SYNAPTIC AND ASSOCIATIVE MECHANISMS MEDIATING PAVLOVIAN CONDITIONING TO UNPREDICTABLE THREATS

A Dissertation

by

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ABSTRACT

Mental illness is one of society's most important and pressing challenges. In particular, trauma-related disorders represent some of the most common and debilitating forms of psychopathology that contribute to substantial societal and economic burden. In the laboratory, Pavlovian fear conditioning has been a powerful experimental model for understanding the associative and molecular underpinnings of fear-related behavior. Despite significant advances, our understanding of the circuits underlying conditioned fear is restricted largely to paradigms that have employed highly predictable experimental conditions, largely due to the fact that early theories of associative learning emphasized the importance of predictive relationships in the formation of associative relationships. However, intolerance to uncertainty is argued to be a common feature across fear and anxiety-related disorders and a better understanding of fundamental mechanisms underlying behavioral responses to unpredictable threats may help inform behavioral and brain techniques for intervention. In the current work, we employed an unpredictable backward (BW) conditioning procedure in rodents to examine both the synaptic and associative mechanisms underlying the acquisition and expression of fear to an unpredictable conditioned stimulus (CS). Specifically, we show that NMDA receptors within the BNST play a privileged role in the acquisition of fear to an unpredictable BW CS, whereas those in the CeA are required for the acquisition of fear to both a predictable and unpredictable CS. Next, we examined the associative structure underlying the expression of BW conditioning; we show that the expression of fear to an unpredictable BW CS is mediated by a contextual fear memory and recruits hippocampal (HPC) neurons to a greater degree than a predictable CS. Importantly, chemogenetic reactivation of a BW-tagged HPC ensemble was sufficient to support freezing behavior in neutral context. Lastly, from a translational

perspective an important finding was that presentation of the BW CS resulted in the reactivation of a HPC contextual fear memory that was sensitive to disruption by protein synthesis inhibition. Overall, these data provide important insight into the brain mechanisms underlying fear to unpredictable threats.

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Contributors

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1.0 INTRODUCTION

1.1 Pavlovian fear conditioning

In the laboratory, Pavlovian fear conditioning has been a cornerstone to translational research on neural circuits underlying emotional learning and memory (LeDoux, 2000; Maren, 2001; Maren and Quirk, 2004). In general, these studies are concerned with understanding the neurobiological and associative mechanisms underlying an animal's ability to learn associations between stimuli in the environment and biologically significant events. In a typical Pavlovian conditioning procedure (Pavlov, 1927) in rodents, for example, an initially neutral stimulus (termed conditioned stimulus, CS), is presented immediately prior to the delivery of an aversive event (termed unconditioned stimulus, US), such as a mild footshock. With one or more pairing between the CS and the US, the animal learns that the CS comes to *predict* the US and will demonstrate fear responses (termed conditioned responses, CR) to presentation of the CS alone. This form of conditioning (termed delay conditioning) is a highly robust form of learning that has been fundamental to our understanding of neurobehavioral processes underlying emotional learning and memory. Although the use of Pavlovian conditioning procedures employing a highly predictable CS has been fundamental to our understanding of the neurobiology underlying conditioned responding to explicit threats, much is less is known about the brain structures and associative mechanisms underlying fear to less predictable threats. Indeed, a key feature of Pavlovian conditioning is its sensitivity to the temporal arrangement of stimuli; it is appreciated that modifications to the temporal organization of the CS and US during fear conditioning shifts the neural circuits and associative mechanisms that are recruited during fear learning.

In the following sections I will first discuss our understanding of the neural circuitry and plasticity mechanisms underlying the acquisition of fear to predictable threat signals. I will then

introduce different variations of Pavlovian conditioning procedures in which the contingency between the CS and US is reduced and discuss how distinct neural circuits may underlie the acquisition and expression of conditioned fear under situations of "unpredictability". Importantly, the recruitment of these structures may provide insight regarding the associative mechanisms underlying the ability of an animal to express fear to an ambiguous threat cue. Accordingly, I will briefly introduce theoretical accounts of associative learning and how those have been used to explain the expression of fear to ambiguous threat signals.

1.2 The amygdala

The neural circuitry mediating standard Pavlovian conditioning consists of a distributed network of cortical and limbic structures (Maren and Quirk, 2004; Herry and Johansen, 2014; Tovote et al., 2015). At the core of this fear circuit is the amygdala, a brain region that has long been known to be critical to both the acquisition and expression of conditioned fear (Maren, 2005; Ressler and Maren, 2019). Anatomically, the amygdala is a node of highly interconnected nuclei that can be broadly be divided into two separate subsystems (LeDoux, 1995; Maren, 2001). The first subsystem, the basolateral complex (BLA), is comprised of the lateral (LA), basolateral (BA), and basomedial (BM) nuclei and is traditionally considered the sensory interface of the amygdala. The second subsystem, the central nucleus of the amygdala (CeA), can be divided into medial (CeM) and lateral (CeL) sectors and is thought to serve as the output station of the amygdala, as it directly interfaces with areas of the hypothalamus and brainstem which coordinate physiological and behavioral conditioned fear responses.

The BLA consists of primarily glutamatergic principal neurons (~80%) and inhibitory neurons (~20%) (McDonald, 1992; Spampanato et al., 2011). Although the cytoarchitecture of the

BLA is relatively consistent within subregions, it can be functionally dissociated based on afferent and efferent connectivity. For instance, the LA subdivision has been a major focus of fear conditioning studies as it has been hypothesized to be the site of convergence for pathways carrying sensory and nociceptive signals in the brain. Information regarding unimodal CSs, such as auditory tones, reaches the LA via direct projections from the auditory thalamus [e.g., medial geniculate nucleus (MGN)] and primary auditory cortex (McDonald, 1998). Interestingly, while it is appreciated that the LA receives nociceptive information, the neural pathways carrying this signal are not completely understood. For instance, neurons in the somatosensory thalamus [e.g., posterior intralaminar nucleus (PIN)] and areas of the brain stem [e.g., parabrachial nucleus and periaqueductal gray (PAG)] are activated by nociceptive signals (such as a footshock US) and affect fear learning (Johansen et al., 2010; Kim et al., 2013a; Han et al., 2015). However, the lack of a monosynaptic projection to the LA raises questions about how these regions might provide a short-latency signal that would be required to temporally match the CS signal. Nevertheless, there is electrophysiological evidence that these types of stimuli excite LA neurons at short latencies (Romanski et al., 1993) and the convergence of these signals is largely considered a mechanism by which stimuli are associated during learning (Romanski et al., 1993; Uwano et al., 1995; Maren, 2005). Indeed, CS and US information has been shown to converge on single LA neurons which is thought to be critical to the induction of associative plasticity (long-term potentiation, LTP) underlying increased efficacy of CS inputs onto LA neurons (Quirk et al., 1995; Maren, 1999; Blair et al., 2001). Consistent with this, studies have shown that fear conditioning occludes experimentally induced LTP within the LA (Tsvetkov et al., 2002) and other studies have shown that the strength of fear memories can be bidirectionally modulated by manipulating the synaptic strength of CS inputs to LA neurons (Nabavi et al., 2014). While these findings indicate that fear

conditioning is related to changes in synaptic strength at LA inputs, an important question is whether or not these changes are necessary for conditioned fear. To this end, it is appreciated that administration of pharmacological agents that impair plasticity within the LA also impair fear conditioning. For instance, the induction of LTP within the LA has been shown to depend on activation of NMDA receptors, and infusions of NMDA antagonists into the BLA prior to learning disrupts both the behavioral and physiological correlates of conditioned fear (Miserendino et al., 1990; Campeau et al., 1992; Fanselow et al., 1994; Maren et al., 1996; Lee et al., 2001; Rodrigues et al., 2001; Goosens and Maren, 2003). Similarly, administration of amnesic agents, which have shown to disrupt LTP, also result in impairments in fear learning (Schafe et al., 1999; Nader et al., 2000; Schafe and LeDoux, 2000; Maren et al., 2003). In total, these studies provide evidence that the LA is a critical site of plasticity underlying the acquisition and storage of explicit fear memories.

The CeA is comprised primarily of GABAergic inhibitory neurons and similar to the BLA can functionally dissociated based on anatomical connectivity. Although the CeM is thought to control the expression conditioned fear via descending projections, it does not receive direct projections from the BLA. Instead, one route by which CS-US information reaches the CeM is through the CeL (Pitkänen et al., 1997; Duvarci and Pare, 2014), which gates the expression of conditioned fear via tonic inhibitory tone over CeM output (Haubensak et al., 2010; Johansen et al., 2011). Thus, according to this view the CeA does not function to encode CS-US information, but rather acts as a passive relay station that mediates the *expression* of conditioned fear (LeDoux et al., 1988; Hitchcock and Davis, 1991; LeDoux, 2000; Maren, 2001).

Despite this predominant view, the CeL has many of the same characteristics that originally implicated the LA as a critical site for learning (e.g., afferent connectivity, plasticity

mechanisms)(Turner and Herkenham, 1991; McDonald, 1998; McDonald et al., 1999; Samson and Paré, 2005), suggesting that it may act in parallel to the BLA to encode aspects of CS-US associations during learning (Balleine and Killcross, 2006). Indeed, early electrophysiological studies revealed that the CeA may be an important locus of plasticity for fear conditioning (Applegate et al., 1982; Pascoe and Kapp, 1985) and more recent studies have confirmed that genetically distinct populations of neurons within the CeL undergo learning-dependent modifications (Ciocchi et al., 2010; Haubensak et al., 2010; Duvarci et al., 2011; Li et al., 2013; Penzo et al., 2014). Although one could argue that neurophysiological changes in the CeL reflect a passive readout of upstream plasticity occurring in the LA, other studies have shown that local administration of pharmacological agents into the CeA prior to fear conditioning results in impairments in long-term memory formation. For instance, administration of NMDA antagonists into the CeA impairs fear learning (Goosens and Maren, 2003; Wilensky et al., 2006), and other studies have shown that administration of protein synthesis inhibitors after learning results in similar deficits (Goosens and Maren, 2003; Wilensky et al., 2006; Shrestha et al., 2020). Moreover, a recent study demonstrated that learning-related plasticity within the LA depends on intact plasticity within the CeA (Yu et al., 2017). In general, these studies suggest that plasticity underlying fear learning may not be restricted to a region, but instead may be distributed throughout a distributed amygdala network (Herry and Johansen, 2014; Fadok et al., 2018; Ressler and Maren, 2019).

1.3 Hippocampus

Another limbic structure located in the medial temporal lobe that has been identified as a critical structure for fear conditioning is the hippocampus (HPC). Although both discretely cued and

contextual fear have shown to depend on the amygdala, the structures that process this information before coming into association with shock differ. For instance, whereas information regarding an auditory CS is thought to be relayed from primary sensory areas of the cortex/thalamus to the amygdala where it is in turn associated with the US, information concerning contextual cues has been shown to require the hippocampus (HPC) (Maren, 2001; Fanselow and Poulos, 2005; Maren et al., 2013). Initial evidence indicating a critical role for the HPC in this capacity came from lesion studies that reported deficits in both the acquisition and expression of contextual fear (Phillips and LeDoux, 1992; Fanselow et al., 1994). Importantly, deficits in contextual fear conditioning after HPC damage appear to reflect a deficit in forming and storing the contextual representation, rather than deficits in forming and storing the fear memory (i.e., context-US association) *per se*. Indeed, as previously mentioned contextual fear is impaired when amygdala function is disrupted (Helmstetter and Bellgowan, 1994; Maren et al., 1996; Muller et al., 1997), suggesting it may be the locus of plasticity for both CS-US and context-US associations (Gale et al., 2004). Moreover, studies have shown the HPC lesion-induced deficits in contextual fear can be eliminated if the animal is pre-exposed to the to-be-conditioned context prior to HPC damage (Young et al., 1994). In this case, conditioning is presumed to proceed normally because the contextual memory was acquired and consolidated elsewhere prior to damage. Indeed, infusions of NMDA-antagonists into the HPC prior to the pre-exposure period ameliorates this facilitative effect, suggesting that plasticity within the HPC (at least initially) may encode the contextual representation rather than a contextual fear memory (e.g., context-US) (Young et al., 1994). Similarly, pre-exposing rats to the conditioning context can eliminate deficits that are typically observed using immediate shock procedures (Fanselow, 1990). The immediate shock deficit is a procedure in which contextual fear is impaired if an animal is delivered a shock US soon after placement into the context (Fanselow,

1986). It is thought that this deficit arises from the inability of the animal to encode a configural representation of the context. In this case, the pre-exposure session presumably allows an animal to form a contextual representation, which is then available to be associated with the US in the amygdala when immediate shock procedures are administered.

