm. In this paradigm, rats received auditory fear conditioning in context A (white chamber) on day 1. On day 2, rats received exposure in context A in the morning and tone extinction in context B (red chamber). On the testing day, rats underwent fear renewal test in either context A or context C (blue chamber) or extinction retrieval test in the same context as extinction context (context B).

CHAPTER II

FEAR RENEWAL PREFERENTIALLY ACTIVATES VENTRAL HIPPOCAMPAL NEURONS PROJECTING TO BOTH AMYGDALA AND PREFRONTAL CORTEX

Introduction

In recent years, considerable effort has been focused on understanding the neural mechanisms underlying the extinction and renewal of learned fear (Bouton et al., 2006; Quirk and Mueller, 2008; Milad and Quirk, 2012; Orsini and Maren, 2012). During extinction, repeated exposure to a conditioned stimulus (CS) gradually decreases the probability and magnitude of the conditioned fear response (CR) (Pavlov, 1927). However, substantial evidence suggests that extinction does not eliminate the fear memory; rather, it generates a new extinction memory that competes with the fear memory for control of behavior (Bouton and Bolles, 1979; Bouton, 1993). Importantly, the extinction memory is highly context-dependent insofar as it is only expressed in the extinction context. That is, if animals encounter the CS outside of the extinction context, the conditioned fear response returns or "renews". The renewal of extinguished fear is a considerable challenge for maintaining long-lasting fear suppression after exposure-

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based therapies for anxiety-, trauma-, and stress-related disorders (Bouton et al., 2001; Rothbaum and Davis, 2003; Vervliet et al., 2013).

Recent studies reveal that a brain circuit involving the hippocampus (HPC), medial prefrontal cortex (mPFC) and basal amygdala (BA) is important for the context-dependence of extinguished fear memories (Herry et al., 2008; Knapska and Maren, 2009; Orsini et al., 2011; Knapska et al., 2012; Orsini et al., 2013). For example, pharmacological inactivation of the hippocampus disrupts context-dependent firing in the amygdala and prevents fear renewal (Corcoran and Maren, 2001; Hobin et al., 2006; Maren and Hobin, 2007). Furthermore, immediate early gene expression in the vHPC, BA and PL is context-dependent (Knapska and Maren, 2009; Orsini et al., 2013), and amygdala neurons receiving PL and vHPC efferents are recruited during fear renewal (Knapska et al., 2012). Lastly, disconnection of the vHPC with either the BA or PL eliminates fear renewal (Orsini et al., 2011). These findings suggest that the hippocampus may gate neural activity in either the PL or BA to regulate the context-dependent expression of fear after extinction (Vlachos et al., 2011). However, the relative contribution of vHPC projections to the BA and PL in this process is not clear.

Neuroanatomical studies have shown that within the vHPC, projections to the BA and mPFC originate from ventral CA1 (vCA1) and the ventral subiculum (vSUB) (Canteras and Swanson, 1992; Pitkanen et al., 2000; Vertes, 2006). Although the majority of vCA1 and vSUB neurons project to either the BA or mPFC, some vHPC neurons project to

both areas (Ishikawa and Nakamura, 2006). These dual-projecting neurons (i.e., vHPC neurons projecting to both PL and BA) may be particularly important for coordinating mPFC and BA activity during memory retrieval, a function suggested by the similar consequences of vHPC-PL and vHPC-BA disconnections on fear renewal (Orsini et al., 2011).

Therefore, in this chapter, we explored the possibility that one circuit is predominantly involved in contextual retrieval than the other in the distributed neuronal network consisting of the vHPC, BA and PL. Specifically, we examined if the vHPC is regulating fear renewal predominantly via direct pathway to the PL or to the BA. At the same time, taking advantage of dual retrograde tracer infusions, we were able to detect if vHPC neurons sending collaterals to both the PL and BA were also preferentially involved in contextual retrieval according to a previous study showing the existence of dual-projecting neurons in vHPC (Ishikawa and Nakamura, 2006). For that, we used fluorescently labeled retrograde tracers (AlexaFluor-conjugated cholera toxin B, CTb) and c-Fos immunohistochemistry to examine retrieval-induced Fos expression in vCA1 and vSUB neurons projecting to the PL and/or BA.

Materials and methods

Subjects. The subjects were 26 Long-Evans male adult rats (200–224 g; Blue Spruce) obtained from Harlan. The rats were individually housed on a 14/10 h light/dark cycle and had access to food and water *ad libitum*. Rats were handled for 5 d before the

experiment. All experimental procedures were performed in accordance with the protocols approved by the Texas A&M University Animal Care and Use Committee.

Behavioral apparatus and contexts. Eight identical observation chambers (30×24×21 cm; Med-Associates) were used in all behavioral sessions. The observation chambers were constructed of two aluminum sidewalls and a Plexiglas ceiling, rear wall, and hinged front door. The floor of each chamber consisted of 19 stainless steel rods that were wired to a shock source and a solid-state grid scrambler (Med-Associates) for the delivery of foot shock. A speaker mounted outside of the grating in one wall of the chamber was used for the delivery of acoustic tone. Additionally, ventilation fans and house lights were installed in each chamber to allow for the manipulation of contexts. Sensory stimuli were adjusted within these chambers to generate three distinct contexts A, B and C. For context A, a 15-W house light mounted opposite to the speaker was turned on, and the room light remained on. Ventilation fans (65 dB) were turned on, cabinet doors were left open, and the chambers were cleaned with 70% ethanol. Rats were transported to context A in white plastic boxes without beddings. For context B, house lights were turned off and overhead lighting was provided by fluorescent red lights. Ventilation fans were turned off, the cabinet doors were closed and the chambers were cleaned with 1% acetic acid. Rats were transported to context B in black plastic boxes without beddings. For context C, both the house lights and the red fluorescent overhead lights were turned on. Ventilation fans were off and the cabinet doors were left open. Black Plexiglas floors were placed on the grid of each chamber and the chambers

were cleaned with 1% ammonium hydroxide. Rats were transported to context C in white buckets with beddings. In each context, stainless steel pans were filled with a thin layer of the respective odors of the contexts and inserted below the grid floor. Each conditioning chamber rests on a load-cell platform that is used to record chamber displacement in response to each rat's motor activity and is acquired online via Threshold Activity software (Med Associates). Before the experiment, all load cell amplifiers were calibrated to a fixed chamber displacement and load-cell amplifier output (-10 to +10 V) from each chamber is digitized and absolute values of the load-cell voltages are computed and multiplied by 10 to yield a scale that ranges from 0 to 100. For each chamber, load-cell voltages are digitized at 5 Hz, yielding one observation every 200 ms. Freezing is quantified by computing the number of observations for each rat that has a value less than the freezing threshold (load-cell activity = 10). Freezing is only scored if the rat is immobile 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, the rats were placed into stereotaxic apparatus (David Kopf Instruments) and 27-gauge injectors were lowered into the BA (A/P: -3.0, M/L: ±5.1, D/V: -9.2 from skull) and PL (A/P: +2.9, M/L: ±1.5, D/V: -3.5 from dura; all units are mm). For PL injection, the stereotaxic arm was lowered at a 15° angle to prevent damage to the superior sagittal sinus. The injector was attached to polyethylene tubing, which was connected to a Hamilton syringe (10 ml) located on an infusion pump. AlexaFluor-594

and AlexaFluor-488 conjugated cholera toxin B (CTb) (Life Technology) were unilaterally infused at a rate of 0.1 µl/min for 4 min (0.4 µl total; 5 µg/µl) into BA and PL, respectively. The injectors remained in the injection sites for 10 min to allow for the diffusion of CTbs. Rats were placed back in their home cages to allow for 1 week of post-operative recovery.

Behavioral procedures. Twenty-six rats were randomly assigned to three different groups: DIFF (*n*= 10), SAME (*n*= 10) and HOME (*n*= 6). I used a three-context (‘‘ABC’’) renewal procedure (Orsini et al 2011) in which one group (DIFF) was conditioned in context A, extinguished in context B and tested in context C and another group (SAME) was conditioned in context A, extinguished in context C and tested in context C. Rats in the HOME control group were conditioned in context A and extinguished either in context B or C (counterbalanced); they were left in their home cages during the test session. The ABC design permits the assessment of fear and Fos expression to an extinguished CS independent of fear to the context in which the CS is tested. That is, the test context (context C) has never been paired with footshock. Moreover, this design allowed us to test all rats in the identical context, hence any differences in behavior or c-Fos expression can be attributed to the meaning of the CS in that context and not the CS or context itself.

After surgery, rats underwent fear conditioning in context A. Conditioning consisted of 5 tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) pairings with 60 s intertrial

intervals (ITIs). Twenty-four hours after the conditioning session, rats underwent fear extinction in context B or context C in which they received 45 tone-alone (30 s ITI) presentations. Prior to 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. Twenty-four hours after extinction, rats were returned to context C for a test session consisting of 5 tone-alone (30 s ITI) presentations. In all behavioral sessions, the chamber position of each animal was counterbalanced.

Immunohistochemistry. Ninety minutes after the first tone of the test session, rats were overdosed with sodium pentobarbital (0.5 ml) and were transcardially perfused with ice-cold 0.01 M PBS (pH 7.4) and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH=7.4). Animals were sacrificed in groups of three, with one rat from each group represented in each squad (one rat from the HOME control group was randomly chosen to be sacrificed along with the SAME and DIFF rats in each squad). Brains were extracted and stored in 4% PFA solution for 18 h at 4 °C and transferred to 30% sucrose. Coronal brain sections (30 μm) were made on a cryostat maintained at -20 °C. Sections containing vCA1 and vSUB were collected every 210 μm.

Immunohistochemistry was performed on free-floating brain sections. The sections were washed two times in 1×Tris-buffered saline (TBS, pH= 7.4) for 10 min followed by a third wash in 1×TBS with 0.1% Tween 20 (TBST, pH= 7.4). Brain sections were then

incubated in 10% normal donkey serum (NDS) in TBST for 1 h at room temperature followed by two washes in TBST for 5 min. Tissue was then incubated in primary antibody solution in TBST with 2% NDS (goat anti-c-Fos antibody at 1:2000; sc-52G, Santa Cruz Biotechnology) for 72 h at 4 °C. Brain sections were then washed three times in TBST for 10 min and were incubated in secondary antibody solution in TBST with 2% NDS (biotinylated donkey anti-goat secondary antibody at 1:200; sc-2042, Santa Cruz Biotechnology) for 2 h at room temperature. After being rinsed in TBST three times for 10 min, brain sections were incubated in streptavidin conjugated AlexaFluor 350 (Streptavidin-AF350 at 1:500; s-11249, Life Technology) in TBST with 2% NDS for 1 h at room temperature. Tissue was washed three times in TBS for 10 min and then was mounted onto subbed slides in 0.9% saline and cover slipped with Fluoromount (Sigma-Aldrich).

Image analysis. Three images for the vCA1 (-5.6, -6.3, and -6.8 mm posterior to bregma) and two images for the vSUB (-6.3, and -6.8 mm posterior to bregma) were taken for the quantification. All images were taken at 10×magnification (660 μm × 876 μm, 0.58 mm^2) with an Olympus BX53 microscope. For each region, single-, double-, and triple-labeled neurons for each fluorophore were counted. Counts for each image of the brain region were averaged.

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) post

hoc tests, which were performed after a significant overall F ratio. All data are represented as means ± SEM. Four rats failed to extinguish (freezing during the last block was > 80% of the freezing in the first block) and another two rats exhibited c-Fos counts that were > 2.5 SD outside the group mean; these rats were excluded from the neuronal and behavioral analyses. Hence, the final group sizes were SAME (n= 8), DIFF (n= 8), and HOME (n= 4).

Results

Freezing behavior during the conditioning session is shown in Figure 2-1. All rats increased their levels of freezing during the conditioning session [main effect of block, $F_{(5, 95)}$= 32.4, p< 0.001], and the levels of freezing did not differ between the groups [main effect of group and group × block interaction, F< 1]. During extinction, rats in all of the groups exhibited high levels of conditioned freezing to the CS and similar reductions in conditioned freezing across the extinction session [main effect of block, $F_{(9, 171)}$= 15.3, p< 0.001; main effect of group and group × block interaction, F< 1.3]. As previous studies have shown, the expression of conditioned freezing to the extinguished CS was context-dependent. Conditioned freezing was low when the extinguished CS was presented in the extinction context (SAME), whereas rats tested outside of the extinction context (DIFF) exhibited higher levels of conditioned freezing [main effect of group, $F_{(1,14)}$= 4.5, p< 0.05]. Importantly, renewal was not attributable to the contextual freezing because baseline freezing to the context was not significantly different between groups (p= 0.4). Moreover, differential freezing among the SAME and DIFF groups was

not attributable to physical differences in the test contexts since all testing was conducted in an identical context with the same CS.

Representative CTb injection sites in PL and BA are shown in Figure 2-2A along with a schematic illustration of maximal and minimal infusions. PL- and BA-projecting neurons in vCA1 and vSUB were labeled with different AlexaFluor-CTb conjugates and c-Fos was visualized with AlexaFluor 350 (Figure 2-2B). For all of the animals in the analysis, the number of CTb-labeled neurons in vCA1 and vSUB are shown in Figure 2-3A. In both hippocampal regions, there were more neurons projecting to BA than PL, and a significantly smaller proportion of the neurons projecting to both areas. This impression was confirmed by a two-way ANOVA with factors of brain region (vCA1 and vSUB) and cell type (PL, BA or PL+BA) that revealed a main effect of neuron type [$F_{(2,6)}$= 113; p< 0.001]. Post hoc comparisons revealed significantly higher number of BA-projecting neurons than PL-projecting neurons in both vCA1 and vSUB (p< 0.0001). In addition, the number of dual-projecting neurons was significantly lower than the numbers of neurons projecting to either PL or BA alone (p< 0.0001). Dual-projecting neurons accounted for roughly 10 % of the total labeled neurons in the ventral hippocampus.

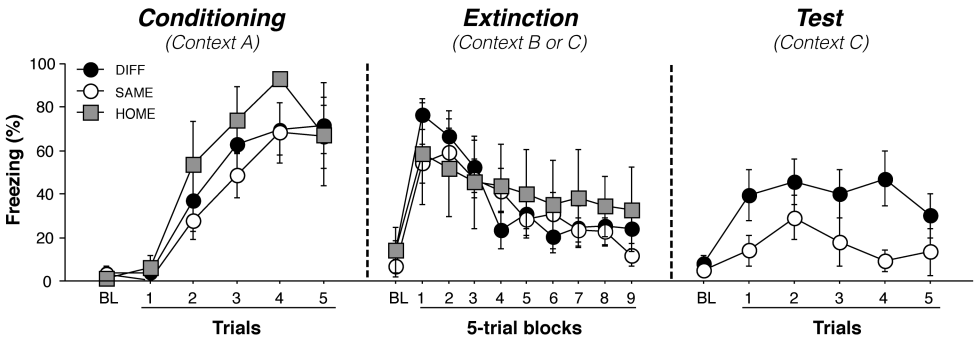

Figure 2-1 Conditioned freezing behavior. Left, Mean (± SEM) percentage of freezing during fear conditioning. Freezing was averaged across the 3 min pre-CS baseline (BL) as well as during each of the five conditioning trials; each trial consisted of the average of freezing during each CS presentation and the subsequent ITI. Middle, Mean (± SEM) percentage of freezing during the 45 tone-alone extinction trials. Freezing was averaged across the BL as well as during the 45 extinction trials; each trial consisted of the average of freezing during each CS presentation and subsequent ITI (data are presented as nine 5-trial blocks). Right, Mean (± SEM) percentage of freezing during the test session, which consisted of 5 tone-alone presentations with 30s ITIs. Freezing was measured during the BL period and during the 5 trials, each of which consisted of a CS presentation and the subsequent ITI. Data are shown for rats that were tested outside the extinction context (DIFF; black circles), tested within the extinction context (SAME; white circles), or not tested at all (HOME; gray squares).

