ROLE OF THALAMIC NUCLEUS REUNIENS IN PAVLOVIAN FEAR CONDITIONING AND EXTINCTION

A Dissertation

by

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ABSTRACT

Stress and anxiety disorders such as posttraumatic stress disorder (PTSD) pose a significant burden to the society. Existing cognitive-behavioral treatments are centered around exposure therapy which, while effective in the clinic, is susceptible to relapse (return of fear). This is because exposure therapy does not erase the underlying fear memory, rather it yields an inhibitory, context-dependent "extinction" memory that competes with the fear memory. Hence, considerable research has focused on understating this context dependence of the extinction memories in an attempt to prevent relapse. However, the mechanism of this fear inhibition is still relatively unknown. When human subjects actively try to suppress memory retrieval, medial prefrontal cortex (mPFC) activation is correlated with hippocampal (HPC) suppression. In rodents, data from a variety of behavioral paradigms have shown that the PFC interacts with the HPC via the thalamic nucleus reuniens (RE). Here I explored the contribution of RE to the conditioning and extinction of conditioned fear in rats. I first show that RE is critical for acquisition and precision of contextual fear memories. Importantly, I show that RE inactivation renders a contextual fear memory that is acquired independently of the hippocampus. I then show that RE is critically involved in both acquisition and expression of fear extinction. Circuit-specific manipulations showed projections form the mPFC to RE are critical for fear extinction. Finally, using intersectional optogenetic methods, I find evidence that RE projections to both the mPFC and HPC are involved in extinction retrieval. Collectively, these data reveal a critical role for RE in contextual memory precision and context-dependent extinction that may be mediated through mPFC-HPC interactions.

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NOMENCLATURE

ACC	Anterior Cingulate Cortex
AMY	Amygdala
ANOVA	Analysis of Variance
BLA	Basolateral Complex of the Amygdala
CeA	Central Amygdala
CeL	Lateral Nucleus of the Central Amygdala
CeM	Medial Nucleus of the Central Amygdala
CNO	Clozapine *N*-oxide
CR	Conditioned Response
CS	Conditioned Stimulus
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
HPC	Hippocampus
IHC	Immunohistochemistry
IL	Infralimbic Cortex
ITCs	Intercalated Cells
LA	Lateral Amygdala
LC	Locus Coeruleus
MD	Mediodorsal Thalamus
mPFC	Medial Prefrontal Cortex
PFC	Prefrontal Cortex
PL	Prelimbic Cortex
PVT	Paraventricular Thalamus
PTSD	Posttraumatic Stress Disorder

RE	Nucleus Reuniens
Rh	Rhomboid Nucleus
US	Unconditioned Stimulus
Xi	Xiphoid Nucleus

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1. INTRODUCTION

1.1 Overview

Learning to contend with threats in the environment is essential for survival. This allows animals, ranging from rodents to humans, to anticipate harm and organize appropriate defensive behaviors in response to threats (Bolles, 1970). These aversive memories are evolutionarily programmed to be rapidly acquired, temporally enduring, and broadly generalized across both familiar and novel contexts (Maren, 2001, 2011; Izquierdo et al., 2016). Although these memories are adaptive in that they allow organisms to anticipate and avoid danger, excessive worry and apprehension can lead to pathological conditions such as panic disorder, generalized anxiety disorder, and post-traumatic stress disorder, to name a few (Bouton et al., 2001; Jovanovic and Ressler, 2010; Liberzon and Ressler, 2016). Currently, treatments for fear and anxiety disorders are centered around pharmacotherapies and cognitive behavioral therapies. However, these therapies often fail to provide long-term relief and have high rates of relapse. Thus, considerable emphasis has been placed on basic and translational research in an effort to 1) understand the brain circuits that govern this fear and anxiety behavior and 2) to develop novel pharmacological drugs or treatment strategies to combat the mental illness.

1.2 Pavlovian fear conditioning

In a laboratory setting, Pavlovian or classical fear conditioning is widely used to study the neurobiology of emotional learning and memory. First developed by Pavlov in 1927, this classical conditioning procedure has proved to be a reliable model to study the neurobiology of learning and memory across different species (Pavlov, 1927; Maren, 2001). Although there are many forms of classical conditioning, I will focus on Pavlovian fear conditioning in rodents. The first step in Pavlovian fear conditioning is the acquisition phase wherein animals are presented with an

innocuous conditioned stimulus (CS), such as a tone, which terminates with the presentation of an aversive unconditioned stimulus (US), such as a footshock. After as little as a single trial, animals learn to associate the innocuous CS with the aversive US and emit a conditioned fear response (CR) to the CS alone. The CR most often measured in rodent subjects is freezing behavior, which is characterized by a lack of bodily movement (except breathing) (Maren, 2001). Freezing CRs are expressed to both the CS, as well as the context in which conditioning occurred (and context can serve to signal shock if the CS is omitted during conditioning). After successful acquisition, fear CRs can be extinguished by repeatedly presenting the CS alone in the absence of the US; a process known as extinction. Following successful extinction training, subjects learn that the CS no longer predicts the US and consequently suppress conditional responding. Importantly, extinction does not result in erasure of the underlying fear memory. Rather, extinction results in the formation of a new inhibitory CS-'no US' memory which competes with the incumbent CS-US memory (Myers et al., 2006; Myers and Davis, 2007). Behavioral therapies that are widely used to treat fear and anxiety are based on extinction learning wherein the patients are repeatedly presented with the traumatic cues in a safe clinical setting until they fear is reduced (Norrholm et al., 2008; Milad et al., 2014; VanElzakker et al., 2014). After extinction, rodents can show high or low fear to the CS depending on the context in which the CS is presented. For example, when the CS is presented in the context in which it was extinguished, they show low fear. Whereas when the same CS is presented outside of the extinction context, the subjects show high fear — a process known as renewal (Bouton et al., 2006; Maren et al., 2013; Chen et al., 2017). This phenomenon is similar to relapse in human patients who often experience the return of fear memories when a traumatic cue is presented outside of the safe clinical setting (Morrison and Ressler, 2014; Bowers and Ressler, 2015). Hence, considerable research has concentrated on elucidating the behavioral and neural mechanisms underlying the context-dependence of extinction memories.

1.3 Fear Circuitry

Over the years, considerable emphasis has been placed on elucidating the neural circuitry mediating the acquisition, expression and extinction of conditioned fear (Maren and Quirk, 2004; Herry et al., 2008, 2010; Johansen et al., 2012; Tovote et al., 2015; Izquierdo et al., 2016). Multiple brain regions are recruited at various stages of Pavlovian fear conditioning and extinction (Maren, 2001, 2011; Maren and Quirk, 2004; Radulovic and Tronson, 2010; Maren et al., 2013; Giustino and Maren, 2015, 2018; Bergstrom, 2016; Wilson and Fadel, 2017; Chaaya et al., 2018). In the following sections, I will focus on three brain regions that are crucially involved in the acquisition and expression of conditioned fear.

1.3.1 The Amygdala

The core of the fear circuit revolves around the amygdala (AMY), which is crucially involved in the acquisition and expression of both fear conditioning and extinction (Maren and Quirk, 2004; Anglada-Figueroa and Quirk, 2005; Herry et al., 2006, 2010; Wilensky et al., 2006; Ehrlich et al., 2009; Maren, 2015; Tovote et al., 2015; Krabbe et al., 2018; Ressler and Maren, 2019). This almond-shaped structure is a node of highly interconnected nuclei and is sub-divided into 1) the basolateral complex (BLA) [consisting of basal nuclei (BA) and the lateral nucleus (LA) of amygdala], 2) the central nuclear group (CeA) and 3) the intercalated cells (ITC's). BLA is predominantly composed of glutamatergic excitatory neurons (80%) and a small population of inhibitory GABAergic interneurons (McDonald, 1992, 1998; Spampanato et al., 2011). On the other hand, both CeA and ITC are mainly comprised of inhibitory interneurons with few projection neurons. These local interneurons spread across the amygdala contribute to the complex microcircuitry within the amygdala.

Functionally, information regarding the CS and the US converges onto LA neurons in the BLA (Quirk et al., 1995; Maren, 2000). Consistent with this, lesions or functional inactivation of

BLA result in impairments in the acquisition and expression of Pavlovian fear conditioning (Maren et al., 1996a, 1996b; Maren, 1999; Goosens and Maren, 2001, 2003; Gale et al., 2004; Anglada-Figueroa and Quirk, 2005; Amano et al., 2011). Furthermore, single-cell electrophysiological recordings in BLA have shown both elevation and suppression of neuronal activity in a separate population of neurons in response to the CS after fear conditioning (Giustino et al., 2019). When animals are presented with two CS's, one of which was paired with an aversive stimulus (CS+) and another which was not associated with any aversive US (CS-), BLA neurons not only show differential activity to CS+ and CS- but also show synaptic plasticity selectively in neurons that encoded for CS+ (Maren et al., 1991; Goosens et al., 2003). Furthermore, when animals are presented with two different CS's encoding for a reward (like sucrose) and an aversive stimulus (like shock), BLA neurons also show selective firing responses (Namburi et al., 2015; Beyeler et al., 2016; Correia and Goosens, 2016; Sengupta et al., 2018). Interestingly, neurons that respond to positive CSs are predominantly striatal-projecting, whereas neurons that encode negative CSs are predominantly CeA-projecting (Namburi et al., 2015; Beyeler et al., 2016, 2018).

In addition to fear conditioning, the BLA is also recruited during fear extinction. For instance, inactivation of both BA and LA sub-regions of BLA results in impairments in extinction learning and expression (Herry et al., 2006; Sotres-Bayon et al., 2007; Laurent and Westbrook, 2008; Sierra-Mercado et al., 2011). Consistent with this, extinction learning also induces changes in CS responses in the BLA. For example, single-unit recording data show that there is a separate population of neurons that respond to high fear states after fear acquisition ("fear neurons") and in low fear after extinction ("extinction neurons") (Herry et al., 2008).

The second key component of the amygdala is the intercalated cells (ITC), which are cell masses spread in between BLA and CeA. They are made of GABAergic cells and control the activity of CeA through feed-forward inhibition. They can be divided into the dorsal ITC which

lies in between the LA and CeA and the ventral ITC which lies in between the BA and CeA. Due to its smaller size and remote positioning, few studies have concentrated on this nucleus. It has been hypothesized that the dorsal ITC regulates fear expression, whereas the ventral ITC could regulate extinction (Duvarci and Pare, 2014). Consistent with his hypothesis, a recent report indicated that fear conditioning induces dopamine-mediated LTP at LA→dorsal ITC synapses, which can inhibit CeA during fear expression (Lee and Kim, 2016). In contrast, c-fos activation studies have indicated ventral ITC nuclei are activated during fear extinction and subsequent retrieval of the extinguished fear in a safe or unsafe context (Knapska and Maren, 2009). Indeed, electrophysiological studies have shown that BA inputs to ITC are potentiated after fear extinction (Royer and Paré, 2002; Li et al., 2011). Functionally, either selective lesions of the ITC or pharmacological inactivation of BLA inputs to the ITC results in extinction impairments (Jüngling et al., 2008; Likhtik et al., 2008; Pape et al., 2010). In addition to projections from BLA, ITC also receives projections from the mPFC (Sesack et al., 1989; Mcdonald et al., 1996). Activation of the infralimbic (IL) division of the mPFC by picrotoxin produces selective increases in firing in ITC (Berretta et al., 2005). Furthermore, stimulation of IL after extinction training causes high frequency bursting in ITC which led to the proposal that IL projections to ITC can gate fear inhibition (Royer and Paré, 2002); (Pinard et al., 2012; Strobel et al., 2015). Together, these reports suggest that the ITC is an inhibitory nucleus that controls CeA activity based on input from BLA or mPFC.

The third and final subdivision of the amygdala is the CeA. Functionally the CeA is divided into a lateral (CeL) and medial (CeM) group. The CeA is widely considered a key output region of the amygdala. Information from BLA/ITC goes to the CeL, which is then transferred to CeM and in turn to the periaqueductal gray (PAG) and hypothalamus to mediate different responses such as freezing and regulation of stress responses, respectively (LeDoux et al., 1988; Paré et al.,

2004; Pape and Pare, 2010). In addition to this information transfer, recent evidence indicates that CeA can also support the learning of fear memories. For example, reversible inactivation of CeA blocks the acquisition of Pavlovian fear conditioning (Wilensky et al., 2006; Zimmerman et al., 2007). Furthermore, fear conditioning produces changes in CS- evoked neural responses within the CeA (Ciocchi et al., 2010; Duvarci et al., 2011; Fadok et al., 2017; Sanford et al., 2017). Interestingly, there is a local inhibitory network within the CeL that controls CeM (Duvarci et al., 2011). Briefly, CeL neurons contain two classes of cells that are distinguished based on the presence of protein kinase C*δ (PKCδ+/PKCδ-)*. Upon fear conditioning, PKCδ- acquires excitatory response and PKCδ+ acquires inhibitory response to the CS. Based on the timing of the activity upon the onset of CS, they concluded that the PKCδ- cells directly inhibit the PKCδ+ cells which then projects to activate the CeM cells for its downstream activity (Ciocchi et al., 2010; Li et al., 2013; Ressler and Maren, 2019).

Even though AMY is considered as a crucial regulator of Pavlovian fear conditioning, it does not function independently. It relies on information coming in from various cortical, sub-cortical and brainstem regions. They range from the mPFC, HPC, bed nucleus of stria terminalis (BNST), ventral tegmental area (VTA), PAG, and locus coeruleus (LC), to name a few (Herry et al., 2010; Giustino and Maren, 2015, 2018; Izquierdo et al., 2016; Goode et al., 2019). I will focus on the mPFC and HPC, which are central to the experiments performed in this thesis.

1.3.2 The Medial Prefrontal Cortex

The rodent mPFC is a midline frontal region that comprises of two major sub-regions namely the prelimbic prefrontal cortex (PL) and the infralimbic prefrontal cortex (IL) with the PL sitting dorsally to the IL. Together, they play a critical role in top-down cortical information processing (Badre and Nee, 2018). Anatomically, the rodent mPFC exhibits a six-layer laminar organization (Caviness, 1975; Uylings et al., 2003; Van De Werd et al., 2010). It is worth noting that in rodents,

mPFC layer IV is poorly defined compared to non-human primates and humans (Uylings et al., 2003; Van De Werd et al., 2010). They are mainly composed of pyramidal projection neurons and local GABAergic interneurons. The PL and IL subregions of the mPFC can be distinguished based on their laminar organization and cytoarchitecture (Krettek and Price, 1977; Van Eden and Uylings, 1985; Heidbreder and Groenewegen, 2003; Van De Werd et al., 2010). Also, they can be distinguished based on their afferent and efferent projection patterns. For example, even though both PL and IL project to the amygdala, they target different populations of neurons (McDonald, 1998; Vertes, 2004; Hoover and Vertes, 2007).

Early lesion studies examining the role of the mPFC in fear conditioning have yielded mixed results. For example, Morgan and colleagues found that mPFC lesions before fear conditioning did not impair fear acquisition and retention. Those animals, however, showed a diminished rate of extinction and deficits in extinction (Morgan et al., 1993). This report is consistent with other mPFC lesion studies that show no changes in defensive responses, aversive stimulus reactivity and long term fear memory (Divac et al., 1984; Holson, 1986). Pharmacological and viral manipulation studies that differentiate between the two sub-regions of mPFC provide a more clear picture of its contribution to Pavlovian fear conditioning and extinction (Giustino and Maren, 2015). For example, lesions or pharmacological inactivation of PL impairs retrieval to a CS or the context after fear conditioning. However, inactivation of PL before the conditioning does not effect on either acquisition or subsequent expression of fear conditioning (Sierra-Mercado et al., 2006, 2011; Corcoran and Quirk, 2007; Laurent and Westbrook, 2009; Kim et al., 2013). When PL is inactivated during fear extinction, animals show decreased fear early in the session which persisted for the end of the session. However, those animals did not show any differences compared to controls on the subsequent drug-free retrieval session (Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). Along these lines, inactivation of PL during extinction retrieval did

not affect responding (Kim et al., 2016). Inactivation of its ventral counterpart, the IL, does not affect fear expression. When IL is inactivated before extinction, some reports show deficits in within-session extinction performance whereas others show normal extinction performance (Sierra-Mercado et al., 2011; Awad et al., 2015; Do-Monte et al., 2015a). Despite this slight discrepancy, all reports show impairments during the subsequent extinction retrieval session (Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011; Do-Monte et al., 2015a; Kim et al., 2016). Furthermore when IL is inactivated after successful extinction, it impairs the consolidation of fear extinction. Finally, when the IL is inactivated during subsequent extinction retrieval, impairs the extinction retrieval while sparring the renewal (Quirk et al., 2000; Kim et al., 2016). These studies led to the view that the PL plays a role in fear expression whereas IL is more involved in fear suppression after fear extinction.

Consistent with the inactivation studies, electrophysiological work has shown a sustained increase in neuronal activity in PL that mirrors the time course of freezing during CS presentation and IL neurons show elevated tone response and increased intrinsic excitability after fear extinction (Burgos-Robles et al., 2007; Santini et al., 2008, 2012; Holmes et al., 2012; Sepulveda-Orengo et al., 2013). Furthermore, in animals that fail to show successful extinction, PL activity was elevated whereas IL activity was suppressed. Studies using immediate early genes such as c-fos or zif268 have also revealed elevated PL activation during high fear states such as fear recall/fear renewal whereas IL showed elevated levels during low fear states after fear extinction (Hefner et al., 2008; Knapska and Maren, 2009; Stern et al., 2013; Fitzgerald et al., 2015b).

It is worth noting that not all reports show different functions of PL and IL in fear conditioning. Specifically, studies using immediate early genes have shown elevated cfos levels in both PL and IL after fear conditioning and some studies show no significant differences between cfos activation levels in PL and IL after fear extinction. Furthermore, single-cell

electrophysiological studies have shown CS-evoked increases in both PL and IL after fear conditioning (Giustino et al., 2016). Interestingly, fear conditioning deficits induced by systemic administration of propranolol results in diminished CS evoked firing in both PL and IL (Fitzgerald et al., 2015a). Additionally, other labs have found no differences between PL and IL either during successful extinction or during a failed extinction in an extinction resistant mice strains (Fitzgerald et al., 2014). This contrasting evidence has led to an alternate theory that suggests PL and IL may covary together and the difference in the input or output patterns contribute in regulating fear expression or in extinction learning which needs to be further investigated (Giustino and Maren, 2015).

1.3.3 The Hippocampus

The hippocampus plays a key role in episodic, spatial and contextual memories (Lisman et al., 2017; Voss et al., 2017; Eichenbaum, 2018). When animals undergo fear conditioning, they not only learn a CS-US association, but they also form a representation of the physical context in which fear conditioning or extinction occurs (Maren, 2001; Maren et al., 2013). Lesions or temporary inactivation of the HPC impair fear to the conditioning context (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren et al., 1996a, 1997; Anagnostaras et al., 1999; Holt and Maren, 1999; Maren and Holt, 2004; Parsons and Otto, 2008). There is some discrepancy in terms of deficits in fear to the CS with some reports reporting deficits to CS in addition to context, whereas others show deficits only to context and not a CS (Phillips and LeDoux, 1992; Maren et al., 1997; Anagnostaras et al., 1999). Interestingly, there is a retrograde temporal gradient associated with this contextual fear deficit. For instance, post-training lesions made 1 day, but not 28 days, after fear conditioning produced deficits in the expression of contextual fear (Anagnostaras et al., 1999, 2001). Pre-training HPC manipulations have yielded mixed results. Temporary inactivation of HPC or impairing neuronal plasticity in HPC before fear conditioning

impairs acquisition of fear to the context (Stiedl et al., 2000; Bast et al., 2003; Quinn et al., 2005; Czerniawski et al., 2012; Ramanathan et al., 2018b). However, pre-training lesions to HPC have no effect in acquisition to the fear the context (Maren et al., 1997; Frankland et al., 1998; Cho et al., 1999; Anagnostaras et al., 2001; Wiltgen et al., 2006). This led scientists to believe that animals can still acquire contextual fear memories without HPC by using a different strategy. This strategy depends on associating individual elements of the context with the US (such as grid floor, smell, etc.) and subsequently exhibiting fear when those elements are present (Rudy and O'Reilly, 1999; Sutherland et al., 2010). This less robust elemental strategy may depend on cortical, amygdaloid and other sensory regions (Rudy and O'Reilly, 1999; Rudy and Matus-Amat, 2005; Sutherland et al., 2010; Zelikowsky et al., 2013; Coelho et al., 2018). This invariably leads to poor discrimination between safe and unsafe contexts. Consistent with this theory, animals that have pre-training HPC lesions have difficulties discriminating between safe and un-safe contexts (Frankland et al., 1998; Cho et al., 1999).

If animals can learn contextual fear without the HPC then what does HPC do? HPC integrates elemental information coming from various input pathways and forms a multimodal, configural representation of the context (Rudy and O'Reilly, 1999; Sutherland et al., 2010). Specifically, when an animal is placed in a context, they explore the environment and acquire sensory and spatial information to form a unified context representation that is assembled in the HPC. Consistent with this idea, Rudy and colleagues performed a series of experiments using an immediate shock paradigm to explain the contribution of HPC in contextual representation. Briefly, when an animal is placed in a context for less than 30 sec before getting a shock, they do not acquire the contextual fear compared to animals that could explore the context for 2 minutes (Fanselow, 1986). This deficit can be overcome by pre-exposure to the context in which they will get shocked (Fanselow, 1990). Inactivation of HPC during this context pre-exposure impairs

contextual fear indicating the role of HPC in contextual representation (Barrientos et al., 2002; Matus-Amat et al., 2004, 2007; Rudy and Matus-Amat, 2005).

As mentioned previously, fear extinction is a context-dependent process and HPC plays a crucial role in fear extinction. Inactivation of the HPC before extinction impairs acquisition of fear extinction and subsequent retrieval of fear in an extinguished context but not in a novel context (renewal) (Corcoran et al., 2005). After successful extinction, inactivation of the HPC produces a different pattern of results. Specifically, inactivation of HPC does not effect in freezing to the CS when retrieved in extinction context, but blocks freezing when it is inactivated before retrieval in a non-extinction context (renewal) (Corcoran et al., 2005; Ji J & Maren, 2005; Ji J & Maren, 2007). This led to the hypothesis that when HPC is inactivated after extinction, the animals lose its ability to show context-specific conditional fear ((Corcoran and Maren, 2001; Corcoran et al., 2005).

1.3.4 Interactions between mPFC, HPC and AMY:

The mPFC, HPC, and AMY interact at different stages of Pavlovian fear conditioning. For example, fear conditioning strengthens the connectivity between PL and amygdala and these interconnections make a key contribution to sustained tone responses and fear expression (Likhtik et al., 2005; Pendyam et al., 2013; Arruda-Carvalho and Clem, 2014; Likhtik and Paz, 2015; Song et al., 2015). Furthermore, electrophysiological studies from primates have shown enhanced synchrony between the dorsal anterior cingulate cortex (dACC), a homolog of rodent PL, and the amygdala. Interestingly, they also show animals that show enhanced synchrony over time were harder to extinguish. In contrast to the PL, IL's input to the amygdala seems necessary for the successful encoding and expression of extinction memories (Cho et al., 2013; Do-Monte et al., 2015; Strobel et al., 2015; Bloodgood et al., 2018; Quirk et al., 2003, 2006). In addition to this, the reciprocal projection from amygdala back to mPFC is also strengthened after fear conditioning (Senn et al., 2014; Arruda-Carvalho and Clem, 2015). HPC projections to the AMY mediates

renewal of extinguished fear memories (Maren and Hobin, 2007; Orsini et al., 2011; Knapska et al., 2012; Hübner et al., 2014; Xu et al., 2016). In addition to the projections to AMY, HPC also influences the mPFC during renewal (Orsini et al., 2011; Wang et al., 2016; Marek et al., 2018). Specifically, a recent report has shown that inactivation of HPC inhibits IL subregion of the mPFC by recruiting local parvalbumin positive (PV+) interneurons in the mPFC during successful renewal (Marek et al., 2018). This leads to the question of whether the mPFC interacts with the HPC during fear conditioning. However, there is a lack of monosynaptic projections from mPFC to HPC. Emerging evidence in recent years has started to show that mPFC recruits a third brain region, the thalamic nucleus reuniens (RE), to mediate its interaction with HPC (Preston and Eichenbaum, 2013; Griffin, 2015; Jin and Maren, 2015).

1.4 Midline Thalamic Nuclei

The thalamus is the largest of all diencephalic structures constituting more than 50 sub-nuclei. As the name suggests, the midline thalamic nuclei are a cluster of nuclei that are situated sub-cortically along the midline. They are mainly comprised of 1) dorsal midline nuclei which include the paraventricular nucleus of the thalamus (PVT), mediodorsal thalamus (MD), and 2) ventral midline thalamus which include the RE, xiphoid (Xi) and rhomboid (Rh) nuclei. The midline thalamic nuclei are an essential part of the "limbic" thalamus and have dense projections to brain regions that are part of the limbic system or the regions that are involved in emotional learning and memory (Cassel and de Vasconcelos, 2015; Vertes et al., 2015). These nuclei have no specified function and they are involved in various cognitive processes. Although the individual projections vary across nuclei, collectively they have rich connectivity to cortical, sub-cortical, striatal and brain stem nuclei which enables the midline thalamus to play a big part in cognition (Mitchell and Chakraborty, 2013; Mitchell et al., 2014; Vertes et al., 2015; Wolff and Vann, 2019). Consistent with the anatomical connectivity, lesions among the midline thalamic nuclei alter a host of

physiological, affective and cognitive functions (Aggleton and Sahgal, 1993; Aggleton et al., 2011; Aggleton, 2014; Mitchell et al., 2014; Cassel and de Vasconcelos, 2015; Kirouac, 2015; Wolff and Vann, 2019). Furthermore, human patients with midline thalamic damage exhibit a variety of deficits ranging from amnesia to loss of executive functions (Braak and Braak, 1991a, 1991b; Aggleton and Sahgal, 1993; Van der Werf et al., 2003b, 2000, 2003a; Schmahmann, 2003; Carlesimo et al., 2011; Mair et al., 2015; Mitchell, 2015; Aggleton et al., 2016).