Despite early studies demonstrating that pretraining lesions of the HPC resulted in robust deficits in contextual fear, more recent studies have demonstrated that animals can acquire contextual fear independent of the HPC (Maren et al., 1997; Frankland et al., 1998; Wiltgen et al., 2006; Gidyk et al., 2020). Based on these findings, it has been suggested that under normal circumstances animals use a hippocampal-dependent configural strategy in which individual elements of the context are assembled into a unified representation before coming into association with the US. Once acquired in this manner, contextual fear is sensitive to post-training HPC lesions. In contrast, when HPC lesions are made before training, animals' default to an HPC-independent strategy which is presumed to involve the animal associating individual elements of the context (e.g., grid floor, smell, etc.) with the US (Rudy and O'Reilly, 1999). A similar pattern of results has recently been reported after inactivation of the thalamic nucleus reuniens (RE), a critical component of the HPC memory system (Ramanathan et al., 2018). It is important to note that fear acquired using this elemental strategy often results in poor discrimination between safe and unsafe contexts (Frankland et al., 1998), which has led to the notion that the HPC may play a privileged role in integrating multiple elements of particular context into a unified representation which may ultimately result in a more precise memory. Despite this prevailing view, there is some evidence that animals can use a configural strategy in the absence of the HPC, which suggests that the brain may have multiple configural learning systems (Fanselow, 2010). Accordingly, it's

possible that the HPC is not necessary for configural learning *per se*, but rather is the most effective memory system for this learning strategy, which contributes to better memory precision.

1.4 Bed nucleus of the stria terminalis

Anatomically the BNST is part of the basal forebrain and constitutes a part of the extended amygdala. It is composed of a number of neurochemically distinct nuclei; how each of these nuclei uniquely contribute to behavior and physiology is relatively unknown, although advances in molecular tools enabling cell-and circuit-specific manipulations have begun to provide some insight (Jennings et al., 2013; Kim et al., 2013b; Dabrowska et al., 2016; Giardino et al., 2018; Xiao et al., 2020). With respect to Pavlovian conditioning, the majority of studies have focused on the anterior located nuclei as these areas are heavily connected with areas of the amygdala (e.g., CeA, BLA) and HPC that play critical roles in fear behavior (Sun et al., 1991; Sun and Cassell, 1993; McDonald et al., 1999; Dong et al., 2001b; Dong and Swanson, 2004). Importantly, the BNST also sends direct projections to areas of the brain such as the and PAG and hypothalamus (Holstege et al., 1985; Gray and Magnuson, 1992; Nagy and Paré, 2008), positioning it well to modulate both the behavioral and physiological expression of fear.

Within the last several decades there has been a significant amount of work conducted in an effort to understand the precise contributions of the BNST to Pavlovian fear conditioning. Importantly, there is now substantial evidence from preclinical rodent models as well as from clinical studies in humans indicating that the BNST may play a dissociable role from canonical amygdala circuits mediating the acquisition and expression of delay conditioning (discussed above). For example, in addition to acquiring fear to a discrete CS, fear conditioning can also occur in the absence of any discrete cue and in the case the environment or "context" serves as the CS.

It is appreciated the BNST lesions do not universally impair behavioral and physiological fear responses. Rather, several lines of evidence suggest a necessary for the BNST in the acquisition/expression of contextual but not discretely cued fear (LeDoux et al., 1988; Lee and Davis, 1997; Resstel et al., 2008; Sink et al., 2013; Goode et al., 2015). For instance, BNST lesions have been shown to disrupt freezing as well as corticosterone responses elicited by a contextual, but not auditory CS (Sullivan et al., 2004).These findings are consistent with a proposed role for the BNST in sustained fear insofar as contextual fear is thought to involve longer duration fear responses relative to those elicited by phasic CS conditioned using delay procedures (Walker and Davis, 2008; Walker et al., 2009). Despite this, contextual fear has been shown to persist independent of the BNST under some circumstances (Hammack et al., 2015; Goode et al., 2020), and others have shown that BNST lesions impair freezing responses to long duration (e.g. 10 minute) but not short duration (e.g. 1 min) auditory tones (Waddell et al., 2006).

1.5 Learning to fear unpredictable threats

Based on these findings our laboratory has argued that the BNST is not involved in the expression of contextual fear or sustained responding *per se*, but rather is engaged by stimuli (whether cues or exteroceptive/interoceptive contexts) that are poor predictors of when the aversive event will occur (Goode and Maren, 2017; Goode et al., 2019, 2020). Despite this appreciation, much of what we understand regarding the neural structures supporting plasticity underlying Pavlovian fear comes from studies of delay conditioning, which employ highly predictable experimental conditions. Indeed, the widespread use of delay conditioning procedures in research today is largely due to the fact that early accounts of associative learning hypothesized that in order for learning to occur this predictive relationship must exist (Kamin and L. J., 1969; Rescorla, 1972).

This was largely based on the observation that learning in other preparations, for example backward (BW) conditioning, was often found to be weaker than that found using delay procedures. In contrast to forward conditioning, during BW conditioning the CS *follows* the delivery of the US, and thus provides little to no information to the organism about when the aversive US will occur. Interestingly, while early studies of Pavlovian conditioning used these procedures as controls, it is now appreciated that animals can acquire fear to an "unpredictable" CS. Although these paradigms have been largely overlooked, they may provide valuable insight into the neural substrates underlying the encoding and expression of fear to less predictable or diffuse threats. Given that uncertainty is argued to be a key component contributing to anxiety, an understanding of these processes will help inform brain and behavioral treatments for anxiety disorders.

To demonstrate a selective role for the BNST in fear to temporally unpredictable threats previous work in our lab has examined its role in the expression of fear to a forward (FW)-trained (delay) or BW-trained CS. A primary advantage of using a FW versus BW paradigm is to generate CSs that differ in their temporal relationship to the shock, but are matched in other aspects (modality, intertrial interval, etc.). Accordingly, comparing the effects of BNST inactivation on the expression of fear to a FW and BW CS allows a direct comparison of a role for the BNST in the expression of fear to a temporally predictable and unpredictable CS, respectively. It is important to note that while extensively trained BW CSs have shown to become conditioned inhibitors (i.e., they reduce conditioned responding to other first-order excitatory cues) minimally trained BW CSs elicit excitatory responding that readily transfers across contexts (Ayres et al., 1987; Bevins and Ayres, 1992; Cole and Miller, 1999; Chang et al., 2003; Prével et al., 2016). Consistent with our hypothesis, we found that inactivation of the BNST resulted in dramatic

impairments in the expression of fear to the BW CS. In contrast, BNST inactivation had no effect on fear expression to the FW CS. Moreover, we also found that relative to a FW CS, retrieval of the BW CS resulted in increased expression of c-Fos within the BNST, which is consistent with other studies that have found elevated BNST activity in response to CS that has been partially reinforced (but not fully reinforced; in this case CS is rendered ambiguous because it only predicts the US on half of the conditioning trials) (Glover et al., 2020). To further confirm that this effect was specifically related to the temporal uncertainty of the BW CS we conducted an additional experiment in which rats were either trained using a delay or randomized procedure (e.g., USs occurred randomly with respect to the CS during conditioning). Again, although the BNST had no effect of the expression of fear to the FW CS, it impaired the expression of fear to the randomized CS. Consistent with this idea, Duvarci and colleagues (Duvarci et al., 2009) used a discriminative fear conditioning paradigm in which one CS signaled the US (CS^+) and another did not (CS^-). Interestingly, they found that a subpopulation of rats exhibited substantial fear to the CS^- which was impaired by BNST inactivation. In contrast, BNST inactivation had no effect on responding to the CS^+. Together these findings together support the notion that the BNST plays a selective role in fear conditioning to temporally unpredictable threats, a finding that is mirrored in the human literature (Alvarez et al., 2011; Naaz et al., 2019).

Although it is clear that backward conditioning recruits circuits distinct from those mediating forward conditioning, the content of learning after BW conditioning (or other procedures that degrade predictability) is poorly understood. For instance, during delay conditioning the convergence of CS and US information within the amygdala is thought to underlie the formation of a direct first-order CS-US association (LeDoux, 2000; Blair et al., 2001; Maren, 2005; Pape and Pare, 2010). This association is ultimately thought to support the expression of

fear such that when an animal is presented with a CS following conditioning, it activates a mental representation of the US, which in turn supports fear responding. However, as previously mentioned, theoretical accounts of learning (e.g., informational accounts) predict that the CS must have a predictive relationship to the US for this to occur (Kamin and L. J., 1969; Rescorla, 1972).

In addition to BW conditioning, trace conditioning is another procedure in which the contingency between the CS and US is reduced by inserting a temporal gap between the stimuli during conditioning. In contrast to delay conditioning, these types of procedures have shown to be particularly sensitive to HPC manipulations (McEchron et al., 1998; Quinn et al., 2002; Chowdhury et al., 2005; Bangasser et al., 2006; Wilmot et al., 2019). The exact contribution of the HPC is still not completely understood, however its involvement has led to the notion that contextual information may be an important contributor to the associative mechanism underlying the expression of fear to less predictable threat signals (Quinn et al., 2002). Indeed, learning theorists have now described several ways in which contextual cues can influence behavior (Holland and Bouton, 1999; Maren et al., 2013). In general, in addition to becoming directly associated with the US, contextual cues can also enter into higher order relations with an explicit CS that appear to be especially important for retrieving the meaning of a CS when it is rendered ambiguous. Consistent with this idea, it has been suggested that fear to a BW CS (and perhaps unpredictable CSs in general) is dependent on an association between the conditioning context and the US. The most straightforward evidence for this comes from a series of studies by Miller and colleagues which demonstrated that excitatory responding to a BW CS was impaired if the animal was exposed or "extinguished" in the conditioning context prior to retrieval (Chang et al., 2003, 2004). In this case, the contextual representation was argued to bridge an indirect association between the CS and the US. In other words, in contrast to the direct CS-US association made in

delay conditioning, in BW conditioning the CS does not become directly associated with the US. Rather, the BW CS serves as a reminder to the animal about the environment or "context" in which the aversive event occurred, which in turn is directly associated with the US. Accordingly, extinguishing fear to the context degrades the associative link between the BW CS and the US and reduces freezing behavior. In line with this idea, a recent study demonstrated that infusion of a protein synthesis inhibitor into the HPC after the retrieval of a trace CS resulted in impairments in contextual freezing (Runyan and Dash, 2005). Importantly, these impairments in contextual fear were evident despite the animal never being exposed or extinguished to the conditioning context itself following conditioning. Again, one possible explanation for these results is that the presentation of the trace CS resulted in an episodic-like recollection of the conditioning context, which in turn was disrupted by protein synthesis inhibition. Although this provides strong support for a role on context in trace conditioning, whether similar associative processes extend to other unpredictable threats, such as a BW CS, is still under investigation.