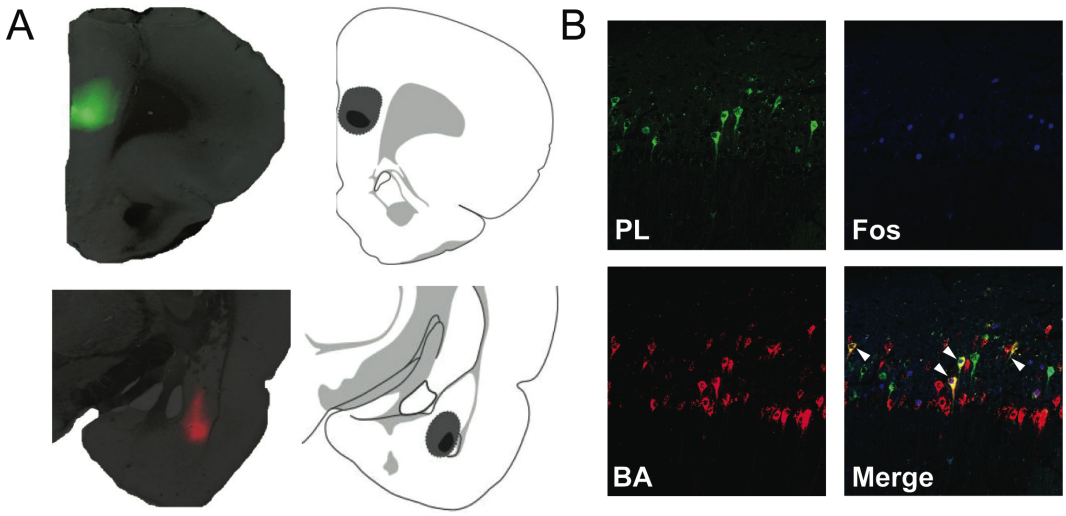

Figure 2-2 CTbs in vHPC after infusions in PL and BA. Alexa fluor conjugated cholera toxin B (CTb) infusion sites within the PL and BA. (A) Representative coronal sections displaying the site of the CTb injections in PL and BA; schematic indicates maximal (dark gray) and minimal (black) spread in each region. (B) Images of representative coronal sections (10×) showing Fos- and CTb-labeling from a representative rat. BA-projecting neurons are red, PL-projecting neurons are green, and dual-projecting neurons (PL+BA, white arrowheads) are yellow; Fos-positive neurons are blue.

Next, Fos expression in all vCA1 and vSUB neurons (regardless of their projection targets) as a function of memory retrieval group (SAME, DIFF, or HOME) was examined. As shown in Figure 2-3, Fos expression in vCA1 and vSUB was influenced by the context in which retrieval testing occurred. In vCA1, the number of Fos-positive neurons in both of the retrieval conditions was similarly elevated relative to HOME controls. However, Fos expression was context-dependent in vSUB. That is, Fos expression in vSUB was greater when the CS was presented outside the extinction context (DIFF) relative to when it was presented in the extinction context (SAME) or in rats that were not tested (HOME). These impressions were confirmed in a two-way ANOVA with factors of brain region (vCA1 and vSUB) and group (DIFF, SAME, and HOME) that revealed a significant main effect of group [$F_{(2,17)}= 4.52$, $p< 0.05$] and a significant group × region interaction [$F_{(2,17)}= 4.47$, $p< 0.05$]. Post-hoc comparisons ($p< 0.05$) revealed that in vCA1 both SAME and DIFF rats exhibited greater levels of Fos expression than rats in the HOME control (but did not differ from one another). In contrast, in vSUB rats in the DIFF condition exhibited significantly greater Fos expression than rats in the SAME or HOME groups (which did not differ from each other).

Whether Fos was expressed in vHPC neurons projecting to PL, BA, or both areas, and whether neurons with different efferent targets exhibited different degrees of Fos expression were next examined. In general, only a small percentage (~5%) of CTb-labeled neurons expressed Fos in the retrieval conditions, a finding that is consistent

with previous reports (Orsini et al 2011). A three-way ANOVA with factors of group (SAME, DIFF or HOME), brain region (vCA1 or vSUB) and cell-type (PL-, BA-, or dual-projecting) revealed only a significant main effect of group [main effect of group, $F_{(2,18)}= 4.26$, $p< 0.05$]; all other main effects and interactions fell short of significance. Because the pattern of Fos expression in CTb-labeled neurons did not differ between vCA1 and vSUB, neurons in these areas were collapsed to simplify the analysis. As shown in Figure 2-3C, Fos expression among CTb-labeled neurons was highest among rats in the DIFF group. Post-hoc comparisons ($p< 0.05$) revealed that DIFF rats (the condition associated with fear renewal) exhibited a greater percentage of Fos-positive CTb-labeled neurons than rats in the HOME control; SAME rats did not differ from the HOME control. These results are congruent with previous results from our laboratory showing renewal-induced increases in Fos expression in BA-projecting neurons in the ventral hippocampus (Orsini et al 2011).

Importantly, inspection of Figure 2-3C reveals that a greater proportion of dual-projecting neurons exhibited Fos expression relative to neurons projecting only to BA or PL when animals renewed their fear (i.e., in the DIFF condition). Planned comparisons in the form of one-way ANOVAs in each behavioral group revealed a significant difference between the cell types in only the DIFF condition [$F_{(2, 14)}= 3.70$, $p< .05$]. Post-hoc comparisons ($p< 0.05$) revealed that dual-projecting neurons were nearly twice as likely to express Fos as neurons projecting to either PL or BA alone. Hence, among the entire population of vCA1 and vSUB neurons that projected to BA or PL, the subset of

neurons projecting to both regions (i.e., the dual-projecting neurons) was more likely to express Fos after fear renewal.

The present results reveal that the renewal of extinguished fear is associated with Fos expression in vCA1 and vSUB neurons projecting to PL and/or BA. Thus, whether there was a relationship between the number of Fos-positive projection neurons in the ventral hippocampus and freezing behavior during the retrieval test was analyzed. As shown in Figure 2-4, there was a strong positive correlation (Pearson $r = 0.57$, $p < .01$) between freezing behavior and the number of Fos-positive projection neurons when the rats in each group were aggregated. This supports the view that the ventral hippocampus plays a key role in fear renewal, and suggests that relapse may be particularly dependent on those neurons projecting to the prefrontal cortex and basal amygdala.

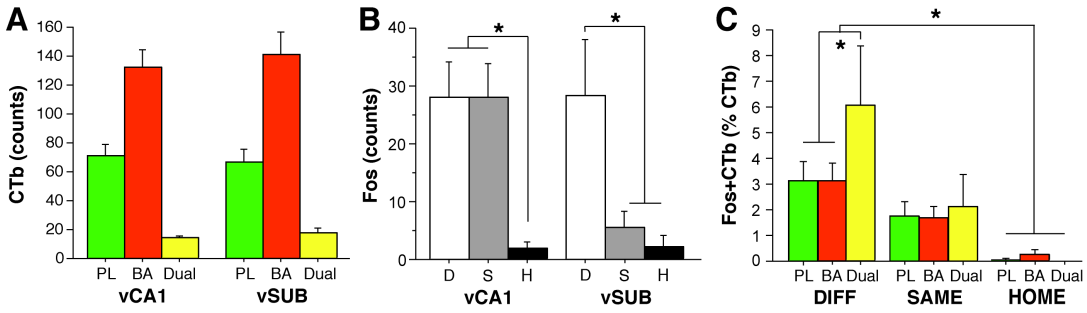

Figure 2-3 C-Fos quantification in PL-, BA- and dual-projecting neurons. (A) Mean (± SEM) cell counts for CTb-positive neurons in vCA1 and vSUB collapsed across retrieval condition. Neurons in both vCA1 and vSUB projected to the prelimbic cortex (PL, green), basal amygdala (BA, red), or both areas (Dual, yellow). (B) Mean (± SEM) cell counts for Fos-positive neurons in vCA1 and vSUB among animals tested outside the extinction context (DIFF, D), inside the extinction context (SAME, S), or untested animals (HOME, H). (C) Mean (± SEM) percentage of Fos-positive projection neurons (PL, BA, or dual-projecting) Fos- and CTb-positive neurons divided by CTb counts alone) in the vCA1 and vSUB of animals in each of the behavioral groups.

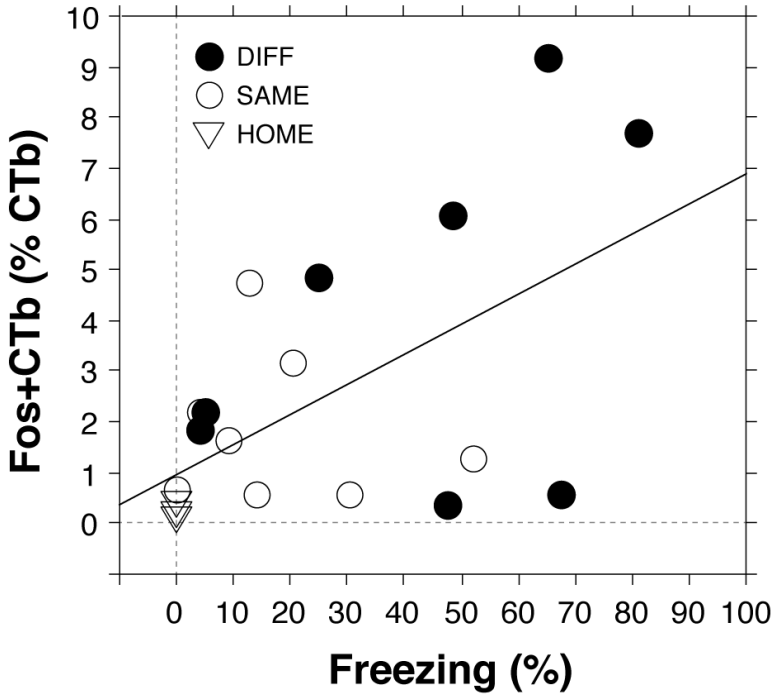

Figure 2-4 Correlation plot. Correlation between the number of Fos-positive CTb-labeled cells in the vCA1 and vSUB with average freezing behavior during the retrieval test among rats in DIFF, SAME, and HOME groups.

Discussion

Data reveal that the retrieval of an extinguished CS induces context-dependent Fos expression in vHPC neurons projecting to the mPFC and BA. The context-dependence of Fos expression in the vHPC among all neurons was greatest in vSUB; Fos expression in vCA1 was not itself context-specific when all Fos-positive neurons in the region were considered. However, when neurons were parsed by their projection targets, it was observed that PL- and BA-projecting neurons in both vCA1 and vSUB exhibited context-dependent Fos expression. Specifically, greater numbers of projection neurons expressed Fos in the renewal condition (DIFF) compared to the extinction condition (SAME). Interestingly, although few in number, dual-projecting neurons with collaterals to both PL and BA were more likely to exhibit Fos expression than neurons projecting only to PL or BA after fear renewal. The greater proportion of dual-projecting neurons expressing Fos during fear renewal suggests that these cells may be particularly important for synchronizing prefrontal-amygdala circuits involved in fear expression (Lesting et al., 2011).

Previous results indicate that fear renewal increases Fos expression (Knapska and Maren, 2009; Orsini et al., 2011) in the vHPC, including in vHPC neurons projecting to the BA. Interestingly, I now show that there are important differences in the context-specificity of Fos expression insofar as neurons in vCA1 are engaged in memory retrieval regardless of where the extinguished CS is encountered, whereas vSUB neurons are Fos-active only in the renewal condition. This suggests that vCA1 may have a

general role in memory retrieval engaged by the presentation of ambiguous stimuli (Hall et al., 2001; Ji and Maren, 2008). In contrast, vSUB neurons may be involved in an associative mismatch process that occurs when an animal encounters a familiar CS in a familiar context for the first time (i.e., a situation that promotes renewal) (Honey et al., 1998; Kumaran and Maguire, 2006; Maren, 2014). Indeed, the vSUB may be particularly important for integrating contextual memories retrieved by the hippocampus with emotional information retrieved by the amygdala to drive context-dependent fear behavior (Canteras and Swanson, 1992).

The crucial role of the hippocampus in contextual memory retrieval has been shown in many previous studies (Good and Honey, 1991; Maren and Holt, 2000; Kennedy and Shapiro, 2004). After extinction, it has been suggested that the ventral hippocampus uses contextual information to ‘‘gate’’ the expression or suppression of fear in a given context (Maren, 2011; Vlachos et al., 2011). Consistent with this view, infusion of the $GABA_A$ agonist muscimol into the ventral hippocampus disrupts context-dependent fear memory retrieval outside of the extinction context (Hobin et al., 2006). Importantly, hippocampal inactivation does not affect fear expression to a non-extinguished CS or disrupt context discrimination (Holt and Maren, 1999; Corcoran and Maren, 2001; Wang et al., 2009). Rather, hippocampal inactivation impairs the retrieval of CS-context associations necessary to guide context-dependent behavior.

We have previously suggested that the ventral hippocampus gates fear behavior through

its projections to the basal amygdala (Orsini et al., 2011; Maren et al., 2013). The ventral hippocampal formation, including vCA1 and vSUB, has robust reciprocal projections with amygdala, a structure that is essential for acquisition and expression of the fear memory (Orsini and Maren, 2012; Duvarci and Pare, 2014). Muscimol infusion in the ventral hippocampus eliminates context-dependent neuronal activity in the amygdala (Maren and Hobin, 2007) and BA neurons that fire during fear renewal receive direct projections from vHPC (Herry et al., 2008). Disconnection of hippocampal projections to the BA also eliminates fear renewal after extinction (Orsini et al., 2011). Moreover, in the present experiment, vCA1 and vSUB neurons projecting to BA expressed Fos after fear renewal. Collectively, these studies indicate that hippocampal projections to the amygdala are essential for context-dependent fear expression.

Another possibility is that vHPC projections to the mPFC regulate the expression of fear after extinction. Both the vCA1 and vSUB project strongly to the mPFC, including PL (Jay and Witter, 1991; Vertes, 2006), which in turn has robust connections with the amygdala (Vertes, 2004). Recent work indicates that disconnecting vHPC projections to PL impairs fear renewal (Orsini et al., 2011) and vHPC inactivation modulates PL spike firing and regulates the expression of extinguished fear (Sotres-Bayon et al., 2012). Moreover, mPFC projections to the amygdala are important for both the expression and suppression of fear after extinction (Likhtik et al., 2008; Ehrlich et al., 2009; Amano et al., 2010; Milad and Quirk, 2012). The present data are consistent with the role of vHPC projections to mPFC in the regulation of extinguished fear insofar as vHPC neurons

projecting to PL exhibited high c-Fos expression after renewal.

Interestingly, vHPC neurons with projections to both the PL and BA (dual-projecting neurons) (Ishikawa and Nakamura, 2006) were engaged to a greater degree than vHPC neurons projecting to either the PL or BA alone. These dual-projecting neurons have similar antidromic latencies to mPFC and amygdala stimulation, which suggests that spiking in vHPC neurons can simultaneously activate both the mPFC and the amygdala (Ishikawa and Nakamura, 2006). Even though the total number of dual-projecting neurons is small, our results suggest that they might play a particularly important role in fear memory retrieval after extinction. Indeed, these neurons may play an important function in synchronizing neuronal activity in the mPFC and BA to overcome extinction-related inhibition and promote conditional responding (Lesting et al., 2011; Marek et al., 2013; Preston and Eichenbaum, 2013; Stujenske et al., 2014). Together with previous data, the present results support the view that the contextual retrieval of emotional memories involves hippocampal coordination of neuronal activity in prefrontal-amygdala circuits that regulate fear expression (Maren et al., 2013).

CHAPTER III

HIPPOCAMPAL-PREFRONTAL PATHWAY BIDIRECTIONALLY REGULATES FEAR RELAPSE AFTER EXTINCTION

Introduction

Fear renewal is a form of relapse in which individuals fail to suppress fear after extinction. That is to say, after successful fear extinction, inhibitory neural circuits actively suppress fear in a safe context (e.g. extinction context). However, in dangerous contexts, a loss of inhibition leads to fear relapse. Hence, extinction is an inhibitory "brake" on the expression of conditioned fear, and the circumstances that result in renewal of fear must release this brake. The loss of inhibitory control that underlies fear renewal is a major challenge to clinical interventions for a host of disorders, including anxiety- and stress-related disorders (Maren et al., 2013).