Recently, several reports have linked the midline thalamus to various learning and memory processes. They range from executive behavior, appetitive behavior, spatial and contextual memory, and working memory to name a few (Das et al., 2005; Mitchell and Chakraborty, 2013; Preston and Eichenbaum, 2013; Griffin, 2015; Mitchell, 2015; Vertes et al., 2015; Do-Monte et al., 2017; Halassa and Kastner, 2017; Choi et al., 2019). These nuclei also play a role in aversive learning and memory. For example, early studies indicated the role of MD in the acquisition of instrumental avoidance behavior in rabbits (Orona and Gabriel, 1983; Gabriel et al., 1989; Buchanan, 1994). In Pavlovian fear conditioning, lesions or functional inactivation of the dorsal midline nuclei produces deficits at various stages of fear conditioning and extinction (Li et al., 2004, 2014; Lee et al., 2011, 2019; Padilla-Coreano et al., 2012; Mátyás et al., 2014; Paydar et al., 2014; Do-Monte et al., 2015b; Penzo et al., 2015). These defects are thought to arise from mPFC and intended for the CeA subregion of the AMY (Bergstrom, 2016; Do Monte et al., 2016). However, the role of its ventral counterpart is poorly studied in the context of Pavlovian fear conditioning. Early evidence using a passive avoidance paradigm indicated that the ventral midline nucleus RE is critical for the acquisition and expression of passive avoidance task (Davoodi et al., 2011). More recently, Xu and Südhof have shown some initial indications that RE could mediate mPFC→HPC interactions in rendering contextual memory specificity during fear conditioning

(Xu and Südhof, 2013). ***Because we are interested in mPFC-HPC interactions, we sought to explore the contribution of RE to Pavlovian fear conditioning and extinction.***

1.5 Nucleus Reuniens

1.5.1 Anatomy

The RE is a ventral thalamic midline nucleus that lies just above the third ventricle. It was first described by Herkenham who showed that it has a strong connection with the HPC (Herkenham, 1978). Later studies confirmed that projections from RE to HPC target both dorsal and ventral CA1, as well as the dorsal and ventral subiculum and the parasubiculum (Wouterlood, 1991; Vertes, 2006). Although these projections are excitatory, they terminate on both excitatory and inhibitory neurons in the HPC (Dolleman-Van der Weel et al., 1997; Dolleman-Van der Weel and Witter, 2000; Anderson et al., 2016). Inputs to RE from HPC also originate from these layers specifically (McKenna and Vertes, 2004). In addition to the HPC, RE has a dense reciprocal projection with both PL and IL (Vertes, 2002). Interestingly, two separate tracing studied have found evidence of bifurcating RE neurons that project to both mPFC and HPC. Specifically, these are collateral neurons that have cell bodies in RE and axonal terminals that project to both mPFC and HPC. They are estimated to be 3-10% of the total projection neurons to these regions (Hoover and Vertes, 2012; Varela et al., 2014). RE also has dense reciprocal projections to other cortical regions. These include orbitofrontal cortex (OFC), entorhinal cortex (EC), retrosplenial cortex (RSC) and anterior cingulate cortex (ACC) (Sesack et al., 1989; Berendse and Groenewegen, 1991; Vertes, 2002, 2004; McKenna and Vertes, 2004; Hoover and Vertes, 2007).

Sub-cortical and brainstem nuclei send sparse projections to RE. They arise from the amygdala, lateral septum, and adjacent basal forebrain nuclei. Inputs from brainstem arise from LC, PAG, and dorsal raphe to name a few. However these projections are not as strong as

projections from cortex or HPC (Vertes, 1991; Krout and Loewy, 2000; Krout et al., 2002; McKenna and Vertes, 2004).

1.5.2 Functional Consideration

RE has come into focus due to its dense reciprocal connections with mPFC and HPC, which has led to the proposal that RE coordinates prefrontal-hippocampal interactions (Vertes et al., 2007; Griffin, 2015; Jin and Maren, 2015a). Interactions between HPC and mPFC are crucial for successful encoding and retrieval of various forms of memory including episodic memory, working memory, spatial memory and contextual memories (Moscovitch et al., 2005; Preston and Eichenbaum, 2013; Jin and Maren, 2015a; Eichenbaum, 2017, 2018). However, they are not reciprocally connected to mediate bi-directional interactions. There are monosynaptic inputs that arise from HPC and terminate in the mPFC. But there is a lack of monosynaptic projection that arises from mPFC which terminates in HPC (Vertes, 2004). Despite the lack of projections, recent evidence indicates that mPFC interacts with via the RE, which is thought to be this essential bridge between mPFC and HPC that enables successful interaction between the two (Vertes et al., 2007; Griffin, 2015; Dolleman-van der Weel et al., 2019). Consistent with this, RE inactivation impairs the performance of tasks that require the combined activation of both mPFC and HPC, such as the radial arm maze or Morris water maze (Hembrook et al., 2012; Cholvin et al., 2013; Layfield et al., 2015; Hallock et al., 2016; Harvey et al., 2017; Maisson et al., 2018). Because the RE has reciprocal connectivity with both mPFC and HPC, it is not possible to distinguish the directionality of the information flow for these deficits. However, electrophysiological recordings have shown that mPFC oscillations lead those in RE, which in turn lead area CA1 in the dorsal HPC, in a goal-directed spatial navigation task (Ito et al., 2015). In a Pavlovian fear conditioning paradigm, Xu and Südhof have shown that RE lesions or reducing synaptic transmission in the mPFC-RE-HPC pathway leads to deficits in fear generalization, whereas excitation of this pathway leads to

enhanced specificity of the fear memories (Xu and Südhof, 2013). These studies provide direct evidence for RE enabling mPFC→HPC interactions.

In addition to mediating mPFC→HPC information flow, RE can also influence behavior that is dependent on either the mPFC or HPC alone. For example, Dolleman-van der Weel and colleagues have shown in a reference memory (RM) version of the Morris water maze task, RE lesions cause enhanced behavioral flexibility while sparing the acquisition and retrieval of this task (Dolleman-van der Weel et al., 2009). Similar results were seen in an mPFC-dependent task as well. In a 5-choice reaction time task, a task dependent on the mPFC, lesions to RE/Rh resulted in more impulsive but not compulsive behaviors (Prasad et al., 2013; Linley et al., 2016).

Finally, recent reports indicate that RE also regulates neuronal synchrony between the mPFC and HPC. Synchrony between brain regions is critical for acquisition, consolidation and performance in a variety of spatial and contextual memory tasks (Buzsáki, 2006; Buzsáki and Watson, 2012; Harris and Gordon, 2015). RE, which has an excitatory influence on both mPFC and HPC, is aptly suited to mediate the synchrony between them. In anesthetized rats, Ferraris and colleagues provided evidence that RE serves to synchronize bursts of gamma activity in CA1 and mPFC. Specifically, they showed synchronized gamma wave activity between CA1 and mPFC in and, interestingly, observed that RE neurons show bursts in firing before the onset of the gamma wave. They further demonstrated that inactivation of RE impairs this gamma coupling (Ferraris et al., 2018). In a separate study, Hauer and colleagues demonstrated that RE coordinates PFC-HPC synchrony during slow-wave sleep, and this had functional consequences for memory consolidation (Hauer et al., 2019). Behaviorally, in a spatial water maze task, Hallock and colleagues have shown that RE inactivation impairs synchrony between mPFC and HPC on trials that were working memory-dependent, but not on working memory-independent trials (Hallock et al., 2016). These results were further confirmed by a study (Ito et al., 2018) that showed enhanced

coordination with the CA1 theta rhythm when rats approached the choice point in a T-maze. Interestingly, they show that this temporal coordination on this circuit was dependent on the supramammillary nucleus (Ito et al., 2018). However, in somewhat contradictory to the pattern of results explained above, Roy et al have reported little to no effect of RE inactivation on theta coupling between mPFC-HPC rather influences coupling at the delta band (Roy et al., 2017). Nonetheless, these studies give indications to RE synchronizing mPFC-HPC interactions in various behavioral tasks.

1.6 Role of RE in Pavlovian Fear Conditioning and Extinction

Overall, there is considerable evidence linking RE to various behavioral processes that involve mPFC and HPC. As mentioned in previous sections, the Pavlovian fear conditioning paradigm is one such task that critically involves these two brain regions. My doctoral work has focused on understanding the role of RE in Pavlovian fear conditioning and extinction, in the context of mPFC-HPC interactions. ***I hypothesize that RE mediates prefrontal-hippocampal interactions involved in the acquisition and expression of Pavlovian fear conditioning and extinction.*** To this end, I employed intracranial pharmacology, in-vivo electrophysiology, chemo- and optogenetic techniques to understand the contribution of RE in mediating prefrontal-hippocampal interaction in Pavlovian fear conditioning and extinction.

2. NUCLEUS REUNIENS IS REQUIRED FOR ENCODING AND RETRIEVING PRECISE, HIPPOCAMPAL-DEPENDENT CONTEXTUAL FEAR MEMORIES IN RATS*

2.1 Introduction

The nucleus reuniens (RE) is a ventral midline thalamic nucleus that interconnects the medial prefrontal cortex (mPFC) and hippocampus (HPC) (McKenna and Vertes, 2004; Vertes et al., 2006). Recent work has shown that lesions or inactivation of the RE reduces synchrony between the mPFC and HPC (Preston and Eichenbaum, 2013; Jin and Maren, 2015a; Hallock et al., 2016; Cholvin et al., 2018). Behaviorally, inactivation of RE produces deficits in tasks that require coordinated activity between the HPC and mPFC (Hembrook and Mair, 2011; Hembrook et al., 2012; Cholvin et al., 2013; Jin and Maren, 2015a; Layfield et al., 2015). For example, RE inactivation impairs performance in spatial working memory task that requires delayed alternation in a T-maze, and these memory deficits are accompanied by reductions in theta coherence between the mPFC and HPC (Layfield et al., 2015; Hallock et al., 2016).

Deficits in spatial working memory associated with RE inactivation may be due to deficits in contextual processing associated with impaired HPC-mPFC interaction (Cassel and Pereira de Vasconcelos, 2015). Consistent with this possibility, chronic inhibition of synaptic transmission in the RE made with a virally expressed tetanus toxin (TetTox) disrupts contextual processing during Pavlovian fear conditioning in mice (Xu and Südhof, 2013). Although RE inactivation did not affect the acquisition or expression of freezing behavior in the conditioning context (or freezing to an auditory cue), it did cause a robust increase in freezing to a novel test context. This

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impairment in contextual discrimination was also obtained after TetTox inhibition of the mPFC or its projections to RE. In addition to its role in contextual encoding, recent work also suggests role for the RE in the consolidation of contextual memories (Vetere et al., 2017; Troyner et al., 2018). Together, these results suggest that the RE is involved in encoding precise context representations that normally limit the generalization of fear from the conditioning context to other, dissimilar contexts.

Once learned, however, the retrieval of precise contextual memories does not appear to require the RE (Xu and Südhof, 2013). Yet there is considerable data in both animals and humans indicating that HPC-mPFC interactions are involved in the retrieval of spatial and contextual memories (Orsini et al., 2011; Preston and Eichenbaum, 2013; Schlichting and Preston, 2016; Wang et al., 2016; Marek et al., 2018). Indeed, recent work has found that reversible optogenetic manipulations of the hippocampus yield robust deficits in both the encoding and retrieval of contextual fear memories (Goshen et al., 2011; Bernier et al., 2017). Moreover, RE inactivation produces robust impairments in spatial working memory (Griffin, 2015; Layfield et al., 2015; Hallock et al., 2016). Hence, intact acquisition and expression of contextual freezing in rats with permanent lesions of the RE (Xu and Südhof, 2013) may be due to compensation by alternate neural systems, which has been observed after hippocampal lesions for example (Maren et al., 1997). Insofar as the RE plays a role in supporting HPC-mPFC interactions involved in both memory encoding and retrieval, we hypothesize that reversible inactivation of the RE will impair both the acquisition and expression of contextual fear.

To examine this question, we temporarily inactivated RE using intra-cranial infusions of muscimol (MUS, a $GABA_A$ agonist) during either the acquisition or retrieval (or both) of Pavlovian fear conditioning in rats. Retention tests were conducted in both the conditioning

context and an alternate context to assess the influence of RE inactivation on context discrimination and generalization; both contextual and auditory freezing were assessed. Importantly, we included control groups in our design to determine whether state-dependent generalization deficits account for performance impairments in MUS-treated rats. Consistent with our hypothesis, we found that the RE is involved in both the encoding and retrieval of context representations that support contextual discrimination. Interestingly, deficits in contextual freezing in animals that underwent fear conditioning after RE inactivation could be rescued by inactivating the RE during retrieval testing. However, the contextual memories acquired under RE inactivation were hippocampal-independent, insofar as contextual freezing in rats conditioned under RE inactivation was insensitive to intra-hippocampal infusions of D,L-amino-5-phosophonovaleric acid (APV). This supports the view that the RE is a component of a hippocampal memory system that prioritizes the encoding of a configural representation of context that ultimately overshadows its underlying elements.

2.2 Results

2.2.1 Inactivation of RE impairs acquisition of contextual conditioning and generalization of conditioned freezing

Figure 2.1 (A and B) illustrates the spread of fluorescently labeled muscimol (TMR-X) in the RE along with an illustration of cannula placements in all the animals included in the analyses (Figure 2.1C). During the fear conditioning session, rats receiving SAL or MUS infusions into the RE exhibited low levels of freezing behavior before the first conditioning trial and increased their freezing behavior across the conditioning trials (Figure 2.2A); there were no differences between MUS- and SAL-treated in the levels of conditioned freezing. These observations were confirmed in a one-way repeated measures ANOVA that revealed a significant main effect of training trial

[$F(5, 545) = 166.9$; $p < 0.0001$] with neither a main effect of drug [$F(1,109) = 3.56$; $p = 0.06$] nor a drug x trial interaction [$F(5,545) = 0.78$; $p = 0.57$]. Hence, RE inactivation did not affect the expression of post-shock freezing during the acquisition of Pavlovian fear conditioning.

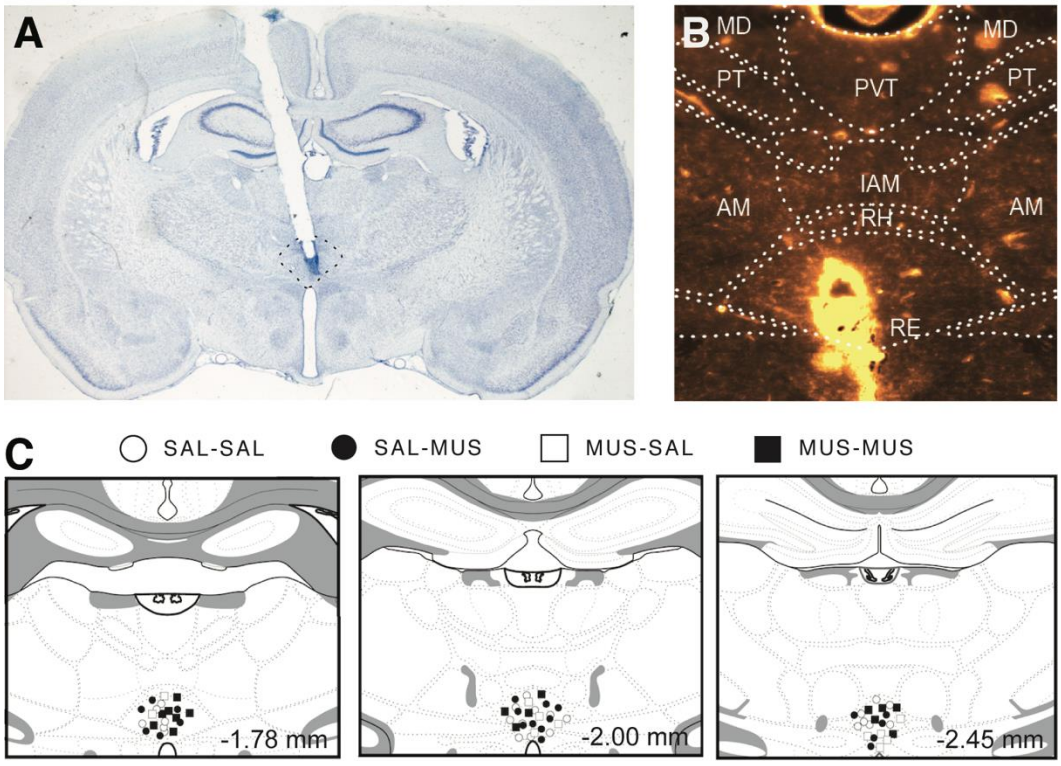

Figure 2.1 Cannula placements in nucleus reuniens (RE). ***A,*** Representative thionin-stained coronal section showing a midline cannula placement in the RE. *B*, Representative darkfield image showing diffusion of fluorescent muscimol (0.3 µl; TMR-X) in the RE. *C*, Cannula placements for all subjects that were included in the analysis across three different levels along the anterior-posterior axis. The distribution of cannula placements within the RE was similar across all of the groups.

Twenty-four and forty-eight hours later, all animals were again infused with either SAL or MUS and placed in either the conditioning context (A) or a novel context (B) for a 10-min retrieval test; the test order in the two contexts was counterbalanced and the infusion assignment for each test was the same. Importantly, this design allowed us to assess the state-dependent effects of RE inactivation on the acquisition, expression, and generalization of conditioned freezing. As shown

in Figure 2.2B, rats conditioned after MUS infusions into the RE (MUS-SAL group) exhibited a substantial impairment in conditioned freezing in the conditioning context. Interestingly, this impairment was absent in animals both conditioned and tested after MUS infusions into the RE (MUS-MUS group). The relatively high level of freezing behavior in the MUS-MUS rats was not simply due to a nonspecific increase in freezing caused by RE inactivation insofar as SAL-MUS animals were no different from SAL-SAL or MUS-MUS animals. All of these observations were confirmed in a two-way repeated measures ANOVA that revealed significant main effects for conditioning drug [$F(1, 107) = 8.28$; $p = 0.004$], testing drug [$F(1, 107) = 6.04$; $p = 0.02$], and time [$F(1, 107) = 8.90$; $p < 0.0001$]. Moreover, there was a trend towards a significant interaction between conditioning and testing drug conditions [$F(1, 107) = 3.52$; $p = 0.06$] (Figure 2.2B). Post-hoc comparisons revealed that freezing among rats in the MUS-SAL group was significantly lower than that in the SAL-SAL ($p = 0.0007$), SAL-MUS ($p = 0.0002$) and MUS-MUS ($p = 0.003$) groups, which did not differ from one another. This reveals that RE inactivation impairs the acquisition of contextual fear conditioning and does so in a state-dependent manner; testing animals in the same state under which they were conditioned resulted in high levels of conditioned freezing.

In contrast, when the animals were tested for their generalization of fear to a novel context a different pattern of results emerged. As shown in Figure 2.2C, rats conditioned after MUS inactivation of the RE exhibited similar and low levels of freezing relative to animals conditioned under SAL. However, MUS infusions before the generalization test increased conditioned freezing. Interestingly, animals conditioned and tested under MUS (MUS-MUS) exhibited the highest level of freezing, and this was manifest early in the test session. Rats conditioned under SAL and tested under MUS (SAL-MUS) showed low levels of freezing at the beginning of the test

that increased over the course of the test to approach those in the MUS-MUS group. These observations were confirmed in a two-way repeated measures ANOVA that revealed a significant main effect of test drug [$F(1,107) = 22.78$; $p < 0.0001$] and time [$F(9,963) = 7.65$; $p < 0.0001$]; there was no interaction between conditioning and test drug [$F(1,107) = 1.1$; $p = 0.29$]. However, there was a significant interaction between test drug and time [$F(9,963) = 2.31$; $p = 0.014$], which indicates that MUS increased the generalization of fear (SAL-MUS), and served as a retrieval cue to support contextual freezing in rats conditioned after MUS infusions in the RE (MUS-MUS). Importantly, increases in freezing were not due to nonspecific effects of MUS on locomotion, insofar as MUS did not decrease locomotor (i.e., load-cell) activity during the pre-trial baseline period on the conditioning day, nor did it affect shock-elicited activity (data not shown).

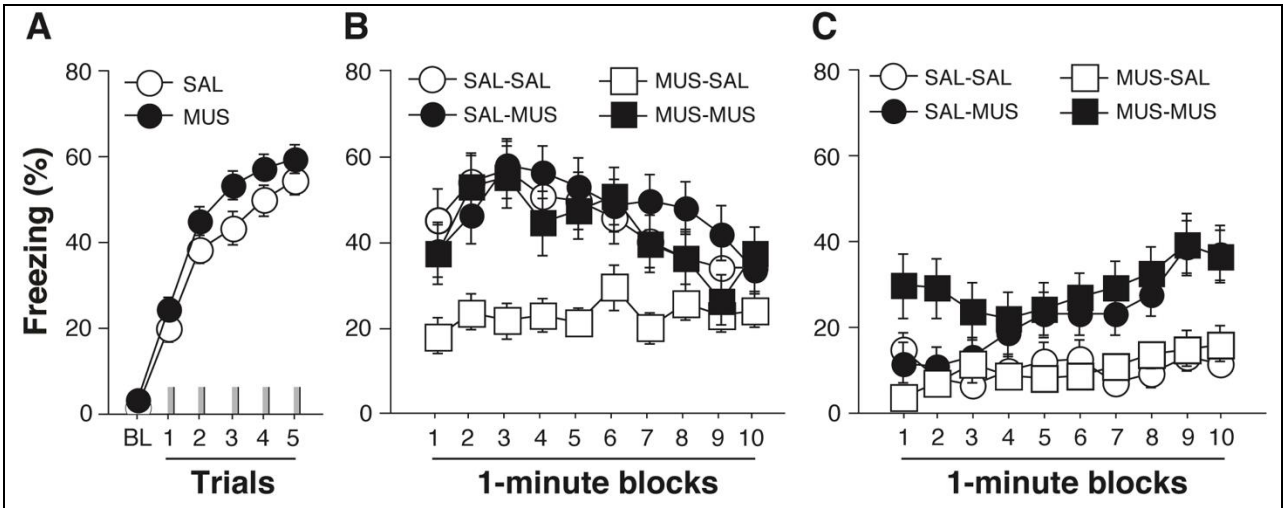

Figure 2.2 Muscimol inactivation of the RE results in a state-dependent impairment in contextual fear conditioning. *A,* Percentage of freezing during the 3-min baseline (BL) and 1-min ITI (intertrial interval) after each conditioning trial (indicated by gray hatch marks on the X-axis) in animals infused with either saline (SAL) or muscimol (MUS) in the RE. *B,* Percentage of freezing during the 10-minute retrieval test in the conditioning context. Animals conditioned after SAL or MUS infusions received the retrieval test after either SAL or MUS infusions in a factorial design that yielded four groups SAL-SAL, MUS-SAL, SAL-MUS, MUS-MUS. *C,* Percentage of freezing during the 10-minute retrieval test in a novel context (the order of the retrieval tests in *B* and *C* was counterbalanced); the groups are the same as those described in *B.* All data are shown as means ± SEMs

2.2.2 RE inactivation impairs contextual discrimination

Because animals received the same drug infusions across both of the context tests, we were able to assess contextual discrimination using a within-subjects analysis. As shown in Figure 2.3A, rats in all the groups exhibited a contextual discrimination and exhibited lower levels of freezing in context B compared to context A. Consistent with this observation, a three-way repeated measures ANOVA with between-subject variables of conditioning and testing drug condition and a within-subject variable of test context revealed a significant main effect of test context [$F(1,107) = 80.48$; $p < 0.0001$]. Furthermore, there was a significant main effect of test drug [$F(1,107) = 17.42$; $p < 0.0001$], but not conditioning drug [$F(1,107) = 2.3$; $p = 0.13$], although there was a trend towards a significant interaction between the drug conditions [$F(1,107) = 3.48$; $p = 0.06$]. Close examination of these data reveals that the SAL-SAL animals exhibited the highest level of contextual discrimination relative to all the other groups.

To examine this possibility more closely, we calculated a discrimination index by computing the ratio between the differences in the average freezing across the 10-min tests (context A - context B) divided by the total freezing in each context (context A + context B). As shown in Figure 2.3B, MUS infusions into RE resulted in lower discrimination scores whether the infusions occurred before conditioning or retrieval testing. A factorial ANOVA revealed a trend towards a significant main effect of conditioning drug [$F(1,107) = 3.62$; $p = 0.06$] and a significant main effect of drug during testing [$F(1,107) = 6.83$; $p = 0.01$]; there was no significant interaction between these factors [$F(1,107) = 0.16$; $p = 0.69$]. Hence, it appears that RE inactivation reduces contextual discrimination when infused either before conditioning or retrieval testing.