Although our understanding of the structures contributing to the acquisition and expression of conditioned fear has advanced considerably over the last couple decades, there are several outstanding questions. For example, although the BNST is appreciated to be an important structure for fear responding to unpredictable threats (Goode and Maren, 2017), many of the early studies involved pretraining lesions of the BNST. This makes it difficult to determine a role for the BNST in different stages of learning (e.g., acquisition, consolidation, expression). Moreover, the role for context in supporting BW conditioning (and trace conditioning) suggests a critical role for the HPC in the expression to freezing to CSs that are poor predictors of aversive outcomes. Accordingly, the current work aims to further our understanding of the synaptic and associative mechanisms underlying the acquisition and expression of fear to an unpredictable BW CS. Given

the established role for NMDA receptors within the amygdala in the encoding of predictable threat signals, along with proposed dissociations between the CeA and BNST in conditioned fear, we first sought to understand the contribution of NMDA receptors within the BNST in the acquisition of fear to a predictable (FW) or unpredictable (BW) CS. In addition, given the proposed role for context in supporting fear to unpredictable threat signals we combined BW conditioning procedures with intracranial pharmacology and sophisticated viral approaches to understand the contribution of the HPC to the expression of a BW CS. **Specifically, we hypothesized that while NMDA receptors within the BNST play a critical role in the *acquisition* of fear to a BW CS, the *expression* of fear to a BW CS would be mediated by a HPC-dependent contextual fear memory.**

2.0 NMDA RECEPTORS IN THE CEA AND BNST DIFFERENTIALLY REGULATE FEAR TO PREDICTABLE AND UNPREDICTABLE THREAT SIGNALS[*]

2.1 Introduction

Anticipating future threats is fundamental to survival—it allows animals to organize behavioral defense systems and prepare for future adversity. However, excessive worry and apprehension are core symptoms of a number of fear-related psychiatric disorders. Consequently, the brain circuits underlying fear and defensive behavior have received significant attention over the past several decades (LeDoux, 2000; Maren, 2001; Maren and Quirk, 2004; Craske et al., 2006; Johansen et al., 2011; Maren et al., 2013; Calhoon and Tye, 2015; Tovote et al., 2015; Lebow and Chen, 2016; Goode et al., 2018).

Pavlovian conditioning (Pavlov, 1927) is a powerful behavioral model for elucidating the neurobiological mechanisms underlying aversive learning and memory. In a typical experiment, rats learn to associate an innocuous conditioned stimulus (CS), such as an auditory tone, with an unavoidable and aversive unconditioned stimulus (US), such as a footshock. Fear conditioning studies have revealed that convergent sensory and nociceptive inputs within the amygdala activate N-methyl-D-aspartate (NMDA) receptors, which are critical for the induction of associative long-term potentiation (LTP) (LeDoux et al., 1990; Romanski et al., 1993; Campeau and Davis, 1995; Fanselow and LeDoux, 1999; Collins and Paré, 2000; Blair et al., 2001; Ressler and Maren, 2019). Although significant work has concentrated on NMDA-dependent plasticity within the basolateral

[*] Reprinted with permission from *Neurobiology of Learning and Memory*
Ressler, R. L., Goode, T. D., Evemy, C., & Maren, S. (2020). NMDA receptors in the CeA and BNST differentially regulate fear conditioning to predictable and unpredictable threats. *Neurobiology of Learning and Memory*, *174*, 107281.

nucleus of the amygdala (BLA), more recent work has demonstrated that NMDA receptor-dependent plasticity within the central nucleus of the amygdala (CeA) is also critical to fear learning (Samson and Paré, 2005; Goosens and Maren, 2003; Wilensky et al., 2006; Ciocchi et al., 2010; Duvarci et al., 2011; Li et al., 2013; Penzo et al., 2014, 2015). Together, these findings suggest that NMDA receptor-mediated plasticity within a distributed network of brain areas may contribute to the formation of CS-US associations during the acquisition of conditioned fear.

Although this work has been fundamental to our understanding of neural mechanisms by which the brain detects and responds to explicit threats, much less is known about how the brain encodes unpredictable threat signals that have been linked to anxiety-like behavioral states in both rodents and humans (Mineka and Hendersen, 1985; Foa et al., 1992; Grillon et al., 2004; Grupe and Nitschke, 2013; Davies and Craske, 2015). Preclinical and clinical work has shown that brain systems coordinating behavioral and physiological fear responses to predictable threats may be dissociable from those coordinating anxiety-like states evoked by uncertain or unpredictable prospective threats. Specifically, this work suggests that although the amygdala is critical for phasic fear responses to predictable threat cues, the bed nucleus of the stria terminalis (BNST) mediates sustained fear states evoked by uncertain threat (Walker and Davis, 2008; Walker et al., 2009; Davis et al., 2010; Alvarez et al., 2011). Although initial studies suggested a role for the BNST in contextual (but not cued) fear (LeDoux et al., 1988; Sullivan et al., 2004; Resstel et al., 2008; Poulos et al., 2010; Zimmerman and Maren, 2011; Hott et al., 2012, 2017; Sink et al., 2013b; Davis and Walker, 2014), more recent work suggests the role of the BNST in fear conditioning may be more nuanced than previously appreciated (Waddell et al., 2006; Hammack et al., 2015; Goode et al., 2019, 2020). In a recent study from our laboratory, we found that reversible inactivation of BNST impaired the expression of fear to discrete CSs that poorly signaled when

shock would occur [e.g., backward (BW) or temporally randomized]; in contrast, this manipulation had no effect on the expression of fear to forward (FW) CSs that reliably predict shock onset (Goode et al., 2019). These data suggest that the BNST may be involved in fear conditioning to temporally unpredictable threat signals (Goode and Maren, 2017).

Despite progress in our understanding of the circumstances in which the BNST is recruited to conditioned fear, very few studies have examined the molecular mechanisms that contribute to BNST-dependent aversive learning. Importantly, it is not known whether NMDA receptors within the BNST contribute to fear conditioning. To explore this question, we compared the effects of NMDA receptor antagonism in the BNST and CeA on the acquisition of FW and BW conditioning. These procedures differ in the degree to which the CS predicts when the US will occur, but equate CS modality, context exposure, the number of conditioning trials, and interstimulus intervals.

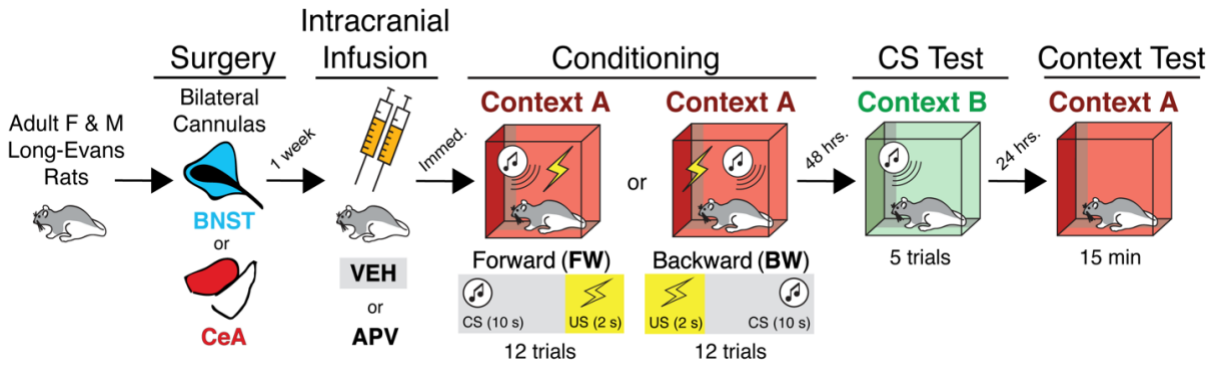

Figure 1. Experimental design (Ressler et al., 2020b). Adult male and female rats were surgically implanted with cannulas aimed at the BNST or CeA. After recovery, animals were infused with VEH or the NMDA receptor antagonist, APV, just prior to FW (CS-then-US; predictable) or BW (US-then-CS; unpredictable) auditory fear conditioning. Two days after conditioning animals were tested off-drug to the CS in the absence of the US. One day later, animals were placed back in the conditioning context (no CS or US) to assess contextual fear conditioning.

2.2 Results

2.2.1 BW, but not FW conditioning, requires NMDA receptors in the BNST

Immediately prior to fear conditioning, animals were infused with the NMDA antagonist APV into either the CeA (CeA-APV) or BNST (BNST-APV); saline (VEH) infusions served as the control. Rats were placed in context A where they underwent forward (FW) or backward (BW) fear conditioning procedures as described above. A summary of the behavioral design is shown in Figure 1 and representative cannula tracts and histological placements are shown in Figure 2.

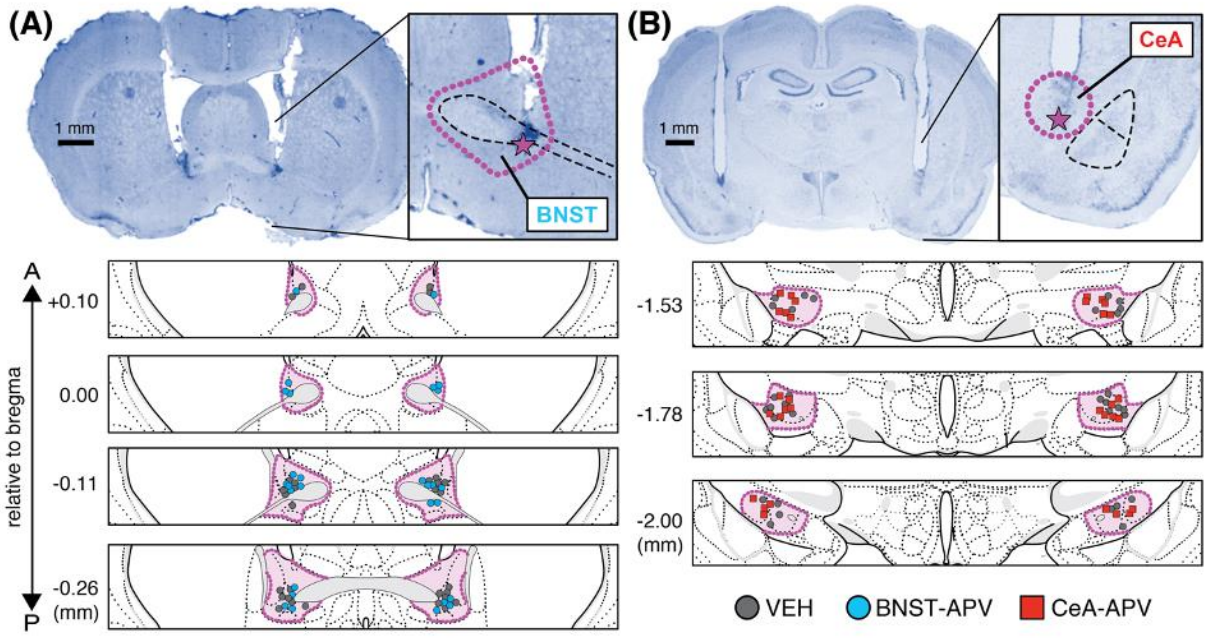

Figure 2. Cannula placements (Ressler et al., 2020b). Representative photomicrographs of thionin-stained coronal sections (40 μm) with bilateral cannula placements aimed at the BNST (A) or CeA (B). Purple dotted lines indicate the approximate borders of the BNST and CeA, respectively. Purple stars denote the location of the injector tip of the cannula tract in the representative tissue. Symbols correspond to injector tips of each animal included in the final analyses

As shown in Figure 3, freezing behavior significantly increased throughout the conditioning session as indicated by a main effect of trial [$F(4, 200) = 144.69$, $p < 0.0001$]. A significant trial ×

drug interaction was detected [$F(8, 200) = 4.06$, $p = 0.0002$]; post-hoc comparisons (Bonferroni's test) revealed that BNST-APV animals exhibited significantly more freezing than CeA-APV ($p = 0.0081$) or VEH animals ($p = 0.0086$) during the conditioning trials, independent of the FW or BW training. Additionally, the analysis revealed a main effect of sex [$F(1, 50) = 5.37$, $p = 0.02$], such that females exhibited higher levels of freezing overall; nonetheless, sex did not interact with any others in the analysis. No other main effects or interactions were detected in the ANOVA (F's < 2.58, p's > 0.06). Thus, both FW- and BW-conditioned rats exhibited robust freezing during conditioning, and this was not impaired by APV infusion into either the BNST or CeA, though freezing during conditioning was somewhat elevated in BNST-APV animals.