Renewal of fear has received considerable attention over the last decade, and several important brain regions have been implicated in the process (Maren, 2011; Maren et al., 2013; Vervliet et al., 2013). Research indicates that rats with hippocampal dysfunction (lesions or inactivation) fail to exhibit fear renewal outside the extinction context (Corcoran and Maren, 2001, 2004; Ji and Maren, 2005; Hobin et al., 2006; Ji and Maren, 2007; Zelikowsky et al., 2012). Interestingly, inactivation of the HPC eliminates neuronal activity associated with renewal in amygdala neurons, indicating that contextual retrieval is modulated by hippocampal control of the amygdala activity

(Hobin et al., 2003; Maren and Hobin, 2007). One possible mechanism of the contextual control is that contextual information presented by the hippocampus gates the expression of appropriate memory (original fear memory vs. extinction memory) associated with a certain stimulus within the amygdala. Indeed, hippocampal lesions or inactivation do not affect freezing to CS *per se* or the ability to discriminate between contexts, but only influences context-dependency of the memories (Maren and Holt, 2004; Corcoran et al., 2005; Zelikowsky et al., 2012). This suggests that the hippocampus itself is not required for expression of CS-associated memory but is necessary to guide expression of the context-appropriate memory. Therefore, an important research question is to decipher the neural circuits by which the hippocampus regulates context-dependent fear memory retrieval.

Considerable work has revealed that the mPFC is another important brain structure involved in context-dependent fear memory retrieval. For example, the mPFC is capable of using contextual information to modulate expression of the memories associated with stimuli; and this modulation can compensate for hippocampal loss in some cases (Kim et al., 2013; Zelikowsky et al., 2013; Sharpe and Killcross, 2015). Importantly, intact direct projections between vHPC and mPFC are necessary for context-dependent fear memory retrieval, which suggests that direct interaction between these two structures is required for context-dependency (Orsini et al., 2011). Anatomical studies have shown that the hippocampus, especially the ventral part, sends massive direct projections to the mPFC, with the CA1 and subiculum being the main output sub-regions (Jay and Witter, 1991;

Hoover and Vertes, 2007). Interestingly, within the mPFC, the PL has been implicated in fear expression as well as fear renewal after extinction (as is shown in Chapter II), whereas the IL is known as the key structure for extinction learning and fear suppression after extinction (Maren, 2011; Milad and Quirk, 2012; Giustino and Maren, 2015). Therefore, in this chapter, we explore the role of vHPC-IL direct interactions in inhibitory control of fear after extinction.

Projections from the vHPC, mainly from vCA1 and subiculum region, synapse on both pyramidal neurons and interneurons in the IL; therefore the IL shows both excitatory and inhibitory responses to hippocampal stimulation (Ishikawa and Nakamura, 2003). However, hippocampal afferents more strongly excite interneurons compared to pyramidal neurons, suggesting that the vHPC is capable of exerting feed-forward inhibition on pyramidal neurons in the IL (Tierney et al., 2004). Thus, we propose that fear renewal after extinction is due to feed-forward inhibition in the IL, which is mediated by direct control of the vHPC. Inhibition of the IL disables its role as an inhibitory brake and leads a return of fear. This is also consistent with the previous finding showed that inactivation of the vHPC disrupts fear renewal (Hobin et al., 2006). Therefore, in this chapter, we ran a series of experiments to test the hypothesis that ventral hippocampal projections to the IL are responsible for fear relapse by inducing feed-forward inhibition in the IL.

Materials and methods

Subjects. Sixty-four adult male rats (200-224 g; Long-Evans; Blue Spruce) obtained from Envigo were used for experiments. The rats were individually housed on a 14/10 h light/dark cycle and had access to food and water *ad libitum*. All experiments were performed during the light cycle. The rats were handled for about 30 s everyday for 5 days before the experiments to habituate them to the experimenters. All experimental procedures were performed in accordance with the protocols approved by the Texas A&M University Animal Care and Use Committee.

Viruses and drugs. AAV5-hSyn-DIO-hM4D(G_i)-mCherry (5.7×10e12 vg/mL), AAV8-hSyn-DIO-hM4D(G_i)-mCherry (6.7×10e12 vg/mL) and AAV5-hSyn-DIO-GFP (6.7×10e12 vg/mL) were from University of North Carolina Vector Core and Addgene. CAV2-Cre (8.7×10e12 pp/mL) was obtained from the Institute of Molecular Genetics of Montpellier (France) and AAV5-CMV-HI-eGFP-Cre-WPRE-SV40 (1.62×10e13 GC/mL) is from University of Pennsylvania Vector Core. Clozapine-*N*-oxide (CNO) was obtained from the National Institute of Mental Health (NIMH; Chemical synthesis and drug supply program). CGP55845 was obtained from Sigma-Aldrich, and picrotoxin was obtained from Tocris Bioscience.

Surgery. For behavioral DREADD and PV/Fos dual-staining experiment, rats were anesthetized with isoflurane (5 % for induction, ~2 % for maintenance), and placed into a stereotaxic instrument (Kopf Instruments). An incision was made in the scalp, the head

was leveled, and bregma coordinates were identified. For the inhibitory vHPC->IL DREADD experiment, rats (*n*=8) were bilaterally injected with CAV2-Cre in IL and AAV8-hSyn-DIO-hM4D(Gi)-mCherry in vHPC. For the excitatory DREADD experiment and PV/Fos dual-staining experiment, rats (*n*=17; 5 for the behavior-only experiment, 12 for the PV/Fos dual-staining experiment) were bilaterally injected with AAV5-CMV-HI-eGFP-Cre-WPRE-SV40 in IL and AAV5-hSyn-DIO-hM3D(Gq)-mCherry in vHPC. Additional rats (*n*=5) received CAV2-Cre in IL and AAV-hSyn-DIO-GFP in vHPC. All the viruses were injected (0.1 μl/min) using an injector connected to polyethylene tube and a Hamilton syringe (10 μl) mounted on an infusion pump (Kd Scientific). Four injections (0.5 μl each injection) were made into the vHPC at two different AP levels (coordinates are shown in A/P, M/L, D/V): -5.2 mm, ±6.0 mm, -6.5 mm; -5.2 mm, ±6.0 mm, -5.3 mm; -6.1 mm, ±6.0 mm, -6.1 mm; -6.1 mm, ±6.0 mm, -5.0 mm (all D/V coordinates are measured from dura). Solely for Cre-dependent experiments, one injection (1.8 μl) was made in the IL in each hemisphere at A/P: +2.8 mm, M/L: ±3.0 mm, D/V: -4.9 mm with a 30° angle. Rats were placed back in their home cages to allow for viral expression for at least 4 weeks. Rats that with unilateral, off-target, or no viral expression were excluded from the analyses.

For the IL microinfusion experiment, rats were anesthetized with isoflurane and were placed into a stereotaxic apparatus as described above. Small holes were drilled in the skull to affix three jeweler's screws and to target bilateral cannulae above the IL. Two stainless-steel guide cannulae (26 gauge, 8 mm; Plastics One) were lowered into bilateral

IL (A/P: +2.7 mm, M/L: 3.0 mm, D/V: -4.9 mm at 30° angle) (*n*=32). Cannulas were secured with jeweler's screws and dental cement. Stainless steel dummy cannula (30 gauge, 9 mm; Plastics One) was placed in each guide cannula and was changed twice prior to behavioral tests. The rats were allowed for 1 week of post-operative recovery in their home cages. Rats that showed off-target cannula placements (including unilateral misses) were excluded from all of the analyses.

Drug delivery. For IL microinfusions, rats were transported to a prep room in the laboratory using white buckets (5-gallon) filled with a layer of bedding. Dummies were removed and stainless steel injectors (33 gauge, 9 mm) connected to tubes were inserted into the guide cannulae for intracranial infusions. Polyethylene tubing connected the injectors to Hamilton syringes (10 μl), which were mounted in an infusion pump (Kd Scientific). Infusions were monitored by the movement of an air bubble that separated the drug or saline solutions from distilled water within the polyethylene tubing. All infusions were made approximately 10 min before either extinction training or retrieval sessions. Infusions were made at a rate of 0.3 μl/min for 1 min (0.3 μl total) and the injectors were left in place for 1 min for diffusion. CGP55845 (10 mM), picrotoxin (0.33 mM), a cocktail of CGP55845 (5 mM) and picrotoxin (0.16 mM) were the actual amount of the drugs infused into the bilateral IL. After infusions, clean dummies were secured to the guide cannulae.

For DREADD experiments, CNO was first dissolved in 2.5% DMSO and then diluted in sterile saline (0.9%) to a concentration of 3 mg/ml immediately before injection. Approximately 30~40 min before testing session, rats received intraperitoneal injection of either CNO (2-3 mg/kg) or saline in the vivarium and then were placed back to their home cages until the start of the behavioral procedures.

Behavioral apparatus and contexts. Behavioral testing was conducted in two distinct rooms in the laboratory. Eight identical conditioning chambers (30×24×21 cm; MED-Associates) in each room were used in all behavioral experiments. The chambers consisted of aluminum sidewalls and Plexiglas ceilings, rear walls, and hinged front doors. The chamber floors consisted of 19 stainless steel rods that were wired to a shock source and a solid-state grid scrambler (Med-Associates) for delivery of footshock. A speaker mounted on one wall of the chamber was used for delivery of the acoustic tone, and ventilation fans and house lights were installed in each chamber to allow for the manipulation of contexts. Each chamber rests on a load-cell platform that is used to record chamber displacement in response to each rat's motor activity and is acquired online via Threshold Activity software (Med-Associates). Absolute values of the load-cell voltages are computed and multiplied by 10 to yield a scale that ranges from 0 to 100. For each chamber, load-cell voltages are digitized at 5 Hz, yielding one observation every 200 ms. 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). Freezing was only scored if the rat is immobile for at least 1 s.

Stimuli were adjusted within these chambers to generate distinct contexts (A and B). For context A, a 15-W house light mounted on the sidewall was turned on, and the white room light remained on. Ventilation fans (65 dB) were turned on, cabinet doors were left open, and the chambers were cleaned with 1% ammonium hydroxide. Rats were transported to context A in white plastic boxes. For context B, house light and white room light were all turned off, and fluorescent red room light was turned on. Ventilation fans were turned off, the cabinet doors were closed and the chambers were cleaned with 1% acetic acid. Rats were transported to context B in black plastic boxes. In each context, stainless steel pans were filled with a thin layer of the respective odors of the contexts and inserted below the grid floor.

Behavioral procedures. After a period of post-operative recovery in their home cages for virus-infused animals, rats underwent fear conditioning in context A on day 1. Conditioning consisted of 5 tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 2 s, 1.0 mA) pairings with 60 s intertrial intervals (ITIs) after 3 min of acclimation to the context (baseline). On days 2-3, rats received context exposure in context A for 35.5 min in the morning, and received 45 CS-alone (30 s ITIs) extinction training in context B in the afternoon (3 min baselines). Twenty-four hours after the final extinction session, Cre-dependent hM4D(Gi)- and GFP-expressing rats received renewal tests in context A, and hM3D(Gq)-expressing rats received extinction retrieval tests in context B. Thirty minutes before each test, rats received systemic injection of CNO (2~3mg/kg) or saline in a within-subjects design. The order of the drug injection was counterbalanced. Test

sessions consisted of 10 min of baseline followed by 5 CS-alone (30 s ITIs) presentations. In all behavioral sessions, the chamber position of each animal was counterbalanced. Rats in PV/Fos dual-staining experiment received the same behavioral procedures as DIO-hM3D(Gq) rats described above. Three rats were excluded from DIO-hM3D(Gq) analysis because they failed to retrieve their extinction memory during extinction retrieval test (freezing >90%).

After at least a week of recovery for cannula-implanted animals, rats underwent fear conditioning in context A as described above. Day 2 consisted of context exposure in the morning (context A) and extinction in the afternoon as described above (context B). Twenty-four hours after the final extinction session, rats underwent a renewal test in context A. All other variables used for conditioning, exposure, extinction and tests are identical to DREADD experiment. Ten min prior to the renewal tests, rats received microinfusion of CGP55845 (10 mM), picrotoxin (0.33 mM), a cocktail of CGP55845 and picrotoxin, or vehicle into IL. Rats received intracranial infusion with 0.6 μl of drug (0.3 μl per hemisphere) over 1 min at a rate of 0.3 μl/min.

Histology. At the conclusion of behavioral testing, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml) and were transcardially perfused with saline and 10% formalin. Brains were extracted and post-fixed in formalin solution for 24 h at 4 °C and transferred to 20% sucrose (4 °C). Brains were flash frozen with dry ice and sectioned (40 μm) on a cryostat maintained at -20 °C. Brain tissue was collected into

wells for immunohistochemistry. For cannula-implanted rats, thionin-stained coronal sections containing IL were imaged on a Leica microscope (MZ FLIII).

Immunohistochemistry. For DREADD animals, immunohistochemistry was performed on free-floating brain sections containing the vHPC. The tissue was washed three times in 1×Tris- buffered saline (TBS, pH 7.4). Brain sections were then incubated in 10% normal donkey serum (NDS) in 1×Tris-buffered saline with Tween (TBST) for 1 h at room temperature followed by two washes in TBST for 5 min. Tissue was then incubated in primary antibody solution in TBST with 2% NDS (rabbit anti-mCherry antibody at 1:2000; Abcam) for 48 h at 4°C. Brain sections were then washed three times in TBST for 10 min and were incubated in secondary antibody solution in TBST with 2% NDS (AF594 conjugated donkey anti-rabbit antibody at 1:200; Abcam) for 2 h at room temperature. Tissue was washed three times in TBS for 10 min and then was mounted on subbed slides in 0.9% saline and cover slipped with Fluoromount. Brain sections containing the IL were wet mounted to microscope slides and coverslipped with Fluoromount for imaging.

For PV/Fos dual-staining, rats were sacrificed 90 min after behavioral tests under CNO or saline administration. 18 h post-fix, brains were moved into 30% sucrose solution for 3 days. 40 μm-thick brain sections were collected for staining. Brain sections were washed three times in TBST and then were incubated in 0.3% H_2O_2 for 15 minutes. The tissue was washed in TBS three times and was incubated in rabbit anti-c-Fos primary

antibody (1:1000; Millipore) overnight. Brain tissue was washed three times in TBS followed by 1 h incubation in a biotinylated goat anti-rabbit secondary antibody (1:1000; Jackson Immunoresearch), amplification with the avidin biotin complex at 1:1000 (ABC; Vector labs), and visualization with 3, 3' diaminobenzidine (DAB) + nickel ammonium sulfate to yield a purple/black nuclear reaction product. The tissue was subsequently incubated in a mouse anti-PV primary antibody (1:5000; Sigma) overnight and biotinylated goat anti-mouse secondary antibody (1:500; Jackson Immunoresearch) for 1 h, amplified with the avidin biotin complex at 1:1000 (ABC; Vector labs), and visualized with 3, 3' diaminobenzidine (DAB) to yield a light brown somatic stain. Slices were mounted and coverslipped with mounting medium (Permount; Sigma), and stored at room temperature until photographed using a Zeiss microscope.

Statistics. Paired t-tests were used to analyze data in DIO-hM4D(Gi), DIO-GFP, and DIO-hM3D(Gq) experiments separately. Unpaired t-tests were used to analyze the PV/Fos dual-staining experiment. IL microinfusion and DREADD behavioral data were analyzed with repeated (one- and two-way) measures analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) post hoc tests, which were performed after a significant overall F ratio. All data are represented as means ± SEM.

Results

Experiment 1: Antagonizing GABA receptors in the IL prevent fear renewal. Prior

work indicates that the IL is involved in extinction encoding and fear suppression after extinction. However, it was not clear whether the IL is responsible for fear renewal after extinction. Our hypothesis was that during fear renewal, the IL fails to exert its inhibitory control accrued after extinction, which leads to a return of fear. To test this hypothesis, we examined whether activation of the IL by antagonizing $GABA_A$ and/or $GABA_B$ receptors prevents fear renewal. To this end, rats first underwent standard auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,140)}$= 28.3, p< 0.0001] and extinction [repeated measures ANOVA, main effect of block, $F_{(9,252)}$= 30.7, p< 0.0001] (Figure 3-1). On the following day, rats received fear renewal test (ABA) with GABA receptor antagonists microinfused into the IL. The results showed that intracranial infusions of picrotoxin (a $GABA_A$ receptor antagonist; n=5), CGP55845 (a $GABA_B$ receptor antagonist; n=10), or both (n=6) into the IL significantly reduced fear renewal as compared to vehicle treatment (n=11; one-way factorial ANOVA, main effect of drug, $F_{(3,28)}$= 12.6, p< 0.0001; post hoc Fisher's PLSD, p< 0.05, p< 0.001, p< 0.0001 for CGP, PTX, and CGP+PTX vs. VEH comparisons, respectively). In other words, enhancing IL activity by antagonizing GABA receptors resulted in disruption of fear renewal. These results reveal that GABAergic transmission in the IL is responsible for fear relapse.