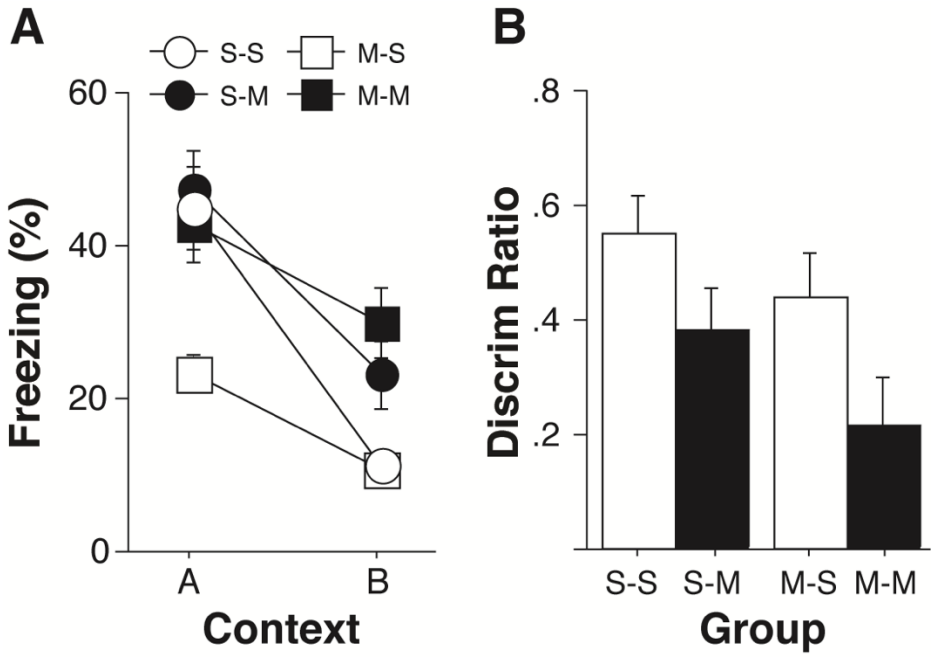

Figure 2.3 Muscimol inactivation of the RE impairs contextual discrimination. *A*, Average percentage of freezing across the 10-minute retrieval tests in the conditioning context (context A) and the novel context (context B) shown in Figure 2. The order of the two retrieval tests was counterbalanced and animals trained under saline (S) or muscimol (M) were tested in both contexts A and B under after either saline or muscimol infusions to yield the four groups described in Figure 2 (e.g., S-S, S-M, M-S, and M-M). *B*, Average discrimination ratios [(context A- context B)/(context A + context B)] calculated on freezing behavior across the 10-minute tests shown in *A*. All data are shown as means ± SEMs

2.2.3 RE inactivation does not impair acquisition or expression of freezing to an auditory CS

Of the subset of animals that received auditory fear conditioning, we determined whether inactivation of RE causes deficits in acquisition or expression of fear to the tone CS. As shown in Figure 2.4 A and B, conditional freezing to the tone was similar in all of the groups. Two-way repeated measures ANOVA revealed no significant main effects or interactions of drug on conditioning [$F(1,52) = 2.6$; $p = 0.12$] or testing [$F(1,52) = 1.77$; $p = 0.19$] across the entire test. Moreover, average freezing during the CS trials (Figure 2.4B) did not differ between the groups [conditioning, $F(1,52) = 3.47$; $p = 0.07$; testing, $F(1,52) = 1.05$; $p = 0.31$]. Overall, these results

indicate that inactivation of RE does not cause deficits in either the acquisition or retrieval of fear to an auditory CS.

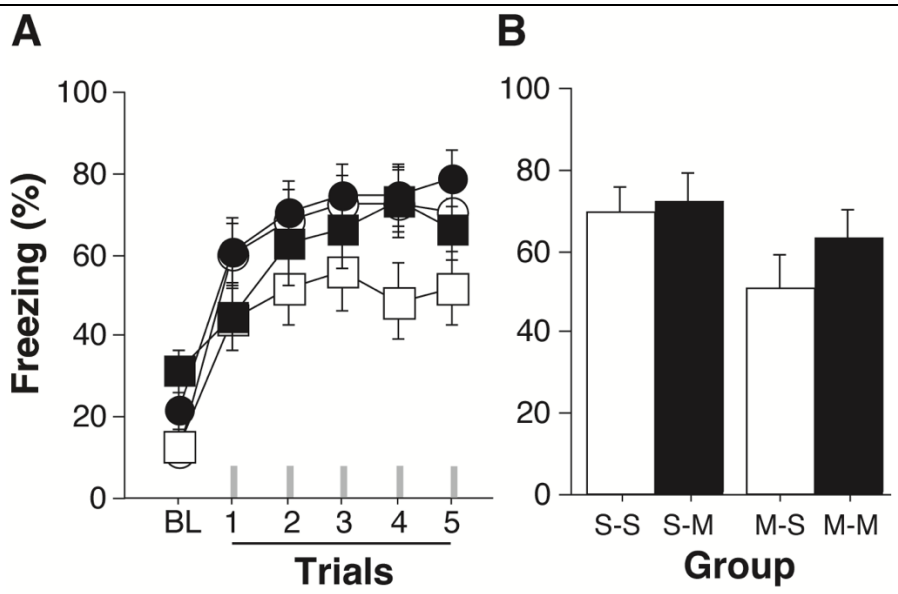

Figure 2.4 Muscimol inactivation of RE did not impair the acquisition or expression of freezing to an auditory conditioned stimulus (CS). *A,* Average percentage of freezing during a 10-min stimulus free baseline (BL) and after five tone presentations (indicated by the grey hatch marks on the x-axis) during an auditory retrieval test. Freezing during each trial represents the average freezing during the CS and intertrial interval. Animals received either saline (S) or muscimol (M) infusions before conditioning and retrieval testing in a factorial design to yield four groups (S-S, M-S, S-M, M-M). *B,* Average percentage of freezing across all five CS trials shown in *A.* All data are shown as means ± SEMs

2.2.4 Contextual memories acquired under RE inactivation are insensitive to hippocampal NMDA receptor antagonism

Animals conditioned and tested under RE inactivation exhibited high levels of freezing in the conditioning context, suggesting that they had acquired a contextual memory. Because considerable evidence indicates that RE inactivation impairs hippocampal-dependent memory encoding (Layfield et al., 2015; Hallock et al., 2016), contextual learning in rats conditioned under RE inactivation may not require hippocampal synaptic plasticity (Stiedl et al., 2000; Quinn et al., 2005; Czerniawski et al., 2012). To test this possibility, we examined whether intra-hippocampal

infusions of the NMDA receptor antagonist, APV, impair contextual conditioning when training occurs under simultaneous RE inactivation.

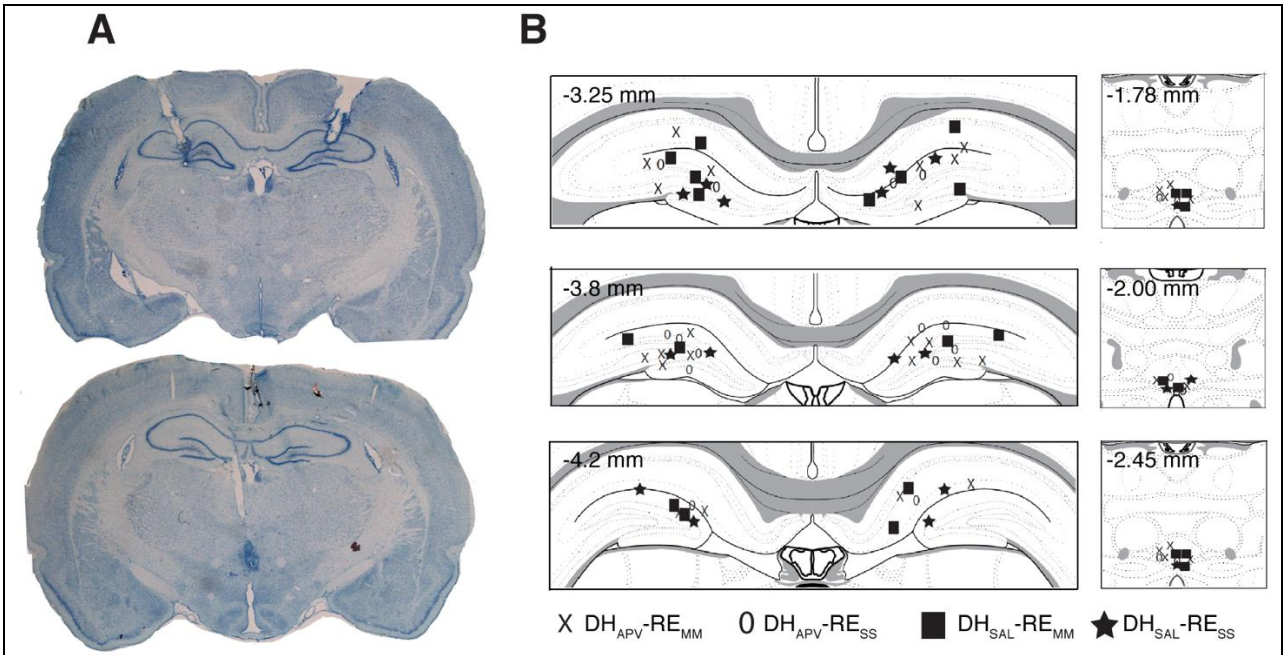

Figure 2.5 Cannula placements in nucleus reuniens (RE) and dorsal hippocampus (DH). *A*, Representative thionin-stained coronal sections showing a midline cannula placement in the RE (left) and DH (right). *B*, Cannula placements for all subjects that were included in the analysis across three different levels along the anterior-posterior axis in both RE (left) and DH (right). The distribution of cannula placements within the RE and DH was similar across all of the groups.

Representative cannula placements in RE or DH along with an illustration of cannula placements in all the animals included in the analyses are shown in Figure 2.5. During fear conditioning, all rats exhibited low levels of freezing behavior before the first conditioning trial and increased their freezing behavior across the conditioning trials (Figure 2.6A). There were no differences between the groups in the levels of conditioned freezing. A one-way repeated measures ANOVA revealed a significant main effect of conditioning trial [$F(5,140) = 32.79$; $p < 0.0001$] with neither a main effect of drug in RE [$F(1,28) = 0.005$; $p = 0.94$] and drug in DH [$F(1,28) = 1.81$; $p = 0.19$] nor a drug x trial interaction [$F(1,28) = 1.17$; $p = 0.29$]. Hence, neither MUS

infusions into RE nor APV into DH affected post-shock freezing during the acquisition of Pavlovian fear conditioning.

Forty-eight hours later, all animals were again infused with either SAL or MUS in RE and placed in the conditioning context (A) for a 10-min retrieval test (the drug infused before retrieval testing was identical to that infused before conditioning); there were no DH infusions before retrieval test. As shown in Figure 2.6B, rats conditioned after APV infusions into the DH and SAL infusions into the RE (DH_{APV}-RE_{SAL}) exhibited a substantial impairment in conditioned freezing in the conditioning context relative to animals receiving SAL infusions into the DH (DH_{SAL}-RE_{SAL}). Interestingly, this impairment was absent in animals that received MUS infusions to RE (DH_{APV}-RE_{MUS}) who froze at levels similar to that in controls (DH_{SAL}-RE_{SAL} and DH_{SAL}-RE_{MUS}) The relatively high level of freezing behavior in the DH_{APV}-RE_{MUS} animals was not simply due to a nonspecific increase in freezing caused by drug infusions in RE insofar as the level of freezing in DH_{APV}-RE_{MUS} group was similar to that in animals conditioned under RE inactivation alone (DH_{SAL}-RE_{MUS}) and saline controls (DH_{SAL}-RE_{SAL}). These observations were confirmed in a two-way repeated measures ANOVA that revealed was a significant interaction between RE and DH drug conditions [$F(1,28) = 5.91$; $p = 0.02$]; there was no main effect of RE drug condition [$F(1,28) = 3.11$; $p = 0.09$] or DH drug condition [$F(1,28) = 1.52$; $p = 0.23$]. Post-hoc comparisons revealed that freezing among rats in the DH_{APV}-RE_{SAL} group was significantly lower than that in the DH_{SAL}-RE_{SAL} ($p = 0.0098$), DH_{SAL}-RE_{MUS} ($p = 0.0432$) and DH_{APV}-RE_{MUS} ($p = 0.0061$) groups, which did not differ from one another. This reveals that whereas contextual conditioning normally requires hippocampal NMDA receptors, contextual memories acquired after inactivation of the RE do not require DH NMDA receptors.

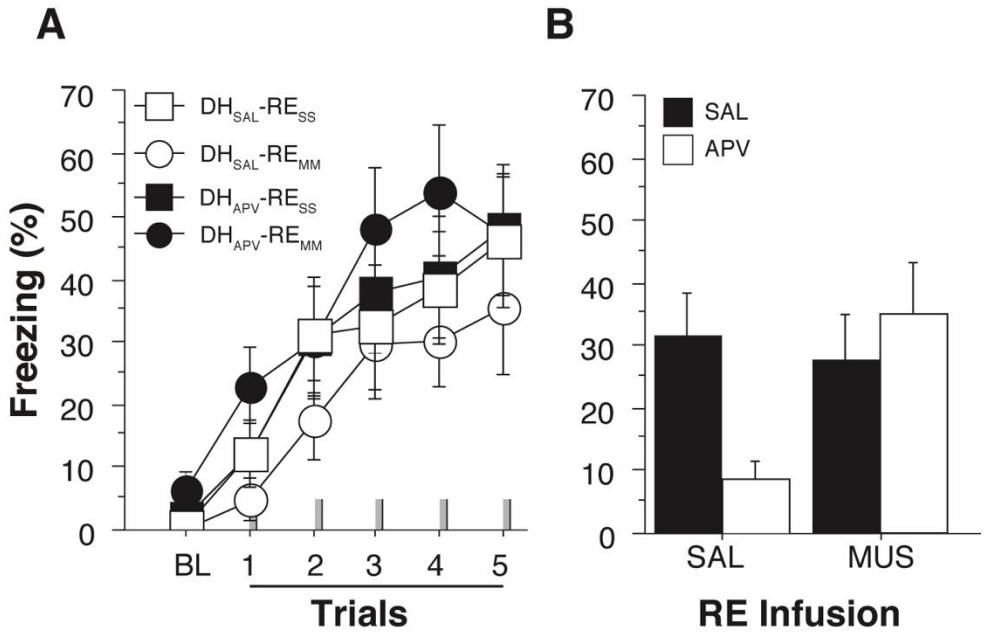

Figure 2.6 Contextual conditioning under RE inactivation is insensitive to NMDA receptor antagonism in the DH. *A,* Percentage of freezing during the 3-min baseline (BL) and 1-min ITI (intertrial interval) after each conditioning trial (indicated by gray hatch marks on the X-axis) in animals infused with either saline (SAL) or muscimol (MUS) in the RE and APV or saline (SAL) infused in DH. *B,* Percentage of freezing during the 10-minute retrieval test in the conditioning context. Animals conditioned after SAL or MUS infusions in RE received the same drug infusions in RE before the retrieval test in a factorial design that yielded four DH_{SAL}-RE_{SAL}, DH_{SAL}-RE_{MUS}, DH_{APV}-RE_{SAL} and DH_{APV}-RE_{MUS}. SAL or APV indicates drug infusions in DH before fear conditioning and SAL or MUS indicate the RE infusions before both conditioning and the retrieval test. All data are shown as means ± SEMs.

2.3 Discussion

The present study examined the role of the RE in the acquisition, expression, and generalization of conditioned freezing behavior in rats. We show that when RE is inactivated before fear conditioning, animals exhibit low levels of contextual freezing when later tested in the conditioning context. Freezing during the conditioning session was not affected by RE inactivation, suggesting that deficits in long-term memory were the result of memory consolidation deficits (though post-training inactivation of the RE would be required to validate this hypothesis; (Vetere et al., 2017). Interestingly, the deficit in contextual conditioning could be completely

rescued by testing animals under RE inactivation, indicating that the contextual memory acquired with the RE inactivated is acquired in a state-dependent manner. That is, the contextual memory acquired in animals conditioned after RE inactivation is not expressed when retrieval testing occurs with a functional RE; rather, this RE-independent memory is only expressed when the RE is again inactivated. RE inactivation alone before retrieval testing did not impair freezing in animals that received SAL infusions before conditioning, which is consistent with other reports showing that pharmacological or optogenetic inactivation of the hippocampal system does not affect retrieval of contextual fear (Holt and Maren, 1999; Matus-Amat et al., 2007; Kheirbek et al., 2013). In addition, inactivation of the RE during retrieval testing increased fear generalization to a novel context and thereby decreased contextual discrimination. RE inactivation during conditioning or retrieval testing did not cause deficits in freezing to an auditory CS. Critically, we found that contextual memories acquired under RE inactivation are hippocampal-independent, insofar as contextual freezing in rats conditioned under RE inactivation was insensitive to intra-hippocampal infusions of the APV. These data reveal that the RE is required for hippocampal-dependent encoding of precise contextual memories to support the discrimination of safe and dangerous contexts.

The finding that RE inactivation impairs the acquisition of precise context memories is consistent with previous findings that RE inactivation leads to deficits in acquisition of passive avoidance and consolidation of contextual fear memories (Davoodi et al., 2011; Vetere et al., 2017; Troyner et al., 2018). Moreover, our results are also consistent with work showing that inactivating synaptic inputs to the RE, particularly from the PFC, leads to context freezing that generalizes across contexts in mice (Xu and Südhof, 2013). Interestingly, Xu and Südhof (2013) did not observe deficits in the acquisition of contextual freezing after RE inactivation, an effect that we

observed in the present study. However, Xu and Südhof (2013) permanently inactivated the RE with irreversible TetTox inhibition of synaptic neurotransmitter release. As we have shown here, memories encoded under RE inactivation can be retrieved as long as retrieval occurs when the RE is offline. In both reports, RE inactivation failed to affect the acquisition or expression of auditory fear conditioning, The fact that freezing to auditory CS is spared after RE inactivation is consistent with previous work from our lab showing that HPC-PFC interactions are involved in contextual regulation of fear memories, rather CS freezing *per se* (Jin and Maren, 2015a, 2015b; Wang et al., 2016; Marek et al., 2018).

The important role for RE in contextual fear conditioning is in line with previous work implicating the RE in HPC-dependent processes, such as spatial and working memory (Cassel and Pereira de Vasconcelos, 2015; Layfield et al., 2015; Hallock et al., 2016). In these tasks, memory relies heavily on contextual processing. During contextual fear conditioning, for example, animals must encode a contextual representation that then comes into association with the aversive US. After acquisition, this memory yields conditioned freezing in the conditioning context and allows animals to discriminate the dangerous conditioning context from other, safe places. What might cause the contextual discrimination deficits observed after RE inactivation? One possibility is that animals without a functional RE use a non-hippocampal system to acquire an "elemental" representation of the context (Maren et al., 1997; Rudy and O'Reilly, 1999; Maren, 2001; Matus-Amat et al., 2004; Rudy and Matus-Amat, 2005). Bereft of a memory system that integrates multimodal sensory information into a unified, configural representation of context, animals with permanent damage to the RE might associate only the most salient features of the conditioning experience (perhaps even the experimenter or transport cues) with shock. These elemental associations would readily generalize across test contexts and fail to support subtle discriminations

between contexts. Consistent with this view, many investigators have found that permanent hippocampal lesions fail to affect contextual conditioning *per se* (Maren et al., 1997; Frankland et al., 1998; Cho et al., 1999; Wiltgen et al., 2006), but impair contextual discrimination (Frankland et al., 1998; Cho et al., 1999).

Consistent with this proposition, we found that contextual memories acquired in animals conditioned after MUS infusions into the RE were not sensitive to intra-hippocampal infusions of APV. In contrast, contextual conditioning was impaired by APV infusions into DH in animals trained after SAL infusions into the RE, consistent with many other reports (Stiedl et al., 2000; Quinn et al., 2005; Czerniawski et al., 2012). These results indicate that RE inactivation forces animals to acquire contextual fear memories in a hippocampal-independent manner, presumably through alternate neural systems that associate contextual elements with the US (Maren et al., 1997; Rudy and O'Reilly, 1999; Barrientos et al., 2002; Matus-Amat et al., 2004; Rudy and Matus-Amat, 2005). It is clear that the amygdala mediates Pavlovian fear conditioning to both discrete and contextual CSs (Goosens and Maren, 2001; Maren, 2001; Wilensky et al., 2006), and it is likely that elements of the context (light, odor, or grid) are associated with the foot-shock US in the amygdala to support contextual conditioning in rats trained after RE inactivation. Previous studies have suggested that cortical regions such as prefrontal cortex, retrosplenial cortex, or perirhinal cortex can process contextual information independently of the hippocampus, and these regions may support context-US associations in the amygdala (Zelikowsky et al., 2013; Heroux et al., 2017; Coelho et al., 2018).

It has previously been argued that the hippocampal memory system, within which the RE is a critical component (Cassel and Pereira de Vasconcelos, 2015; Griffin, 2015; Jin and Maren, 2015a), functions to acquire configural representations of context that enable context conditioning

and discrimination (Maren et al., 1997; Frankland et al., 1998; Rudy and O'Reilly, 1999; Matus-Amat et al., 2004). Interestingly, in the absence of the hippocampal system, animals can acquire context memories using a non-hippocampal system that associated context elements with an aversive US to yield conditional freezing. However, this impoverished representation of context leads to considerable generalization, particularly across highly similar contexts. Under normal circumstances, hippocampal-dependent configural leaning overshadows the elemental learning system, which results in the encoding of context memories that later require the hippocampal system for retrieval (Sutherland et al., 2010). In the context of the present results, this model suggests that the RE functions as a critical component of the hippocampal memory system involved in encoding precise contextual representations during fear conditioning. More specifically, our data reveal that 1) the RE functions to encode contextual memories that support contextual discriminations and 2) when conditioning occurs during RE inactivation, memories of conditioning are formed, but are only accessible when retrieved again under RE inactivation. Interesting, we also show that RE inactivation does not prevent the retrieval of contextual memories *per se* and causes inappropriate and generalized fear to safe contexts. Altogether, these results are consistent with the proposal that conditioned freezing to an aversive context can be supported by either a configural representation encoded by the hippocampal system or by an elemental representation encoded outside the hippocampus (Maren et al., 1997; Rudy and O'Reilly, 1999; Anagnostaras et al., 2001; Chang and Liang, 2017). Configural representations normally overshadow the elemental representation of context to dominate performance during retrieval. Similar to the hippocampal lesions, RE inactivation impairs configural encoding of context representations, rendering an elemental memory of the conditioning experience that is inhibited when the hippocampal system is active at the time of retrieval.

By this account, animals conditioned after RE inactivation encode an elemental representation of context (e.g., context *A'*) that comes into association with the US and is only retrieved when the animal encounters context *A'* in the future. After fear conditioning, contextual freezing in animals conditioned under RE inactivation will only be expressed in context *A'*, which requires RE inactivation to be experienced. Moreover, this account assumes that configural representations of the context, which are encoded by the intact brain, are not only insufficient to retrieve the elemental *A'* association, but in fact inhibit its retrieval. Ultimately, this leads to both deficits in the contextual freezing and poor contextual discrimination. Overall, the present data reveal that the RE is critical for contextual processes involved in fear conditioning and suggest that it is a critical hub for HPC-mPFC interactions involved in learning and memory.

2.4 Materials and Methods

2.4.1 Subjects

One hundred and sixty-eight experimentally naïve adult male Long-Evans rats (Blue-Spruce; 200–224 g; 50–57 days old; RRID:RGD_5508398) were obtained from a commercial supplier (Envigo, Indianapolis, IN). The rats were individually housed in cages within a temperature- and humidity-controlled vivarium and kept on a 14:10 h light: dark cycle (lights on at 07:00 hours) with *ad libitum* access to food and water. All experiments took place during the light phase of the cycle. Rats were handled for one minute per day for 5 days to habituate them to the experimenter before any surgical procedures or behaviors were carried out. All experiments were conducted at Texas A&M University with approval from its Animal Care and Use Committee.

2.4.2 Surgical procedure

One week before the behavioral testing, 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 cannulas to target RE and the dorsal hippocampus (DH). For Experiment 1, RE was targeted using a single guide cannula (8 mm, 26 gauge; Plastics One) implanted at a 10° angle on the midline (A/P: -2.0 mm, M/L: -1.0 mm, D/V: -6.7 mm from dura; coordinates were measured from bregma). For Experiment 2, three guide cannulas were implanted to target RE (midline) and DH (bilateral) in the same animal. The DH was targeted using two guide cannulas (4 mm, 26 gauge; Plastics One) implanted at a 20° angle (A/P: -3.7 mm, M/L: -3.5 mm, D/V: -3.2 mm from dura; coordinates were measured from bregma). The cannulas were affixed to the skull with dental cement and stainless-steel dummy cannulas (31-gauge, 9 mm for the RE and 5 mm for the DH; Plastics One) were inserted into the guides. Rats were allowed to recover for a period of 7 d after surgery before behavioral testing during which the dummy cannulae were replaced twice.

2.4.3 Drug infusions

For microinfusions, rats were transported to a prep room in the laboratory using white buckets (5-gallon) filled with a layer of bedding. The dummies were removed and stainless-steel injectors (31-gauge, 9 mm for RE and 5 mm for DH) connected to polyethylene (PE) tubing were inserted into the guide cannulas for intracranial infusions. The PE tubing on each injector was connected to a Hamilton syringe (10 μl), which was 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 PE tubing. All infusions were made approximately 10 min before either the conditioning or retrieval testing sessions. Muscimol (MUS; 0.1 μg/μl) and APV (10 μg/μl)

were dissolved in sterile saline (SAL). Infusions were made at a rate of 0.1 μl/min for 3 min (0.3 μl total; 0.03 μg MUS in RE and 3 μg APV per hemisphere in DH) and the injectors were left in place for 2-3 min for diffusion. After the infusions, clean dummies were inserted into the guide cannulae and the animals were transported to the conditioning chambers for the behavioral sessions.

2.4.4 Behavioral apparatus and procedure

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, 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 sec. 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 1.5% acetic acid. Rats were transported to context B in black plastic boxes.

2.4.5 Behavioral procedure: Experiment 1

The experiment was run in two replications with half of the animals undergoing a contextual conditioning fear procedure (n = 64) and the other half undergoing an auditory fear conditioning procedure (n = 64). There were no statistical differences (all F's < 1.2; p > 0.3) across these replications in the effects of RE inactivation on the acquisition or expression of freezing during conditioning or the context retrieval tests, so the data from these sessions were collapsed. Conditional freezing to the tone CS was assessed in only the cohort that underwent auditory fear conditioning.

Approximately one week after surgery, the animals were randomly assigned to one of four groups: SAL-SAL, SAL-MUS, MUS-SAL, and MUS-MUS. On day 1 rats received microinfusions of either MUS or SAL and were subjected to either contextual or auditory fear conditioning in context A. The conditioning session consisted of a 3-min baseline followed by 5 footshock unconditioned stimuli (US; 1 mA, 2 sec) equally spaced with 70 second inter-trial intervals (ITI). In half of the animals, the US was preceded by a 10-sec auditory conditioned stimulus (CS; 2 kHz, 80 dB). Twenty-four hours later, rats again received microinfusions of MUS or SAL and were placed in either the conditioning context (A) or a novel context (B) for a 10-min stimulus-free retrieval test to assess conditioned freezing and its generalization, respectively. Each rat was tested in each context in a counterbalanced fashion across two days and the infusion group assignment was the same for each test. Rats that received auditory fear conditioning were tested identically, except that five CS-alone test trials (30-sec ISI) were delivered after the 10-min baseline (generalization test) period in context B.