Forty-eight hours following conditioning, rats were placed into a novel context (B) and presented with five tone-alone presentations to assess retention of fear memory to the CS. As shown in Figure 3, intra-BNST APV selectively impaired freezing responses to the BW CS; it had no effect on freezing behavior to the FW CS. In contrast, intra-CeA APV resulted in robust impairments in freezing behavior to both the FW and BW CS. Analysis of freezing behavior across the entire session (including the baseline) revealed a main effect of trial [$F(5, 250) = 43.27$, $p < 0.0001$] (as freezing increased across the session) and a significant main effect of conditioning procedure [$F(1, 50) = 13.91$, $p = 0.0005$], such that freezing to the FW CS was higher overall than BW freezing. The ANOVA also revealed a significant main effect of drug treatment [$F(2, 50) = 17.50$, $p < 0.0001$] and a significant trial × conditioning procedure × drug interaction [$F(10, 250) = 2.40$, $p = 0.01$]. Post-hoc comparisons revealed that rats in the BNST-APV group that underwent BW conditioning showed significantly less freezing during the retention test than VEH-treated rats ($p = 0.001$). Conversely, rats in the BNST-APV group that underwent FW conditioning showed no difference relative to VEH-treated rats ($p = 0.85$). Note that these data suggest that the higher

levels of freezing observed in BNST-APV animals during conditioning did not translate into higher levels of conditioning fear at recall.

In contrast to these effects, FW and BW animals that received intra-CeA APV showed significantly less freezing than FW-VEH (p < 0.0001) and BW-VEH (p = 0.001) groups, respectively. CeA-APV rats also differed from BNST-APV animals in the FW condition (p = 0.0004), but not in the BW condition (p = 0.87). To examine whether the observed effects were specific to CS-evoked freezing, we ran a separate factorial ANOVA of the post-BL trials (Figure 3). This analysis revealed a main effect of conditioning procedure [$F(1, 50) = 28.93$, $p < 0.0001$]

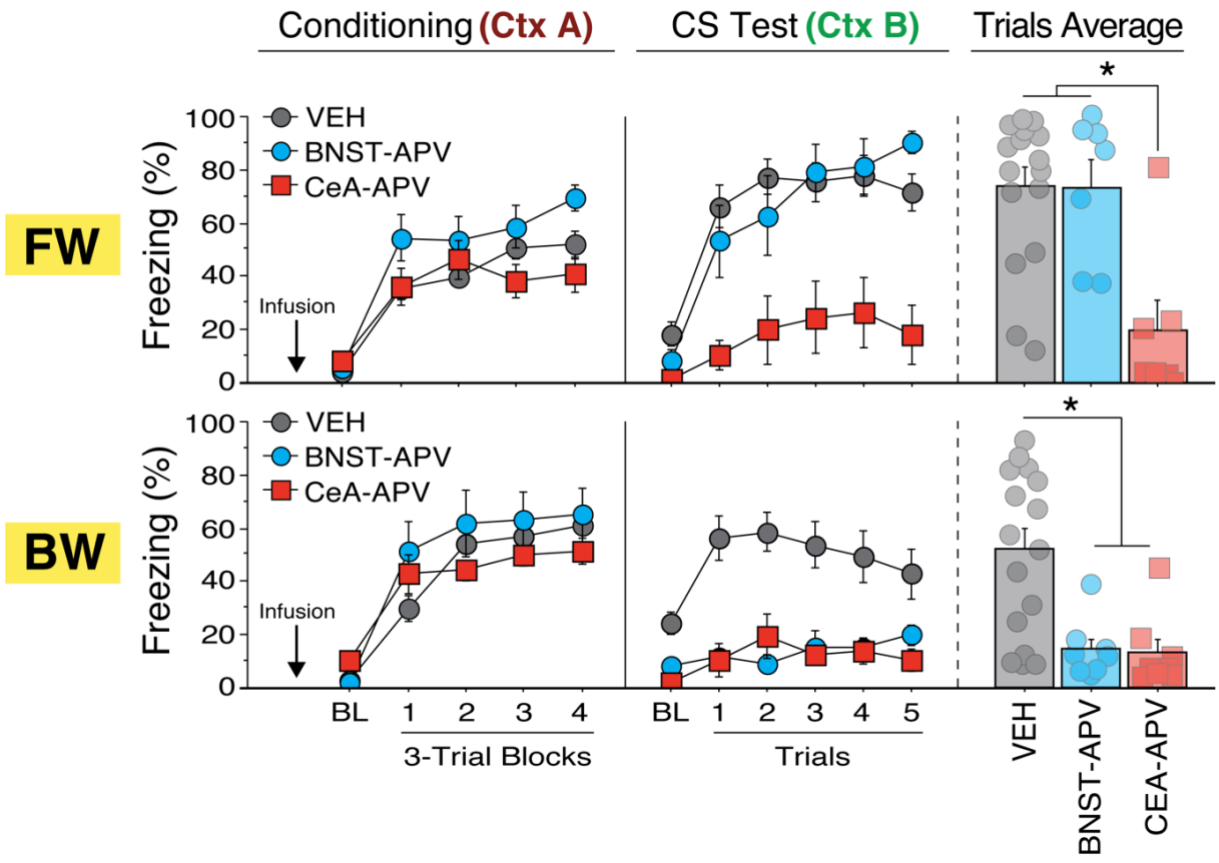

Figure 3. Intra-BNST or intra-CeA infusion of APV impairs auditory fear conditioning (Ressler et al., 2020b). Mean percentage freezing (±SEM) during FW (top panels) or BW (bottom panels) conditioning (each trial block consisting of the three tones and their intertrial intervals), retrieval to the CS alone, and mean CS responding during test trials 1-5 (consisting of each tone and its intertrial interval). * = p < 0.05.

and a main effect of drug [$F(2, 50) = 8.85$, $p = 0.0005$] and a significant conditioning procedure × drug interaction [$F(2, 50) = 5.97$, $p = 0.005$]. Post-hoc comparisons indicated that although BW conditioning was reduced by APV infusion into either the CeA ($p = 0.0006$) or BNST ($p = 0.001$), FW conditioning was only reduced by intra-CeA ($p < 0.0001$), but not intra-BNST ($p = 0.85$), APV. Lastly, there was a significant trial × sex interaction [$F(5, 250) = 2.88$, $p = 0.02$] with male rats exhibiting higher levels of freezing than females; sex did not interact with any other variables and there were no other main effects or interactions (F's < 1.54, p's > 0.22). Hence, the predictive relationship between the CS and the US regulates the role for BNST NMDA receptors in fear conditioning, whereas CeA NMDA receptors are involved in FW and BW fear conditioning. Moreover, APV-induced deficits on the retention of conditioned fear were not associated with a failure to express freezing during the conditioning session.

2.2.2 Acquisition of contextual fear requires NMDA receptors in both the CeA and BNST

Twenty-four hours after the CS retention test, rats were returned to the conditioning context (A) to examine the impact of NMDA receptor antagonism on freezing to contextual cues (Figure 4). Because we found no main effect of conditioning procedure (FW or BW) and no significant interactions between conditioning procedure and any other variable (sex or drug) in the analysis (F's < 1.37, p's > 0.25) we collapsed this factor for the analysis (Figure 4A). Freezing behavior was significantly reduced in rats that received intra-cranial infusion of APV relative to VEH, independent of brain region. Repeated measures ANOVA revealed a main effect of drug [$F(2, 56) = 11.88$, $p < 0.0001$], a main effect of time [$F(14, 784) = 9.27$, $p < 0.0001$], and a time × group interaction [$F(28, 784) = 2.93$, $p < 0.0001$]. No other main effects or interactions were detected

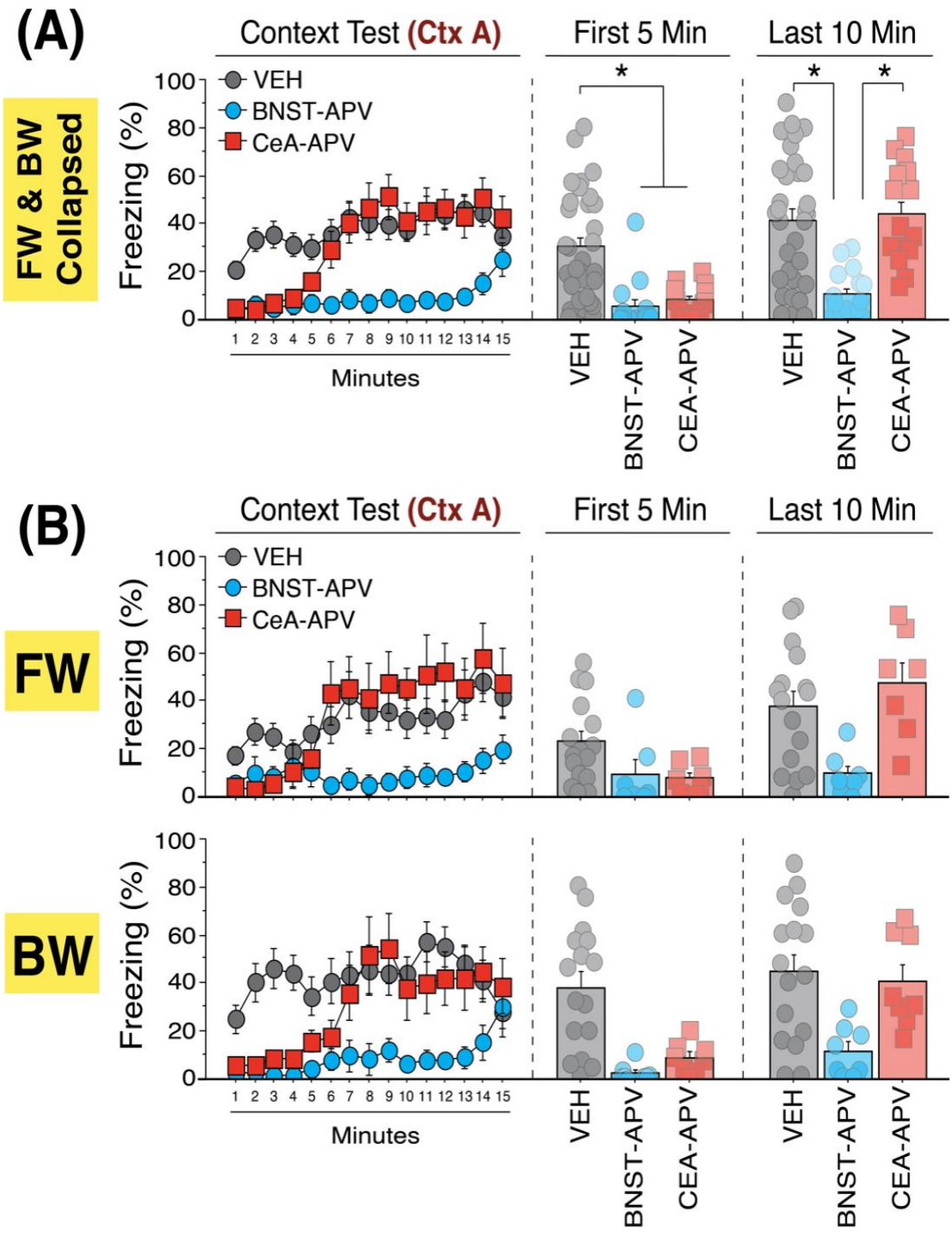

Figure 4. Intra-BNST or intra-CeA infusion of APV impairs contextual fear conditioning (Ressler et al., 2020b). (A) Mean percentage freezing (±SEM) during each minute of retrieval to the conditioning context, collapsed across FW and BW training. Mean percentage freezing of minutes 1-5 (First 5 Min) and minutes 6-15 (Last 10 Min) are shown in the bar graphs. (B) Mean percentage freezing during each minute of retrieval to the conditioning context, split by animals trained under FW or BW conditions. * = $p < 0.05$.