Experiment 2: Silencing IL-projecting neurons in the vHPC prevents fear renewal.

In Experiment 1, we showed that increasing IL activity by antagonizing GABA receptors prevented fear renewal. Previous research has shown that inhibition of the IL using

$GABA_A$ receptor agonist muscimol disrupts extinction retrieval, suggesting that GABAergic signaling within the IL can bidirectionally modulate fear after extinction. However, the neural circuit underlying the bidirectional modulation is not clear yet. Here, we hypothesized that the bidirectional modulation on fear relapse depends on direct vHPC projections to the IL. In other words, during fear renewal, the vHPC is modulating activity of the IL, and silencing the vHPC→IL pathway can prevent return of fear after extinction.

To test the hypothesis, we used circuit-specific DREADD approach to specifically express inhibitory engineered G-protein coupled receptors (hM4D[Gi]) in IL-projecting neurons within the vHPC. One group of rats expressed control virus (DIO-GFP) in vHPC→IL pathway while the other group of rats expressed inhibitory DREADD virus [DIO-hM4D(Gi)] in the same pathway. GFP control rats underwent normal auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,20)}= 6.7$, $p< 0.001$] and extinction [repeated measures ANOVA, main effect of trial, $F_{(9,36)}= 8.5$, $p< 0.0001$] (Figure 3-2, C). During the test, freezing to the extinguished tone after CNO injection did not differ from that after saline injection in GFP control rats (n=5; repeated measures ANOVA, $F< 1$, $p= 0.64$; paired t-test for average freezing, $t_{(4)}< 1$, $p= 0.66$). This indicates that the CNO alone does not change freezing behavior. Similar to GFP control rats, DIO-hM4D(Gi) rats showed normal conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,35)}= 7.3$, $p< 0.0001$] and extinction [repeated measures ANOVA, main effect of trial, $F_{(9,63)}= 18.4$, $p< 0.0001$] (Figure 3-2, D). However, different from

control rats, silencing vHPC→IL projections by injecting CNO significantly reduced freezing to the extinguished CS compared to saline treatment (n=8; repeated measures ANOVA, main effect of drug, $F_{(1,6)}$= 7.3, p< 0.05; paired t-test for average freezing, $t_{(7)}$= 2.6, p< 0.05); and the baseline freezing was not affected by CNO (paired t-test, $t_{(7)}$= 1.7, p= 0.13). This indicates that vHPC→IL direct pathway is required for fear renewal after extinction.

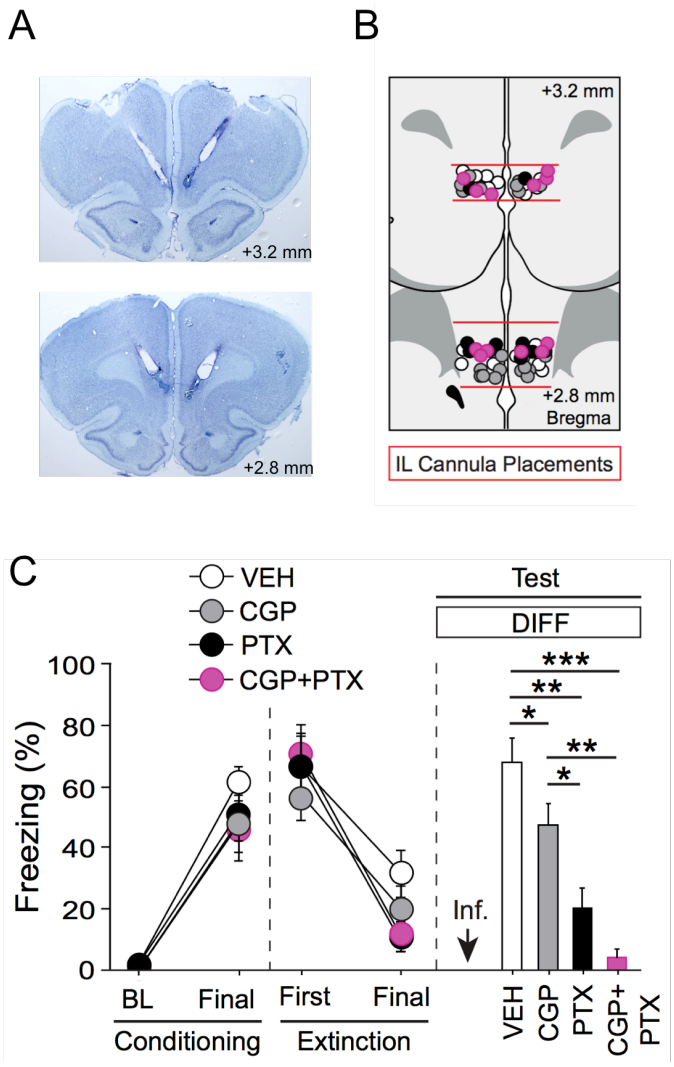

Figure 3-1 Antagonizing GABA receptors in the IL during fear renewal. (A) Representative images of bilateral IL cannula placements. (B) Schematic images of bilateral IL cannula placements in each group. (C) (Conditioning) Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and 1-min ITI (intertrial interval) following the last CS-US pairing during conditioning. (Extinction), Percentage of freezing (mean ± SEM) during the first and last extinction blocks (each block represents average of 30-s ITI freezing of 5 extinction trials) for the extinction training session. (Test), Percentage of freezing (mean ± SEM) for 30-s ITIs during the entire 5 CS test session. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$.

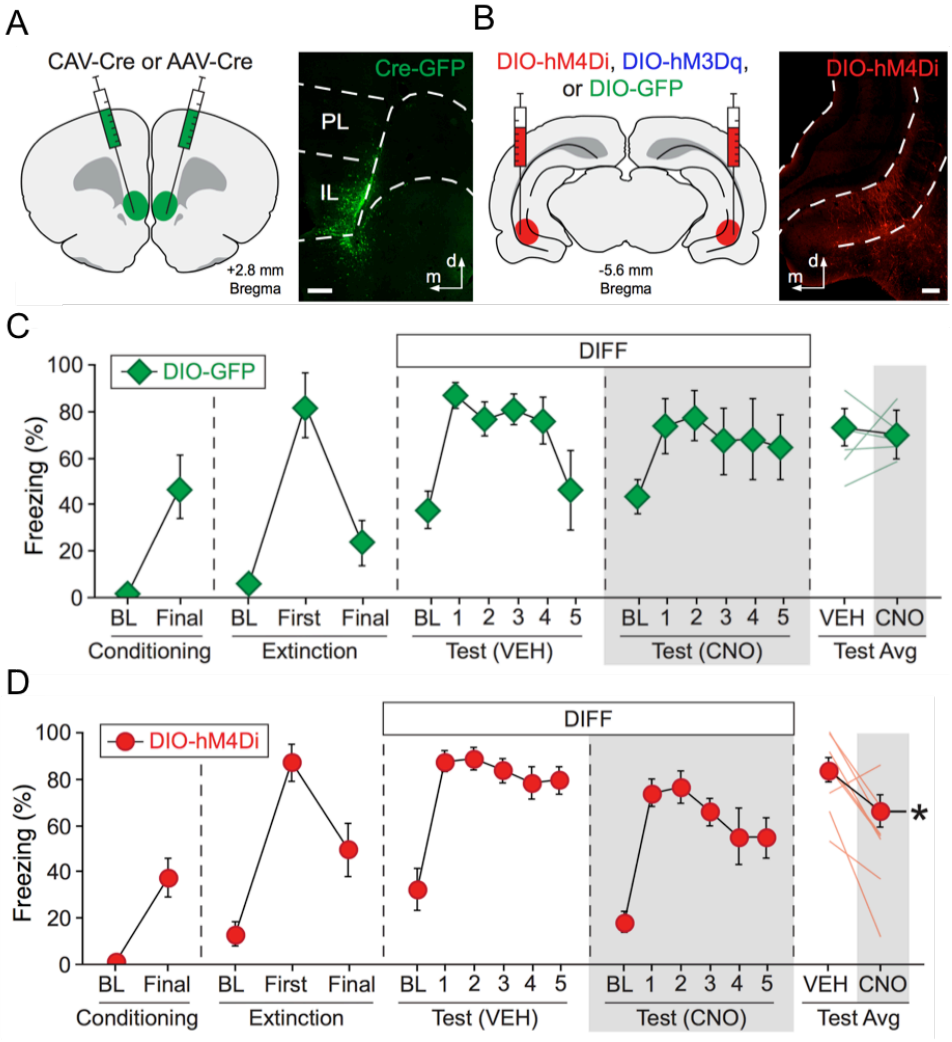

Figure 3-2 Silencing vHPC-IL pathway during fear renewal. (A) A schematic image of CAV2-Cre or AAV5-Cre infused into the IL (left); infusion sites confirmed by co-infused AAV8-hSyn-GFP (right). (B) A schematic image of Cre-dependent (DIO) viruses expressing hM4D(Gi)-mCherry, hM3D(Gq)-mCherry, or GFP infused into the vHPC (left); mCherry expression in vHPC→IL neurons in CA1 region (right). (C) Conditioned freezing in DIO-GFP control rats. (D) Conditioned freezing in DIO-hM4D(Gi)-mCherry rats. (Conditioning), Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and the last 1-min ITI (intertrial interval) following CS-US pairings during the fear conditioning session. (Extinction), Percentage of freezing (mean ± SEM) during the 3-min baseline and the first and last extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (Test), Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during a renewal test either after saline (white background) or CNO (gray background) injection. Shaded panel represents average ITI freezing (mean ± SEM) and each grey line represents an individual rat. *$p < 0.05$.

Experiment 3: Activating IL-projecting neurons in the vHPC induces fear relapse in the extinction context. In Experiment 2, we showed that inactivation of the vHPC→IL direct pathway prevented fear renewal. If the pathway is responsible for fear relapse after extinction, we were curious if activation of the same pathway is sufficient to cause fear relapse. Thus, in Experiment 3, we examined whether activating vHPC→IL pathway in the extinction context, where fear to the CS should be low, can induce fear relapse. To this end, we first expressed excitatory DREADD (hM3D[Gq]) in vHPC→IL pathway. Rats then received fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,20)}$= 6.7, p< 0.001] and extinction [repeated measures ANOVA, main effect of trial, $F_{(9,36)}$= 2.9, p< 0.05] as previously described (Figure 3-3). During the within-subjects extinction retrieval tests (ABB), CNO injection prior to the test increased freezing to the tone, (n=5; repeated measures ANOVA, main effect of drug, $F_{(1,4)}$= 13.2, p< 0.05; paired t-test for average freezing, $t_{(4)}$= -3.5, p< 0.05) without affecting baseline freezing (paired t-test, $t_{(4)}$= -1.4, p= 0.24). The result indicates that activation of vHPC→IL projections is sufficient to impair expression of extinction memory and lead to a relapse of fear in the extinction context.

Experiment 4: vHPC→IL pathway mediates activity of interneurons in the IL. In Experiments 1-3, we showed that GABAergic transmission within the IL is involved in fear renewal, and that direct vHPC projections to the IL bidirectionally modulate fear relapse. However, these results do not provide direct evidence that GABAergic signaling in the IL is indeed modulated by vHPC→IL pathway. Thus, in this experiment, we

examined whether manipulation of the vHPC→IL pathway modulates activity of IL interneurons by combining DREADD and immunohistochemistry. As is mentioned above, projections from the vHPC synapse on interneurons, thereby generating feed-forward inhibition within the IL (Ishikawa & Nakamura, 2003; Tierney et al., 2004). Among several types of the cortical interneurons that could potentially mediate the feed-forward inhibition, we specifically looked at activity of parvalbumin (PV^+) interneurons in response to vHPC→IL activation since PV^+ interneurons have been reported to regulate fear expression within the mPFC (Courtin et al., 2014).

Similar to Experiment 3, a group of rats received infusion of excitatory DREADD (hM3D[Gq]) in vHPC→IL pathway. A different group of rats received infusion of a control virus (DIO-mCherry) in the same pathway. Then, all rats underwent fear conditioning, extinction and between-subjects extinction retrieval tests (ABB). Prior to the test, rats either received either CNO injection or vehicle injection. Virus control (rats expressing mCherry control virus received CNO injection) and drug control (rats expressing hM3Dq virus received vehicle injection) groups were collapsed into a single control group for the convenience of the analysis. Ninety minutes after the retrieval test, rats were perfused and their tissue was processed for analyses.

We first looked at $mCherry^+$ DREADD neurons in the vHPC. Fos analysis revealed that after CNO injection, a significantly higher percentage of ventral hippocampal $mCherry^+$ neurons in hM3Dq-CNO rats expressed c-Fos compared to the control rats (Figure 3-4,

A; unpaired t-test, $t_{(10)}$= 6.5, p< 0.0001). This indicates that CNO injection successfully activated hM3D(Gq)$^+$ neurons in the vHPC, and that CNO injection or hM3Dq expression alone did not produce the same effect in vHPC neurons. Importantly, elevated c-Fos expression in hM3Dq$^+$ neurons was accompanied by a significant increase in the number of c-Fos-positive PV$^+$ neurons in the IL, but not the PL (Figure 3-4, B; unpaired t-tests, IL: $t_{(10)}$= 2.3, p< 0.05; PL: $t_{(10)}$= -1.4, p= 0.2). Moreover, the percentage of c-Fos$^+$hM3Dq$^+$ neurons is positively correlated with the percentage of Fos$^+$PV$^+$ neurons within the IL (r= 0.5, p= 0.07). The results suggest that activation of vHPC→IL pathway is responsible for increased GABAergic activity within the IL.

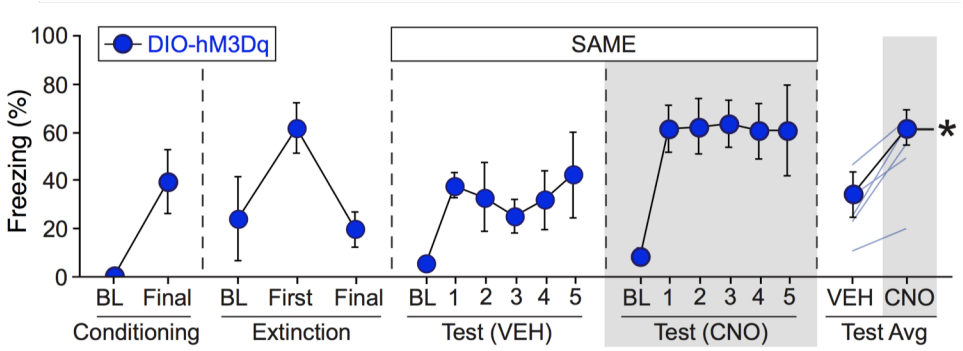

Figure 3-3 Activating vHPC-IL pathway during extinction retrieval. Conditioned freezing in DIO-hM3D(Gq)-mCherry rats was shown. (Conditioning), Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and the last 1-min ITI (intertrial interval) following CS-US pairings during the fear conditioning session. (Extinction), Percentage of freezing (mean ± SEM) during the 3-min baseline and the first and last extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (Test), Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during an extinction retrieval test either after saline (white background) or CNO (gray background) injection. Shaded panel represents average ITI freezing (mean ± SEM) and each grey line represents an individual rat (n=5). *p< 0.05.