2.4.6 Behavioral procedure: Experiment 2

This experiment was run in two replications with equal representation of subjects in each group. After recovery from surgery, animals were randomly assigned to one of four groups: DH_{SAL}-RE_{SAL}, DH_{SAL}-RE_{MUS}, DH_{APV}-RE_{SAL} and DH_{APV}-RE_{MUS}. Animals received either SAL or APV infusions into the DH and either SAL or MUS into the RE before conditioning; all animals were then tested after an infusion of SAL or MUS into the RE (the RE drug infusion during testing was the same as that during conditioning). This design permitted us to determine whether contextual conditioning under RE inactivation requires NMDA receptors in the DH. After DH and RE infusions on day 1, the conditioning session consisted of a 3-min baseline followed by 5 footshocks unconditioned stimuli (US; 1 mA, 2 sec) equally spaced with 70 second inter-trial intervals (ITI). Forty-eight hours after conditioning, rats again received microinfusions of MUS or SAL in RE and were placed in the conditioning context (A) for a 10-min stimulus-free retrieval test to assess contextual freezing.

2.4.7 Histological procedures

Upon completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus, 100 mg/kg) and perfused transcardially with 0.9% saline followed by 10% formalin. The brains were extracted from the skull and post-fixed in a 10% formalin solution for 24 h followed by a 30% sucrose-formalin solution where they remained for a minimum of 72h. After the brains were fixed, coronal sections (40 μm thickness) were made on a cryostat (−20 °C), mounted on subbed microscope slides, and stained with thionin (0.25%) to visualize cannula placements (see Figure 2.1A for sample cannula placement).

2.4.8 Data analysis

For Experiment 1, three rats died or were sacrificed before completion of the experiment and were excluded from the analysis. Of the 125 remaining animals, fourteen were excluded due to poor cannula placements resulting in the following group sizes: SAL-SAL (n = 29), SAL-MUS (n = 27), MUS-SAL (n = 31), MUS-MUS (n = 24) for the conditioning session and context retrieval tests. Freezing to the tone was assessed in roughly half the number of subjects: SAL-SAL (n = 16), SAL-MUS (n = 13), MUS-SAL (n = 16), MUS-MUS (n = 11). For Experiment 2, two rats died or were sacrificed before completion of the experiment; of the remaining 40 animals, eight were excluded due to poor cannula placements or inadvertent lesions associated with cannula placement or drug infusions. One animal that exhibited average freezing that was greater than two standard deviations above its group mean was excluded resulting in the following group sizes: DH_{SAL}-RE_{SAL} (n = 9), DH_{APV}- RE_{SAL} (n = 9), DH_{SAL}-RE_{MUS} (n = 7), DH_{APV} - RE_{MUS} (n = 7). All behavioral data (mean ± SEM) represent the average percentage of freezing behavior during one-minute intervals during each session. During the tone retrieval test, freezing was averaged across both the auditory CS and the subsequent 30-sec ISI (CS+ITI). Freezing during the CS+ITI period is highly correlated with freezing to the CS itself and is less susceptible to competition by active CS-elicited orienting responses. Two-way analysis of variance (ANOVA) and repeated-measures ANOVA were used to assess general main effects and interactions (α = 0.05). 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.

3. PREFRONTAL PROJECTIONS TO THE THALAMIC NUCLEUS REUNIENS MEDIATE FEAR EXTINCTION*

3.1 Introduction

Learning to contend with threats in the environment is essential to survival. It allows animals, whether rats or humans, to anticipate harm 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 to name a few (Rosen and Schulkin, 1998; Maren et al., 2013). 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 (Vervliet et al., 2013; Goode and Maren, 2014; Milad et al., 2014). Therefore, considerable research has explored the neural circuits that govern these forms of learning. In the laboratory, Pavlovian fear conditioning and extinction procedures are widely used to study the neural basis of emotional memory. Briefly, animals learn an innocuous conditioned stimulus (CS) predicts an aversive unconditioned stimulus (US). After fear conditioning, animal exhibit conditioned fear responses (CRs), such as freezing, to presentation of the CS alone. Repeated presentation of the CS alone (i.e., extinction training) ultimately reduces conditioned responses (Maren, 2001; VanElzakker et al., 2014). Importantly, extinction represents new learning and does not erase the original fear memory. Fear to an extinguished CS returns under many circumstances, including when the CS is encountered outside of the extinction context, a phenomenon termed renewal

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Ramanathan KR, Jin J, Giustino TF, Payne MR, Maren S (2018a) Prefrontal projections to the thalamic nucleus reuniens mediate fear extinction. Nat Commun 9:4527.

(Bouton, 2004; Dunsmoor et al., 2015). Because extinction learning is at the heart of clinical interventions, such as exposure therapy, that are aimed to treat stress- or trauma-related disorders such as PTSD, many patients are prone to fear relapse (Vervliet et al., 2013; Goode and Maren, 2014).

Decades of research have implicated the hippocampus (HPC), medial prefrontal cortex (mPFC), and basolateral amygdala (BLA) in the encoding and context-dependent expression of extinction memories (Maren et al., 2013; Giustino and Maren, 2015). Recently, we have shown that the renewal of fear to an extinguished CS activates ventral hippocampal (vHPC) neurons projecting to both the mPFC and BLA (Orsini et al., 2011; Jin and Maren, 2015b; Wang et al., 2016). Importantly, functional disconnection of the vHPC and either the prelimbic (PL) prefrontal cortex or BLA impairs fear renewal (Orsini et al., 2011). These studies support a circuit model in which vHPC projections to the mPFC and BLA facilitate the retrieval of CS-US memories when an extinguished CS is encountered outside the extinction context (Orsini et al., 2011; Jin and Maren, 2015b). 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. For example, the mPFC projects to midline thalamic nuclei that relay information to both the hippocampus and amygdala (McKenna and Vertes, 2004; Vertes et al., 2007): mPFC projections to the midline paraventricular nucleus, in particular, have been implicated in the expression of conditioned fear (Padilla-Coreano et al., 2012; Do-Monte et al., 2015b). In addition, the mPFC projects to the nucleus reuniens (RE), a midline thalamic nucleus

that is well positioned to mediate mPFC influences on hippocampal function (Vertes et al., 2007; Griffin, 2015; Jin and Maren, 2015a). Lesions or inactivation of the RE impair forms of memory that require both the mPFC and HPC (Hembrook et al., 2012; Layfield et al., 2015; Hallock et al., 2016), including goal-directed spatial memory (Ito et al., 2015) and contextual fear memories (Xu and Südhof, 2013; Vetere et al., 2017). Given the crucial role of the RE in mediating mPFC-HPC interactions, we sought to determine whether it also plays a role in the encoding and retrieval of context-dependent extinction memories. Using Pavlovian fear conditioning and extinction procedures in rats, we show that pharmacological inactivation of the RE dramatically increases freezing behavior during both the encoding and later retrieval of an extinction memory. This extinction impairment was not state-dependent. This pattern of extinction deficits was reproduced by selective pharmacogenetic silencing of mPFC neurons (or their terminals) projecting to the RE. Taken together, these data reveal a novel role for the prefrontal-reuniens circuit in the inhibition of fear after extinction. This circuit may function to oppose fear expression after threat has passed.

3.2 Results

3.2.1 RE inactivation impairs encoding of extinction.

To explore the role of RE in fear extinction, we first examined whether reversible inactivation of the RE with the $GABA_A$ agonist muscimol would impair the acquisition and later retrieval of the extinction memory. Because mPFC-HPC circuits have been implicated in contextual processing (Maren et al., 2013; Marek et al., 2018), we were particularly interested in whether RE inactivation might influence the context-dependence of the extinction memory. To this end, we examined the effects of RE inactivation on freezing during within-subject retrieval tests conducted in the extinction (ABB) and conditioning (ABA) contexts. Rats were first implanted with a single midline cannula targeting the RE (Figure 3.1a). After recovery from surgery, rats underwent fear

conditioning, extinction, and retrieval testing (Figure 3.1b). During fear conditioning (Figure 3.1c, left), rats exhibited low levels of freezing behavior before the onset of the first conditioning trial, and an increase in freezing across the conditioning trials [repeated measures ANOVA, main effect of trial, $F(5, 115) = 36.7$, $p < 0.001$]. The levels of freezing did not differ between the drug groups [$F < 1.8$], indicating that rats in each group acquired similar levels of conditioned fear. The following day, rats received intra-RE infusions of either saline (SAL) or muscimol (MUS) immediately before an extinction training session (45 CS-alone trials) that was conducted in a context different from that used for conditioning. During this session (Figure 3.1c, middle), both groups of rats exhibited robust conditioned freezing to the CS in the earliest trial block, and saline-treated rats exhibited a within-session decrease in freezing that is typical of extinction learning. However, inactivation of the RE completely eliminated this within-session decrement in freezing [repeated measures ANOVA, main effect of drug, $F(1, 23) = 14.86$, $p = 0.0008$; drug x trial interaction $F(9, 207) = 6.46$, $p < 0.0001$].

Twenty-four hours after extinction, rats were tested for their fear to the extinguished CS in both the extinction (retrieval) and conditioning (renewal) contexts. As shown in Figure 3.1c (right), rats extinguished under muscimol showed greater levels of CS-elicited freezing compared to control rats and this was particularly pronounced in the extinction context; both groups of rats renewed fear to the extinguished CS outside the extinction context. These observations were confirmed in a repeated measures ANOVA, which revealed main effects of drug [$F(1,23) = 5.14$; $p = 0.03$] and test context [$F(1,23) = 16.85$; $p = 0.0004$]. Although there was not a reliable drug x test interaction [$F(1,23) = 0.89$; $p = 0.36$], planned comparisons revealed a significant difference between SAL and MUS groups during the retrieval session ($p = 0.011$) but not in the renewal

session (p = 0.226). These results reveal that RE inactivation causes a deficit in the acquisition of fear extinction.

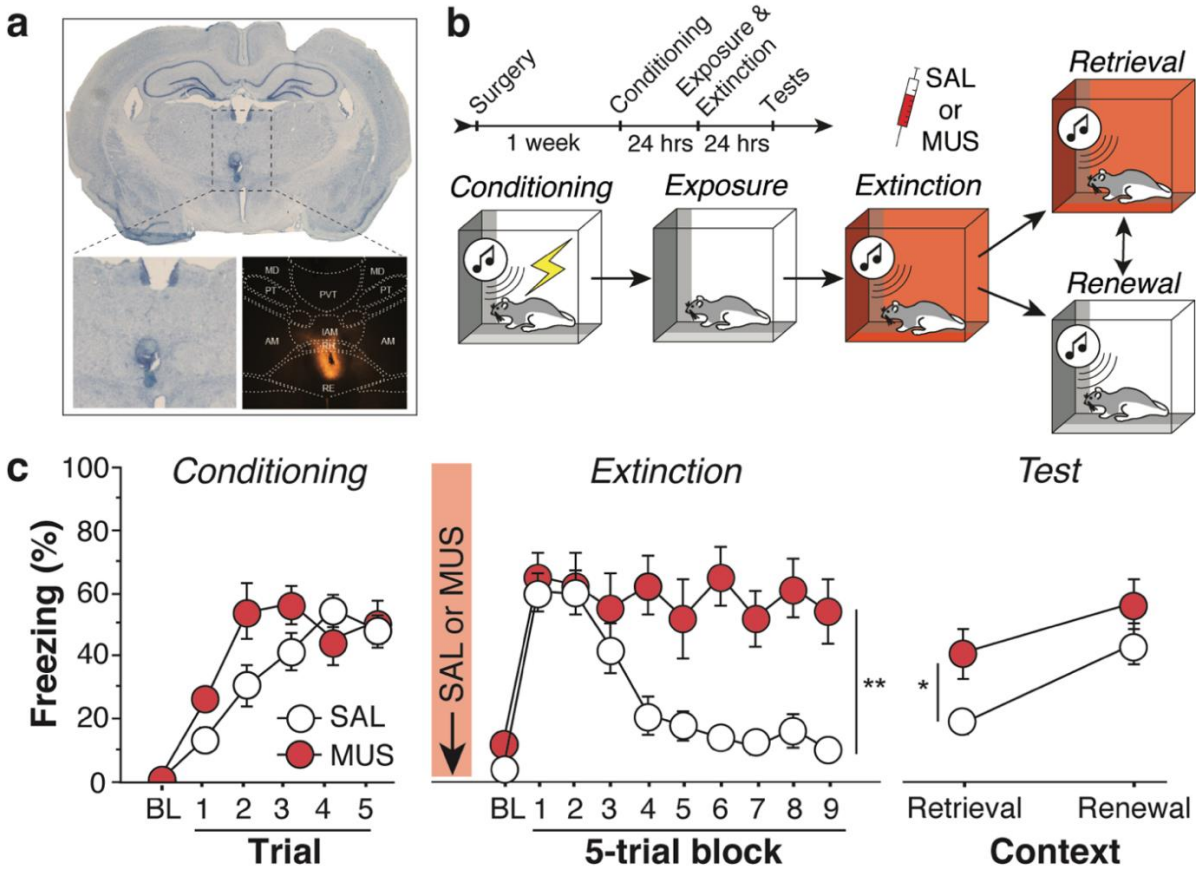

Figure 3.1 Muscimol inactivation of RE impairs encoding of fear extinction. a Representative thionin-stained coronal section showing cannula placement in the RE. The darkfield image shows diffusion of TMRx muscimol in the RE. b Schematized behavioral design. c (Conditioning, left) Percentage of freezing during the 3-min baseline (BL) and 1-min interstimulus interval (ISI) following each CS-US pairing during the fear conditioning session. (Extinction, middle), Percentage of freezing during the 3-min baseline and 30-s ISIs across 9 extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction session. Arrow indicates the timing of saline (SAL; white circles; n = 14) or muscimol (MUS; red circles; n = 11) infusion before the extinction session. (Retrieval tests, right), Average percentage of freezing for 5 CS test trials in the extinction (retrieval) and conditioning (renewal) contexts. All data are means ± s.e.m.s; *p < 0.05; **p < 0.01; one-way factorial and repeated measures ANOVA.

3.2.2 RE inactivation impairs extinction retrieval, but not fear renewal.

Next, we sought out to explore the role of RE in extinction retrieval. Importantly, we wanted to see whether the context dependent extinction impairment seen in previous experiment was relative to the extinction context and not just physical change between two contexts. To this end, we compared the effects of RE inactivation on extinction retrieval in rats that underwent extinction in either the conditioning context (COND) or a novel context (NOVEL); all rats were then tested in their respective extinction contexts (AAA or ABB) and then in a novel renewal context (C) (see Figure 3.2 a for behavioral paradigm). Animals were first implanted with cannulas targeting RE and, after recovery from surgery, underwent fear conditioning in context A Figure 3.2). On Day 2, animals were extinguished in either the conditioning context (COND) or a novel context (NOVEL). During the extinction session, rats showed high levels of CS-elicited freezing early in the session, but it dramatically decreased by the end of the session indicating successful extinction [repeated measures ANOVA, main effect of trial $F_{(1,11)} = 13.07$; $p = 0.0041$]. Freezing in rats extinguished in the conditioning context was significantly higher than that in rats extinguished in the novel context [repeated measures ANOVA, main effect of group $F_{(1,11)} = 11.24$; $p = 0.007$], which reflects a summation of context and CS fear in the conditioning context.

On subsequent days, animals received infusions of SAL or MUS (counterbalanced, within-S's design) and retrieval test in the extinction context followed by a test in a third novel context (renewal test). During the retrieval test (Figure 3.2b, right), MUS infusions in to the RE increased freezing to the extinguished CS independent of the extinction procedure; MUS infusion did not affect the renewal of freezing outside the extinction context. These observations were confirmed in a one-way repeated-measures ANOVA that revealed a main effect of drug [$F_{(1,11)} = 37.57$; $p < 0.001$], but no effect of extinction context [$F_{(1,11)} = 0.79$; $p = 0.39$] or drug x context interaction

[$F_{(1,11)}$ = 2.44; p = 0.15]. During the renewal session, a one-way repeated measures ANOVA revealed no main effect of drug [$F_{(1,11)}$ = 2.98; p = 0.12] or group [$F_{(1,11)}$ = 0.21; p = 0.65] and no interaction between the two variables $F_{(1,11)}$ = 2.13; p = 0.13].

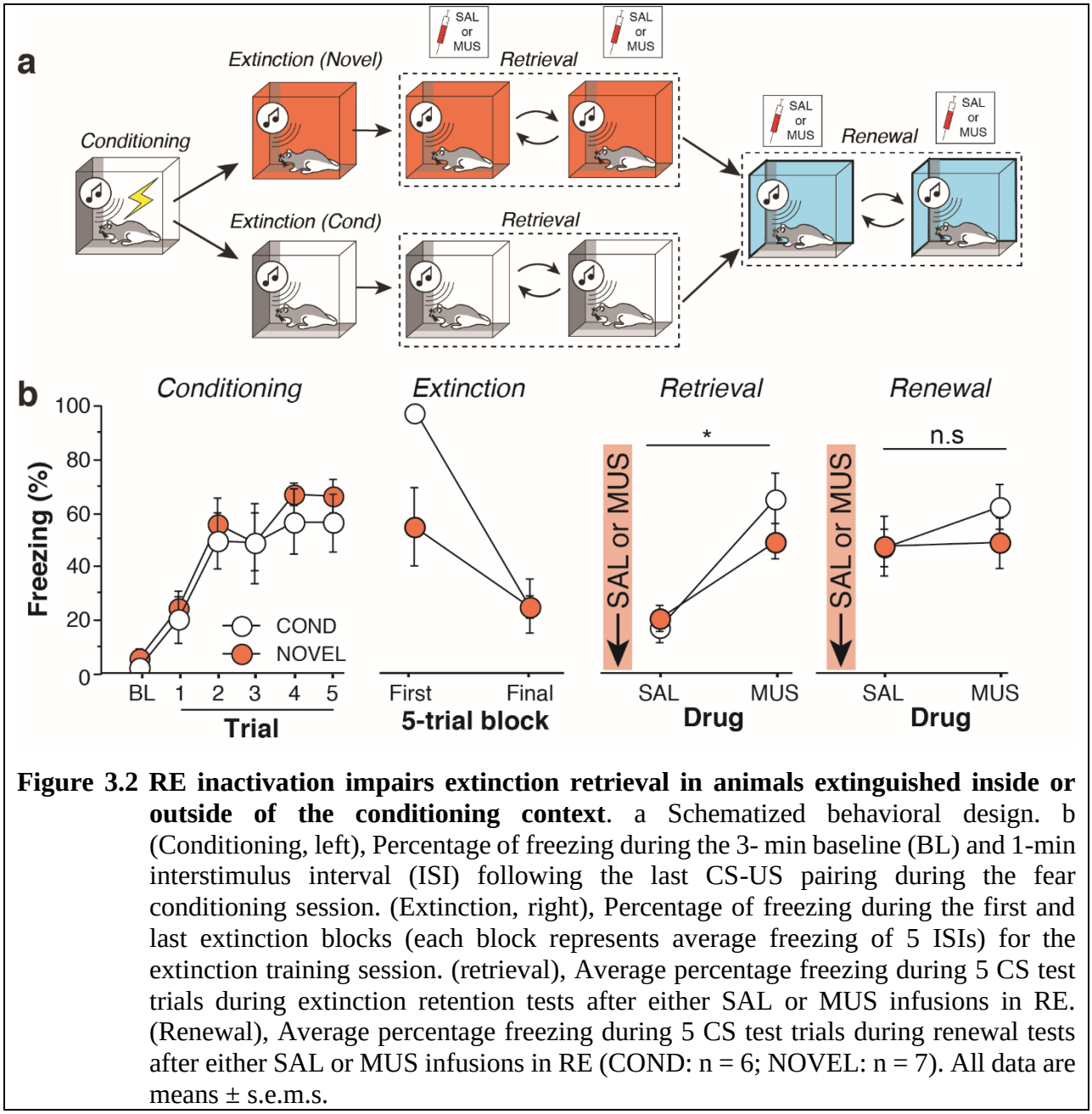

Figure 3.2 RE inactivation impairs extinction retrieval in animals extinguished inside or outside of the conditioning context. a Schematized behavioral design. b (Conditioning, left), Percentage of freezing during the 3- min baseline (BL) and 1-min interstimulus interval (ISI) following the last CS-US pairing during the fear conditioning session. (Extinction, right), Percentage of freezing during the first and last extinction blocks (each block represents average freezing of 5 ISIs) for the extinction training session. (retrieval), Average percentage freezing during 5 CS test trials during extinction retention tests after either SAL or MUS infusions in RE. (Renewal), Average percentage freezing during 5 CS test trials during renewal tests after either SAL or MUS infusions in RE (COND: n = 6; NOVEL: n = 7). All data are means ± s.e.m.s.

3.2.3 Muscimol-induced extinction impairments are not state-dependent.

The previous results reveal that MUS infusions into the RE impair both the encoding and retrieval of fear extinction but did not affect fear renewal. It is possible that this pattern of results is due to

a shift in interoceptive (i.e., drug) context between extinction and retrieval testing that itself causes fear renewal (Bouton et al., 1990). To examine this possibility, we conducted an experiment in which RE inactivation occurred before both the extinction and retrieval sessions. If the interoceptive context associated with RE inactivation is critical for the expression of extinction, then animals that are extinguished and tested after RE inactivation should show normal extinction retrieval.

To examine this possibility, rats were implanted with a single midline cannula targeting the RE and after recovery from surgery underwent fear conditioning, extinction, and retrieval testing. Muscimol was infused in RE before both extinction and retrieval sessions. During the extinction session (Figure 3.3, middle), we replicated our previous observation that RE inactivation impairs within-session extinction compared to saline controls [repeated measures ANOVA, main effect of group, $F_{(2,60)} = 12.8$; $p < 0.001$]. During retrieval testing (Figure 3.3, right), animals extinguished and tested under RE inactivation continued to exhibit an extinction impairment relative to SAL-treated controls and exhibited levels of fear comparable to that in rats that did not undergo extinction. These observations were confirmed in an ANOVA performed on the average CS-elicited freezing during the test [main effect of group, $F_{(2,60)} = 4.8$; $p < 0.05$]. Post-hoc comparison revealed that SAL-treated rats differed from both MUS-treated and No-Ext controls, which did not differ from one another. Importantly, these data indicate the extinction retrieval deficits in muscimol-treated rats are not due to a drug-shift induced renewal, because extinction deficits were observed in animals extinguished and tested in the same drug state. These results indicate that encoding and retrieval deficits after MUS infusions into RE are not due to state-dependent generalization deficits.

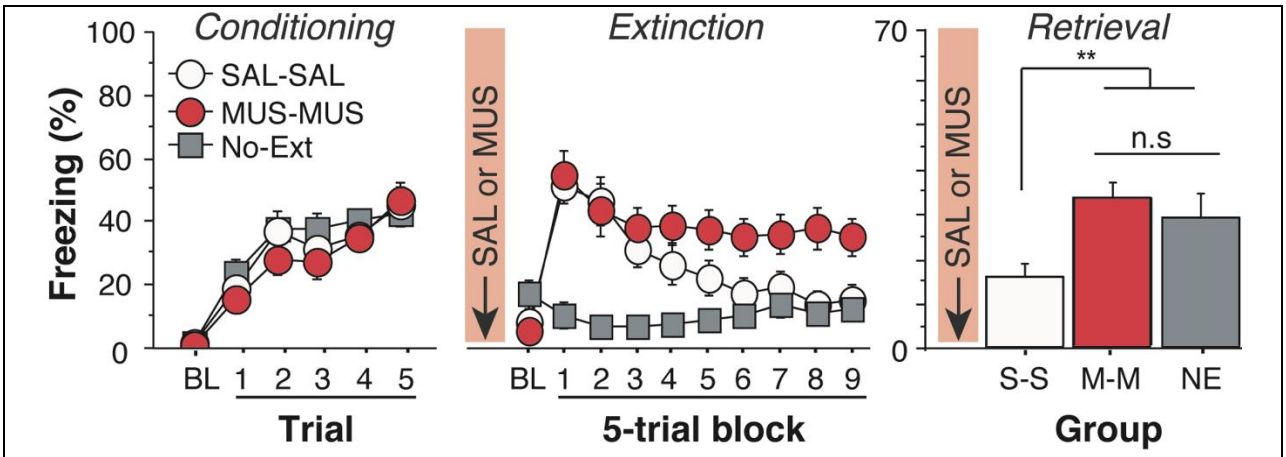

Figure 3.3 Muscimol-induced extinction impairments are not state-dependent. (Conditioning, left), Percentage of freezing during the 3-min BL and 1-min ISI following each CS-US pairing during the fear conditioning session. (Extinction, middle), Percentage of freezing during the 3-min BL and 30-s ISIs across the extinction session. (Retrieval, right), Percentage of freezing for the 5 CS test trials in the extinction (retrieval) context. Arrows indicate the timing of muscimol (MUS; red circles; n = 20) or saline (SAL; white circles; n = 20) infusions into RE before either extinction training or retrieval testing. No-Ext rats (grey squares; n = 23) were placed in the extinction context during but did not receive CS presentations. All data are means ± s.e.m. * p <0.05; ** p <0.01; one-way factorial and repeated measures ANOVA.

3.2.4 Extinguished CSs increase single unit firing in the RE.

The previous data indicate that extinguished CSs increase Fos expression in the RE in both the extinction and renewal contexts. However, Fos expression has low temporal resolution and integrates neuronal activity elicited by both the context and CS during retrieval testing. It is therefore possible that RE neurons respond differentially to CSs presented in the extinction and renewal contexts. To examine this possibility, we made single-unit recordings from RE neurons in freely behaving rats using a within-subject design. A schematic illustration of the behavioral paradigm is shown in Figure 3.4a. Briefly, animals were implanted with a microwire bundle targeting RE (see Figure 3.4b for representative electrode placements). After recovery from surgery, animals underwent auditory fear conditioning followed 24 hours later by extinction training.