(F's < 0.87, p's > 0.59). Post-hoc comparisons across the entire context test revealed that pre-conditioning APV infusions in the BNST significantly reduced contextual freezing relative to both VEH ($p < 0.0001$) and CeA-APV ($p < 0.0001$) rats. Conversely, CeA-APV animals did not differ across the entire session relative to VEH animals ($p = 0.02$). Interestingly, rats in the CeA group exhibited impairments in early, but not late, periods of the context test. To examine this further, we collapsed the session into an early period (i.e., the first 5 mins) and the remaining duration (i.e., the last 10 minutes) (Figure 4). Repeated measures ANOVA of freezing during these periods revealed a main effect of time [$F(1, 56) = 40.07$, $p < 0.0001$], main effect of drug [$F(2, 56) = 12.49$, $p < 0.0001$] , and a drug × time interaction [$F(2, 56) = 10.66$, $p < 0.0001$]. No other main effects or interactions were observed (F's < 0.78, p's > 0.46). Post hoc comparisons showed that both BNST-APV ($p < 0.0001$) and CeA-APV ($p = 0.0002$) animals exhibited significantly less freezing than VEH-treated rats during the early time period. Conversely, freezing deficits were only observed in the BNST-APV group at the later time points relative to VEH ($p < 0.0001$) and CeA-APV ($p = 0.0001$). This pattern of deficits was similar when the FW and BW experiments are considered independently (Figure 4B). In total, these data suggest that NMDA receptors in the BNST and CeA make critical (but distinct) contributions to the acquisition of contextual fear (Figure 5).

2.3 Discussion

Here we demonstrate a dissociable role for the BNST in the acquisition of conditioned freezing to unpredictable and predictable threat stimuli. Specifically, NMDA receptor antagonism in the BNST prior to fear conditioning significantly reduced freezing to an unpredictable BW CS, but not a predictable FW CS. NMDA receptor antagonism in the BNST also broadly reduced

contextual freezing. Conversely, APV administration into the CeA prior to conditioning disrupted conditioned freezing to both the FW and BW CS. Although contextual freezing was also disrupted by intra-CeA APV, the impairment was restricted to early portion of the test session, whereas intra-BNST APV impaired freezing during throughout the test. Altogether these data reveal dissociable roles for NMDA receptors in the CeA and BNST in the acquisition of conditioned fear to predictable and unpredictable threats (Figure 5).

Anatomically the BNST is well positioned to integrate information from the amygdala (Krettek and Price, 1978; Sun et al., 1991; Dong et al., 2001), hippocampus (Cullinan et al., 1993), and prefrontal cortex (PFC) (McDonald et al., 1999; Hoover and Vertes, 2007), structures that provide contextual and nociceptive information that may be critical to learning-related plasticity in this region. Moreover, efferent projections to the hypothalamus and periaqueductal gray (Holstege et al., 1985; Gray and Magnuson, 1992; Nagy and Paré, 2008) position the BNST to potentially elicit or modulate defensive responses in the presence of threat. Indeed, substantial work in both humans and rodents has implicated the BNST in conditioned fear (Sullivan et al., 2004; Grillon and Morgan, 1999; Duvarci et al., 2009; Somerville et al., 2010; Alvarez et al., 2011; Zimmerman and Maren, 2011; Hott et al., 2012, 2017; Davis and Walker, 2014; Goode et al., 2015; Hammack et al., 2015; Herrmann et al., 2016; Marcinkiewcz et al., 2016; Asok et al., 2018; Luyck et al., 2018, 2020; Bjorni et al., 2020; Williams and Lattal, 2020).

In line with these data, a recent report from our lab demonstrated a role for the BNST in the expression of fear to unpredictable – but not predictable – threat signals (Goode et al., 2019). Specifically, this study demonstrated that the expression of fear to a BW (but not FW) CS is attenuated by muscimol infusions into the BNST; similar results were obtained if the CS was trained with randomized onset of the US. Based on these findings, we and others have argued that

the BNST is involved in the expression of conditioned fear to threat signals that poorly predict US onset (Goode and Maren, 2017; Luyck et al., 2019; Miles and Maren, 2019). The current results extend these findings and show that NMDA receptors in the BNST are necessary for both backward fear conditioning to a discrete CS, as well contextual conditioning. These findings are also supported by recent work that observed deficits in contextual fear learning (as well in its reconditioning) after pharmacological inactivation of the BNST (Williams and Lattal, 2020) (Williams et al., 2019). Given that other studies have shown that BNST neurons exhibit experience- and NMDA-receptor-dependent plasticity (Vyas et al., 2003; Dumont et al., 2005; Kash et al., 2008a, 2008b, 2009; McElligott et al., 2010; Conrad et al., 2011; Wills et al., 2012; Haufler et al., 2013; Daldrup et al., 2016; Glangetas et al., 2017; Bjorni et al., 2020; Salimando et al., 2020), our data suggest that NMDA receptor-dependent plasticity in the BNST is critical to encoding CSs that poorly predict US onset. In line with this, a recent paper found that the spontaneous activity of BNST neurons is maximal during the period immediately after delivery of an aversive footshock during early conditioning trials, when the footshock is unexpected (Bjorni et al., 2020). Interestingly, this study found little evidence in support of a role for the BNST in cued (forward) fear conditioning. Instead, the authors argued that, because responsive neurons exhibited firing rate changes during the post-shock period, when only contextual stimuli were present, these changes may be associated with contextual fear conditioning. Indeed, our current study supports this idea insofar as intra-BNST APV was shown to selectively affect the acquisition of fear to a BW CS, which occurs at the time BNST neurons exhibit the largest changes in firing rate.

In contrast to the BNST, APV administration into the CeA resulted in deficits in conditioned freezing to both the predictable FW and unpredictable BW CS. Several studies have

shown that genetically distinct populations within the CeA undergo learning-dependent modifications following fear conditioning (Ciocchi et al., 2010; Duvarci et al., 2011; Li et al., 2013; Penzo et al., 2014; Fadok et al., 2017; Sanford et al., 2017) and plasticity within the BLA has been shown to rely on activity within the CeA (Yu et al., 2017). Thus, although plasticity within the CeA may be important for fear conditioning to both predictable and unpredictable threat cues, it is also possible that NMDA receptor antagonism within the CeA indirectly affects learning related plasticity in other brain regions (e.g., BNST) that, in turn, mediate dissociable forms of fear learning.

Prior research in both rodents and humans has suggested that while the CeA mediates phasic forms of fear expression, the BNST may instead control sustained fear states, which are often attributed to unpredictability (Davis et al., 2010). Indeed, there is evidence that the BNST and CeA may mediate different aspects of conditioned fear (Walker and Davis, 2008; Walker et al., 2009; Davis et al., 2010), but other studies have suggested these regions have overlapping or perhaps complementary functions (Sullivan et al., 2004; Fox et al., 2015; Gungor and Paré, 2016). The results of the current study are consistent with the proposed role of the CeA in phasic fear responses, insofar as the effects of NMDA antagonism within the CeA were restricted to the early portions of the context test. Interestingly, this freezing deficit was observed during an early part of the test (~5 min) that is similar to the length of the pre-shock baseline. As time passes in conditioned context, the uncertainty of shock onset may increase and become independent of the CeA. Given the deficit in freezing to the BW CS in the CeA-APV animals (as well as the low freezing of BNST-APV animals in the early portion of the context test), these findings also suggest that CeA-dependent plasticity is required for some aspects of BNST-dependent defensive behaviors (serving complementary roles). Nonetheless, these and other findings (Resstel et al.,

2008; Mobbs et al., 2010; Choi et al., 2012; Shackman and Fox, 2016) suggest that BNST activity may not be limited to sustained responses alone, and can influence the rapid onset of defensive behaviors, at least in some cases.

Given the evidence for sexual dimorphisms in the anatomy of the BNST (Allen and Gorski, 1990; Hines et al., 1992), along with its well appreciated role in contextual fear, there has been significant interest in understanding how these neuroanatomical differences may contribute to differences in fear and anxiety. In particular, several studies have shown that males and females exhibit differences in conditioned fear to contexts, but not discrete CSs (Maren et al., 1994; Markus and Zecevic, 1997; Barker and Galea, 2010). Note that these effects may depend in part on the behavioral measure (e.g., freezing), insofar as female rats exhibit active defensive behaviors (e.g., "darting"; (Gruene et al., 2015) under some conditions. Given this, it's possible that a lack of cued freezing in APV-infused animals in the current study reflects a change in fear response modality (e.g., darting versus freezing), as opposed to a true memory impairment. Although we did not conduct a formal analysis to examine darting behavior in the present study, we have failed to observe this behavior in current (unpublished) and past work (Maren et al., 1994), suggesting that the effects of APV in the present study were specific to an impairment in the acquisition of the cued fear memory. Although the current results suggest that NMDA receptors within the BNST and CeA play similar roles in fear conditioning in males and females, it is possible that different signaling mechanisms [e.g., neurosteroids; (Nagaya et al., 2015; Acca et al., 2017)], particularly within the BNST, may contribute to sex-related differences in fear learning.

It should be noted that there are several limitations to the current study. As a whole, the BNST is composed of several different subdivisions with unique neurochemical signatures, each of which are thought to play unique roles in fear and anxiety related behaviors (Jennings et al.,

2013; Kim et al., 2013; Daniel and Rainnie, 2016; Gungor and Paré, 2016; Lebow and Chen, 2016; Giardino et al., 2018; Yamauchi et al., 2018; Xiao et al., 2020). In the present study, our histological analysis revealed that infusions sites were not restricted to any particular subregion of the BNST. Thus, our study is limited by the fact that we cannot attribute a role for NMDA receptors in the current procedures to any particular subdivision of the BNST. Additionally, although we assume that NMDA receptor antagonists influenced performance by disrupting learning-related synaptic plasticity, we cannot rule out the possibility that intracranial APV infusions impaired basal synaptic transmission (Maren and Fanselow, 1995; Maren et al., 1996). In addition, one could argue that the use of a BW conditioning procedure in the current study resulted in forward trace conditioning after the first trial, and therefore was not a truly "unpredictable" training procedure. Although a role for the BNST in trace conditioning has not been established, it may be required on the basis that trace conditioning degrades the temporal relationship between the CS and the US.

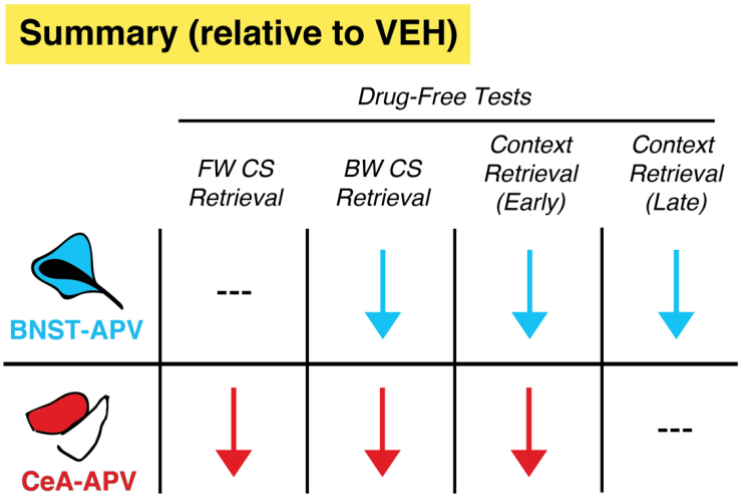

Figure 5. Summary of the effects of intra-BNST or intra-CeA administration of APV on fear conditioning (Ressler et al., 2020b). Arrows indicate significant reductions in freezing levels relative to VEH animals in the current study, whereas hash marks denote no significant changes. BNST-APV and CeA-APV animals exhibited dissociable as well as overlapping deficits in learning.