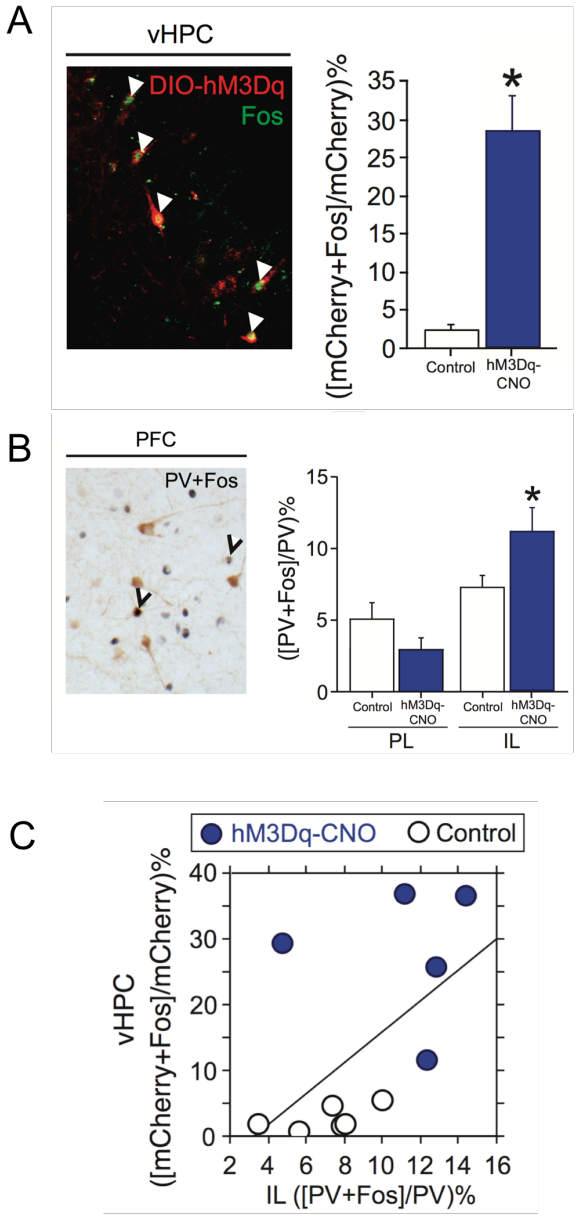

Figure 3-4 Fos expression in PV-positive neurons in IL after activating vHPC-IL pathway. (A) hM3D(Gq)$^{+}$ neurons (mCherry) and Fos^{+} nuclei (GFP) in vHPC (left); percentages of Fos^{+}hM3D(Gq)$^{+}$ neurons within hM3D(Gq)$^{+}$ neurons after VEH or CNO injection (right). (B) PV^{+} neurons (brown in soma) and Fos^{+} neurons (purple/black in nuclei) in PL and IL (left); percentages of Fos^{+}PV^{+} neurons within PV^{+} neurons (right). (C) Correlation between percentage of Fos^{+}PV^{+} neurons in the IL and percentage of Fos^{+}hM3D(Gq)$^{+}$ neurons in the vHPC in all rats.

Discussion

The present data reveal that the vHPC→IL pathway is critical for bidirectional modulation of fear relapse. First, we found that increasing IL activity by infusing GABA receptor antagonists into the IL prevented fear renewal. Then we showed that silencing vHPC direct projections to the IL also prevented fear renewal, and that activation of the same circuit was sufficient to induce fear relapse in the safe context. Finally, we showed that the vHPC→IL regulation on fear relapse depends on its effect on PV^+ interneurons within the IL. Together, the results indicate that vHPC→IL pathway is responsible for fear relapse after extinction.

Although the role of the IL in extinction retrieval has been challenged recently (Do-Monte et al., 2015), studies have shown strong evidence that the IL is crucial in extinction retrieval (Kim et al., 2016; Lingawi et al., 2016). Researchers have reported that the CS-evoked response of IL neurons increased *in vivo* during retrieval of extinction memory (Milad and Quirk, 2002). Moreover, optogenetic inactivation of the IL impairs extinction retrieval (Kim et al., 2016), and microinfusion of $GABA_A$ and $GABA_B$ agonists prior to extinction retrieval induced fear relapse (Sangha et al., 2014). This evidence clearly demonstrates that the IL activity mediates extinction retrieval. Now we show that antagonizing $GABA_A$ and $GABA_B$ receptors within the IL prevents fear renewal. This, together with previous data, indicates that suppressed activity in the IL is causing fear relapse, and increasing its activity prevents renewal. C-Fos studies support the idea by showing that activity of IL neurons, including amygdala-projecting

IL neurons, increased during extinction retrieval but remained low during fear renewal (Knapska and Maren, 2009; Orsini et al., 2011). Thus, failure to inhibit fear during renewal might be due to suppressed activity of the IL. Indeed, the IL has been shown to exert its inhibitory role in fear expression via direct projections to the ITC interneurons in the amygdala (Berretta et al., 2005; Likhtik et al., 2008; Amano et al., 2010; Amir et al., 2011). Another study emphasized the role of IL→BMA circuit in mediating fear suppression (Adhikari et al., 2015). Even though the circuit mechanism of the IL→amygdala regulation is not entirely clear, these studies together indicate that the IL is required for fear suppression, and release of this inhibitory control results in fear relapse.

Moreover, we show in the present study that vHPC→IL pathway can bidirectionally modulate the expression of extinguished fear. Indeed, the importance of hippocampal→prefrontal direct pathway has been identified in previous work regarding stress- and anxiety-related behaviors (Padilla-Coreano et al., 2016; Tripathi et al., 2016). However, only a few studies specifically looked at the role of hippocampal control on IL regarding fear extinction and relapse. For instance, one study showed that histone acetylation in HPC→IL is involved in fear extinction (Stafford et al., 2012), and another study suggested that BDNF (brain-derived neurotrophic factor) in HPC→IL is a key factor for fear extinction (Peters et al., 2010), but the circuit mechanism of hippocampal modulations on the IL in fear suppression and relapse has not previously been reported. Prior work has suggested that the vHPC gates context-dependent expression of fear after

extinction (when the meaning of the CS is ambiguous) (Hobin et al., 2006). Ventral hippocampal inactivation disrupts fear renewal without affecting freezing *per se* or the ability to discriminate the test context from the extinction context (Holt and Maren, 1999; Corcoran and Maren, 2001; Wang et al., 2009). Instead, the vHPC is involved in processing contextual information that used to retrieve the meaning of CSs in a certain context; therefore, the meaning of a CS is understood with reference to the context in which it is retrieved (Maren et al., 2013). This leads us to believe that the inhibitory control of the IL is modulated by contextual information processed by the vHPC.

Previous work has emphasized the role of the PL in actively driving fear renewal. For example, Fos is elevated in the PL during fear renewal, and disconnection between the vHPC and PL disrupts renewal (Knapska and Maren, 2009; Orsini et al., 2011; Zelikowsky et al., 2013; Jin and Maren, 2015a; Sharpe and Killcross, 2015). Renewal is the result of both increased fear expression and a simultaneous reduction of fear suppression. Thus, understanding the mechanism underlying the failure to inhibit fear in renewal situations is crucial. Anatomically, the vHPC projects to the entire mPFC but with the vHPC→IL projections outnumbered vHPC→PL (Wang et al., 2016). Although ventral hippocampal inputs to the IL are generally glutamatergic, increasing evidence suggests that the vHPC ultimately inhibits IL output via GABAergic feedforward inhibition (Ishikawa and Nakamura, 2003; Tierney et al., 2004). We also showed that vHPC→IL activation lead to increased activity in PV^+ interneurons within the IL but not PL. This further indicates that fear renewal after extinction is caused by feed-forward

inhibition in the IL, which releases inhibitory brake and leads to fear relapse. Taken together, in this chapter, we provide circuit mechanism underlying direct ventral hippocampal control on the infralimbic cortex in fear renewal.

CHAPTER IV

PREFRONTAL PROJECTIONS TO THE THALAMIC NUCLEUS REUNIENS MEDIATE CONTEXT-DEPENDENT EXTINCTION RETRIEVAL

Introduction

Learning to contend with threats in the environment is essential to survival. It allows us to anticipate danger and organize appropriate defensive behaviors in response to threat. However, aversive learning can become maladaptive and lead to pathological conditions such as panic disorder, anxiety and post-traumatic stress disorder (Maren et al., 2013; VanElzakker et al., 2014). Of course, fear memories are evolutionarily programmed to be rapidly acquired, temporally enduring, and broadly generalized across both familiar and novel contexts. In contrast, procedures that reduce fear and anxiety, such as exposure therapy, tend to produce fear suppression that is often slow to develop, short-lived, and context-dependent (Maren, 2011; Milad and Quirk, 2012). Therefore, considerable research has explored the neural circuits that govern these forms of learning. Pavlovian fear conditioning and extinction procedures are widely used to study the neural basis of aversive memory. Animals learn a neutral conditioned stimulus (CS) predicts an aversive unconditioned stimulus (US). After fear conditioning, animals exhibit conditioned fear responses (CRs), such as freezing, to presentation of the CS. Repeated presentation of the CS alone (i.e. extinction training) ultimately reduces conditioned responses (Maren, 2001). Importantly, extinction represents new learning and does not erase the original fear memory; indeed, fear to an extinguished CS returns

under many circumstances, including when the CS is encountered outside of the extinction context (Bouton, 1993).

Decades of research have implicated the hippocampus (HPC), medial prefrontal cortex (mPFC), and amygdala in the encoding and context-dependent expression of extinction memories (Maren et al., 2013; Giustino and Maren, 2015). Previous work from our lab has shown that fear renewal activates ventral hippocampal (vHPC) neurons projecting to both the mPFC and amygdala (Jin and Maren, 2015a; Wang et al., 2016). Importantly, functional disconnection of the vHPC and prelimbic (PL) prefrontal cortex or basal amygdala (BA) impairs fear renewal (Orsini et al., 2011). These studies support a circuit model in which vHPC projections to the mPFC and amygdala facilitate the retrieval of CS-US memories when an extinguished CS is encountered outside the extinction context (Orsini et al., 2011; Jin and Maren, 2015a). However, when the CS is encountered in the extinction context, the retrieval of fear memories must be suppressed in order to dampen fear responses, such as freezing, to the CS. Recent work in humans suggests that "retrieval suppression" might be mediated by prefrontal cortical projections to the hippocampus (Anderson et al., 2016).

Anatomically, the mPFC does not project directly to the HPC, but it can influence the HPC through indirect projections (Vertes, 2006; Vertes et al., 2007; Jin and Maren, 2015b). Indeed, it has been recently appreciated that the nucleus reuniens (RE) of the midline thalamus is well positioned to mediate prefrontal cortical control on

hippocampal function (Vertes et al., 2007; Cassel et al., 2013; Griffin, 2015; Vertes, 2015; Vetere et al., 2017). Indeed, lesions or inactivation of the RE impair forms of memory that require both the mPFC and HPC (Lopez et al., 2009; Loureiro et al., 2012; Cholvin et al., 2013; Layfield et al., 2015). For example, inactivation of the RE disrupts hippocampal-prefrontal synchrony during goal directed navigation (Ito et al., 2015; Hallock et al., 2016). Moreover, the mPFC→RE→HPC pathway is involved in the context specificity of fear memories (Xu and Sudhof, 2013). Given the crucial role of the RE in mediating prefrontal-hippocampal interactions and memory processes, we sought to determine whether it also plays a role in the contextual retrieval processes that regulate the expression of fear after extinction. We found that pharmacological inactivation of the RE with the $GABA_A$ receptor antagonist, muscimol, impaired context-dependent extinction retrieval. The deficit was reproduced by selectively silencing mPFC projections to the RE using chemogenetic approach.

Materials and methods

Subjects. One hundred twenty-nine adult male rats (200-224 g; Long-Evans Blue Spruce) obtained from Envigo were used for the experiments. The rats were individually housed on a 14/10 h light/dark cycle and had access to food and water *ad libitum*. All experiments were performed during the light cycle. The rats were handled for 30 s everyday for 5 days before the experiments to habituate them to the experimenters. All experimental procedures were performed in accordance with the protocols approved by the Texas A&M University Animal Care and Use Committee.

Viruses and drugs. AAV8-hSyn-DIO-hM4D(G_i)-mCherry (titer≥ 4×10^{12} vg/mL) was obtained from University of North Carolina Vector Core and Addgene. CAV2-Cre (titer: 8.7×10^{12} pp/mL) was obtained from the Institute of Molecular Genetics of Montpellier. Clozapine-*N*-oxide (CNO) was provided by the National Institute of Mental Health (NIMH; Chemical synthesis and drug supply program) and muscimol ($GABA_A$ receptor agonist) was from Sigma.

Surgery. For muscimol microinfusion experiments, rats were anesthetized with isoflurane (5% for induction, ~2% for maintenance), and placed into a stereotaxic instrument (Kopf Instruments). An incision was made in the scalp, the head was leveled, and bregma coordinates were identified. Small holes were drilled in the skull to affix three jeweler's screws and to target a single midline cannula (8 mm, 26 gauge; Plastics One) above the RE. The cannula was implanted at a 10° angle on the midline (A/P: -2.05- 2.15 mm, M/L: +1.0 mm, D/V: -6.7- 6.9 mm from dura; coordinates were measured from bregma). The cannula was affixed to the skull with dental cement, and a stainless-steel dummy cannula (30 gauge, 9 mm; Plastics One) was inserted into the guide cannula. Rats were allowed to recover for a period of 7 d after surgery before behavioral testing.

For DREADD experiments targeting the mPFC→RE circuit, rats were bilaterally infused with AAV8-hSyn-DIO-hM4D(Gi)-mCherry into the mPFC (including PL and

IL), and CAV2-Cre into the RE. Within the mPFC, two infusions (1.0 µl each) were made in the IL (A/P: +2.7- 3.0 mm, M/L: ±0.5- 0.75 mm, D/V: -4.4 mm from dura) and PL (A/P: +3.0 mm, M/L: ±0.75 mm, D/V: -3.2 mm from dura) respectively. A single infusion (1.0- 1.2 µl) was made in the RE (A/P: -2.15 mm, M/L: +1.0 mm, D/V: -6.9 mm from dura) at a 10° angle. For experiments specifically targeting RE→vHPC circuit, AAV8-hSyn-DIO-hM4D(Gi)-mCherry or AAV8-hSyn-DIO-mCherry was infused into the RE, and CAV2-Cre was bilaterally infused into the vHPC. A single infusion was made in the RE as previously described. For the vHPC, three infusions (0.5 µl each) were made in three coordinates (A/P: -5.6 mm, M/L: ±5.4 mm, D/V: -6.4 and -5.1 mm; A/P: -6.3 mm, M/L: ±5.5 mm, D/V: -5.0 mm; all DVs were from dura). The infusion rate of all infusions was 0.08-0.1 µl/min. Rats were allowed to recover for at least 4 weeks after surgery before behavioral testing.

Drug delivery. For RE microinfusions, rats were transported to a prep room in the laboratory using white buckets (5-gallon) filled with a layer of bedding. Dummies were removed and stainless steel injectors (33 gauge, 9 mm) connected to tubes were inserted into the guide cannulae for intracranial infusions. Polyethylene tubing connected the injectors to Hamilton syringes (10 µl), which were mounted in an infusion pump (Kd Scientific). Infusions were monitored by the movement of an air bubble that separated the drug or saline solutions from distilled water within the polyethylene tubing. All infusions were made approximately 10 min before retrieval sessions. Muscimol was diluted in sterile saline to a concentration of 0.1 µg/µl. Infusions were made at a rate of

0.3 µl/min for 1 min (0.3 µl total, 0.03 µg muscimol) and the injectors were left in place for 1 min for diffusion. After infusions, clean dummies were secured to the guide cannulae.

For DREADD experiments, CNO was first dissolved in 2.5% DMSO and then diluted in sterile saline (0.9%) to a concentration of 3 mg/ml immediately before injection. Approximately 30~40 min before retrieval session, rats received intraperitoneal injection of either CNO (3 mg/kg) or saline in the vivarium and then were placed back to their home cages until the start of the behavioral procedures.

Behavioral apparatus and contexts. Sixteen identical rodent conditioning chambers (30 × 24 × 21 cm; Med-Associates, St Albans, VT) were used in all behavioral sessions. Each chamber consisted of two aluminum sidewalls and a Plexiglas ceiling and rear wall, and a hinged Plexiglas door. The floor consisted of 19 stainless steel rods that were wired to a shock source and a solid-state grid scrambler (Med-Associates) for the delivery of footshocks. A speaker mounted on the outside of the grating in one aluminum wall was used to deliver auditory stimuli. Additionally, ventilation fans and house lights were installed in each chamber to allow for the manipulation of contexts. Each conditioning chamber rests on a load-cell platform that is used to record chamber displacement in response to each rat's motor activity and is acquired online via Threshold Activity software (Med-Associates). For each chamber, load-cell voltages are digitized at 5 Hz, yielding one observation every 200 ms. 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). Freezing was only scored if the rat is immobile for at least 1 s. Stimuli were adjusted within conditioning chambers to generate two distinct contexts in two distinct behavioral rooms. For context A, a 15-W house light was turned on, and the room light remained on. Ventilation fans (65 dB) were turned on, cabinet doors were left open, and the chambers were cleaned with 1% ammonium hydroxide. Rats were transported to context A in white plastic boxes. For context B, house lights were turned off and fluorescent red room light was turned on. The cabinet doors were closed and the chambers were cleaned with 1~1.5% acetic acid. Rats were transported to context B in black plastic boxes.