Twenty-four hours after extinction, the rats were received an unsignaled remainder shock in context A to facilitate the return of freezing during the renewal test [main effect of trial, $F_{(2,1)}$ = 125.05; p = 0.0075]. On the subsequent day, rats were subjected to a within-subject testing procedure wherein the extinguished CS was presented in either the extinction context (retrieval) or a novel context (renewal); single-unit recordings were made during both tests and the same neurons tracked across sessions. During the retrieval tests (Figure 3.4d), rats showed lower levels of freezing in the extinction context relative to the renewal context, though this was not statistically reliable [$F_{(1,2)}$ = 17.10; p = 0.053 for trial 1]. During the retrieval tests we recorded from a total of 27 neurons in RE. The basal firing rate of these neurons was significantly higher in the retrieval (2.88 ± 0.17 Hz) than the renewal (2.42 ± 0.22 Hz) [paired t test; $t_{(26)} = -2.3$, $p < 0.03$]. Among this population of cells, seven neurons (25%) exhibited significant increases in firing to the tone CS (defined as an increase in firing rate > 1.96 standard deviations above the 500 ms pre-CS baseline). Interestingly, RE neurons exhibited greater CS-evoked firing within 200 ms of CS onset in the extinction context relative to that in the renewal context (Figure 3.4c, d). This observation was confirmed in a one-way repeated measures ANOVA that revealed a main effect of test [$F_{(1,6)}$ = 15.67; p = 0.008] indicating that neurons in RE showed greater CS-evoked responses to an extinguished CS in the extinction context relative to the renewal context.

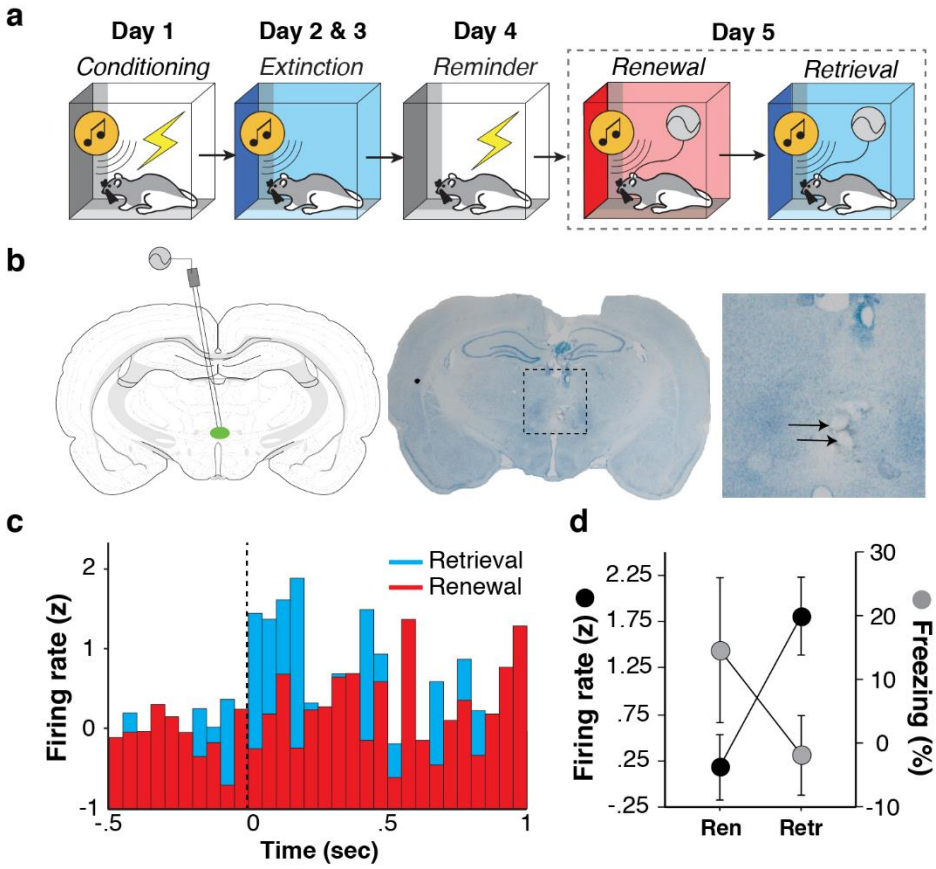

Figure 3.4 Extinction increases CS-elicited spike firing in RE. a Schematic behavioral design. **b** Representative coronal sections showing electrode placements in RE. **c** Average normalized firing rate among RE neurons (n = 7) across five CS presentations in either the retrieval (blue bars) or renewal context (red bars). Firing rate was binned (50 ms) during a 500 ms pre-CS period and a 1-sec post-CS period. d Average firing rate of RE neurons over 5 trials during the 1 sec of tone onset (black circles) and percentage of freezing for the 5 trials during testing in retrieval and renewal context (grey circles). All data are means ± s.e.m.s * p <0.05; ** p <0.01; repeated measures

3.2.5 Silencing RE projectors in the mPFC impairs extinction encoding.

The mPFC plays a critical role in extinction learning and retrieval. The RE receives a heavy input from the mPFC and this may represent a critical functional input regulating fear extinction. Here we sought to determine whether mPFC projections to the RE are involved in the acquisition and retrieval of fear extinction. Rats received injections of AAV5-Cre in the RE and AAV8-hSyn-DIO-hM4D(G_i)-mCherry in the mPFC 4-5 weeks prior to behavioral training (see Figure 1B for

behavioral design and Figure 3.5a for viral expression). Twenty-four hours after auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,160)} = 35.6$; $p < 0.001$] (Figure 3.5b, left), rats received systemic injections of either SAL or CNO and underwent fear extinction. As shown in Figure 3.5b (middle), CNO administration increased CS-elicited freezing during the extinction session [repeated measures ANOVA, main effect of drug $F_{(1,32)} = 4.15$; $p = 0.05$ and main effect of trials $F_{(1,32)} = 46.25$; $p < 0.0001$].

During retrieval testing (Figure 3.5b, right), all animals exhibited low levels of freezing in the extinction context and increased freezing to the CS in the renewal context [repeated measures ANOVA, main effect of test $F_{(1,32)} = 17.57$; $p = 0.0002$]. Interestingly, rats that previously received CNO during extinction training showed higher levels of freezing compared to SAL-treated rats during both of the retrieval tests [repeated measures ANOVA, main effect of drug, $F_{(1,31)} = 8.23$; $p = 0.007$]. Post-hoc comparisons revealed that CNO-injected animals showed elevated levels of freezing compared to SAL-injected animals during both retrieval ($p = 0.031$) and renewal ($p = 0.011$) sessions. These results are consistent with the effects that we previously showed with RE inactivation alone and reveal that projections from the mPFC to the RE are involved in extinction learning. Furthermore, this effect was not simply a performance effect of CNO (e.g., non-specific increases in freezing), insofar as pre-CS baseline freezing during extinction training was not affected by CNO [unpaired t-test $t_{(32)} = 0.18$; $p=0.85$] and the extinction impairments were manifest during the drug-free retrieval tests.

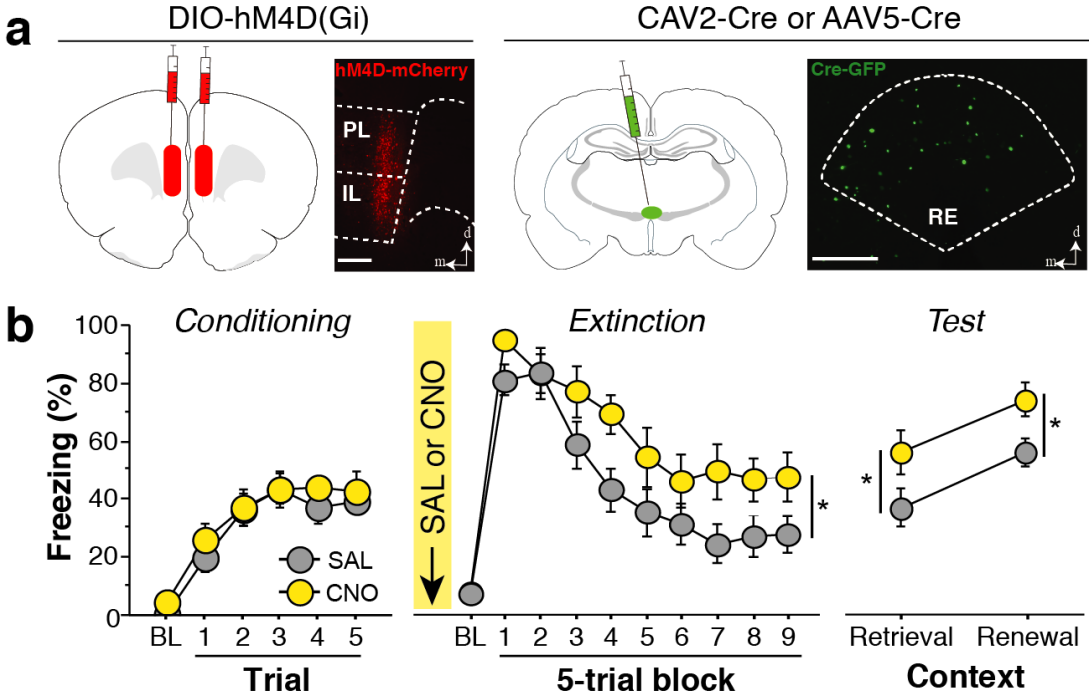

Figure 3.5 Silencing mPFC→RE projectors impairs encoding and expression of fear extinction. a Representative images of Cre-dependent DREADD expression; AAV8-hSyn-DIO-hM4D(G_i)-mCherry in the mPFC and AAV5-Cre-eGFP virus in the RE. **b** (Conditioning, left), Percentage of freezing during the 3-min baseline (BL) and 1-min interstimulus interval (ISI) following each CS-US pairing during the fear conditioning session. (Extinction, middle), Percentage of freezing during the 3-min baseline and 30-s ISIs across 9 extinction blocks (each block represents average freezing of 5 extinction trials) for the extinction training session. (Test, right), Percentage of freezing for 5 CS test trials in the extinction (retrieval) and conditioning (renewal) context. The arrow indicates the timing of the CNO (CNO; yellow circles; *n* = 15) or saline (SAL; white circles; *n* = 19) injections before the extinction session.

3.2.6 Silencing mPFC terminals in RE impairs extinction retrieval.

Next, we examined whether mPFC projections to RE also mediate the retrieval of extinction memories. In spite of using intersectional DREADD strategy to silence mPFC projections to RE, There is a chance that it might influence mPFC output to other brain areas insofar it has been shown that mPFC neurons send collateral projections to medio-dorsal thalamus and reticular thalamus (Cornwall and Phillipson, 1988). To specifically manipulate mPFC projections to RE, we expressed inhibitory DREADDs (or a blank control) in the mPFC and microinfused CNO into

the RE to inactivate mPFC terminals (Lichtenberg et al., 2017). Furthermore, we tried to isolate the contribution of both PL and IL to the retrieval deficit. A schematic illustration of the behavioral paradigm is shown in Figure 3.6a. Rats received injections of AAV8-hSyn-hM4D(G_i)-mCherry or AAV8-hSyn-GFP in either PL or IL or both and were implanted with cannula targeting the RE five weeks after viral infusions. Viral infusions in the mPFC produced robust terminal expression in the RE.

One week after cannula implantation, rats underwent auditory fear conditioning [repeated measures ANOVA, main effect of trial, $F_{(5,240)} = 50.72$; $p < 0.001$, no main effect of group $F_{(3,48)} = 0.604$, $p = 0.61$] and three sessions of extinction training [repeated measures ANOVA, main effect of trials, $F_{(1,48)} = 132.45$; $p < 0.0001$, no main effect of group $F_{(3,48)} = 0.28$; $p = 0.84$] (Data not shown). 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 (Figure 3.6c). That is, rats were tested after receiving infusions either SAL or CNO in two counterbalanced tests in the extinction context, which were conducted over two days. As shown in Figure 7c, CNO infusion into RE increased conditional freezing to the extinguished CS in animals expressing inhibitory DREADDs in either the PL, IL or both areas; CNO did not affect freezing in blank controls. These observations were confirmed in a repeated measures ANOVA that revealed a main effect of drug [$F_{(1,48)} = 17.74$; $p = 0.0001$], without a main effect of group, [$F_{(3,48)} = 1.73$; $p = 0.17$] or a group x drug interaction [$F(3,48) = 2.02$; $p = 0.12$]. Planned comparisons revealed that CNO infusions did not result in any changes in freezing in rats receiving blank GFP virus $p = 0.74$ confirming that CNO-induced increases in freezing are not due to nonspecific effects of the drug. However, CNO infusions increased freezing in all three groups expressing inhibitory DREADDs in the mPFC: PL+IL ($p = 0.022$), PL ($p = 0.012$), and IL ($p = 0.046$). These results indicate that

both prelimbic and infralimbic prefrontal projections to the RE are involved in the retrieval of extinguished fear memory.

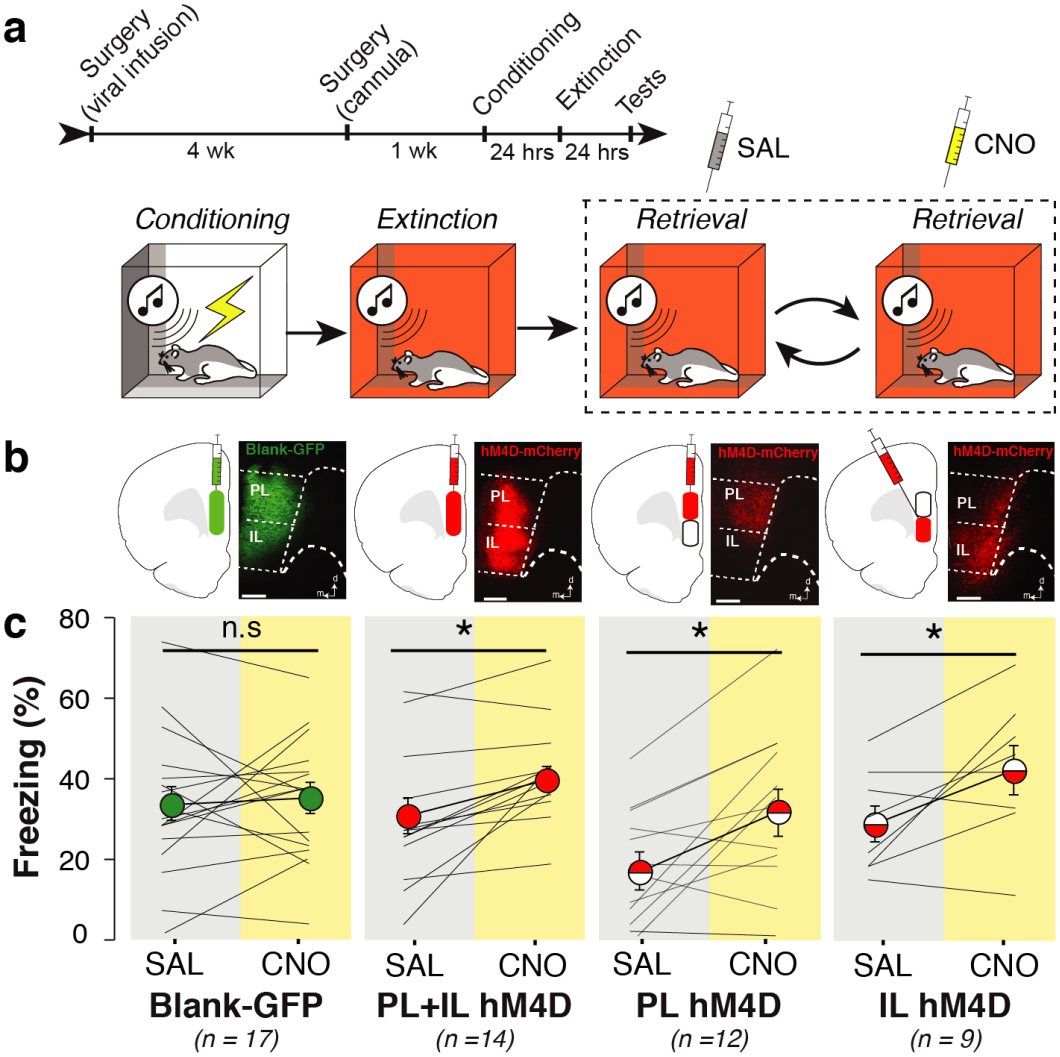

Figure 3.6 Silencing mPFC terminals in RE impairs extinction retrieval. a Schematic behavioral design. **b** Representative images of viral expression in the mPFC. c Average percentage of freezing during the retrieval tests. Freezing during each test is averaged across five CS test trials either SAL or CNO infusions in RE. Scale bars represent 0.5mm. All data are means ± s.e.m.s * p <0.05; ** p <0.01; repeated measures ANOVA.

3.3 Discussion

Here we have demonstrated for the first time that the nucleus reuniens of the midline thalamus is required for both encoding and retrieving extinction memories. Extinction training or retrieval testing increased the activity of RE neurons and inactivation of the RE or its projections from the mPFC produced deficits in extinction memory. Taken together, the present study reveals a novel role for the prefrontal-thalamic circuits in fear extinction and suggests the RE is a key structure mediating prefrontal top-down inhibitory control of fear inhibition that is crucial for extinction.

The fact that the RE is critically involved in extinction learning and recall is in line with previous work demonstrating the importance of the midline thalamus in both memory and emotion (Davoodi et al., 2011; Cassel et al., 2013; Xu and Südhof, 2013; Ito et al., 2015; Pereira de Vasconcelos and Cassel, 2015; Hallock et al., 2016; Linley et al., 2016). Importantly, a recent study demonstrated that the RE is important for maintaining the specificity of contextual fear memory (Xu and Südhof, 2013). Specifically, the authors showed that RE inactivation caused an overgeneralization of conditional fear to contexts other than the one in which shock was encountered, but did not affect fear recall in the original conditioning context or auditory fear expression (Xu and Südhof, 2013). Interestingly, both contextual conditioning and the extinction of fear to an auditory CS rely heavily on contextual processing. That is, contextual fear conditioning requires the acquisition of a contextual representation that comes into association with an aversive US. As a result, conditioned fear is expressed in the place where shock is encountered, but not in other places. Similarly, extinction involves learning that a CS is not reinforced in a particular context. In this case, the suppression of fear to a CS after extinction occurs in the extinction context, but not in other places; in other words, fear to an extinguished CS renews outside the extinction context (Maren et al., 2013). In both cases, deficits in contextual

specificity—knowing what happened where—would result in both overgeneralized fear after context conditioning and an inappropriate renewal of fear after extinction. In both cases, fear is expressed in otherwise safe contexts. Together, these data suggest that the RE and its connections with the mPFC might be involved in the inhibition of fear in safe contexts. Importantly, RE or mPFC-RE projections were not involved in mediating the renewal of fear to an extinguished CS outside the extinction context.

Previous studies have demonstrated that the renewal of extinguished fear requires the hippocampus and its projections to the mPFC (Orsini et al., 2011; Jin and Maren, 2015b; Wang et al., 2016; Marek et al., 2018). HPC inactivation or disconnections of the HPC and mPFC disrupted fear renewal, but did not affect the expression of extinction (Hobin et al., 2006; Orsini et al., 2011). This reveals that direct HPC-mPFC projections are not involved in fear inhibition, but rather contribute to the excitation of fear to an extinguished CS outside the extinction context. In the present study, we have shown that direct mPFC inputs to the RE are crucial for fear extinction. Indeed, the RE has been suggested to be a critical hub that interconnects the mPFC and hippocampus (Vertes et al., 2007; Griffin, 2015). Prefrontal projections to the RE are involved in fear memory generalization (Xu and Südhof, 2013), goal-directed spatial navigation (Ito et al., 2015), motivation and reward-related behavior (Zimmerman and Grace, 2016), and spatial working memory (Griffin, 2015; Hallock et al., 2016). Anatomically, there are strong reciprocal projections between the RE and the mPFC and hippocampus (Varela et al., 2014), and the RE is important for synchronizing local field potentials in this circuit (Roy et al., 2017; Kafetzopoulos et al., 2018). This suggests that the mPFC-RE interactions we find are important for extinction learning might ultimately be mediated through RE projections to the hippocampus. Indeed, interactions between the mPFC and HPC are involved in a number of emotional and cognitive

processing fear and anxiety including in extinction (Bouton, 2004; Milad et al., 2007; Adhikari et al., 2010; Maren et al., 2013; Bukalo et al., 2014; Jin and Maren, 2015a; Wang et al., 2016; Marek et al., 2018).

Indeed, a role for mPFC-RE-HPC circuits in the inhibition of conditioned fear in safe contexts might be an example of a broader role of this circuit in *retrieval suppression*. For example, humans can actively suppress recalling a particular memory, either by being instructed to do so in the laboratory or spontaneously when confronted with a reminder of a trauma, for example. Interestingly, functional neuroimaging work indicates that retrieval suppression is associated with an increase in activity in the mPFC, but a suppression of activity in the hippocampus (Anderson et al., 2016). It has been suggested that RE might coordinate this inhibitory influence of the mPFC on hippocampal memory retrieval. In the context of the present work, this mechanism might eliminate interference between fear and extinction memories, by suppressing the retrieval of the fear memory in otherwise safe contexts. That is, during extinction retrieval, when an extinguished CS is encountered in a safe context, a retrieval suppression process mediated by projections of the mPFC to the HPC via the RE might prevent retrieval of the fear memory. Alternatively, RE projections to the amygdala, including the basolateral and basomedial amygdala (Vertes, 2006), might allow for both the mPFC and HPC to exert integrated contextual control over the expression of fear.

In conclusion, the present study suggests that the prefrontal inputs to the nucleus reuniens are critically involved in fear extinction. Because the reuniens interferes with neuronal activity in both the prefrontal cortex and hippocampus during extinction retrieval, the reuniens may mediate retrieval suppression by relaying prefrontal inputs to the hippocampus, thus disruption of the pathway leads to fear relapse in otherwise safe context. Preventing return of fear is at the core of

exposure therapy; therefore, future studies are needed to understand how dysfunction in prefrontal-reuniens circuits underlies psychopathology associated with stress- and trauma-related events.

3.4 Methods

3.4.1 Subjects

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 every day 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.

3.4.2 Viruses and drugs.

AAV8-hSyn-DIO-hM4D(G_i)-mCherry (titer $\geq 4\times10^{12}$ 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 and AAV5-CMV-HI-eGFP-Cre-WPRE-SV40 (titer: $0.64\text{-}1.42\times10^{14}$ GC/mL) was from University of Pennsylvania Vector Core. AAV-8-hsyn-hM4D(Gi)-mCherry (titer: 3×10^{12} vp/mL) and AAv8-hSyn-eGfp (titer: 3×10^{12} vp/mL) was obtained from Addgene. 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.

3.4.3 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(G_i)-mCherry into the mPFC (including PL and IL), and AAV5-Cre-eGFP into the RE. Within the mPFC, two infusions (1.0 μl each) were made in the IL (A/P: +2.7 mm, M/L: ±0.5- 0.5 mm, D/V: -4.4 mm from dura) and PL (A/P: +2.7 mm, M/L: ±0.5 mm, D/V: -3.2 mm from dura) respectively. A single infusion (1.0- 1.2 μl) was made in the RE (A/P: -2.05 mm, M/L: +1.0 mm, D/V: -6.9 mm from dura) at a 10° angle.

For the terminal inactivation experiment, rats were bilaterally infused with AAV8-hSyn-EGFP into PL&IL using the coordinates mentioned above. For the active virus groups, targeting of PL and PL+IL groups was done using the coordinated mentioned above. However, for the IL group the following coordinate was used in order to limit the damage to PL (A/P=+2.7, M/L= ± 2.0, D/V= -4.9 from dura at 30° angle).

3.4.4 Drug delivery

For RE microinfusions, rats were transported to an infusion using white buckets (5-gallon) from the vivarium. Dummy cannula was removed from the implanted guides and stainless-steel injectors (33-gauge, 9 mm) connected to tubes was 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. Muscimol was diluted in sterile saline to a concentration of 0.1μg/μl. For terminal inactivation experiment, CNO dissolved in SAL (with 2.5% DMSO) at 1mM concentration Infusions were made at a rate of 0.1 μl/min for 3 min (0.3 μl total) 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 extinction or testing 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.

3.4.5 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.

3.4.6 Behavioral procedures.

For muscimol inactivation experiments, approximately 1 week after surgery, rats underwent fear conditioning, extinction, and retrieval testing in either the conditioning context (Context A) or the extinction context (Context B). 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 (ISIs). On the following day, rats underwent fear extinction in which they received a 3 min stimulus free BL followed by 45 tone-alone presentations (30 s ISIs). 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 a subsequent 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 ISIs). Rats received microinfusions of SAL or MUS into the RE prior to either 10-min before extinction training or 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 assess the state-dependence of RE inactivation, rats underwent fear conditioning, extinction and extinction retrieval testing as previously described. One group of rats received MUS infusions in the RE before both extinction training and the retrieval test and a second group of rats received SAL infusions before both extinction and retrieval test. A third group of rats (No-Ext) also received SAL infusions, but they did not receive CS presentations during the extinction session. To access whether the retrieval is affected in same context, the animals were conditioned and extinguished as described above. On the following two days, rats received either infusions of SAL or MUS (counterbalanced across days) prior to the retrieval test in the extinguished context to test the strength of the extinguished memory. On the subsequent two days, rats received either infusions of SAL or MUS (counterbalanced across days) prior to the retrieval test in a novel non-extinguished context to test their fear renewal.

For the intersectional DREADD experiments, rats underwent auditory fear conditioning, extinction, retrieval testing 4-5 weeks after surgery as previously described. Rats received SAL or CNO injections either 30 min before extinction training or retrieval testing. For the encoding experiment, retrieval tests were conducted in both the conditioning context (context A, renewal) and the extinction context (context B, retrieval). For the retrieval, experiment animals were only tested in the extinction context (context B, retrieval) using a within-subjects procedure in which each animal served as its own control. That is, each rat received either a SAL or CNO 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.

For the terminal DREADD experiment, animals underwent surgery for viral infusions into either PL or IL or both. Five weeks after this surgery, animals underwent a second surgery to

implant cannula targeting the RE. One week after the second surgery, rats underwent auditory fear conditioning, extinction, retrieval testing as previously described. Rats received infusions of SAL or CNO in RE 10 min before the retrieval testing using a within-subjects procedure in which each animal served as its own control.

3.4.7 Electrophysiological recordings.

For the *in-vivo* electrophysiological recording experiment, a modified rodent conditioning chamber (30 x 24 x 21 cm) was used for the extinction and testing sessions. This chamber was modified to allow for awake, behaving recordings. One week after recovery from surgery, rats (n = 3) underwent auditory fear conditioning in context A in which they were presented with 3 CS-US (60 sec ITI) pairings after a 3 min stimulus-free baseline period. On the subsequent two days, rats underwent identical extinction sessions in context B in which they were presented with 45 CS-alone trials (30 sec ITI) after a 3 min stimulus-free baseline period. Twenty-four hours after the final extinction session, rats received a single, weak unsignaled reminder shock (2 sec, 0.5 mA) in context A after a 3-min baseline period.