Lastly, BW conditioning resulted in lower levels of conditioned freezing relative FW conditioning. Given this observation, one could argue that the BNST NMDA receptor antagonists produce impairments with procedures that produce weak, but not strong, fear conditioning. However, we and others have demonstrated that BNST lesions or inactivation reduce the expression of conditioned freezing even when those levels are high (Goode et al., 2015, 2019, 2020; Hammack et al., 2015). Based on these findings, we have argued that magnitude or duration of freezing is not predictive of BNST involvement (Goode and Maren, 2017). With regard to the CeA, future studies will need to be conducted to determine whether its participation is unique to BW conditioning, or whether it also plays a role in the acquisition of conditioned fear to truly unpredictable threat signals. Altogether, the present results build on previous research demonstrating that an extended network of brain structures mediate different forms of fear conditioning. Moreover, these results reveal for the first time that NMDA receptors in the BNST are necessary for the acquisition of conditioned fear to unpredictable threats, including contextual fear. Although further electrophysiological studies will be needed to determine whether plasticity within the BNST does indeed mediate the learning of conditioned fear to unpredictable threats, the current results suggest that NMDA receptors within the BNST are critical for aversive learning and memory within the extended amygdala.

2.4 Material and Methods

2.4.1 Subjects

For all experiments, adult male and female Long-Evans rats (200-250 g upon arrival; Envigo; Indianapolis, IN) were used (n = 64, equal numbers of male and females per group prior to exclusions). Rats were individually housed in clear plastic cages in a climate-controlled vivarium on a fixed light/dark cycle (lights on at 7:00 a.m. and off at 9:00 p.m.). All behavioral experiments

were conducted during the light phase of the cycle. All group assignments were randomized for cage position in the vivarium and male and female rats were housed together (individual, alternating cages) in the vivarium. Animals had access to standard rodent chow and water ad libitum. For five consecutive days prior to the start of surgery animals were handled by experimenters (~1 min/day). All procedures were conducted in accordance with the US National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC).

2.4.2 Surgical procedures

One week prior to behavioral testing, rats were transported to the surgical suite and anesthetized with isoflurane (5% for induction and 1-2% for maintenance) and placed into a stereotaxic instrument (Kopf Instruments). Hair was clipped from the top of the rodent's head and povidone-iodine was applied. A small incision was made in the scalp and the skull was leveled by placing bregma and lambda in the same horizontal plane. Small holes were drilled into the skull for placement of jeweler's screws and bilateral stainless-steel guide cannulas (8 mm for BNST; 10 mm for CeA; 26 gauge; Plastics One); the cannulas were inserted bilaterally into either the BNST or CeA. All coordinates (in mm) were relative to bregma. For the BNST, cannulas were implanted at a 10° angle (directed at the midline) at the following coordinates: anteroposterior (A/P), -0.15; mediolateral (M/L), ±2.65; dorsoventral (D/V), -6.15 from dura. For the CeA, cannulas were implanted at the following coordinates (no angle): A/P, -1.8 mm; M/L, ±3.9 mm; D/V, -7.9 mm from dura. Dental cement was used to secure the guide cannulas to the skull and stainless-steel dummies (9 mm for BNST; 11 mm for CeA; 31 gauge; Plastics One) were inserted into the guide cannulas. Topical antibiotic (Triple Antibiotic Plus; G&W Laboratories) was applied to the

surgical site and one Rimadyl tablet (2 mg; Bio-Serv) was provided for post-operative pain management. Animals were given a minimum of one week to recover prior to the beginning of behavioral training.

2.4.3 Behavioral apparatuses

Behavioral testing was conducted in two separate rooms within the laboratory each containing eight standard rodent conditioning chambers (30 × 24 × 21 cm; Med Associates), which were housed in sound attenuating cabinets. Each chamber consisted of aluminum side walls with a ceiling, rear wall, and front-hinged door made of Plexiglas. Grid floors in the chambers were composed of 19 stainless steel rods (4 mm in diameter; spaced 1.5 mm apart) that were connected to a shock source and a solid-state grid scrambler for delivery of the footshock US (Med Associates). A speaker was mounted within each chamber for delivery of the auditory CS and ventilation fans and a house light were used to generate distinct contexts as needed. Digital cameras were positioned above each conditioning chamber to record and remotely inspect behavior. Freezing behavior served as an index of conditioned fear. For unbiased measurements of freezing behavior, each chamber rested on a load-cell platform that was sensitive to cage displacement produced by each animal's movements (Maren, 1998). Load-cell voltages ranging from -10 to +10 V were collected and digitized at 5 Hz during behavioral testing, yielding one observation every 200 ms. Load-cell voltages were converted values ranging from 0-100 with lower values indicating less cage displacement. Based on prior work, freezing bouts were defined as values of ≤10 for a period of 1 or more seconds (i.e., 5 observations) (Maren, 1998). For each behavioral session, freezing behavior (shown as a percentage of each period, see Results and figures for details) was calculated for the baseline (prior to CS presentation), the CS, and the intertrial interval. Stimuli

within each testing room were manipulated to generate two unique contextual settings. For "Context A", a 15 W house light was turned on within each chamber and overhead red fluorescent room lights were turned on. Each chamber was wiped down with 3.0% acetic acid prior to each behavioral session and chamber doors remained open throughout the duration of each test. White plastic transport boxes were used to move animals to and from the vivarium and Context A. For "Context B", the house light remained off, white overhead fluorescent lights were turned on, and a mounted ventilation fan was used in each chamber to provide constant background noise (65 dB). Chamber doors remained closed during testing and chambers were wiped down with 1% ammonium hydroxide prior to each behavioral test session. Rats were transported to and from Context B in black plastic boxes with clean sawdust bedding.

2.4.4 Drug infusions

Prior to behavioral testing, and in the week following surgery, animals were acclimated to the intracranial drug infusion process. Animals were transported to the infusion room from the vivarium in 5-gallon buckets and the dummies were removed from the guide cannulas and replaced with clean ones. This procedure was conducted twice, on separate days, prior to drug infusions. On the conditioning day, rats were transported to the infusion room and dummy guides were removed. Stainless steel injectors (33 gauge; 9 mm for BNST; 11mm for CeA) were connected to polyethylene tubing (PE-20; Braintree Scientific); the other end of the tubing was connected to a Hamilton syringe (10 µl; Hamilton Scientific) which was mounted on an infusion pump (KD Scientific). For all infusion procedures, the NMDA receptor antagonist, D,L-2-amino-5-phosphonovalerate (APV; Tocris Biosciences), was dissolved in physiological saline to a concentration of 10 µg/µl; saline served as a vehicle (VEH) control. This concentration of APV

robustly disrupts fear conditioning when infused into the amygdala (Maren et al., 1996; Goosens and Maren, 2003). APV also produces behavioral effects when injected into the BNST, albeit in different behavioral tasks (Liu et al., 2009; Lungwitz et al., 2012; Glangetas et al., 2017). All infusions were made immediately (~10 min) prior to the start of conditioning. For all infusions, animals received bilateral infusions of 0.275 □l of APV or VEH at a rate of 0.275 □l/min. Injectors remained in the guide cannulas for 1 minute after the infusion to allow for diffusion. Once injectors were removed, clean dummies were inserted into the guides.

2.4.5 Behavioral procedures and exclusions

An overview of the behavioral procedures is shown in Figure 1. Male and female rats were randomly assigned in equal numbers (e.g., 4 males, 4 females) to receive either forward (FW) or backward (BW) conditioning and vehicle (VEH) or APV infusions into either the CeA or the BNST. Vehicle-treated animals were ultimately collapsed into a single group (VEH) insofar as there were no main effects of brain region in VEH-treated rats for any of the sessions [F's < 1.77, p's > 0.19]. This yielded the following factors and groups for the analyses: training procedure (FW or BW), sex (female or male), and drug treatment (VEH, CeA-APV, or BNST-APV). One animal was excluded because it became ill, and two animals had off-target cannula. This yielded the following group sizes: FW-VEH (n = 16), BW-VEH (n = 16); FW-CeA-APV (n = 7); BW-CeA-APV (n = 8); FW-BNST-APV (n = 7); BW-BNST-APV (n = 8). For conditioning, FW- and BW-conditioned animals were trained in alternating squads; drug assignments and sex were counterbalanced for chamber position for all sessions. Prior to conditioning (day 1) animals were infused intracranially with APV or VEH into CeA or BNST and immediately placed into context A. For FW conditioning, after a 5-minute baseline period, rats received 12 trials in which an

auditory CS (10 s, 2 kHz, 80 dB) immediately preceded an aversive footshock US (2 sec, 1 mA); each trial was separated by a 60-sec intertrial interval (ITI). Rats remained in the chamber for 1 min after the final trial (19 min total for the entire session). For BW conditioning, these parameters were identical to FW conditioning except the order of the CS and US were reversed (Goode et al., 2019).

Forty-eight hours after conditioning (day 3), all animals underwent a drug-free CS retrieval test. Rats were transported from the vivarium in squads of eight and placed into context B (drug assignment and sex were counterbalanced for chamber position) and after a 3-minute baseline period they received 5 presentations of the CS (in the absence of shock); each presentation was separated by a 60-sec interstimulus interval (ISI). Animals remained in the chamber for 1 minute after the last CS presentation (session duration was 8 min 50 sec) and were returned to their home cages after the test.

Twenty-four hours after CS retrieval (day 4) rats were again transported in squads of 8 and placed in the conditioning context (A) to assess contextual freezing in a drug-free test session (15 minutes). Rats were returned to their home cages immediately after the test.

2.4.6 Histological procedures

Upon completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml, i.p.) and perfused transcardially with physiological saline followed by 10% formalin. Brains were extracted and stored overnight (at 4° C) in 10% formalin after which they were transferred to a 30% sucrose-formalin solution for a minimum of 3 days. After fixation and cryoprotection, brains were flash frozen on dry ice and sections containing either CeA or BNST were collected using a cryostat (Leica Microsystems) at -20° C. Coronal sections (40 μm thick)

were mounted on subbed microscope slides and stained with thionin (0.25%) for cannula tract visualization. Glass coverslips were mounted on the slides using Permount mounting medium (Fisher Scientific). Coronal sections were imaged at 10× using a Leica Microscope (MZFLIII) with Leica FireCam software. Only animals with bilateral placement of injector tips within the borders of the BNST or CeA were included in the final analyses (shown in the figures). Localization of injector tips were determined by an experimenter blind to the group assignments of the subjects.

2.4.7 Statistics

All behavioral data were analyzed with repeated measures ANOVA (StatView, SAS Institute) with variables of training procedure (FW or BW), sex (female or male), and drug treatment (VEH, CeA-APV, or BNST-APV) ($\alpha = 0.05$). Bonferroni's test was used for post-hoc analyses. Results are shown as means (±SEM).

3.0 COVERT CAPTURE AND ATTENUATION OF A HIPPOCAMPAL-DEPEDENT FEAR MEMORY[**]

3.1 Introduction

Cognitive behavioral therapies, such as prolonged exposure therapy, are widely used treatments for a number of debilitating fear-related and anxiety disorders (Craske et al., 2014). Similar to extinction learning in rodents, prolonged exposure therapy attempts to extinguish maladaptive fear responses by exposing patients to trauma-related stimuli (often using imaginal exposure) in a safe environment. Despite efficacy in most patients, clinical interventions are nonetheless susceptible to relapse. Accordingly, there is significant interest in developing therapeutic strategies that selectively target and eliminate traumatic fear memories.

Studies in rodents have shown that consolidated fear memories become labile upon retrieval and undergo a protein synthesis-dependent phase of reconsolidation (Nader et al., 2000). Memory attenuated in this way may be less susceptible to relapse (Duvarci and Nader, 2004) suggesting an effective therapeutic strategy to provide long-term relief (Kindt et al., 2009). Although reconsolidation-based therapies have high therapeutic potential (Phelps and Hofmann, 2019), translating findings from experimentally controlled situations to real-world clinical scenarios is a challenge. In animal models, for example, contextual fear memories are reactivated by direct exposure to shock-associated contexts. In patients, however, these memories must be retrieved indirectly using trauma-related cues or imaginal exposure. Although the development of virtual reality exposure therapy holds promise for enhancing exposure-based treatment outcomes

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Ressler R.L., Goode T.G., Kim S., Ramanathan K.R., & Maren S. (2021) Covert capture and attenuation of a hippocampal-dependent fear memory

in humans (Eshuis et al., 2020), a critical question is whether reactivation using indirect reminders yields episodic retrieval of traumatic fear memories that are sensitive to reconsolidation manipulations (Soeter and Kindt, 2015).