Behavioral procedures. For muscimol inactivation test (Experiment 1), approximately 1 week after surgery, rats underwent fear conditioning, extinction, and retrieval testing in either the conditioning context (ABA) or the extinction context (ABB). Auditory fear conditioning consisted of five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) pairings with 60 s intertrial intervals (ITIs). On the following day, rats underwent fear extinction in which they received 45 tone-alone presentations (30 s ITIs) followed by 3 min baseline. Prior to the extinction session, rats were exposed to the conditioning context for 35 min 30 s to extinguish fear associated with the context. On the following two days, rats received a retrieval test in the conditioning context to assess fear renewal and another test in the extinction context to assess extinction retrieval. Each test consisted of a 10-min stimulus-free baseline period followed by and 5 CS presentations

(30 s ITIs). Rats received microinfusions of MUS or SAL into the RE prior to retrieval testing. The test order was counterbalanced such that half of the rats received the renewal test first and the others received the retrieval test first.

To determine whether extinction retrieval testing activates the RE, we examined Fos expression in the RE after the procedure (Experiment 2). Rats underwent fear conditioning and extinction as previously described (in this experiment the animals received three days of extinction training because they exhibited particularly high levels of freezing). After all extinction sessions, a group of rats underwent ABA renewal test (DIFF) and a second group received ABB extinction retrieval test (SAME), while a third group of rats (HOME) was staying in their home cages during behavioral testing. Ninety-minutes after testing, all rats were sacrificed and perfused for c-Fos analysis. We also explored whether RE inactivation effected Fos expression in brain regions involved in extinction that interact with RE (Experiment 3). For this experiment, rats received conditioning and extinction as previously described followed by an extinction retrieval test in the extinction context after the infusion of MUS or SAL infused into the RE. Then all the rats were perfused 90 min after the retrieval test.

For DREADD experiments (Experiments 4 and 5), 4-5 weeks after surgery, rats underwent auditory fear conditioning, extinction, retrieval testing as previously described. Rats received CNO or SAL injections either 30 min before extinction retrieval testing. Animals were tested in the extinction context (ABB) using a within-subjects

procedure in which each animal served as its own control. That is, each rat received either a CNO or SAL injection before each of two extinction retrieval tests conducted over two days; test order was counterbalanced such that half of the animals received SAL in their first test whereas the other half received CNO in their first test.

Immunohistochemistry. Rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml) and were transcardially perfused with ice-cold saline and 10% formalin. Brains from the muscimol experiments were extracted and stored in 30% sucrose-formalin) at 4 °C. Brains from the Fos and DREADD experiments were extracted and stored in 10% formalin for up to 24 h and then transferred to 30% sucrose at 4 °C for at least 48 hours. Coronal brain sections (40 μm) were made on a cryostat (-20 °C). For the muscimol experiments, brain sections were mounted on subbed slides and stained with thionin staining (0.25% thionin) to visualize cannula placements. For the DREADD experiments, sections were also mounted on subbed slides and coverslipped using fluoromount (Diagnostic Biosystems) to visualize viral expression. Brain sections from the Fos experiments were washed three times in TBST and then were incubated in 0.3% H_2O_2 for 15 minutes. The tissue was washed in TBS three times and was incubated in rabbit anti-c-Fos primary antibody (1:1000; Millipore) overnight. Brain tissue was washed three times in TBS followed by 1 h incubation in a biotinylated goat anti-rabbit secondary antibody (1:1000; Jackson Immunoresearch), amplification with the avidin biotin complex at 1:1000 (ABC; Vector labs), and visualization with 3, 3’ diaminobenzidine (DAB)+nickel ammonium sulfate to yield a purple/black nuclear

reaction product. Stained brain sections were mounted on subbed slides and coverslipped with Permount, and stored at room temperature until photographed using a Zeiss microscope (Axio Imager).

Data analysis. In RE inactivation experiments, rats with cannula targeting outside of the RE have been removed from the analyses. In DREADD experiments, rats with unilateral or no expression of DIO-DREADD in mPFC or RE were excluded from the analyses. All freezing data represent freezing behavior during intertrial periods (ITIs). Data were analyzed with analysis of variance (ANOVA), paired and unpaired t-tests. Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio for ANOVA. All data are presented as means ± SEM.

Results

Experiment 1: RE inactivation impairs extinction retrieval but not fear renewal. In the first experiment, we examined the role of the RE in the retrieval of extinguished fear. Because we have previously observed that hippocampal manipulations influence the context-dependent retrieval of extinction, we examined the effects of RE inactivation on both extinction retrieval and fear renewal. Freezing behavior during the conditioning session is shown in Figure 4-1. As before, there were low levels of freezing before conditioning, but freezing behavior significantly increased across the conditioning trials [repeated measures ANOVA, main effect of trial, $F_{(5,\ 145)}= 26.3$, $p< 0.0001$]. The

following day the rats received extinction training in a different context. Rats showed high levels of CS-elicited freezing early in the session, but it dramatically decreased by the end of the session [repeated measures ANOVA, main effect of trial, $F_{(9, 261)}$= 53.0, p< 0.0001], indicating successful within-session extinction. There were no group differences observed during the conditioning and extinction sessions [Fs< 1.6]. Twenty-four hours after extinction, rats received a retrieval test in a context different from that in which extinction was performed (i.e., a renewal test); extinction retrieval was tested in the extinction context the following day. As shown in Figure 4-1, RE inactivation did not affect fear renewal [repeated measures ANOVA, main effect of drug, $F_{(1,29)}$< 1, p= 0.9], but produced a dramatic increase in CS-elicited freezing during the extinction retrieval test [repeated measures ANOVA, main effect of drug, $F_{(1,29)}$= 12.1, p< 0.01]. Importantly, the increases of freezing produced by RE inactivation were not due to nonspecific reductions in locomotor activity insofar as both pre-CS baseline freezing and fear renewal were unaffected by RE inactivation. These results indicate that the RE is specifically required for extinction retrieval.

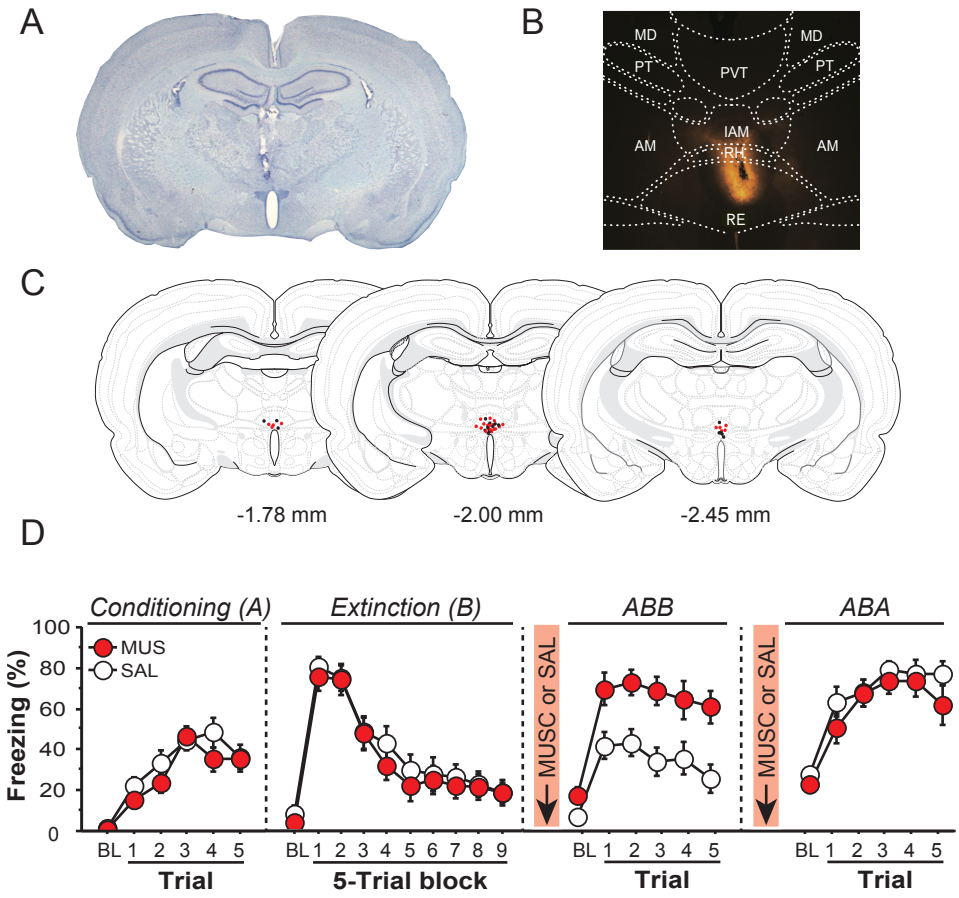

Figure 4-1 RE inactivation during retrieval tests. (A) Representative thionin-stained coronal section showing cannula placement in the RE. (B) Representative darkfield image showing diffusion of TMRx muscimol in the RE. (C) RE cannula placements in all subjects in the experiment (red: MUS; black: SAL). (D) (Conditioning) Percentage of freezing (mean ± SEM) during the 3 min baseline (BL) and 1 min ITI (intertrial interval) following each CS-US pairing during the fear conditioning session. (Extinction) Percentage of freezing (mean ± SEM) during the 3-min baseline and 30-s ITIs across 9 extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (ABB and ABA) Percentage of freezing (mean ± SEM) during retrieval and renewal tests in10-min BL period and 30-s ITIs for 5 CS test trials. Arrows indicate the timing of muscimol (MUS; *n*= 15) or saline (SAL; *n*= 16) injection before the onset of the CS tests.

Experiment 2: Extinction retrieval does not preferentially increase Fos expression in the RE. The previous results indicate that RE inactivation impairs retrieval of extinction memories. Here we sought to determine whether RE neurons are activated (as indexed by c-Fos immunohistochemistry) during these behavioral sessions. To this end, we examined Fos expression in the RE after extinction retrieval and fear renewal tests. Rats underwent auditory fear conditioning followed by three sessions of extinction training; the animals were sacrificed 90 min after the end of the retrieval session. Interestingly, there was an increase in Fos^+ RE neurons after both the extinction retrieval and fear renewal tests, relative to home-cage controls [one-way ANOVA, main effect of group, $F_{(2,25)}= 3.8$, $p< 0.05$; post hoc Fisher's PLSD, $p< 0.05$ for HOME vs. DIFF or SAME, $p= 0.9$ for DIFF vs. SAME]. These results reveal that RE neurons are engaged by both extinction retrieval and fear renewal.

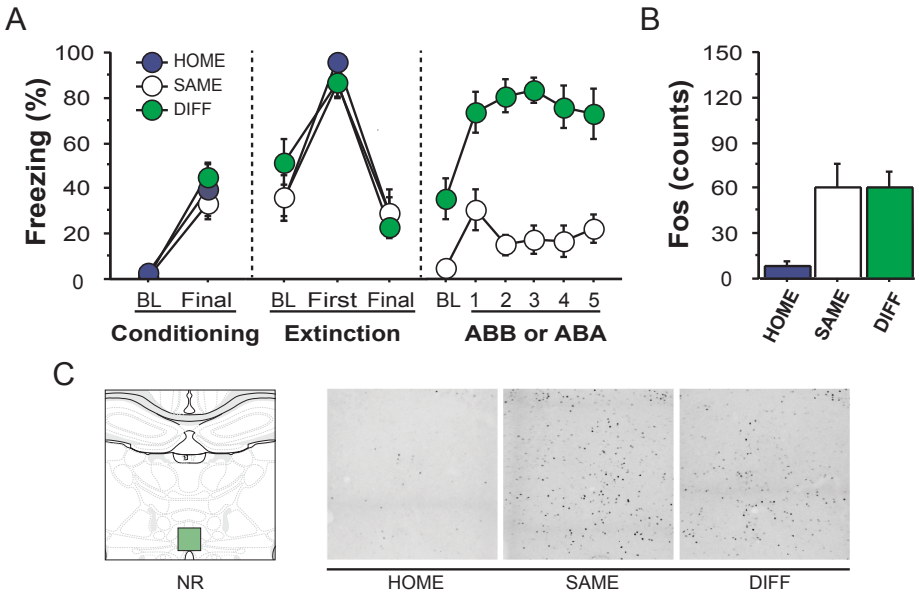

Figure 4-2 Fos expression in the RE during retrieval of extinction. (A) (Left) Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and the last 1-min ITI (intertrial interval) following CS-US pairings during the fear conditioning session. (Middle), Percentage of freezing (mean ± SEM) during the 3-min baseline and the first and last extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (Right), Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during an extinction retention test. (B) Number of c-Fos positive neurons in the RE in rats received extinction retrieval test (Same; *n*= 11), fear renewal test (Diff; *n*= 11) or home control (Home; *n*= 6). (C) Representative images of the RE selected for c-Fos counting.

Experiment 3: RE inactivation blunts retrieval-induced Fos expression in the mPFC and vHPC. The present experiments indicate that the RE is necessary for extinction retrieval and that neuronal activity in the RE is elevated during this process. Interestingly, the RE is an important subcortical interface between the mPFC and HPC, two brain areas that have been implicated in extinction. In this experiment, we explored whether the RE influences mPFC and HPC activity after extinction retrieval. Rats received auditory fear conditioning and extinction. Twenty-four hours after extinction, rats received either SAL or MUS infusions into the RE. As in Experiments 1 and 2, RE inactivation significantly impaired extinction retrieval [repeated measures ANOVA, main effect of drug, $F_{(1,25)}= 5.7$, $p< 0.05$]. Analyses of Fos expression in the mPFC and vHPC revealed that muscimol infusion in the RE during extinction retrieval significantly decreased c-Fos expression in both the mPFC and vHPC compared to saline group that showed successful extinction retrieval [unpaired t-test, mPFC: $t_{(25)}= 2.7$, $p< 0.05$; vHPC: $t_{(25)}= 2.9$, $p< 0.01$]. This suggests that RE inactivation may impair extinction retrieval by disrupting activity within and interactions between the mPFC and vHPC.

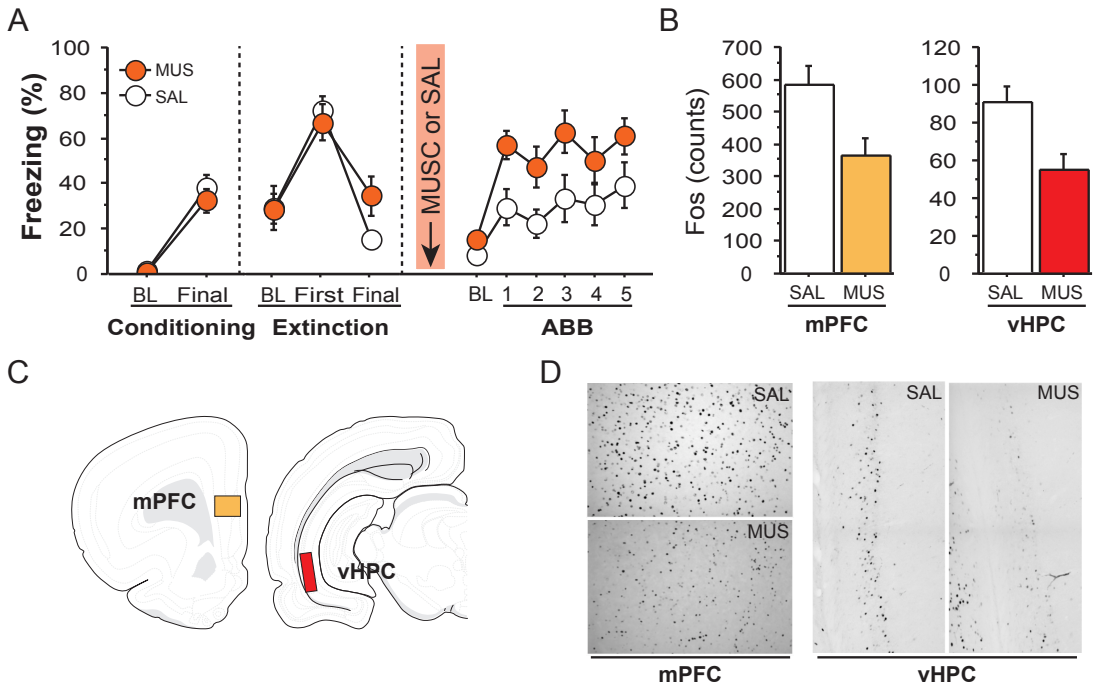

Figure 4-3 Fos expression in the mPFC and vHPC following RE inactivation. (A) (Left) Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and the last 1-min ITI (intertrial interval) following CS-US pairings during the fear conditioning session. (Middle), Percentage of freezing (mean ± SEM) during the 3-min baseline and the first and last extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (Right), Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during an extinction retention test. Arrow indicates the timing of saline or muscimol injection before the onset of the CS test. (B) Number of c-Fos positive neurons in the mPFC and vHPC in rats received saline (SAL; *n*= 12) or muscimol (MUS; *n*= 15) before the onset of tests. (C) Representative schematic images of mPFC and vHPC regions for c-Fos counting. (D) Fos^{+} neurons in the mPFC and vHPC after DAB staining.