On the fifth and final day of the experiment, rats received a dual-test session for extinction retrieval (context B) and fear renewal (context C). These sessions consisted of a 3 min baseline period followed by presentation of 5 CS-alone trials (30 sec ITI). Three minutes after the final CS the recording system was paused and rats were temporarily placed in a 5-gallon buck with bedding (the headstage cable remained connected), allowing us to record signal from the same neurons over both retrieval tests. During this time, the experimenters quickly changed the contextual layout of the recording chamber (i.e., swapping from context B to context C). Rats were then placed back into the recording chamber and underwent a second retrieval session in the new context. This dual

testing session enabled us to record CS-elicited activity in the same single-units in both the retrieval and renewal contexts.

Extracellular single-unit activity and freezing behavior were automatically recorded with a multichannel neurophysiological recording system (OmniPlex, Plexon, Dallas, TX). Wideband signals recorded on each channel were referenced to one of two ground wires, amplified (8,000x), digitized (40 kHz sampling rate), and saved on a PC for offline sorting and analysis. After high-pass filtering (600-6000 Hz), waveforms were sorted manually using 2D principal component analysis (Offline Sorter, Plexon). Only well-isolated units with a signal-to-noise ratio greater than 3 standard deviations were used in our analysis. We then imported sorted waveforms and their timestamps to NeuroExplorer (Nex Technologies, Madison, AL) for further analysis.

3.4.8 Histology

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 animals in the RE muscimol experiments were extracted and stored in 30% sucrose-formalin at 4 °C. Brains from animals in the 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 animals only implanted with RE cannula, brain sections were mounted on subbed slides and stained with thionin staining (0.25% thionin) to visualize cannula placements. For animals expressing viruses in the mPFC, the sections were mounted on subbed slides and coverslipped using fluoromount (Diagnostic Biosystems) to visualize viral expression.

3.4.9 Data analysis.

For the RE muscimol experiments, 16 out of 152 rats were excluded from the analysis because RE cannula were misplaced or the animals did not complete the experiment. This yielded the following group sizes: encoding experiment (MUS=11, SAL=14), retrieval experiment (COND= 6; NOVEL= 7) and state experiment (MUS-Ext= 20, SAL-Ext= 20, SAL-no Ext= 23). For the intersectional DREADD experiments, 6 of 40 rats had incomplete or unilateral mPFC expression of AAV-hM4Di. This yielded the following group sizes: encoding experiment (SAL= 19, CNO:15). For the terminal DREADD experiments, 28 out of 80 rats had incomplete or unilateral mPFC expression of AAV-hM4Di and/or had misplaced RE cannulae and were excluded from the analyses. This yielded the following group sizes: Blank-gfp= 17; PL+IL DREADD= 14; PL DREADD= 12; IL DREADD= 9. All freezing data represent freezing behavior during the interstimulus intervals (ISIs). Data were analyzed using analysis of variance (ANOVA), and post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall *F* ratio in the ANOVA. For some analyses, paired or unpaired *t*-tests were used. All data are represented as means ± s.e.m.

4. NUCLEUS REUNIENS MEDIATES THE EXTINCTION OF CONTEXTUAL FEAR CONDITIONING*

4.1 Introduction

Decades of research in both rats and humans indicate that the hippocampus (HPC) and medial prefrontal cortex (mPFC) play essential roles in the encoding and retrieval of episodic memories (Preston and Eichenbaum, 2013; Eichenbaum, 2017; Barry et al., 2019; Clewett et al., 2019). In recent years it has become apparent that reciprocal interactions between the HPC and mPFC are critical for these memory functions (Preston and Eichenbaum, 2013; Jin and Maren, 2015a). Anatomically, the HPC has robust projections to the medial prefrontal cortex (mPFC) that can support these functions, but there are no direct projections from the mPFC to the HPC (Vertes, 2004). Rather, recent work suggests that the mPFC projects to the HPC indirectly via the nucleus reuniens (RE), a ventral midline thalamic nucleus that synchronizes local field potentials in the HPC and mPFC and mediates forms of learning and memory that depend on HPC-mPFC interactions (Hembrook et al., 2012; Cholvin et al., 2013; Varela et al., 2014; Griffin, 2015; Ito et al., 2015; Jin and Maren, 2015a; Hallock et al., 2016; Zimmerman and Grace, 2016; Barker and Warburton, 2018; Maisson et al., 2018) .

Consistent with a role for RE in hippocampal-dependent learning, we have recently shown that pharmacological inactivation of the RE impairs the acquisition of contextual, but not auditory,

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Ramanathan KR, Maren S (2019) Nucleus reuniens mediates the extinction of contextual fear conditioning. Behav Brain Res 374:112114.

fear conditioning in rats (Ramanathan et al., 2018b). Moreover, inactivation of the RE during retrieval testing impairs the specificity of contextual memory and increases the generalization of fear to novel contexts (Xu and Südhof, 2013; Vetere et al., 2017; Ramanathan et al., 2018a, 2018b; Troyner et al., 2018; Silva et al., 2019). Interestingly, RE inactivation does not prevent the formation of context memory, but the memories formed under RE inactivation do not require the hippocampus (Ramanathan et al., 2018b). This suggests that non-hippocampal systems acquire context memory under RE inactivation and that these memories lack the precision of a memory normally encoded by the hippocampus.

The HPC plays a role not only in contextual conditioning, but also is critical for the context-dependent extinction of fear (Corcoran et al., 2005; Ji and Maren, 2005, 2007; Sierra-Mercado et al., 2011; Zelikowsky et al., 2012). For example, we have recently shown that projections from the HPC to the mPFC mediate the context-dependent renewal of fear that occurs when an extinguished CS is presented outside the extinction context (Orsini et al., 2011; Jin and Maren, 2015b; Wang et al., 2016; Marek et al., 2018). In contrast, either inactivating the RE or pharmacogenetically silencing mPFC projections in RE prevents both the encoding and retrieval of extinction memory while sparing renewal (Ramanathan et al., 2018a)). Together, this work suggests that the RE may be required for processing contextual information during extinction learning, information that is critical for generating context-appropriate defensive responses to a CS that has predicted both danger and safety.

Although our previous work implicates the RE in the contextual processes that support contextual fear conditioning on the one hand, as well as extinction of an auditory CS on the other, it is not clear whether the extinction of context fear memories *per se* requires the RE. To address this question, we employed contextual fear conditioning paradigm and temporarily inactivated RE

using muscimol (MUS; $GABA_A$ agonist) either prior to encoding or retrieval (or both) of contextual fear extinction. Consistent with our previous work (Ramanathan et al., 2018a), we found that inactivation of the RE impaired both the acquisition and expression of contextual fear extinction. Despite this impairment, animals extinguished after RE inactivation exhibited savings during a subsequent drug-free extinction session. Taken together, these results indicate that inactivation of RE is required for the inhibition of freezing behavior after the extinction of contextual fear.

4.2 Results

4.2.1 Histological analysis

A photomicrograph of a sample thionin stained section is shown in Figure 4.1a, and cannula placements for all the animals included in the study is shown in Figure 4.1b. Of the 64 animals that started the experiment, four animals either died or had a broken injector and were unable to complete the study. Of the remaining 60, animals that had cannula placements either rostrally or extending beyond the RE were excluded from the analysis. This yielded the following group sizes: SAL-SAL (N=12), SAL-MUS (N=11), MUS-SAL (N=9), MUS-MUS (N=9).

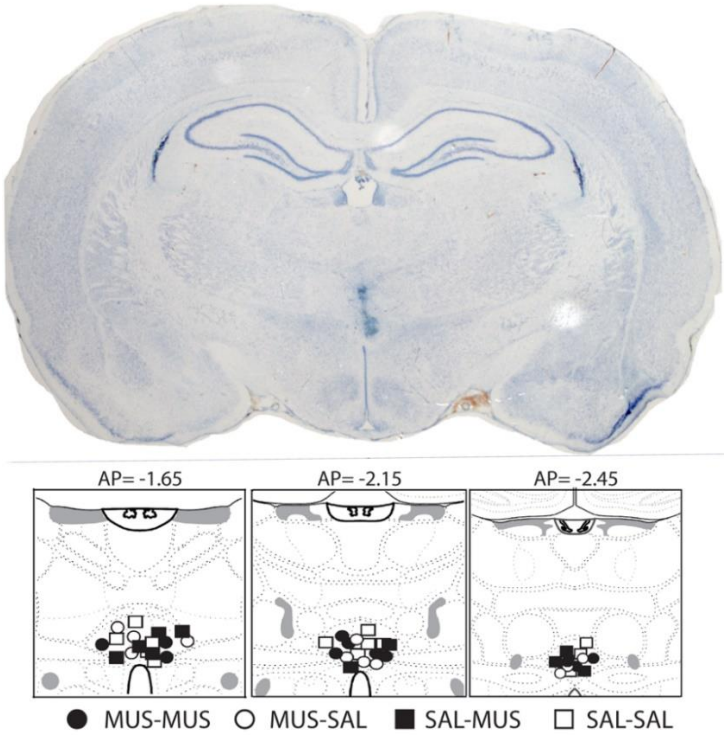

Figure 4.1 Histology, A, representative thionin-stained coronal section of midline thalamus showing cannula placement (arrow) in RE. B, Cannula placements of all the subjects included in the analysis. The cannula placements were similar across the groups.

4.2.2 Inactivation of RE impairs encoding and retrieval of fear extinction

To explore the contribution of the RE to the acquisition and expression of context extinction memories, we reversibly inactivated RE with intracranial infusions of MUS either before the extinction session, a subsequent retrieval test, or both. This yielded a 2×2 factorial design that enabled us to determine whether the effects of RE inactivation on extinction are state-dependent, as has previously been observed (Ramanathan et al., 2018b).

During fear conditioning (Figure 4.2a), animals exhibited low levels of freezing during the 3-min baseline and subsequently showed an increase in freezing across 5 conditioning trials. There were no differences between the levels of fear acquisition across groups indicating that all groups have acquired fear conditioning to a similar extent. These results were confirmed by a two-way repeated measures ANOVA which revealed a significant main effect of conditioning trial

[$F(5,195) = 41.58$, $p < 0.0001$], but no effects of either the prospective extinction [$F(1,37) = 0.08$, $p = 0.77$] or retrieval [$F(1,37) = 0.549$, $p = 0.46$] drug conditions or their interaction [$F(1,37) = 0.07$, $p = 0.78$].

Twenty-four hours after fear conditioning, animals received microinfusions of either SAL or MUS into the RE 10 min prior to a 35-min context extinction session. For clarity, we collapsed animals in the SAL-SAL and SAL-MUS groups ("SAL") and animals in the MUS-SAL and MUS-MUS groups ("MUS"), because the drug assignment for the subsequent extinction retrieval test did not interact with the extinction effects. As shown in Figure 4.2b , SAL-infused rats showed high levels of freezing early in the extinction session that decreased across the session. In contrast, rats receiving intra-RE MUS infusions exhibited impaired freezing early in the session and an increase in freezing across the extinction session. These observations were confirmed by a two-way repeated measures ANOVA which revealed a significant main effect of drug [$F(1,39) = 15.76$, $p = 0.0003$] and a significant drug*time interaction [$F(6,222) = 15.82$, $p < 0.0001$]. This indicates that inactivation of the RE impaired within-session (short-term) extinction memories.

Twenty-four hours after the extinction session, animals again received microinfusions of SAL or MUS to RE 10 min prior to the 35-min extinction retrieval session to assess the strength of their extinguished fear memories. As shown in Figure 4.2c, animals that received SAL during extinction and SAL during the retrieval test (SAL-SAL) exhibited the lowest levels of freezing during the retrieval test. In contrast, animals that received MUS during the extinction session and SAL during the retrieval test (MUS-SAL) exhibited an impairment in extinction retrieval early in the test but reduced their freezing across the retrieval test. RE inactivation prior to the retrieval test also impaired both extinction retrieval and extinction learning during the retrieval test (i.e., the

second extinction session). Rats that underwent extinction after SAL infusions and received MUS prior to the test session (SAL-MUS) exhibited an increase in freezing across the test. The impairments in extinction encoding and retrieval were not due to shifts in drug state in the SAL-MUS and MUS-SAL groups, because matching drug state across the two sessions (MUS-MUS) did not restore extinction performance. In fact, MUS-MUS animals exhibited the largest impairments in extinction retrieval. These observations were confirmed by a two-way repeated measures ANOVA which revealed significant main effects of drug condition during extinction [$F(1,37) = 6.65$, $p = 0.014$] and drug condition during the retrieval test [$F(1,37) = 17.05$, $p = 0.0002$] on conditioned freezing during the test session; there was no interaction between the drug conditions across the extinction and retrieval sessions [$F(1,37) = 0.067$, $p = 0.81$]. In other words, RE inactivation impaired the formation of long-term extinction memory and inhibited extinction retrieval and extinction learning during the retrieval session (i.e., the second extinction session).

The nature of the extinction impairment produced by RE inactivation is more clearly shown in the early part of the extinction retrieval session before new extinction learning had occurred. Figure 4.3 shows the average freezing in each of the four groups during the first 15-min of the retrieval test. In this graph it is clear that RE inactivation prior to extinction, extinction retrieval, or both increased freezing relative to SAL-SAL controls. This observation was confirmed by a two-way factorial ANOVA that revealed a main effect for drug condition during extinction [$F(1,37) = 6.15$, $p = 0.018$] and drug condition during retrieval [$F(1,37) = 4.99$, $p = 0.032$] and no interactions between these conditions [$F(1,37) = 2.01$, $p = 0.16$]. Planned comparisons revealed that freezing in SAL-SAL group was significantly lower than MUS-MUS ($p = 0.0018$), MUS-SAL ($p = 0.008$), SAL-MUS ($p = 0.001$), which did not differ from each other (all p's >0.47). Taken

together, these data reveal that temporary inactivation of RE impairs both encoding and retrieval of contextual fear extinction. Furthermore, these deficits were not state-dependent.

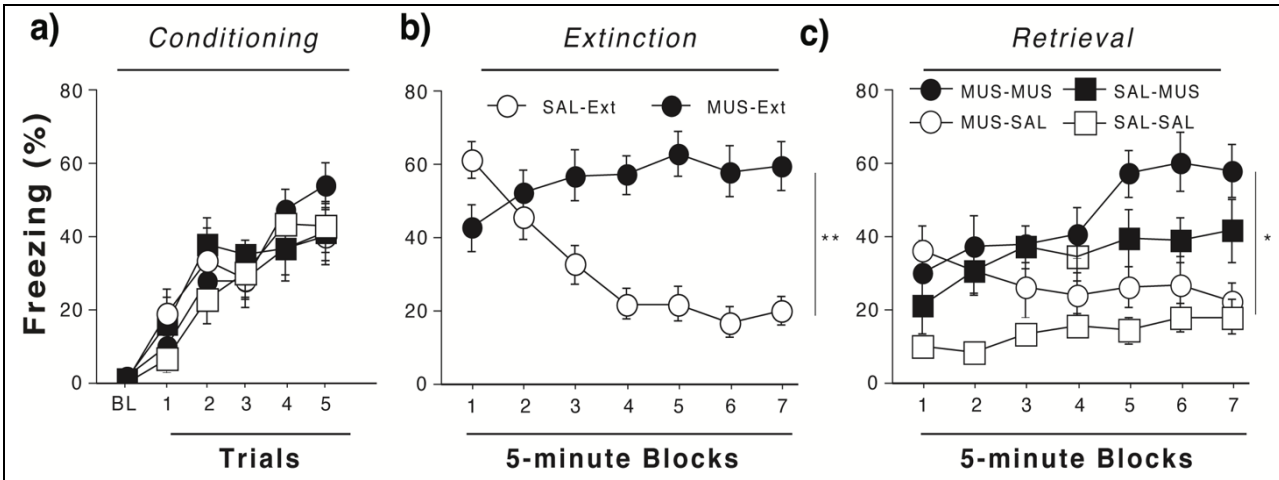

Figure 4.2 Inactivation of RE impairs encoding and expression of contextual fear extinction. *A*, Percentage of freezing during the 3-min baseline period prior to the onset of first conditioning trial (BL) followed by a 70-sec ISI after each conditioning trial. B, Percentage of freezing during the 35-min stimulus-free extinction session. Animals received infusions of SAL of MUS into the RE 10 min. prior to the extinction session. C, Percentage of freezing during the 35-min stimulus-free extinction retrieval session. Animals received infusions of SAL of MUS into the RE 10 min. prior to the retrieval session yielding a factorial design with four groups: SAL-SAL, SAL-MUS, MUS-SAL, and MUS-MUS. All data are means ± SEMs.

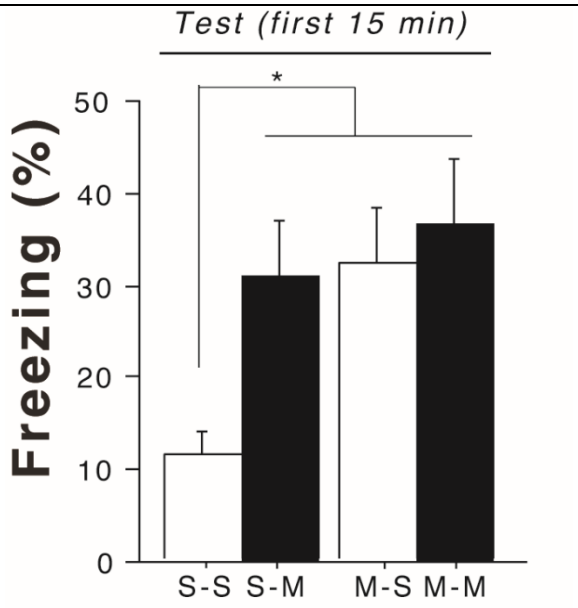

Figure 4.3 Inactivation of RE impairs both encoding and expression of contextual fear extinction. Average percentage of freezing during the first 15 min of extinction test. Animals received infusions of SAL or MUS into the RE 10 min. prior to both extinction and retrieval session yielding a 2×2 factorial design with four groups: SAL-SAL, SAL-MUS, MUS-SAL, and MUS-MUS. All data are means ± SEMs

4.2.3 Inactivation of RE during extinction does not result in savings of extinction

We determined whether animals extinguished under RE inactivation exhibited savings when extinguished once again during the retrieval test (the test constituted a second extinction session). To this end, we compared freezing during the retrieval test session (which constituted a second extinction session) for animals originally extinguished under saline (SAL-Ext 1) or muscimol (MUS-Ext 1) with freezing in the saline controls during the first extinction session [SAL-Naive]. As shown in Figure 4.4, animals extinguished under RE inactivation (MUS-Ext 1) exhibited a substantial extinction impairment relative to controls (SAL-Ext 1). Importantly, the rate of extinction in animals extinguished under MUS was similar to that of naïve rats (SAL-Ext 1) suggesting an absence of savings in animals extinguished under RE inactivation. These observations were confirmed by a two-way repeated measures ANOVA, which revealed a

significant main effect of group [$F(2,41) = 4.25$, $p = 0.021$] and group*time interaction [$F(12,246) = 9.15$, $p < 0.0001$]. This main effect of group was driven by differences between the SAL-Naive and SAL-Ext 1 groups (post-hoc comparisons $p = 0.006$). Furthermore, the group*time interaction reveals that the rate of extinction was different among the groups, and animals extinguished after intra-RE infusions (MUS-Ext 1) showed a slower rate of extinction than naïve (SAL-Naive) rats. To estimate the rate of extinction in animals undergoing extinction, we determined the block in which animals reached 50% of their initial freezing level during the second extinction session. There was no difference in the average time to half-max in the MUS-Ext 1 (11.33 ± 2.032 min.) and SAL-Naive (10.39 ± 1.13 min.) groups [$F(1,30) = 0.184$, $p = 0.67$]. This confirms that RE inactivation during the initial extinction session does not result in behavioral savings expressed during the second extinction session.

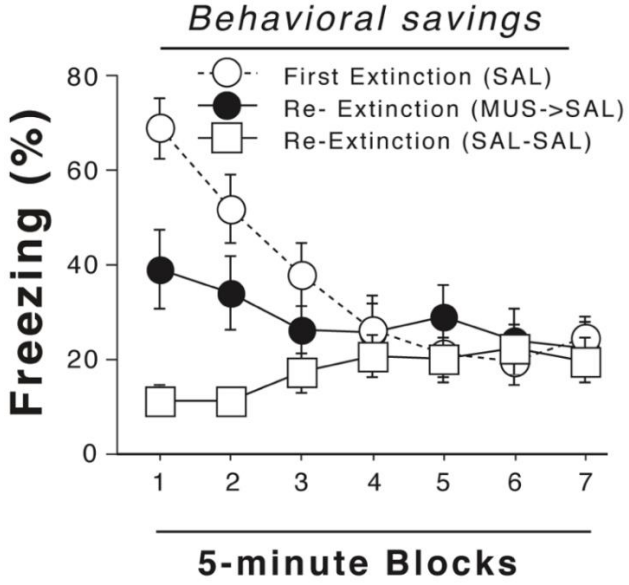

Figure 4.4 RE inactivation does not yield extinction savings. Between-session comparison of freezing during a second extinction session in animals that received initial extinction training under either saline [SAL-Ext 1] or muscimol [MUS-Ext 1]; freezing of naïve rats in their first extinction session under SAL [SAL-Naive] are shown to determine whether there was savings in rats extinguished under MUS. All data are means ± SEMs.

4.3 Methods

The present study examined the role of RE in acquisition and expression of contextual fear extinction. We show that temporary inactivation of RE impairs both acquisition and expression of contextual fear extinction. Together, the present results are consistent with recent reports indicating the role of RE in acquisition and expression of both fear conditioning and extinction (Vetere et al., 2017; Ramanathan et al., 2018a, 2018b; Troyner et al., 2018; Silva et al., 2019). Specifically, we have recently shown that inactivation of RE prevents both the acquisition and expression of extinction to an auditory CS (Ramanathan et al., 2018a). The effects of RE inactivation are not due to performance effects, such as a nonspecific increase in freezing, because neither expression of freezing to the CS (prior to extinction) nor renewal of fear to the extinguished CS were affected by RE inactivation (Ramanathan et al., 2018a). Furthermore, we have previously shown that MUS infusions are confined to the ventral midline nuclei, particularly RE and rhomboid nucleus, limiting the possibility that the spread of drug to adjacent thalamic nuclei accounts for these effects (Ramanathan et al., 2018a). We now show that RE inactivation also impairs acquisition and expression of contextual fear extinction, an effect that was not due to a state-dependent generalization deficit in extinction.

Interestingly, in the current report we observed that RE inactivation produced a decrement in contextual freezing early in the context extinction session, suggesting that RE inactivation impaired retrieval of the context memory formed during conditioning. This finding contrasts with a recent report from our laboratory in which we found no effect of RE inactivation on freezing in the conditioning context (Ramanathan et al., 2018b). The reason for this disparity is not clear, although animals in our previous report had prior experience with the infusion procedure before the context retrieval test, whereas the animals in the present experiment first experienced the

infusion procedure before this test. It is possible that the novelty of the infusion procedure and RE inactivation produce some impairments in memory retrieval that are insufficient alone to yield a deficit in contextual freezing, but together produce a retrieval deficit. Alternatively, there may have been differences in the distribution of cannula placements within the RE across the studies that yielded different effects on context freezing.

The involvement of the RE in the encoding and retrieval of context memories (Ramanathan et al., 2018b), as well as in fear extinction (Ramanathan et al., 2018a) reveals a broad role for RE in learning and memory processes that involve interactions between the HPC and mPFC. For example, inactivating either the infralimbic region of the mPFC (a homologue of human subgenual anterior cingulate cortex (Giustino and Maren, 2015), the ventral HPC (Zelikowsky et al., 2012), or lesions of the dorsal HPC (Ji and Maren, 2005), produce robust impairments in extinction learning with an auditory CS. Several studies show that both the HPC and mPFC are similarly involved in encoding contextual fear extinction (Ji and Maren, 2005; Fischer et al., 2007; Do-Monte et al., 2010; Radulovic and Tronson, 2010). In addition, the RE has a critical role in coordinating information flow between the mPFC and HPC (Vertes et al., 2007; Cassel et al., 2013). Hence, the effects of RE inactivation on extinction learning and retrieval may be mediated by a loss of coordinated neuronal activity in the HPC and mPFC. Consistent with this idea, inactivation of RE leads to impairments in tasks that require coordinated activity between mPFC and HPC (Hembrook et al., 2012; Cholvin et al., 2013; Layfield et al., 2015), such as spatial working memory (Preston and Eichenbaum, 2013; Jin and Maren, 2015a; Eichenbaum, 2017). Moreover, lesions or functional inactivation of RE causes deficits in spatial maze tasks (Hallock et al., 2016; Maisson et al., 2018), acquisition and specificity of contextual fear (Xu and Südhof, 2013; Vetere et al., 2017; Ramanathan et al., 2018a, 2018b) and context-dependent retrieval of

extinguished fear memories (Ramanathan et al., 2018a). Furthermore, recent literature from both anesthetized and freely behaving rodents shows that RE plays a critical role in mediating mPFC-HPC synchrony, which is critical for various memory related processes (Hallock et al., 2016; Ferraris et al., 2018; Ito et al., 2018). The present results supplant this literature by showing that RE is also critically involved in both acquisition and expression of contextual fear extinction.

The involvement of the RE extinction learning and retrieval suggests that it may have an important role in the inhibition of fear. When animals are presented with an extinguished CS or placed in a context that has undergone extinction, they actively suppress the original fear memory and consequently exhibit reduced freezing. Given the role for the RE in coordinating information flow between the HPC and mPFC (Griffin, 2015), the RE may function to suppress retrieval of fear memories in the extinction context thereby reducing freezing behavior. Support for this hypothesis comes work in humans that shows that mPFC coordinates the suppression of memory retrieval by the hippocampus (Anderson et al., 2016). That is, when subjects are asked to actively suppress retrieval of a memory, it results in increased BOLD activity in dorsolateral prefrontal cortex that correlates with decreased HPC activity. Because the dorsolateral prefrontal cortex is connected to the HPC through RE, it was concluded that the RE mediates this top-down inhibition—a phenomenon termed retrieval-induced suppression (Kesner and Churchwell, 2011; Benoit and Anderson, 2012; Anderson et al., 2016). This contrasts with the role for monosynaptic projections from the HPC to the mPFC, which are critical for the renewal of fear responses outside of the extinction context (Orsini et al., 2011; Jin and Maren, 2015b; Wang et al., 2016; Marek et al., 2018).