To accomplish "covert" memory retrieval in rats, we utilized a backward (BW) fear conditioning procedure (Goode et al., 2019). Critically, this procedure does not require returning the animal to the conditioning context in order to retrieve an aversive memory of that place. In this procedure, rats are placed into a novel chamber and presented with several trials in which an aversive footshock unconditioned stimulus (US) is immediately followed by the presentation of an auditory conditioned stimulus (CS). In this procedure, the CS does not become directly associated with the US, but nevertheless evokes conditioned fear (in this case, freezing behavior). It does so by reactivating a memory of the conditioning context and indirectly retrieving a memory of the aversive US (Chang et al., 2003). Given the critical role for the hippocampus in contextual fear memory (Maren et al., 2013), we hypothesized that a backward CS reactivates a contextual fear engram in the hippocampus in the absence of re-exposure to the conditioning context. This would allow for the capture and manipulation of an indirectly retrieved contextual fear memory, similar to the way in which a clinician might use an incidental reminder to facilitate the episodic recollection of a traumatic experience in the clinic.

3.2 Results

3.2.1 Effects of context extinction on fear to a forward or backward CS

To demonstrate that conditioned freezing to a backward CS is mediated by fear to the conditioning context, animals underwent forward or backward conditioning followed by extinction of the conditioning context (Fig. 6a). We hypothesized that context extinction would undermine freezing

to the backward but not forward CS. During conditioning (Fig. 6b), all rats exhibited low freezing prior to the first trial which increased throughout the duration of the session [repeated measures: main effect of trial; [$F(4, 112) = 99.7$, $p < 0.0001$]. On the following two days, half of the rats in each group were placed into the conditioning context (A; 'Ext'), while the other half were simply exposed to a novel context (C; 'No Ext') for an equivalent amount of time. As expected, freezing behavior in rats exposed to the conditioning context was elevated initially and decreased across days; rats exposed to the neutral context showed low levels of freezing behavior in both sessions. A repeated measures ANOVA revealed a main effect of time [$F(1, 28) = 14.4$, $p = 0.0007$], a main effect of extinction procedure [$F(1, 28) = 10.2$, $p = 0.003$], and a significant time × extinction interaction [$F(1, 28) = 14.6$, $p = 0.0007$]. Importantly, there were no statistical differences between groups in average freezing during the second day of extinction (p's > 0.11; Fig 6b).

Twenty-four hours after the final extinction session all rats were tested for conditioned freezing to the forward or backward CS (Fig. 6b). Analysis of freezing across the five test trials (excluding baseline) revealed a main effect of trial [repeated measures: $F(4, 112) = 8.00$, $p < 0.0001$], a main effect of conditioning procedure [$F(1, 28) = 54.3$, $p < 0.0001$], and a main effect of extinction procedure [$F(1, 28) = 12.3$, $p = 0.002$]. Importantly, the analysis also yielded a significant trial × conditioning procedure × extinction procedure interaction [$F(4, 112) = 2.82$, $p = 0.028$], suggesting that the effects of context extinction differentially affect freezing to the backward and forward CSs. Indeed, planned comparisons revealed that although extinction of the conditioning context had no effect of freezing to the forward CS, it dramatically impaired freezing to the backward CS ($p = 0.005$; Fig. 6b). Together these data support the hypothesis that the retrieval of fear to a backward CS is mediated by contextual fear.

3.2.2 Effects of CS exposure on c-Fos activity in the dHPC

Next, we asked whether the backward CS engages the dorsal hippocampus (dHPC), a brain region known to be important for both contextual fear and higher-order conditioning (Maren et al., 2013). Three experimental groups were compared: rats conditioned and tested to a forward CS ('FW'), rats conditioned and tested to a backward CS ('BW'), and rats conditioned to either a forward or backward CS (evenly split) but remaining in their homecage during the retrieval session ('NoTest'). Prior to conditioning rats underwent a habituation session in what would later be the test context. This session was conducted in an effort to bias c-Fos expression towards cells activated by CS retrieval rather than the test context. Twenty-four hours after habituation, rats underwent forward or backward conditioning in a distinct context (Fig. 6c). Freezing was low during the baseline period and increased significantly across the duration of the session [main effect of trial: $F(4, 76) = 143.3$, $p < 0.0001$; Fig. 6d]. Although the analysis revealed a significant trial × conditioning procedure interaction [$F(4, 76) = 2.54$, $p = 0.047$), post hoc comparisons indicated that there were no statistical differences between any of the groups across the conditioning session (p's > 0.47). Twenty-four hours after conditioning rats received a retrieval test in a safe context; control rats (NoTest) remained in their homecage and were perfused alongside retrieval animals (Fig. 6c-d). During retrieval, freezing was low prior to the first trial and was significant increased by CS presentation in both forward and backward conditioned rats to a similar extent [main effect of trial; repeated measures: $F(1, 21) = 18.6$, $p = 0.0003$; no other main effects or interactions ($F < 2.98$, p's > 0.09).

Ninety minutes after the retrieval test, rats were sacrificed for c-Fos immunohistochemistry and c-Fos-positive (c-Fos+) nuclei were counted in three dHPC subregions (Fig. 6e). As shown in Fig. 6f, presentation of either the forward or backward CS increased the number of c-Fos+ cells in

the dHPC relative to NoTest controls. One-way ANOVAs comparing c-Fos counts within each region revealed significant main effects of group in dCA1 [$F(2, 20) = 12.90$, $p = 0.0003$], dDG [$F(2, 20) = 3.61$, $p = 0.04$], and a trend in dCA3 [$F(2, 20) = 3.47$, $p = 0.051$]. Within the dCA1, both the forward and the backward CS produced similar increases in the number of c-Fos+ cells relative to NoTest controls (BW vs NoTest, $p < 0.0001$; FW vs NoTest, $p = 0.004$), whereas within the dentate gyrus (DG) the backward CS produced greater increases in the number of c-Fos+ cells relative to all of the other groups (BW vs NoTest, $p = 0.027$; BW vs FW, $p = 0.037$; Fig. 6f). These findings reveal that the dHPC is engaged during fear retrieval, and that the DG may be preferentially engaged by the retrieval of a backward CS.

3.2.3 Impact of CS exposure on c-Fos activity in a dHPC fear engram

An important question is whether DG cells active during backward conditioning are, in turn, reactivated by presentation of the backward CS during a retrieval test. To examine this possibility, we infused the dHPC with a viral cocktail (AAV-Fos-tTA and AAV-TRE-hM3Dq-mCherry) to achieve activity-dependent expression of designer receptors exclusively activated by designer drugs (DREADDs) tagged to a fluorescent reporter protein (hM3Dq-mCherry; Fig. 7a-b). To restrict tTA-dependent expression of hM3Dq-mCherry to a training experience, rats were maintained on a doxycycline (DOX) diet until conditioning.

Prior to conditioning, rats were given a brief exposure session in which they were habituated to the retrieval context and were immediately taken off DOX to open a cell labeling window for the conditioning session (see Fig. 7c for behavioral schematic). Two days later, all rats underwent BW conditioning and were immediately placed back on DOX. Conditioning was similar to previous experiments [main effect of trial: $F(4, 40) = 71.5$, $p < 0.0001$; no other main

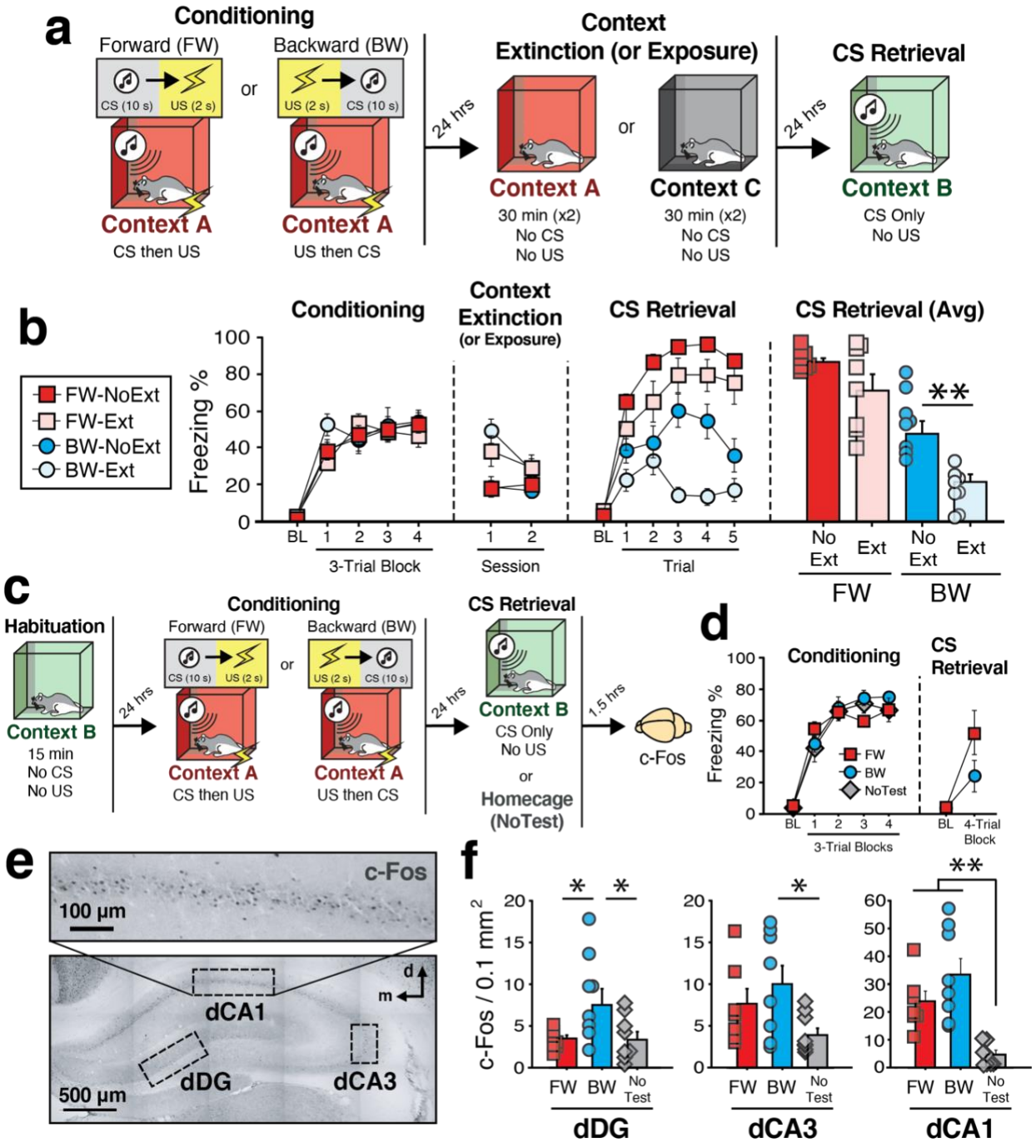


Figure 6. Fear to a backward CS is mediated by a contextual fear memory and engages the dorsal hippocampus (Ressler et al., 2020a). (a) Behavioral schematic. (b) Freezing behavior during conditioning, extinction, and retrieval testing. For conditioning, the left panel depicts mean percentage freezing for each group during the 5 min baseline period (BL) and across each conditioning block. For extinction, data are shown as mean percentage freezing across the whole session for each day. For CS retrieval, data represent mean percentage freezing during the 5 min BL and across each test trial (each trial is composed of the 10 s CS and the 60 s ISI). The right panel depicts average freezing across all test trials. While extinction of the conditioning context

did not significantly impact freezing to the FW CS, it significantly reduced freezing elicited by the BW CS (BW-NoExt vs. BW-Ext, p = 0.005). Groups: [FW-NoExt (n=8); FW-Ext (n=8), BW-NoExt (n=8), BW-Ext (n=8)]. (c) Behavioral schematic. (d) Freezing behavior during conditioning and retrieval. For conditioning, left panel depicts freezing during the 5 min baseline (BL) period and across each conditioning block. For retrieval, right panel depicts average freezing across four retrieval trials (each trial composed of 10 s CS and 60 s ISI). Animals were sacrificed for c-Fos analyses 90 min after the first retrieval trial. (e) Representative photomicrograph depicting c-Fos labeling and regions counted within the dHPC. (f) Mean c-Fos positive cells for each of the quantified regions (standardized to 0.1 mm^2). In the CA1 region, retrieval of both the BW and FW CS resulted in elevated levels of c-Fos expression relative to controls (BW vs. NoTest, p < 0.0001; FW vs. NoTest, p = 0.004), whereas in DG the BW CS resulted in increased levels of c-Fos relative to all other groups (BW vs. NoTest, p = 0.027; BW vs. FW, p =0.037). Groups: [FW (n=8); BW (n=7); NoTest (n=8)]. All data are represented as means ± s.e.m.