Experiment 4: Silencing RE projectors in the mPFC impairs extinction retrieval.

Next, we were curious if the retrieval impairment caused by RE inactivation was due to a disruption of prefrontal projections to RE. Rats underwent auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,30)}$= 9.0; p< 0.001] and three sessions of extinction [repeated measures ANOVA, main effect of session, $F_{(2,12)}$= 16.0; p< 0.001]. On the following two days after the last extinction session, rats received extinction retrieval tests using a within-subjects design in which each animal served as its own control. That is, rats were tested after receiving either SAL or CNO on two counterbalanced tests in the extinction context, which were conducted over two days. When tested after CNO administration, rats exhibited elevated levels of freezing compared to SAL treatment [repeated measures ANOVA, main effect of drug, $F_{(1,6)}$= 7.2; p< 0.05]. Average freezing during the extinction retrieval tests (Figure 4-4C, rightmost panel) also revealed significantly higher levels of freezing after CNO compared to SAL treatment [paired t-test, $t_{(6)}$= -2.5, p< 0.05]. These results indicate that prefrontal projections to the RE are involved in retrieval of extinction memory.

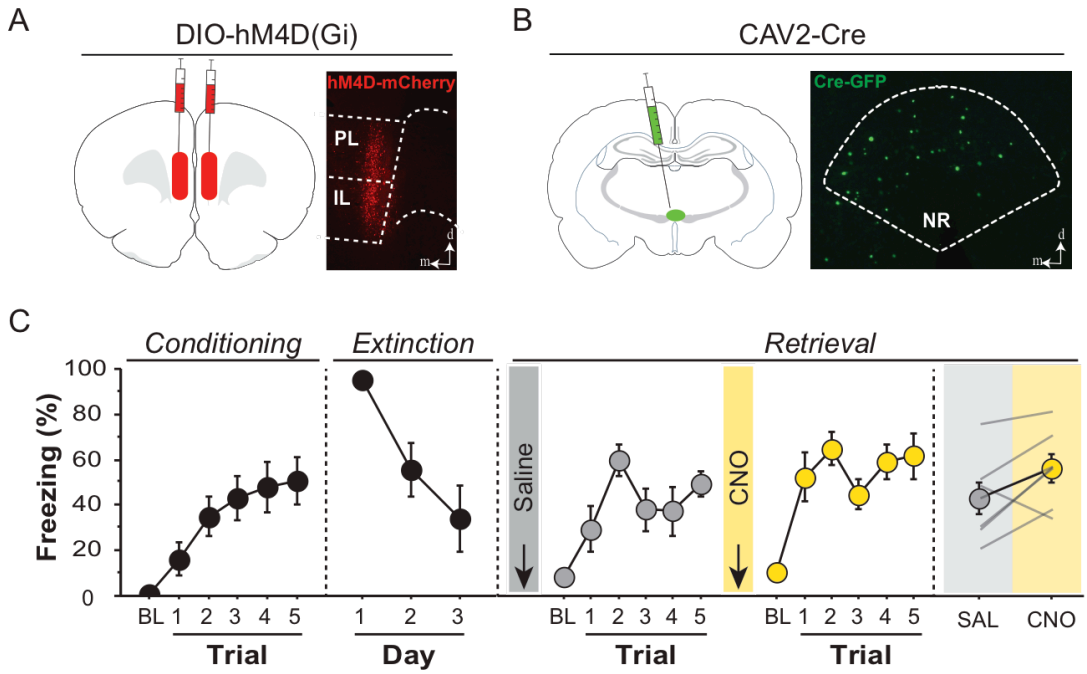

Figure 4-4 Silencing of mPFC-RE projections during extinction retrieval. (A and B) Representative images of Cre-dependent DREADD expression; AAV8-hSyn-DIO-hM4D(G_i)-mCherry in the mPFC and AAV5-Cre-eGFP virus in the RE. (C) (Conditioning) Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and 1-min ITI (intertrial interval) following each CS-US pairing during the fear conditioning session. (Extinction) Percentage of freezing (mean ± SEM) during three days of extinction (each day represents average freezing of first 5 extinction trials during that extinction session). (Retrieval) Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during an extinction retention test. Arrows indicate CNO or saline injection before the onset of tests. Shaded panel represents average ITI freezing (mean ± SEM) and each grey line represents an individual rat (n= 7; p < 0.05).

Experiment 5: Silencing vHPC projectors in RE does not affect extinction retrieval. Finally, we examined if the prefrontal modulation on the RE during extinction retrieval relays to the HPC via direct projections from the RE to vHPC. Thus, in this experiment, we detected whether silencing of RE→vHPC projections influence extinction retrieval. To this end, two groups of rats received fear conditioning, extinction and retrieval test, and all the data were separated by groups. One group of the rats express DIO-hM4D(Gi) in RE→vHPC pathway, the other group of rats express control DIO-mCherry virus in RE→vHPC. Rats received auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,35)}$= 6.3; p< 0.001 for mCherry control; $F_{(5,100)}$= 22.7; p< 0.0001 for RE→vHPC], and three sessions of extinction [repeated measures ANOVA, main effect of session, $F_{(2,14)}$= 27.3; p< 0.001 for mCherry control; $F_{(2,40)}$= 68.5; p< 0.0001 for RE→vHPC]. On the following two days, rats received extinction retrieval tests in within-subject manner in which they received counterbalanced injections of CNO and saline prior to tests. Test results showed that in all groups, rats showed similar levels of freezing when administered with CNO and saline [repeated measures ANOVA, main effect of drug, Fs< 1]. Average freezing during entire extinction retrieval tests also showed no difference after CNO administration (p> 0.05). These results indicate that RE projections to the vHPC are not required for extinction memory retrieval.

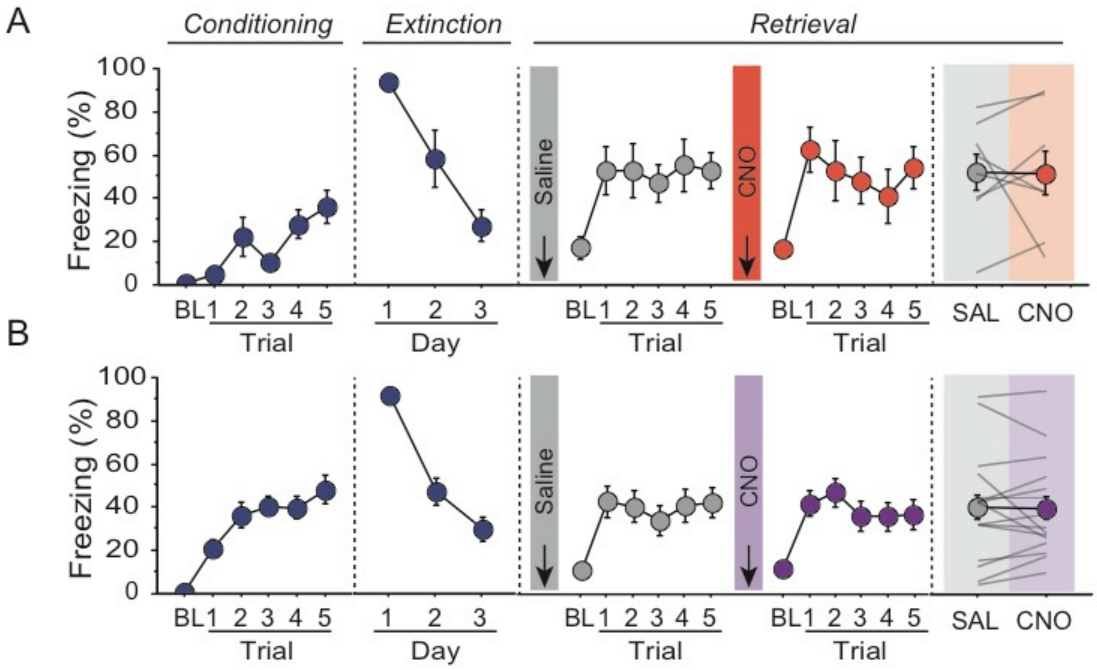

Figure 4-5 CNO inactivation of RE-vHPC during extinction retrieval test. Top panel shows data from rats expressing mCherry control virus in the pathways (RE→vHPC; *n*= 8), bottom panel shows data from rats expressing DIO-hM4D(Gi) virus in RE→vHPC (*n*= 21). (Conditioning), Percentage of freezing (mean ± SEM) during the 3-min baseline (BL) and 1-min ITI (intertrial interval) following each CS-US pairing during the fear conditioning session. (Extinction), Percentage of freezing (mean ± SEM) during three days of extinction (each day represents average freezing of first 5 extinction trials during that extinction session). (Retrieval), Percentage of freezing (mean ± SEM) for the 10-min BL period and 30-s ITIs for 5 CS test trials during an extinction retention test. Arrows indicate CNO or saline injection before the onset of tests. Shaded panel represents average ITI freezing (mean ± SEM) and each grey line represents an individual rat.

Discussion

In the present study, we demonstrated that the RE is required for retrieval of extinction, and that inactivation of the RE attenuates neuronal activity in the mPFC and vHPC, two brain areas crucial for fear extinction. Further, we showed that the mPFC projections to RE are involved in extinction retrieval. Taken together, the present study discovered an important role of the RE in fear extinction and suggests its potential role as a key structure mediating prefrontal top-down inhibitory control on fear expression.

The fact that the RE is critically involved in extinction recall is in line with previous work demonstrating the importance of the RE in learning and memory (Davoodi et al., 2009; Duan et al., 2015; Ito et al., 2015) as well as emotional processing (Davoodi et al., 2011; Xu and Sudhof, 2013; Kafetzopoulos et al., 2017; Sierra et al., 2017). A recent study demonstrated that the RE, as an essential relay structure for the mPFC→RE→HPC circuit, is important for maintaining specificity of contextual fear memory (Xu and Sudhof, 2013). Specifically, the authors showed that intact RE is required in contexts where fear should be inhibited; therefore, fear generalized to a safe context when the RE was inactivated. In line with this, we showed that RE inactivation impaired extinction retrieval. RE inactivation did not affect fear recall in the original conditioning context (Xu and Sudhof, 2013) or disrupt auditory fear expression (fear renewal), suggesting that the role of the RE might be specific to inhibitory control on fear memory. Potential mechanisms underlying inhibitory role of the RE in aversive memory retrieval by relaying prefrontal cortical projections to the hippocampus has been suggested

(Anderson et al., 2016); however, very few studies have been conducted regarding its inhibitory role in either animals or humans. Interestingly, both fear generalization and fear extinction rely heavily on contextual processing. That is, fear is limited to the original conditioning context, and is not expressed in other contexts (e.g. novel context). Similarly, extinction memory is highly context-dependent (Bouton, 2004). This suggests that the RE might be involved in inhibitory control on fear memory that requires contextual modulation. Previous studies from our lab have demonstrated that contextual regulation of fear memory retrieval requires hippocampal regulation on the mPFC (Orsini et al., 2011; Jin and Maren, 2015a; Wang et al., 2016). However, inactivation of the HPC or disconnections between the HPC and mPFC only disrupts fear renewal, but not extinction retrieval (Hobin et al., 2006; Orsini et al., 2011). This reveals that the direct hippocampal-medial prefrontal interaction is not required for extinction retrieval, thus suggesting a potential role of the indirect medial prefrontal-hippocampal interactions in extinction retrieval.

Indeed, in our c-Fos experiment, we demonstrated that silencing the RE blunted neuronal activity in the mPFC and vHPC, two key brain regions for fear extinction. Specifically, the infralimbic (IL) prefrontal cortex has been reported as the primary brain area for encoding as well as retrieval of fear extinction (Kim et al., 2016), and the vHPC is associated with extinction encoding as well as contextual regulation during and after extinction (Corcoran et al., 2005; Hobin et al., 2006; Orsini et al., 2011). In line with these findings, prior work has shown elevated c-Fos expression during extinction

retrieval in both the IL and vHPC, indicating successful extinction retrieval accompanies increased activity of both brain regions (Knapska and Maren, 2009). Thus, blunted activity of the mPFC (including IL) and vHPC caused by RE inactivation could explain extinction retrieval deficit in our study. Interestingly, the Fos results indicate that the RE is not only crucial for fear suppression but also involved in fear expression. Indeed, a recent study indicated that the RE is one of the hub structures involved in fear memory consolidation (Vetere et al., 2017). Specifically, the authors first detected a neural network underlying fear memory recall by examining c-Fos activity in different brain areas, and found that the RE is one of the highly-connected hub areas (Wheeler et al., 2013). Subsequent work showed that chemogenetic silencing of the RE disrupts fear memory consolidation in a greater degree than silencing other brain areas (Vetere et al., 2017). Together with our results, these data suggest that the RE is a hub of in a distributed circuit regulating the conditioning and extinction of fear; thus it is crucial to figure out what specific brain circuits including the RE are involved in a extinction retrieval.

In the present experiments, we have shown that direct mPFC inputs to the RE are crucial for extinction retrieval. Indeed, the RE has been suggested as a very important brain region for relaying prefrontal information to other brain areas, including the hippocampus. Direct prefrontal regulation on the RE is involved in fear memory generalization (Xu and Sudhof, 2013), goal-directed spatial navigation (Ito et al., 2015), motivation and reward related behavior (Zimmerman and Grace, 2016) and spatial

working memory (Hallock et al., 2016). These studies place the RE as the primary structure for relaying mPFC→HPC information or mediating hippocampal-prefrontal synchrony. Anatomically, this is due to strong reciprocal projections between the RE and these brain areas (Varela et al., 2014). More importantly, because of the lack of direct mPFC→HPC projections, the RE is positioned well for indirectly relaying the prefrontal information to hippocampal areas (Vertes et al., 2007). Functionally, the midline thalamic areas are proposed to adjust the activity level of cortical areas via cortico-thalamo-cortical pathways instead of simply relaying sensory information (Groenewegen and Berendse, 1994; Saalmann, 2014). Thus, our results showing a crucial role of prefrontal control on the RE in fear suppression is consistent with these studies.

However, results from the last experiment suggest that the RE is not responsible for relaying the information to the HPC during extinction retrieval. This is unexpected given the fact that the RE has been reported to relay indirect mPFC-HPC interactions in a number of situations including maintaining contextual specificity (Xu and Sudhof, 2013). Nevertheless, the result is somewhat consistent with previous studies showing successful extinction retrieval with ventral or dorsal hippocampal inactivation (Corcoran and Maren, 2001; Hobin et al., 2006). Thus, it is possible that the RE indeed relays medial prefrontal top-down control to a different brain region. For example, the RE might be able to communicate directly to the amygdala during extinction retrieval. The amygdala is the key structure for both fear and extinction, and its sub-nuclei, including basolateral and basomedial amygdala, receive sparse but direct inputs from the RE

(Vertes, 2006). Or, it is possible that the RE sends information back to the mPFC, resulting in a cortico-thalamo-cortico circuitry between cortical areas and the RE (Sherman and Guillery, 2002). Indeed, prior work has demonstrated pronounced excitatory actions on the mPFC generated by RE (Di Prisco and Vertes, 2006; Eleore et al., 2011). Finally, we could not rule out the possibility that the DREADD system failed to work in the reuniens, including its projections to other areas, as we expected. The heterogeneity of DREADD expression in different cell types and brain regions have been reported (Smith et al., 2016). Moreover, the ability of the DREADD receptors to modulate in different brain regions is also different. Thus, further validation of DREADD modulation in the reuniens should be accompanied by future study. Collectively, identifying brain areas receiving mPFC→RE regulations in extinction retrieval is essential for understanding how the circuits contribute to maladaptive fear in anxiety and post-traumatic stress disorder.