The role for the RE in regulation of Pavlovian fear extinction adds to a growing body of literature that the midline thalamic nuclei are critical hubs for regulating emotional learning and

memory. For instance, it has previously been shown that the paraventricular thalamic nucleus and its projections to the central nucleus of the amygdala are involved in the expression and consolidation of conditional fear (Padilla-Coreano et al., 2012; Li et al., 2014; Do-Monte et al., 2015b; Penzo et al., 2015; Do Monte et al., 2016). The mediodorsal nucleus of the thalamus also plays a role in both fear conditioning and extinction (Li et al., 2004; Lee et al., 2011; Mátyás et al., 2014; Paydar et al., 2014). Beyond the midline nuclei, several other thalamic nuclei including the zona incerta and reticular thalamic nuclei have been implicated in associative memory processes (Chou et al., 2018; Zhou et al., 2018; Dong et al., 2019; Venkataraman et al., 2019). Importantly, the current findings extend this and other work (Xu and Südhof, 2013; Vetere et al., 2017; Ramanathan et al., 2018a, 2018b; Troyner et al., 2018) and reveal a critical role for RE in mediating memory retrieval processes that underlie fear extinction.

4.4 Materials and Methods

4.4.1 Subjects

Sixty-four experimentally naïve adult male Long-Evans rats (Blue-Spruce; 200–224 g) were obtained from a commercial supplier (Envigo, Indianapolis, IN). The rats were individually housed in cages within a temperature- and humidity-controlled vivarium and kept on a 14:10 h light: dark cycle (lights on at 0700 hours) with *ad libitum* access to food and water. All experiments took place during the light phase of the cycle. Rats were handled for 1 minute a day for 5 days to habituate them to the experimenter before any surgical procedures or behaviors were carried out. All experiments were conducted at Texas A&M University with approval from its Animal Care and Use Committee.

4.4.2 Surgical procedure

One week before the behavioral testing, 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 RE using a cannula (8 mm, 26 gauge; Plastics One) above the RE. A single guide cannula was implanted at a 10° angle on the midline (A/P: -2.15 mm, M/L: -1.0~1.05 mm, D/V: -6.6- 6.8 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 during which the dummy cannulae were replaced twice.

4.4.3 Behavioral apparatus

Eight 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, 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. Additionally, ventilation fans and house lights were provided ambient noise and light, respectively, and the chambers were cleaned with 1% ammonium hydroxide as part of the context. 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 sec. Rats were transported to in white plastic boxes from the vivarium to the conditioning chambers.

4.4.4 Drug infusions

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 was 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 both extinction and extinction test sessions. Muscimol (MUS) was diluted in sterile saline (SAL) to a concentration of 0.1 μg/μl. Infusions were made at a rate of 0.1 μl/min for 3 min (0.3 μl total, 0.03 μg muscimol) and the injectors were left in place for 2-3 min for diffusion. After infusions, clean dummies were inserted into the guide cannula and the animals were transported to chambers for the behavioral sessions.

4.4.5 Behavioral procedure

This experiment was in two separate replications with equal representation of groups on each replication (n = 8). Sixty-four animals were randomly assigned to a 2X2 factorial design with variables of drug condition (SAL or MUS) during extinction and retrieval yielding sixteen subjects in each group. After recovery from surgery animals were subjected to contextual fear conditioning in context A where they were presented with 5 footshocks (US; 1mA, 2 sec) equally spaced with 70 second intervals (interstimulus intervals; ISI). Twenty-four hours later, rats received microinfusions of SAL or MUS into the RE and were placed in context A for 35 mins in a stimulus free session for extinguishing contextual fear memory. On the next day, rats again received

microinfusions of SAL or MUS into the RE and were placed in context A for 35 mins stimulus free session to test the strength of extinguished fear memory.

4.4.6 Histology

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 were then extracted and stored in 10% formalin for up to 24 h and then transferred to 30% sucrose-formalin solution at 4 °C for at least three days. Coronal brain sections of RE (40 μm) were made on a cryostat (-20 °C) and mounted on a subbed microscope slide. To check the cannula placements, the brain sections were stained with thionin staining (0.25% thionin) to visualize cannula placements.

4.4.7 Data analysis

All behavioral data (mean ± SEM) are represented by the average percentage of freezing behavior during 1 min intervals during the conditioning session and 5-min block during for the extinction and retrieval tests. All the data in Figure 4.2 & 4.4 were analyzed as two-way repeated measures ANOVAs with between-subject factors of drug condition during extinction and retrieval testing and a within-subject variable of time. For Figure 4.3, the data was analyzed as a two-way ANOVAs with between-subject factors of drug condition during extinction and retrieval testing. Post hoc comparisons in the form of Fisher's protected least significant difference tests were performed after a significant overall F-ratio in the ANOVA ($p < 0.05$ for both main effects and interactions). Statistical analyses were performed with StatView version 5.0.1 (SAS Institute) running under an open-source PowerPC.

5: NUCLEUS REUNIENS PROJECTIONS TO THE MEDIAL PREFRONTAL CORTEX AND HIPPOCAMPUS MEDIATE THE RETRIEVAL OF EXTINGUISHED FEAR MEMORIES

5.1 Introduction

Bidirectional prefrontal-hippocampal interactions are crucial for the encoding and retrieval of various memories and nucleus reuniens (RE), a ventral midline thalamic nucleus, is at the center this interaction (Preston and Eichenbaum, 2013; Jin and Maren, 2015a; Eichenbaum, 2017). It is widely thought that the hippocampus (HPC) interacts with medial prefrontal cortex (mPFC) through its monosynaptic projections. The mPFC can influence the HPC via the RE, which interconnects the two structures (Vertes et al., 2007; Griffin, 2015; Jin and Maren, 2015a; Dolleman-van der Weel et al., 2019). In addition to this relay function, recent evidence also suggests that RE can influence oscillatory synchrony in the HPC and mPFC. Prefrontal-hippocampal synchrony is essential for memory and cognition with RE seeming to mediate this communication through synchronizing slow, gamma and/or theta oscillations (Hallock et al., 2016; Roy et al., 2017; Ferraris et al., 2018; Dolleman-van der Weel et al., 2019; Hauer et al., 2019).

Consistent with this, electrophysiological studies have shown that RE has an excitatory influence in HPC and is thought to impose slow-wave oscillations on CA1 pyramidal cells (Dolleman-Van der Weel et al., 1997; Dolleman-Van der Weel and Witter, 2000). In anesthetized rats, RE lesions cause significant reductions in PFC-HPC coherence in the theta and beta bands. Consistent with this, Roy and colleagues found that RE inactivation resulted in decreased coherence in activity between mPFC and HPC at 2-5 Hz without affecting coupling at theta

frequency (Roy et al., 2017). In a more recent study, Hauer and colleagues have found that RE neurons track and maintain phase relationships with mPFC and HPC. After RE inactivation, this phase coherence was lost, which impaired memory consolidation (Hauer et al., 2019). In a spatial water maze task, Hallock and colleagues have shown that RE lesions reduce the oscillatory synchrony between mPFC-HPC in a working memory task, while having no effects on a task that does not require working memory (Hallock et al., 2016). Finally, in a T-maze alternation task, both mPFC and RE showed enhanced coordination with the CA1 subregion of HPC and phase locked at theta rhythm (Ito et al., 2015, 2018; Ito, 2018). Taken together, this evidence points to the crucial role of RE in coordinating cortico-hippocampal synchrony.

Synchronous neuronal activity between the mPFC and the HPC is directly implicated in fear and anxiety like behaviors. For example, neuronal synchrony at theta frequency range (4-10 Hz) is involved in anxiety related processes (Adhikari et al., 2010, 2011; Likhtik et al., 2014; Padilla-Coreano et al., 2016, 2019). Importantly, Adhikari and colleagues have shown enhanced HPC-PFC synchrony in anxiogenic environments, such as in the open arm in an elevated plus maze (Adhikari et al., 2010). Using optogenetic stimulation, Padilla-Coreano and colleagues have shown that oscillatory stimulation at 8 Hz but not 2,4 and 20 Hz enhanced synchrony in the HPC-PFC circuit (Padilla-Coreano et al., 2019). This enhanced synchrony was associated with increased time spent in open arms in an elevated plus maze. In a Pavlovian fear conditioning paradigm, Lesting and colleagues have shown enhanced theta coupling between mPFC and HPC during extinction learning (Lesting et al., 2011).

All of these reports support the idea that HPC synchronizes with the mPFC to actively inhibit defensive responses and engage exploratory behavior. Consistent with this, my previous work has demonstrated a critical role for RE in fear inhibition, which is central to fear extinction

(Ramanathan et al., 2018a; Ramanathan and Maren, 2019). Hence it is possible that RE coordinates this neuronal synchrony to actively inhibit fear after extinction in a safe context. Supporting this hypotheses, unpublished data from our lab show that retrieval deficit associated with RE inactivation resulted in blunted c-fos levels in both mPFC and HPC.

As a first step to explore this idea, we examined the contributions of RE projections to mPFC and HPC during recall of extinguished fear memories. However, silencing RE projections to mPFC might influence HPC and vice versa since 3-10% RE neurons send collateral projections to mPFC and HPC (Hoover and Vertes, 2012; Varela et al., 2014). To specifically target these projections, I employed a novel intersectional optogenetics strategy (INTERSECT), wherein the expression of opsins is conditional on two different recombinases (Fenno et al., 2014). Briefly, we infused viruses expressing retrograde Cre and retrograde Flp in mPFC and HPC respectively (counterbalanced). I then expressed inhibitory IC++ under the conditions of either Flp^{ON}/Cre^{ON} or Flp^{OFF}/Cre^{ON} or Flp^{ON}/Cre^{OFF} to selectively isolate collateral projections or individual projections to mPFC and HPC alone. For example, if I infused retrograde Cre in mPFC and retrograde Flp in HPC, infusing IC++ which is conditional on Flp^{ON}/Cre^{ON} would express IC++ proteins in collateral projections that project to both mPFC and HPC whereas infusing IC++ virus conditional on Flp^{OFF}/Cre^{ON} and Flp^{ON}/Cre^{OFF} would result in expression of IC++ opsin in RE neurons projecting to mPFC and HPC respectively. In this way, I could assess the individual contributions of these projections during extinction retrieval which may contribute to synchronizing mPFC-HPC activity.

5.2 Results

5.2.1 Histology

A sample photomicrograph of a Cre, Flp, and IC++ expression in mPFC, HPC and RE respectively is shown in Figure 5.1A. Of the 50 animals that started the experiment, 14 animals had either insufficient or misplaced viral expression or had fiber placements either rostrally or extending beyond the RE and were excluded from the analysis. This yielded the following group sizes: RE→mPFC (N=9), RE→HPC (N=9), mPFC← RE→HPC (Collateral) (N=7), controls (N=11).

5.2.2 Inactivation of RE projections to either medial prefrontal cortex or hippocampus (or both) impairs the retrieval of fear extinction

To specifically manipulate RE projections to mPFC and HPC (or both), we expressed Cre- or Flp-recombinase viruses in mPFC and HPC (counterbalanced) and inhibitory IC++ (or a blank control) in the RE. Four to five weeks after virus infusions, we implanted optic fibers in RE and shined blue light at 350nm into the RE to inactivate these projections. A schematic illustration of the behavioral paradigm is shown in Fig. 5.2A.

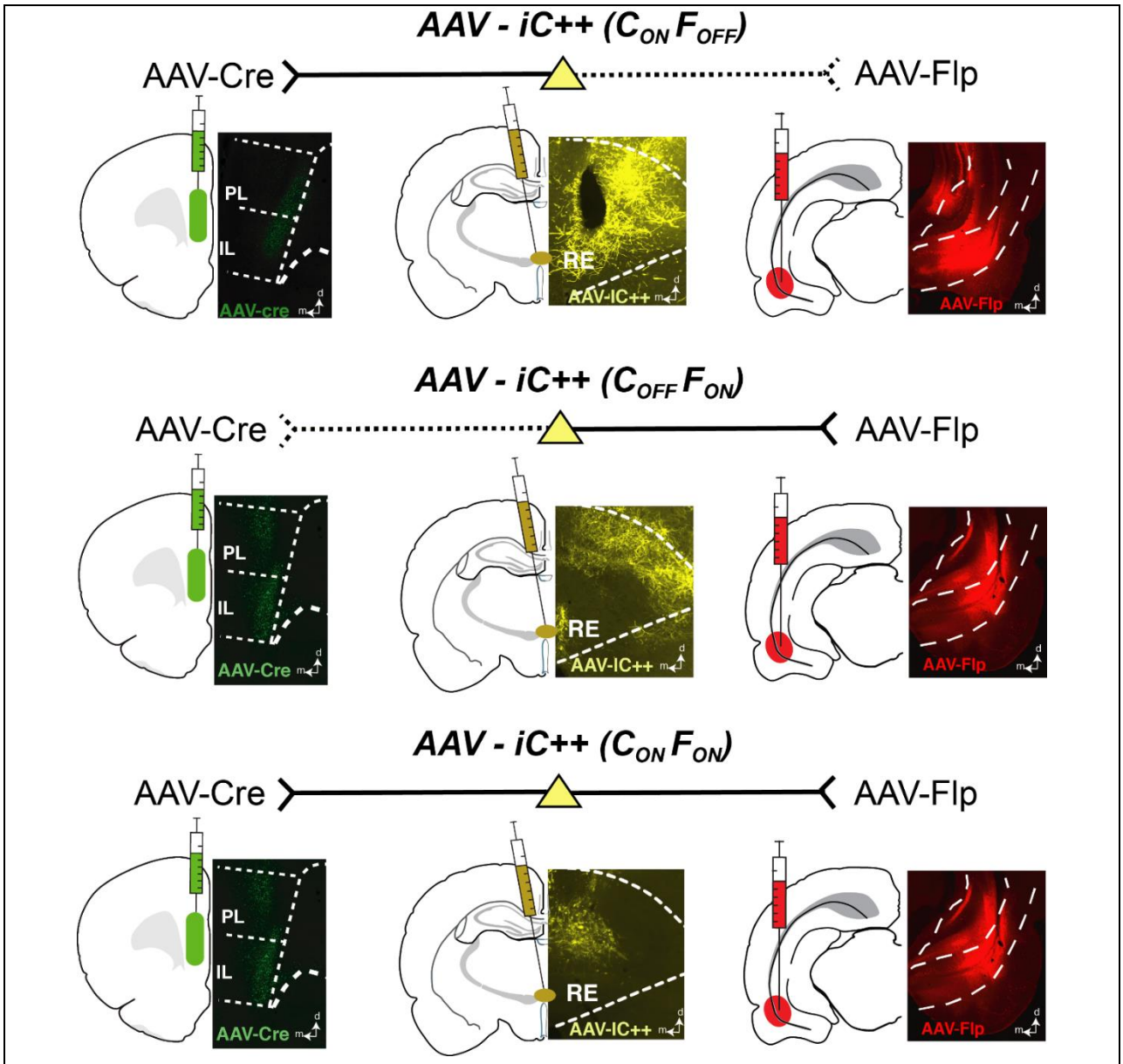

Figure 5.1 Histology: A representative stained image of IC++ virus in RE (middle panel) and representative retrograde Cre and Flp recombinases in mPFC (left panel) and HPC (right panel). Different kind of IC++ virus induced different patterns of yfp expressions in RE.

During fear conditioning (Figure 5.2 B, left panel), animals exhibited low levels of freezing during the 3-min baseline and subsequently showed an increase in freezing across 5 conditioning trials. There were no differences between the levels of fear acquisition across groups indicating

that all groups have acquired fear conditioning to a similar extent. These results were confirmed by a repeated measures ANOVA which revealed a significant main effect of conditioning trial [$F(5,160) = 64.276$, $p < 0.0001$], but no effect of group [$F(3,32) = 1.323$, $p = 0.28$]. On the subsequent days, rats were placed in context B and were presented with one or two sessions of extinction which comprised of 45 CS- alone trials. During this session (Figure 4.2B, middle), both groups of rats exhibited robust conditioned freezing to the CS in the earliest trial block and rapidly decreased across session. These results were confirmed by a repeated measures ANOVA which revealed a significant difference between first and final block of 5 CS trails [$F(3,32) = 159.51$, $p < 0.0001$] and no main effect of group [$F(3,32) = 0.273$, $p = 0.844$]. 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 (Figure 4.2A). That is, rats were tested for fear to the CS in the extinction context with or without light stimulation in RE in two counterbalanced tests in the extinction context, which were conducted over two days. As shown in the figure, light stimulation in RE increased conditioned freezing to an extinguished CS in animals expressing inhibitory IC++ virus but not the control animals which does not exhibit IC++. This observation was confirmed by a repeated measures ANOVA which revealed a main effect of trials [$F(3,32) = 7.67$, $p = 0.009$] and a trend towards significant trial*group interactions [$F(3,32) = 2.38$, $p = 0.087$]. Planned comparisons using Fisher's PLSD revealed a significant increase in freezing after optogenetic inhibition of RE in animals expressing active virus ($p < 0.001$), but not in the animals that did not have active IC++ opsin (p=0.40). Importantly, there was no main effect of either test order [$F(1,27) = 0.$, $p = 0.8$] or its interaction with the retrieval testing [$F(1,27) = 1.08$, $p = 0.31$] indicating that the effects seen above is not interfered by the order in which the animals underwent tests (light on vs lights off).

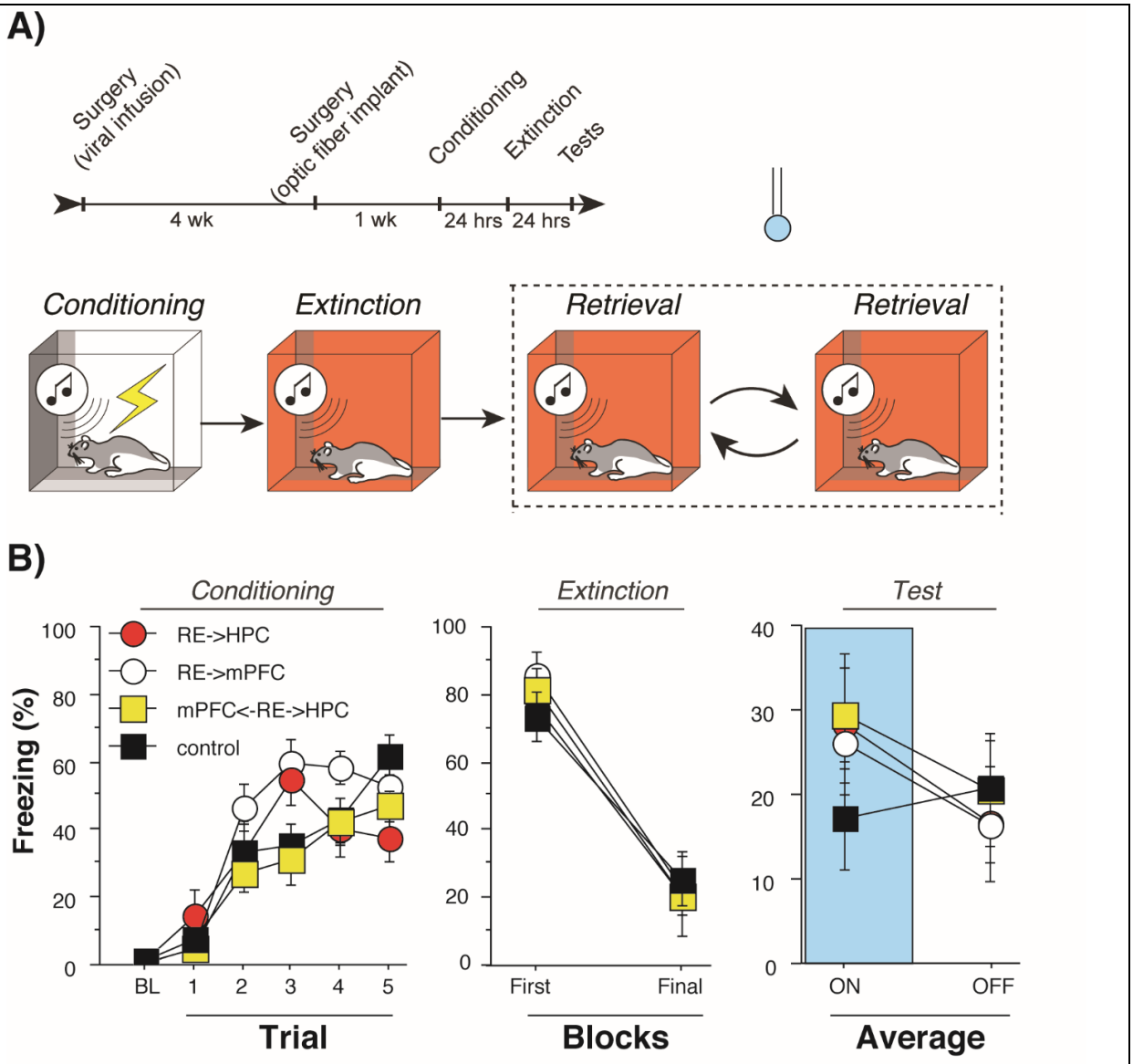

Figure 5.2 Silencing RE projections to mPFC or HPC or both impairs extinction retrieval. a Schematic behavioral design. **b** (Conditioning, left), Percentage of freezing during the 3-min baseline (BL) and 1-min interstimulus interval (ISI) following each CS-US pairing during the fear conditioning session. (Extinction, middle), Percentage of freezing during the first and final 5 CS trial blocks during extinction. (Test, right), Average freezing for 5 CS test trials in the extinction context with light inactivation of RE (ON) and no light (OFF) (renewal) context. All data are means ± s.e.m.s.

5.3 Discussion

In this current study, we show that inactivation of RE projections to either mPFC or HPC (or both) impairs extinction retrieval. Our results cannot be attributed to nonspecific light induced increase

or viral leak since 1) the animals infused with control YFP virus did not show retrieval deficits and 2) animals that did not receive one (or both) of the recombinase virus did not have any viral expression in RE. Our current results are consistent with previous reports from our lab showing RE is critical for encoding and retrieval of contextual and cued fear extinction (Ramanathan et al., 2018a; Ramanathan and Maren, 2019). Furthermore, our results are consistent with the current literature indicating the role of RE in aversive learning and memory processes (Davoodi et al., 2011; Xu and Südhof, 2013; Vetere et al., 2017; Ramanathan et al., 2018a, 2018b; Troyner et al., 2018; Ramanathan and Maren, 2019; Silva et al., 2019; Moscarello, 2020; Quet et al., 2020).

My current result showing inactivation of RE projections to both mPFC and HPC impairs retrieval is not surprising because RE was previously shown to have an excitatory influence on both of these brain regions (Dolleman-Van der Weel et al., 1997; Dolleman-van der Weel et al., 2017). Recently, Vetere and colleagues have shown that RE inactivation results in a global reduction of neural activity after fear conditioning. They indicated that RE could be part of a global hub which when inactive during learning will have deficits far and beyond mPFC or HPC (Vetere et al., 2017). Along the same lines, we have previously noticed a robust decrease in c-fos counts in both mPFC and ventral HPC after RE inactivation induced impaired extinction retrieval (unpublished).

The involvement of RE and its projections to mPFC and HPC in extinction retrieval suggests a broader role for RE in coordinating mPFC and HPC activity during learning and memory. Lesions or functional inactivation of either mPFC or HPC produces extinction deficits that are similar to the deficits observed with RE inactivation (Corcoran and Maren, 2001; Maren and Holt, 2004; Corcoran et al., 2005; Laurent and Westbrook, 2008, 2009; Santini et al., 2008; Giustino and Maren, 2015)(Ramanathan et al., 2018a; Ramanathan and Maren, 2019). Consistent

with our idea of RE coordinating activity between mPFC and HPC, various reports have shown that inactivation RE leads to impairments in tasks that require coordinated activity between these regions such as spatial working memory (Layfield et al., 2015; Barker and Warburton, 2018; Maisson et al., 2018).

Long range oscillatory synchrony is known to be involved in various behavioral and memory processes (Buzsáki, 2006; Buzsáki and Watson, 2012; Buzsáki et al., 2012; Harris and Gordon, 2015). Because synchrony between mPFC and HPC is implicated in episodic or spatial working memory processes (Jones and Wilson, 2005; Siapas et al., 2005; Sigurdsson et al., 2010; Hallock et al., 2016; Padilla-Coreano et al., 2019). This synchrony is either mediated by the monosynaptic HPC→mPFC projections or indirectly through RE (Hallock et al., 2016; Eichenbaum, 2017; Ferraris et al., 2018; Padilla-Coreano et al., 2019). Functionally, this HPC-PFC synchrony helps in organizing memories in the context in which they were experienced (Preston and Eichenbaum, 2013; Eichenbaum, 2017, 2018). Because fear extinction is a context-dependent memory (Maren and Quirk, 2004; Maren et al., 2013), it is no surprise that enhanced HPC-mPFC synchrony is associated with successful extinction (Lesting et al., 2013). Because we have previously shown that RE is involved in contextual fear inhibition, we can speculate that RE may coordinate and synchronize mPFC-HPC activity to actively inhibit fear after extinction.

Our results add to a growing body of literature suggesting that RE coordinates mPFC and HPC activity during learning and memory (Vertes et al., 2007; Cassel et al., 2013; Griffin, 2015; Mei et al., 2018; Dolleman-van der Weel et al., 2019; Jayachandran et al., 2019). Importantly, our results add to growing body of evidence indicating a nuanced role for RE in learning and memory processes beyond just mPFC-HPC relay (Prasad et al., 2013; Jankowski et al., 2014; Hallock et al., 2016; Vetere et al., 2017; Ferraris et al., 2018; Ramanathan et al., 2018b; Viena et al., 2018;

Hauer et al., 2019). Further studies are essential to isolate the contribution of RE in neuronal synchrony during fear inhibition.

5.4 Methods

5.4.1 Subjects

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 every day 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.

5.4.2 Viruses and drugs

rAAV-EF1a-Flp--mCherry (titer $\geq 7\times10^{12}$ vg/mL), AAV5-Cre-gfp (titer $\geq 4\times10^{12}$ vg/mL) and pAAV-hSyn-Con/Fon-EYFP (titer $\geq 1\times10^{13}$ vg/mL) was obtained from Addgene. CAV2-Cre (titer: 8.7×10^{12} pp/mL) was obtained from the Institute of Molecular Genetics of Montpellier. AAV8-hSyn-Con/Fon-IC++-YFP, AAV8-hSyn-Con/Foff-IC++-YFP and AAV8-hSyn-Coff/Fon-YFP (all titers $\geq 5\times10^{12}$ vg/mL) were obtained from Dr. Karl Deisseroth.

5.4.3 Surgery

For virus infusion surgery, 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. Rats were bilaterally infused with either retrograde Flp or retrograde Cre viruses into the mPFC (A/P: +2.7 mm, M/L: ±0.5- 0.5 mm, D/V: -4.4 mm for PL and D/V: -5.1 mm IL) or hippocampus (including dorsal and ventral) in a counterbalanced fashion. Furthermore, in the same surgery the inhibitory opsin virus

(IC++) was infused into the RE (A/P: -2.05 mm, M/L: +1.0 mm, D/V: -6.9 mm from dura) at a 10° angle.

For optic fiber implant surgeries, four weeks after viral infusions 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 RE using an optic fiber (10 mm, Thor labs) at a 10° angle on the midline (A/P: -2.15 mm, M/L: -1.0~1.05 mm, D/V: -6.6- 6.8 mm from dura; coordinates were measured from bregma). The fiber was affixed to the skull with dental cement,

5.4.4 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. 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 closed, and the chambers were cleaned with 1% ammonium hydroxide. Rats were transported to context A in white plastic boxes.

For the extinction and the subsequent test sessions in context B, a modified rodent conditioning chamber (30 x 24 x 21 cm) was used for optogenetic light stimulation. Optogenetic stimulation consisted of continuous 450 nm light (Dragon blue laser; 5-8mW power) delivered using MED-PC (Med Associates). For context B, red room lights were turned on, cabinet doors were open, and the chambers were cleaned with 1.5% Acetic acid solution. Rats were transported to context B in black transport boxes covered with beddings.

5.4.5 Behavioral procedures

Animals first underwent surgery for viral infusions into mPFC, RE and HPC. Five weeks after this surgery, animals underwent a second surgery to implant optic fibers targeting RE. Approximately 1 week after surgery, rats underwent fear conditioning, extinction, and retrieval testing. Briefly, 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 (ISIs) in context A. On the following days, rats underwent fear extinction in which they received a 3 min stimulus-free baseline (BL) followed by 45 tone-alone presentations (30 s ISIs). This extinction procedure was repeated until the freezing levels reduced to 20%. On the following two days, rats received a retrieval test in the extinction context to assess extinction retrieval. Each test consisted of a 3-min stimulus-free baseline period followed by and 5 CS presentations (30 s ISIs). Rats received laser stimulation of RE through the optic fibers on one of the days (counterbalanced) 10 sec before the onset of 1st tone and continued until the end of the retrieval session. This resulted in a within-subjects' procedure wherein each animal served as its own control.

5.4.6 Histology

Rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.8 ml) and were transcardially perfused with ice-cold saline and 10% formalin. Brains 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 (30 μm) were made on a cryostat (-20 °C). One set of the mPFC, RE and HPC sections was mounted on subbed slides and coverslipped using fluoromount (Diagnostic Biosystems) to visualize viral expression. A separate set of sections were collected in a 12 well plates and stored in 1X PBS with 0.01% sodium azide solution until immunohistochemistry (IHC) could be performed.

5.4.7 Immunohistochemistry

Immunohistochemistry was done in free floating RE brain sections to visualize the yfp reporter protein. The procedure performed was similar to our previous reports. Briefly, tissues were rinsed in 1X PBS followed by 1X PBST for 10 minutes each. The tissues were then blocked with 5% normal donkey serum (NDS) for 90 minutes. The tissues were washed with 1X PBS after which they were incubated overnight with the primary antibody (goat anti-gfp polyclonal 1:2000) in 1X PBST and 1% NDS. Next day, the tissues were further washed in PBS and were incubated in secondary antibody for 90 minutes. After incubation in secondary, they were washed in PBS and were stored at 4 °C until they were wet mounted in subbed slides to look at the fluorescence under the microscope.

5.4.8 Data analysis

Fifteen out of 50 rats were excluded from the analysis due to either poor viral expression or failure in targeting RE with the optic fiber. This yielded the following group sizes: RE→mPFC (N=9), RE→HPC (N=9), mPFC← ← RE→HPC (Collateral) (N=7), controls (N=11). All freezing data represent freezing behavior during the auditory tone and interstimulus intervals (Tone+ISIs). Data

were analyzed using analysis of variance (ANOVA), and post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall *F* ratio in the ANOVA. All data are represented as means ± s.e.m.

6 DISCUSSION

6.1 Summary

Overall, my doctoral work has demonstrated that RE is a crucial node in aversive fear memory circuitry that regulates the acquisition and expression of both Pavlovian fear conditioning and extinction. We examined RE because 1) RE has dense reciprocal connectivity with mPFC and HPC, 2) it directly affects mPFC-HPC interactions and synchrony in various behavioral paradigms, and 3) early indications suggest RE might play some role in aversive fear memories. RE likely influences this learning and memory process by giving a contextual control and enables the animals to not show fear in a context that was not associated with the aversive stimulus.

Specifically, in Chapter 2 we first wanted to explore whether RE is involved in Pavlovian fear conditioning. To this end, we implanted cannula targeting RE and pharmacologically inactivated RE before either acquisition or retrieval (or both) of Pavlovian fear conditioning using Muscimol. We show that pharmacological inactivation of RE before fear conditioning impairs the retrieval of the fear to the context in which they were conditioned. When the animals that had RE inactivated during conditioning was inactivated again during retrieval, they showed normal fear memory. When RE was inactivated before retrieval after normal fear conditioning, animals showed normal fear retrieval in the conditioned context. When RE was inactivated before placing the animals in a novel context, they generalized their fear in the novel context which was not associated with any aversive stimulus. This resulted in impaired discrimination across contexts. Inactivation of RE had no effects in either acquisition of retrieval of fear to the CS. These effects that we noticed were similar to HPC lesion/ impairment studies (Frankland et al., 1998; Sparks et al., 2011). Interestingly, the contextual freezing in rats conditioned under RE inactivation was insensitive to intrahippocampal infusions of the NMDA receptor antagonist

aminophosphonovalerate (AP-5). This shows that imprecise contextual memories acquired under RE inactivation are learned independently of the hippocampus. Collectively, these data reveal that the RE is required for encoding of precise contextual memories to support the discrimination of safe and dangerous contexts.

In Chapter 3, we explored whether RE is involved in the extinction of Pavlovian fear conditioning. We first inactivated RE before extinction and showed that RE inactivation resulted in deficits in encoding of fear extinction. In the subsequent drug free testing sessions, RE inactivated animals showed higher freezing in safe extinction context but not in the conditioning context (renewal). Next, when we inactivated RE during extinction retrieval, we show impaired retrieval in the extinction context but not in novel context (renewal). Consistent with these pharmacological studies, RE neurons showed enhanced firing activity to the CS in the extinction context compared to the conditioning context. Finally, using circuit specific manipulations, we show that this RE deficits indeed comes from mPFC as inactivation of this mPFC→RE pathway mirrored the results observed with the pharmacological inactivation. These results suggest that RE (and its input from mPFC) provides crucial information that is essential for inhibition of fear in the safe context which is essential for fear extinction.

Because the deficits we see in the previous experiments are predominantly context mediated, we then questioned the role of RE in contextual extinction. To this end, we inactivated RE during encoding or retrieval (or both) of contextual fear extinction. Similar to the results seen in previous chapter, we show that inactivation of RE impaired both encoding and retrieval of fear extinction. Furthermore, unlike the effects seen during conditioning the effects seen during extinction were not state-dependent.

In addition to RE mediating mPFC→HPC information flow, various reports suggest that RE can mediate mPFC-HPC synchrony (Preston and Eichenbaum, 2013; Hallock et al., 2016; Ferraris et al., 2018; Ito et al., 2018; Kafetzopoulos et al., 2018; Hauer et al., 2019). As a first step to explore this possibility, we used intersectional optogenetics strategy to isolate RE projections to mPFC, HPC and collateral projections to both mPFC and HPC to explore its contributions in extinction retrieval. Consistent with the previous results, inactivation of any of these pathways impaired freezing to the CS in the extinguished context.

6.2 Role for nucleus reuniens in mediating mPFC-HPC interactions

RE has come into focus due to the reciprocal connectivity between mPFC and HPC, thus acting as a relay between these structures. Bidirectional mPFC-HPC interactions are crucial for the encoding and retrieval of various memories and RE is at the center this interaction (Preston and Eichenbaum, 2013; Jin and Maren, 2015a; Eichenbaum, 2017). However, there still exists a void in our understanding of the complex role played by the RE in this mPFC-HPC communication. This is partly due to the fact that mPFC-HPC interactions play a role in executive level processing which are not clearly tapped by the existing rodent behaviors. Furthermore, it is harder to interpret the thinking process behind the performance of a rodent in any given behavioral task. Hence, there is a need to 1) understand the contribution of mPFC-HPC interactions in memory process and 2) incorporate results across behavioral paradigms to understand its complex role memories.

McClelland and colleagues have suggested that new memories are initially represented in the hippocampus and during consolidation they form a network which are linked to similar existing memories that are represented in the neocortex (McClelland et al., 1995). This overwrites or updates existing memory associations. Support for this proposal in rodent came from the study of Tse and colleagues. They trained rats to locate different flavored foods in different locations of an

open field. Once they acquired this initial associations, they showed that new associations could be learnt in as low as single trial. They further show that hippocampal lesions blocked the acquisition of this food-location learning. However, when the hippocampus was lesioned 24 hrs (or later) after this acquisition, they fail produce deficits in retention of this learnt food-location memories (Tse et al., 2007, 2011). Along these lines, Preston and Eichenbaum had suggested a dynamic prefrontal-hippocampal interplay in encoding and retrieval of memories (Preston and Eichenbaum, 2013). Once the hippocampus initially encodes a memory, it interacts with various cortical areas during the consolidation process to support the subsequent retrieval of information about "what" occurred based on "where" an event occurred or vice versa. This is part of the hippocampal processing (Eichenbaum, 2017). Once acquired, the retrieval of a memory based on a cue requires the prefrontal leading the hippocampus. Due to the lack of projection from mPFC to HPC, it is achieved through either perirhinal cortex or through nucleus reuniens. Depending on the kind of memory, it recruits either of these brain regions. For example, Jeyachandran and colleagues performed a series of experiments to look at the top down influence of mPFC on HPC in sequence memory retrieval. When they inactivated mPFC→RE pathway, the animals show deficits in working memory retrieval strategy whereas when they inactivated mPFC→ perirhinal cortex, animals show deficits in temporal context memory retrieval strategy. This pattern of results indicates differential recruitment of RE or perirhinal cortex in supporting mPFC-HPC interactions in memory retrieval. (Jayachandran et al., 2019) In relation to recruitment of RE, many other reports indicated deficits in spatial working memories (Griffin, 2015). Typically, spatial working memory tasks are designed in a way that a spatial cue signals the retrieval of a memory (food or object location) that which result in the subjects performing an action to access that food or object location. RE is at the center of this as lesions to RE produces deficits in spatial working memory

tasks. Furthermore, the deficits are specific to the trails that require the working memory component while sparring the performances in trials that does not require this working memory component (Hembrook and Mair, 2011; Hembrook et al., 2012; Cholvin et al., 2013; Griffin, 2015; Layfield et al., 2015; Hallock et al., 2016; Maisson et al., 2018).

Once a memory is formed, not only its retrieval but also any updates to this memory requires mPFC interactions with HPC. Recent theory indicates that mPFC guides HPC during the acquisition or retrieval of new information when incorporated into an existing knowledge (van Kesteren et al., 2010; Preston and Eichenbaum, 2013; Schlichting and Preston, 2014, 2015, 2016; Eichenbaum, 2017). As reported in the experiments done by Tse and colleagues, after the animal learns the food-location associations in an open field initially, when the already paired food is presented in new location, mPFC leads the activity of HPC (Tse et al., 2011). Furthermore, in an associative learning paradigm wherein a subject learns A→B, learning of new A→C association also require mPFC guiding the HPC. Importantly, this mPFC→ HPC interaction is recruited in choosing between the outcomes B and C based on the cues available to the animal. RE is also important in this process. In a T-maze task in which the rodents were asked to alternate between the choice of arms to access a food reward Ito and colleagues have showed that during the choice phase of the trial, mPFC leads the activity of RE which leads the activity of HPC (Ito et al., 2015; Ito, 2018).

Finally, after the initial associative A→B learning, withholding the response B or actively inhibiting the retrieval of the event B in presence of the cue A can involve mPFC interacting with HPC (Anderson et al., 2016; Hu et al., 2017). For example, when human subjects are asked to suppress a previously learnt action in response to a cue (go- no go paradigm) or asked to actively suppress the recollection of a associative memory in response to a cue (think- no think paradigm),

brain imaging data demonstrates an increased activity in right dlPFC and a concurrent suppression of activity in right HPC indicating that PFC is exerting its influence to suppress the activity of HPC when the subject is actively trying to suppress a memory retrieval (Kikuchi et al., 2010; Anderson et al., 2016; Gagnepain et al., 2017; Guise and Shapiro, 2017). Since mPFC is not monosynaptically connected with HPC, either RE or entorhinal cortex (EC) can mediate this prefrontal suppression of HPC (Anderson et al., 2016).

6.3 Placing the nucleus reuniens in fear memory circuitry

Both mPFC and HPC are highly implicated at various stages of the Pavlovain Fear conditioning and extinction process (Corcoran and Maren, 2001; Corcoran et al., 2005; Ji and Maren, 2007; Sierra-Mercado et al., 2011; Giustino and Maren, 2015). Hence, it is not surprising that RE , which interconnects the mPFC and HPC, is also implicated in this Pavlovian conditioning. Based on the results summarized above, we can say that during fear conditioning, RE mediates contextual inhibition on the fear memory which renders the fear memory specificity. When an animal acquires a fear memory, they not only acquire the CS-US relationship, they also acquire the spatial representation of the context associated with the memory. This spatial representation is encoded in the dorsal HPC (Frankland et al., 1998; Matus-Amat et al., 2004; Sparks et al., 2011; Chang and Liang, 2017). After this initial learning, animals can discriminate between safe vs unsafe contexts based on the spatial cues and can actively suppress their fear responses in the safe context which is not associated with the shock (Wiltgen et al., 2006; Jasnow et al., 2017; Asok et al., 2018). However, the input to HPC that mediates this spatial representation remained elusive (Sutherland et al., 2010). Data from the current thesis along with other reports now indicate that RE is indeed a critical region that enables the spatial representation of the context in the HPC which result in encoding the precise location in which the aversive event happened (Xu and Südhof, 2013;

Ramanathan et al., 2018b; Troyner et al., 2018; Quet et al., 2020). RE does so by turning on the HPC through its excitatory projections to CA1. After this initial acquisition, whenever an animal is placed in a context that is not associated with the shock, the animal relies on the prefrontal input to HPC to convey that all the spatial cues are not present and the animal should not be afraid in this safe context. A lack of RE stops this information flow and causes the animal to generalize the fear in a safe context (Xu and Südhof, 2013; Ramanathan et al., 2018b).

Similar inhibitory role for RE can be seen with fear extinction as well. After the animals are fear conditioned with CS-US associations, the animal learns to be afraid to the CS. During fear extinction when the animals are presented with CS alone trials after fear conditioning, they form an updated inhibitory CS-no US extinction memory. This actively competes with the conditioned fear memory (Herry et al., 2010; Furini et al., 2014). As mentioned in the previous section, this memory updating requires mPFC guiding HPC activity for successful learning of fear extinction. When RE is inactive, the mPFC cannot guide the HPC with this memory updating and the animal does not acquire the inhibit extinction memory. Once acquired this updated CS-no US association is then stored in mPFC. After successful fear extinction, any subsequent retrieval of the CS requires mPFC to interact with HPC enabling the CS-context association. Thus when CS is presented in an extinguished context the animal can show low fear. A lack of RE disturbs this CS-context safety association, thus leading to high fear which are similar to freezing levels when the animals are presented with CS in a non-extinguished context.

Alternatively, it is possible that RE might mediates its suppression of fear in safe contexts by retrieval suppression. After the initial fear conditioning, whenever the animal is placed in context that is devoid of all the spatial/contextual cues that is associated with the shock, they should actively suppress their fear responses that are associated with some of the elements that are present

in this safe context that may be associated with the shock. A lack of RE prevents the cortical suppression of HPC to suppress the contextual fear memories. Similarly, after fear extinction there is a competition between the safe CS- no US memory and unsafe CS-US memory. Context plays an important role in favoring one over the other (Maren et al., 2013). Without a functional RE, the animal cannot suppress the CS-US memory in a safe context thus exhibiting high fear in a safe context similar to an unsafe context while sparring the fear response in the unsafe context (since there's no behavioral suppression in a high fear state) (Ramanathan et al., 2018a).

How does RE complete the fear memory circuitry? The presence of RE would complete a closed loop HPC-mPFC circuit that bidirectionally regulates fear after fear extinction based on where the CS is retrieved. When the CS is presented in a non-extinction context, HPC suppresses the mPFC through the parvalbumin interneurons (Orsini et al., 2011; Jin and Maren, 2015b; Wang et al., 2016; Goode et al., 2018; Marek et al., 2018). However, when the same CS is presented in the extinction context, the mPFC engages RE to 1) suppress HPC's activity (Xu and Südhof, 2013; Ramanathan et al., 2018a). The HPC in turn can regulate its downstream targets such as amygdala and PAG (Maren and Hobin, 2007; Orsini et al., 2011; Xu et al., 2016) and 2) coordinating synchrony between mPFC and HPC which is previously shown to be correlated with fear extinction (Lesting et al., 2011, 2013).

6.4 Behavioral and Theoretical Considerations

In the last section I discussed about the neurocircuitry of fear inhibition and how RE fits into the circuitry. Here in this section, I will present some behavioral and theoretical perspective of how RE could regulate fear inhibition that underlie fear extinction. Before proceeding, I would like to present some fundamental basis of Pavlovain conditioning which was experimented for last century. When an CS is paired with an US, the physical presentation of CS activates a mental the

mental CS representation. This CS representation then activates the US representation associated with the physical presentation of the US. The activation of this US representation triggers the conditioned response (CR). Since the presentation of CS activates a sequence of associations that results in CR, this initial CS-US learning is called as excitatory learning (Rescorla and Wagner, n.d.). During the extinction session when the CS is played without the US, in early trials, the CS still activates the US representation. However, due to the lack of the US creates a new inhibitory US representation. These two competing associations produces no net effect and hence a gradual reduction in the CR's. In addition to this associations, the animals also learn about the relationship between the CS and the context. The associative value of the context gains significance and the context will regulate the dominance of excitatory or inhibitory US representations (Bouton, 2004; Bouton et al., 2006). There are a number of theories regarding the learning of extinction. Here in this section I briefly talk about those theories and how we can deduce whether if RE mediates fear inhibition through one of this process.

6.4.1 Conditioned inhibition

After the initial CS1-US pairing, when a neutral second stimulus (CS2) is presented in conjunction with the CS1 without the US, the animal will gradually reduce its CR. In this situation, CS2 will acquire the ability to acquire the ability to suppress or inhibit the CR elicited by CS1. That is, when CS1 is presented alone, the animals will exhibit high CR whereas when CS1 is presented along with CS2, the animals will actively suppress their CR. This phenomenon is termed as "conditioned inhibition" and CS2 becomes "conditioned inhibitor" (Rescorla, 1969). Bouton and colleagues argued that context by itself can be a cue and will gain some associative value. Hence, it is possible that the context can act as a conditioned inhibitor that when presented in conjunction with CS

signals safety, hence fear inhibition (Bouton, 2002). On the contrary when the animals are presented with CS in a different context, they show high CR.

Here in this thesis, I have shown that inactivation of RE impairs the acquisition of fear to the context, hence it is possible that RE might regulate this conditioned inhibition mediated by the context. We can test this hypothesis by a summation or a retardation test. When a CS acquires inhibitory value, it does not re-acquire a competing excitatory association as a neutral CS. That is, after successful extinction, if the animal is fear conditioned to the context in which it was extinguished, the animals would acquire this contextual fear to a lesser degree compared to a neutral context. We can test whether the RE mediates this context dependent conditioned inhibition by employing a 2X2 experimental design wherein the animals are animals are fear conditioned to a CS in context A and extinguished in B. After successful extinction, animals can be reconditioned in either context B or context C with RE activation (or SAL controls). If RE indeed regulates this conditioned inhibition, then inactivation of RE during re-conditioning should make the animals acquire the fear similar to SAL controls in context C and higher than the SAL controls in context B. Since RE plays a role in acquisition of fear to a neutral context, RE inactivation and conditioning in context C should impair the reacquisition similar to SAL controls in context B.

6.4.2 Retrieval Suppression

In the previous sections I have mentioned about the role of mPFC-HPC interactions in retrieval suppression. In a Pavlovian fear conditioning paradigm, after successful extinction when the animals are presented with the CS in a safe context, the animal has to suppress the excitatory US representation elicited by the CS in order to exhibit low fear (CR). The extinction results I observed in this set of experiments does give some indications regarding retrieval suppression. To directly assess that, we can design an experiment wherein we can condition we can condition the animals

to 2 different CS's in context A and extinguish each CS's in context B & C (counterbalanced). Subsequently we inactivate RE and test their fear to the CS's in the 2 contexts (counterbalanced). If RE mediates retrieval suppression, then the extinguished CS's across the contexts should show low fear whereas the MUS animals should show similar levels of high fear across the contexts.

6.4.3 Occasion setting

Occasion setter is a stimulus that modulates the ability of other stimulus to control the behavior. This stimulus can provide information about whether CS will be followed by US or not. In simple terms, the CS will provide information regarding when the US is incoming whereas the occasion setter provides information regarding whether the US is incoming (Fraser and Holland, 2019). For example, if we pair a CS1 (A) with a US (A+) whereas when CS2 is presented in CS1 (BA) which is not paired with a US (BA-) then the stimulus B will acquire the inhibitory properties and will prevent the expression of CR's. In simple terms this is similar to the conditioned inhibition. However, it differs in few critical ways: 1) in conditioned inhibition, the first learning is always excitatory and when the CS1 is presented in compound with CS2, the CS2 learns inhibitory properties. Whereas the theory behind occasion setting states that we can interchange the order and the animal can still learn this CS2 inhibitory properties. Furthermore, as the name suggests, in conditioned inhibition the CS2 is always inhibitory whereas in occasion setting the CS2 can be designed to set an occasion for CS1 be both excitatory and inhibitory. 2) Unlike conditioned inhibition, the cue that sets the occasion (occasion setter) is not susceptible to either the summation or retardation tests (Trask et al., 2017).

If the RE mediates the fear inhibition through the context acting as a negative occasion setter that gives an inhibitory meaning to the initially well-trained excitatory CS, we can test them by employing the same experiment suggested in the conditioned inhibitor section. Here, if RE is

indeed mediating through occasion setter, then one would expect the results that are opposing to the results that are expected in conditioned inhibitor. That is, in SAL controls reacquisition to the extinguished context should be similar to the reacquisition in novel contexts and MUS inactivation should impair this contextual acquisition.

6.5 Future directions

In this dissertation, we have shown that inactivation of RE and its efferent projections from mPFC or any of its projections to mPFC and HPC (and both) impairs inhibition of fear in the extinction context which is necessary for fear extinction. One question that we can ask is could we suppress the fear in the conditioning context. To explore this possibility, we can employ activity dependent tagging approach to tag the cells during the low fear extinction retrieval and activate those cells in the high fear renewal context to suppress the fear.

In Chapter 2 we show that RE acts as a rheostat to turn on or off the HPC during initial contextual fear acquisition. The mechanism that governs this is poorly understood. To better understand this, we can photo-tag the neurons in RE that project to HPC and record from those neurons during fear conditioning and retrieval. Alternatively, we can record from HPC during fear conditioning/retrieval after RE inactivation to better understand the results we saw in Chapter 2.

In Chapter 5, we have shown some preliminary evidence that RE can influence the mPFC-HPC neuronal synchrony. One obvious follow-up study is to record the local field potentials (LFP's) from mPFC and HPC during extinction retrieval after RE inactivation. This is an important experiment that can tell us the dynamics of mPFC-HPC synchrony and how RE can affect that. Another simple addition to this experiment would be to test them in renewal condition after RE inactivation. All the results we have seen with RE inactivation were specific to extinction context

but not in a renewal context. Hence, this experiment can help us understand the LFP's during high fear state and why RE inactivation does not affect renewal specifically.

To compliment findings from Chapter 5, we could infuse two different retrograde opsins in mPFC and HPC to photo-tag the RE neurons that project to mPFC/HPC/both and record from those neurons during extinction retrieval. This experiment will give us a clear idea of what kind of information these neurons encode.

Finally, the midline thalamic nuclei have a high presence of collateral projections incoming from cortical regions or collateral efferent projections rising from midline thalamus to various cortical and subcortical regions. We have some preliminary indications that mPFC (both PL and IL) send rich collateral projections to paraventricular thalamus (PVT) and RE. While RE is shown to be involved in fear inhibition, PVT is previously shown to be involved in high stress/ fear expression states (Fear excitation) (Do-Monte et al., 2015b; Penzo et al., 2015; Do Monte et al., 2016; Chen and Bi, 2018). Hence, it will be interesting to understand the contribution of these collateral projections in this dynamic control of high (renewal) and low fear (extinction retrieval) states. To this end, we can employ a dual retrograde tracing approach to isolate mPFC projections to PVT and RE (including collaterals) and see what percentage of these populations are activated during these high/low fear retrievals after fear extinction. Alternatively, we can employ intersectional optogenetics strategy (described in Chapter 5) and inhibit these projections to determine the behavioral consequences of these collateral projections.

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