CHAPTER V

CONCLUSIONS

Summary of findings

It has been suggested that a distributed neural network consisting of the hippocampus, medial prefrontal cortex (mPFC) and the amygdala is essential for contextual retrieval. In the current dissertation, we explored the neuronal mechanisms underlying ventral hippocampal modulation of the medial prefrontal cortex and amygdala during fear renewal. We also determined whether indirect medial prefrontal control of the ventral hippocampus (vHPC), via the nucleus reuniens (RE), is also involved in contextual retrieval.

In Chapter II, we asked whether prelimbic (PL)-, basal amygdala (BA)- or dual-projecting neurons within the vHPC are preferentially involved in fear renewal. We combined cholera toxin subunit b (CTb) infusions in the PL and BA with c-Fos immunohistochemistry in the vHPC projection neurons to examine whether different pathways show different levels of neuronal activity during fear renewal and extinction retrieval. As expected, ventral hippocampal projections to both the PL and BA were preferentially involved in fear renewal, but there was no difference between PL- and BA-projecting neurons in c-Fos level during fear renewal. This indicates that there is no preferred pathway between vHPC→PL and vHPC→BA in terms of expression of the fear after extinction. Interestingly, although few in number, dual-projecting vHPC

neurons were more likely to show c-Fos expression than neurons projecting only to PL or BA during fear renewal, suggesting that the vHPC dual-projection neurons might be important for synchronizing medial prefrontal and amygdala activity during fear expression.

We then explored whether vHPC projections to IL are important for context-dependent memory retrieval in Chapter III. We used circuit-specific DREADD approach to test if the circuit is necessary for fear renewal. The results revealed that vHPC→IL pathway was required for fear renewal, and activation of the same circuit was sufficient to induce fear relapse in the safe (extinction) context. We then showed that the vHPC→IL pathway exerted such a role by inducing feedforward inhibition within the IL, the key structure for fear suppression. This means that the vHPC inputs to the IL dampen inhibitory control of the IL during extinction retrieval and thus lead to fear renewal.

Next, we demonstrated that the indirect medial prefrontal pathway to the vHPC is also involved in context-dependent fear memory retrieval. We showed that inactivation of either the RE or mPFC→RE pathway disrupted context-dependent extinction retrieval when tested in the extinction context. However, contrary to our hypothesis, inactivation of the RE→HPC did not affect extinction retrieval, suggesting that the medial prefrontal control on the RE does not relay to the vHPC. Taken together, findings in the current dissertation suggest important roles of both direct and indirect hippocampal-medial prefrontal interactions in contextual fear memory retrieval.

Hippocampal-prefrontal control in contextual retrieval

Accumulating data in the last decade provide knowledge about the individual roles of the HPC, amygdala, and mPFC in context-dependent memory retrieval. For example, neurons within the amygdala, including both the LA and BA, show context-dependent neuronal firing (Hobin et al., 2003; Herry et al., 2008). Hippocampal inactivation eliminates context-dependent firing within the LA and disrupts renewal of fear (Corcoran and Maren, 2001; Hobin et al., 2006; Maren and Hobin, 2007). Furthermore, disconnections of vHPC and PL or BA disrupt fear renewal, indicating that the interactions between the vHPC and the amygdala or mPFC are crucial for contextual retrieval. However, the circuit mechanism underlying the interactions was not clear.

Results from Chapter II and III now suggest that ventral hippocampal direct projections to the PL, BA or IL are recruited during fear renewal. However, the functional mechanism of each pathway underlying contextual retrieval is distinct. Our result is consistent with Orsini and colleagues, who showed BA-projecting vHPC neurons exhibiting higher c-Fos activity during fear renewal compared to extinction retrieval (Orsini et al., 2011). Herry and colleagues (2008) also showed that "fear neurons", neurons that only fire during fear renewal, within the BA receive input preferentially from the vHPC while no "extinction neurons" receive vHPC input (Herry et al., 2008). These findings together suggest that ventral hippocampal projections to the BA are preferentially involved in fear expression after extinction but not in fear suppression. Recently, Xu and colleagues (2016) used circuit specific approach to show that ventral

hippocampal projections to the CeA, but not the BA, are responsible for fear renewal (Xu et al., 2016). This is surprising given the fact that previous c-Fos, recording and disconnection studies all suggest a crucial role of the vHPC→BA circuit in fear renewal. The first possible reason for the discrepancy could be the technical issue. The BA and CeA are adjacent to each other; therefore, lesions, tracer or viral infusions in either area will inevitably affect the other one. These manipulations could also have affected interactions between the BA and CeA. Second, it was not clear if the CeA-projecting vHPC neurons also project to the PL, which is crucial for fear renewal (Orsini et al., 2011). Our data, as well as another study, showed that vHPC neurons send collaterals to both the amygdala and mPFC (Ishikawa and Nakamura, 2006), therefore it is possible that the effect was partially driven by the collaterals. Finally, it is possible that the BA→vHPC projections are also involved in the process as Xu and colleagues suggested. Neuroanatomical studies showed reciprocal projections between the BA and vHPC, as such the vHPC-projecting BA neurons might also involve in fear renewal. However, this idea has not been tested yet thus needs further study.

Another important finding in Chapter II is that BA and PL dual-projecting vHPC neurons are preferentially activated during fear renewal. Anatomy and physiology of the dual-projecting vHPC neurons has been identified before (Ishikawa and Nakamura, 2006). Recently, Kim and Cho conducted a more in-depth investigation on these dual-projecting vHPC neurons (Kim and Cho, 2017). The authors showed that dual-projecting vHPC neurons make excitatory synapses on both the mPFC and BA neurons, and

activation of these dual-projecting neurons induces action potentials in amygdala-projecting mPFC neurons. Simultaneous activation of axon collaterals in the mPFC and amygdala induces synchronization between these two areas and further facilitates expression of the fear after extinction (Lesting et al., 2011). This is consistent with our finding showing the dual-projecting neurons preferentially involved in fear renewal.

The ventral hippocampal projections to PL and IL are crucial for fear renewal, but in different ways. For example, lesion, inactivation and c-Fos studies showed that the PL is actively involved in fear renewal after extinction (Orsini et al., 2011; Kim et al., 2013; Zelikowsky et al., 2013; Jin and Maren, 2015a; Sharpe and Killcross, 2015; Fu et al., 2016). That is, fear renewal induces c-Fos expression in the PL and decreasing its activity disrupts renewal. Importantly, interactions between the PL and vHPC are required for renewal and PL-projecting vHPC neurons show increased activity during the process (Orsini et al., 2011). These findings suggest that the PL receives direct modulation from the vHPC during renewal given that there is no direct projection back from the PL to the vHPC. Contrary to the PL, IL neurons showed increased activity during extinction retrieval, and inactivation of the IL leads to failure of extinction retrieval, suggesting that the IL is required for fear suppression after extinction (Sierra-Mercado et al., 2006; Burgos-Robles et al., 2007; Knapska and Maren, 2009). Indeed, IL neurons project to amygdala GABAergic ITC neurons, the inhibitory neurons that limit excitatory input from the BA and therefore reduce CeA-mediated fear responses (Berretta et al., 2005; Likhtik et al., 2008; Amano et al., 2010). Accordingly, fear

renewal is due to active dampening of IL inhibitory control. In line with this idea, we showed in Chapter III that activation of the IL by infusing GABA receptor antagonists disrupted fear renewal. This further confirms that the renewal is due to suppressed activity of the IL, and fear renewal can be avoided by releasing the suppression. Importantly, we further showed that the suppression of the IL activity is due to direct ventral hippocampal inputs. That is, circuit specific inactivation of the vHPC→IL pathway disrupted fear renewal, and inactivation of the same circuit in the safe context was sufficient to drive fear relapse. These results indicate that the vHPC→IL pathway is actively dampening fear suppression by reducing inhibitory control of the IL. The opposite effect of the ventral hippocampal modulation on the PL and IL is probably due to anatomical and physiological differences between the circuits. Studies suggest that IL-projecting vHPC neurons outnumber PL-projecting neurons; thus, the impact of the vHPC modulation on the mPFC will be greater through the IL (Wang et al., 2016). Further, vHPC neurons project to both excitatory and inhibitory neurons in the mPFC, therefore, activation of the vHPC produces both excitation and feedforward inhibition within the mPFC (Tierney et al., 2004). Importantly, due to the greater feedforward inhibition than excitation within the IL, vHPC activation ultimately leads to suppressed activity in IL neurons (Tierney et al., 2004). We further showed that circuit-specific activation of the vHPC→IL pathway resulted in increased level of c-Fos activity in PV-interneurons within the IL but not in PL. This further indicates that vHPC→IL pathway is responsible for fear relapse due to suppressed activity within the IL by increasing GABAergic transmission. Taken together, the data suggest that the vHPC is responsible

for fear relapse by increasing activity of the PL and BA while simultaneously dampening fear suppression by inducing feedforward inhibition in the IL.

The role of the reuniens in extinction retrieval

Because interactions between the HPC and mPFC are important in contextual retrieval, we examined if the indirect pathway from the mPFC to HPC via RE is also involved in contextual retrieval. We selected the RE as a candidate relay structure for several reasons. First, it has been shown that the RE receives inputs from the mPFC, mainly from the PL and IL (Vertes, 2002). More importantly, the RE is the principal, or virtually the only, source of the thalamic input to the hippocampus (Vertes et al., 2007). This led us to believe that the RE might represent important relay between the mPFC and HPC. Secondly, the RE has been reported to be involved in tasks that require activity of both the mPFC and HPC (Loureiro et al., 2012; Cholvin et al., 2013; Layfield et al., 2015). Lastly, studies have shown that the RE is a hub brain region in fear network and indeed, the RE controls indirect mPFC→HPC modulation in generalization of contextual fear (Xu and Sudhof, 2013; Xu et al., 2016). For these reasons, in Chapter IV we tested our hypothesis that the mPFC→RE→HPC is crucial for contextual retrieval.

Our results suggest that the RE is required for extinction retrieval but not fear renewal. Given that previous work has shown that the RE is responsible for fear memory precision (Xu and Sudhof, 2013), it is not surprising that the RE inactivation interferes with retrieval of context-dependent extinction memory. Moreover, as we expected, result

showed that silencing of the mPFC→RE pathway disrupted extinction retrieval. However, the same manipulation in the RE→HPC pathway failed to show retrieval deficit, indicating that the RE receives medial prefrontal control but does not relay the information to the HPC during extinction retrieval. There are a few explanations for the results. In our experiments, we have manipulated mPFC→RE and RE→HPC pathways separately. Although there is evidence that the mPFC fibers make contacts on RE neurons projecting to the HPC (Vertes et al., 2007), the percentage of the overlapping neurons in all HPC-projecting neurons is not clear. It might give us more precise results if we could manipulate HPC-projecting neurons that receive mPFC inputs. Another explanation is related to the fundamental question of whether or not the HPC itself is required for extinction retrieval. Both the dorsal and ventral HPC are suggested to play a role in expression of the contextual or cued fear, but inactivation of neither of them influences extinction retrieval (Corcoran and Maren, 2001; Hobin et al., 2006). Discordantly, several studies reported increased activity of the vHPC neurons during extinction retrieval (Knapska and Maren, 2009; Orsini et al., 2013). Thus, how HPC neurons contribute to the extinction retrieval still needs further study. Finally, if the target of mPFC→RE top-town inhibitory control is not the HPC, it is possible that other brain regions, such as the amygdala, receive the modulation. As previous studies show that interactions between the mPFC and amygdala are important for fear suppression, the RE could either directly or indirectly involved in the interaction given the fact that it has connections with both the areas (Sotres-Bayon and Quirk, 2010; Griffin, 2015; Likhtik and Paz, 2015). Taken together, we show that the medial prefrontal control on the RE is

important in top-down inhibitory control on fear, but the target region of the control still need further exploration (Figure 5-1).

Future directions

The results of the current work (Chapter II) as well as previous studies suggest that vHPC→BA is preferentially involved in fear renewal. However, the idea was challenged by recent findings that the circuit is indeed responsible for expression of context fear but not fear renewal (Xu et al 2016; Kim and Cho 2017). As such, a necessary experiment to explain the discrepancy would be to test if reciprocal vHPC-BA circuits are necessary for fear renewal. To test the hypothesis, we could use circuit-specific DREADD inactivation approach to selectively silence either pathway during fear renewal in the ABA design. I hypothesize that silencing of either pathway would result in low levels of fear.

In Chapter III, we presented the results that the vHPC→IL pathway is responsible for IL feedforward inhibition and subsequent fear relapse. We showed that activation of the vHPC→IL pathway induced fear return in extinction context by increasing activity of PV-interneurons within the IL. However, in Chapter III, these results were not followed by experiment examining the effect of direct manipulation on the PV-interneurons during fear renewal. Accordingly, an important next step would be to specifically inactivate PV-interneurons within the IL prior to fear renewal test. This could be addressed by injecting Cre-dependent hM4D(Gi) virus into the IL of PV-Cre transgenic

rats to specifically inactivate PV-interneurons during fear renewal. The experiment would explain if increased activity of PV-interneurons within the IL is indeed responsible for fear renewal. Further, using immunohistochemistry, we should be able to detect if the behavioral effect is accompanied by increased c-Fos activity in IL excitatory neurons and decreased activity in CeM fear-output neurons.

Lastly, we demonstrated that the mPFC→RE modulation on extinction retrieval did not relay to the HPC. If the RE is relaying indirect mPFC→HPC modulation, then other possibilities are the RE sends information back to the mPFC thereby generating closed mPFC-RE loop or the RE interferes with mPFC-amygdala interaction. The ideas could be tested via circuit-specific DREADD inactivation of the RE→mPFC or RE→BLA pathway during extinction retrieval. It would also be interesting to test if the RE is involved in extinction encoding as well given the important role of the mPFC in extinction and the role of the RE in maintaining contextual specificity.

In conclusion, studying the role of each pathway within a relatively broad fear and extinction networks is crucial for both pre-clinical animal research as well as future brain region- and neuronal circuit-specific therapeutic interventions in pathological fear and anxiety treatment.

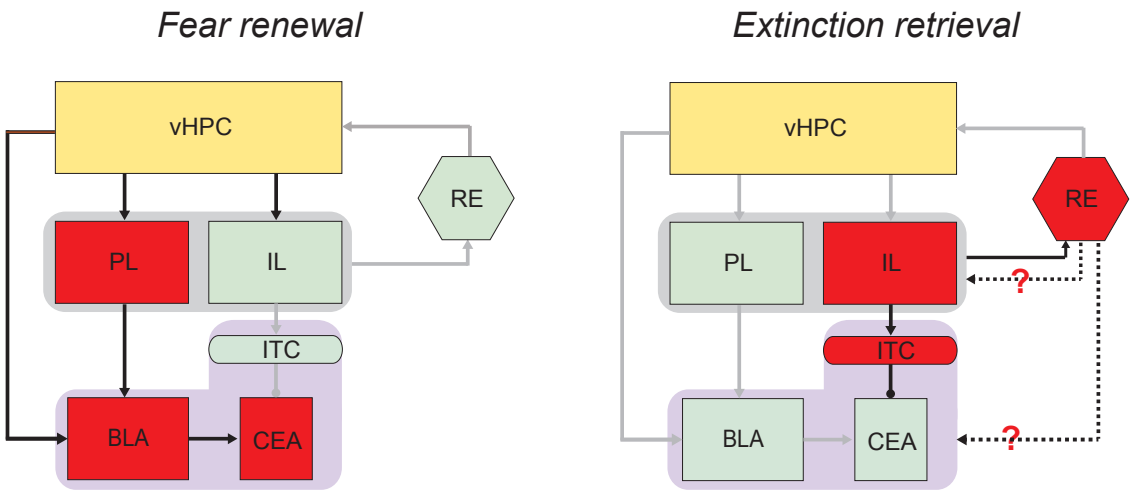

Figure 5-1 Neural circuits for context-dependent memory retrieval. The context-dependence of fear memory involves a neural circuit that includes the ventral hippocampus, medial prefrontal cortex (PL and IL), amygdala and the reuniens. Specifically, the hippocampus projects directly to the BLA, PL and IL, and these projections may be crucial for the renewal of fear expression in response to an extinguished conditional stimulus. Importantly, direct projections from the hippocampus to the IL mediate feedforward inhibition within the IL in response to an extinguished conditional stimulus in renewal context. These circuits together activate CEA fear output neurons thereby generating fear responses. During extinction retrieval, the IL activates ITC neurons thereby inhibiting CEA fear output neurons. Furthermore, the RE receives direct modulation from the mPFC during extinction retrieval, but the roles of the RE projections to the mPFC or amygdala in extinction retrieval needs further study.

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