effects or interactions F's < 0.29, p's > 0.74)]. The next day, half of the rats were given a backward CS ('Ret') retrieval session to examine the extent to which cells activated within the DG during conditioning (mCherry) were reactivated by the presentation of the BW CS (overlapping endogenous c-Fos protein); the other half of rats served as controls and were simply exposed to the retrieval context for an equivalent amount of time (Fig. 7c). Note that animals did not receive drug injections for this test; hM3Dq-mCherry labeling was simply used as a proxy for dHPC activity at conditioning. Analyses of freezing across the five-trial retrieval test revealed no differences between groups [no main effect of group or trial × group interaction; (F's < 1.89, p's > 0.11)]. However, closer inspection of the data revealed that average freezing across the first two trials was significantly elevated in rats that were presented with the CS [main effect of group; repeated measures: $F(1, 10) = 4.97$, $p = 0.049$]. Importantly, although we found no differences between groups in the overall number of cells activated by conditioning (hM3Dq-mCherry+) or CS retrieval (c-Fos+), rats that received backward CS presentation during the retrieval test displayed a significant increase in the percentage of cells that were double-labeled [Fig. 7b,f; factorial ANOVA: $F(1, 10) = 9.53$, $p = 0.01$]. This suggests that presentation of the backward CS resulted in the reactivation of neural ensembles within the DG that encode contextual representations during conditioning.

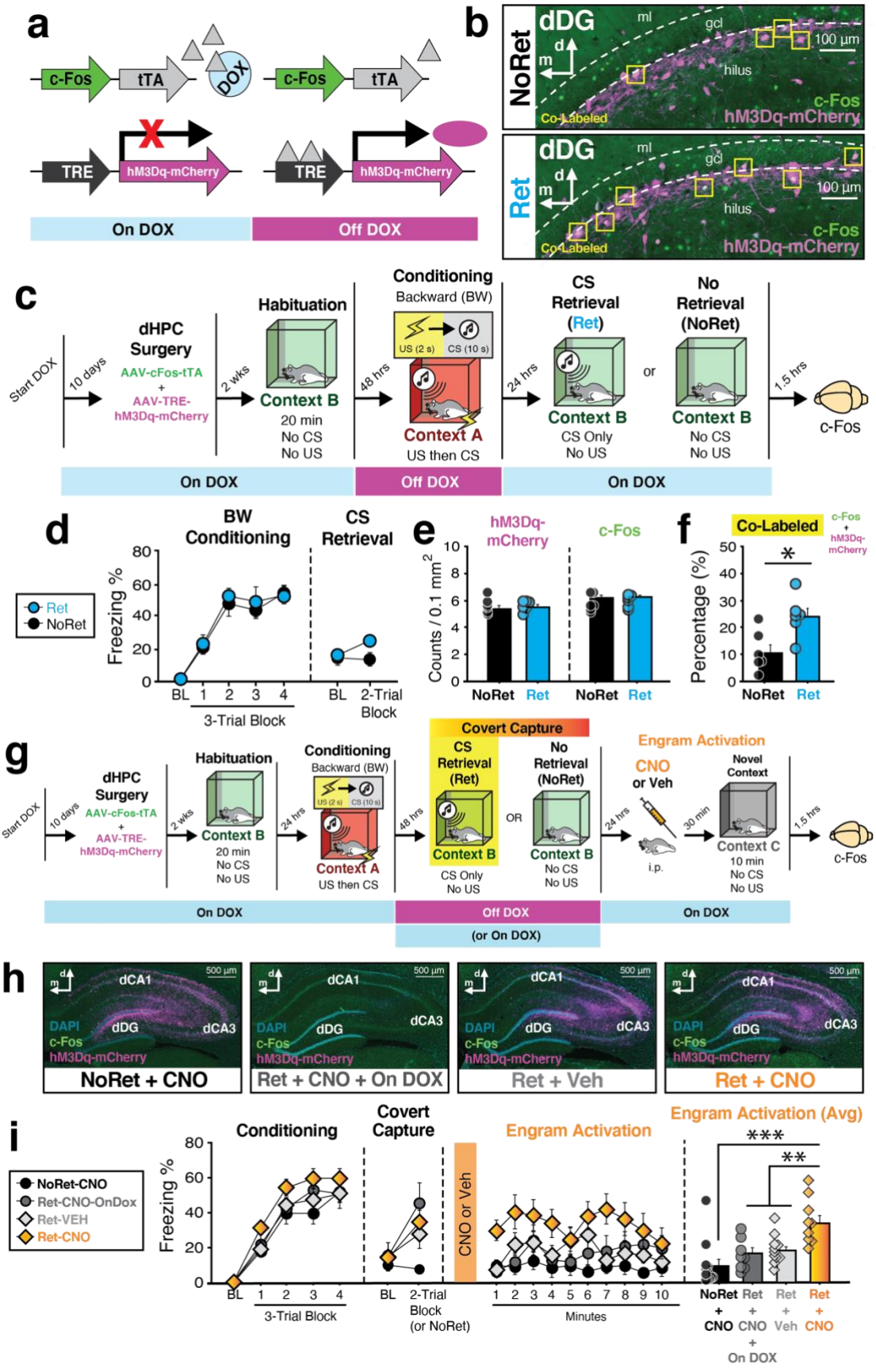
3.2.4 Chemogenetic activation of a BW-tagged fear ensemble

These experiments suggest that the backward CS functions as an indirect retrieval cue to covertly reactivate a hippocampal-dependent contextual fear memory. If so, chemogenetic activation of an HPC ensemble captured during presentation of the backward CS should be sufficient to drive conditional fear in a safe context, as has been demonstrated for direct reactivation of HPC ensembles (Liu et al., 2012). Accordingly, rats were injected with the same viral cocktail noted above to achieve DOX-regulated and c-Fos-dependent expression of the chemogenetic actuator hM3Dq-mCherry in the HPC. Prior to conditioning, and while on the DOX diet, all rats were habituated to the retrieval context in an effort to minimize the animal's tendency to generalize fear across contexts (Fig. 7g). The next day, all rats underwent backward conditioning. All groups exhibited reliable conditioning [main effect of trial; repeated measures: $F(4, 144) = 145.3$, $p < 0.0001$]. There were no other significant main effects or interactions (F's < 1.8, p's > 0.15). After conditioning, rats were immediately returned to their home cages and the DOX diet was replaced with normal chow.

Two days later, rats were given a retrieval session in which they were presented with the backward CS to tag and capture HPC ensembles and were immediately placed back on DOX. Analyses of freezing behavior across the five-trial retrieval session revealed a significant main effect of trial [repeated measures: $F(5, 180) = 13.4$, $p < 0.0001$], a significant main effect of group [$F(3, 36) = 4.00$, $p = 0.015$], and a significant trial × group interaction [$F(15, 180) = 3.41$, $p < 0.0001$]. Similar to our previous experiment, we found that freezing was maximal during the first two retrieval trials and was significantly elevated in rats that were presented with a CS [Fig. 7i; main effect of Ret vs NoRet; repeated measures: $F(1, 38) = 11.7$, $p = 0.002$]. Importantly,

presentation of the backward CS increased hM3Dq-mCherry expression in animals tested off the DOX diet relative to control rats that remained on DOX throughout the duration of the experiment [main effect of group; factorial ANOVA: $F(3, 16) = 41.55$, $p < 0.0001$]. Post hoc analyses confirmed that rats that remained on DOX were statistically different than all other groups (p's < 0.0001; Fig. 7h and Fig. 9).

Twenty-four hours later, rats received systemic injections of either VEH or the DREADD ligand, clozapine-N-oxide (CNO, 3 mg/kg), to activate the captured HPC ensemble; freezing responses were assessed during a 10-minute test session in a novel context. As shown in Fig. 7i, CNO increased freezing behavior in rats that received the backward CS off DOX (Ret-CNO) relative to all of the other control groups. A repeated measures ANOVA revealed a main effect of group [$F(3, 36) = 7.94$, $p = 0.0003$; no other significant main effects or interactions (F's < 1.6, p's > .14)]. Post hoc comparisons confirmed that freezing behavior in the Ret-CNO group was significantly elevated relative to controls (p's < 0.005). This indicates that chemogenetic reactivation of the backward-tagged neuronal ensemble in the HPC is sufficient to drive conditional freezing. Moreover, although activation of the tagged neuronal ensemble increased both the endogenous levels of c-Fos as well as the total number of cells positive for c-Fos and hM3Dq-mCherry (co-labeled) within the DG (Fig. 9), only rats in the Ret-CNO group exhibited increased levels of freezing behavior, suggesting cells tagged during BW retrieval and activated at test represented a contextual fear memory.


a
c-Fos
tTA
DOX
TRE
hM3Dq-mCherry
On DOX
Off DOX
b
NoRet
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dDG
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hilus
100 µm
Co-Labeled
c-Fos
hM3Dq-mCherry
c
Start DOX
10 days
dHPC Surgery
AAV-cFos-tTA + AAV-TRE-hM3Dq-mCherry
2 wks
Habituation
Context B
20 min
No CS
No US
48 hrs
Conditioning
Backward (BW)
US (2 s)
CS (10 s)
Context A
US then CS
24 hrs
CS Retrieval (Ret)
Context B
CS Only
No US
or
No Retrieval (NoRet)
Context B
No CS
No US
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BW Conditioning
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dHPC Surgery
AAV-cFos-tTA + AAV-TRE-hM3Dq-mCherry
2 wks
Habituation
Context B
20 min
No CS
No US
24 hrs
Conditioning
Backward (BW)
Context A
US then CS
48 hrs
Covert Capture
CS Retrieval (Ret)
Context B
CS Only
No US
OR
No Retrieval (NoRet)
Context B
No CS
No US
Engram Activation
CNO or Veh
i.p.
24 hrs
30 min
Novel Context
Context C
10 min
No CS
No US
1.5 hrs
c-Fos
On DOX
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dCA1
dDG
dCA3
DAPI
c-Fos
hM3Dq-mCherry
500 µm
NoRet + CNO
Ret + CNO + On DOX
Ret + Veh
Ret + CNO
i
NoRet-CNO
Ret-CNO-OnDox
Ret-VEH
Ret-CNO
Freezing %
Conditioning
Covert Capture
CNO or Veh
Engram Activation
Engram Activation (Avg)
***
**
BL
1 2 3 4
3-Trial Block
2-Trial Block (or NoRet)
1 2 3 4 5 6 7 8 9 10
Minutes
NoRet + CNO
Ret + CNO + On DOX
Ret + Veh
Ret + CNO

Figure 7. Chemogenetic activation of a BW-tagged neural ensemble drives freezing behavior (Ressler et al., 2020a) (a) Schematic of the viral strategy (b) Representative images (20×) from the dentate gyrus. Yellow squares indicate cells that are doubled labeled for hM3Dq-mCherry (in purple) and c-Fos (in green). (c) Behavioral schematic. (d) For conditioning, the left panels depict mean percentage freezing behavior for each group during the 5 min baseline (BL) period and across each conditioning block. For retrieval, right panel depicts average freezing during the 3 min baseline period and across the first two retrieval trials (each trial composed of 10 s CS and 60 s interstimulus interval) Note that while rats in the NoRet group did not receive any CS presentations, the 2-trial block is defined as an equivalent amount of time (i.e., 140 sec following baseline or the equivalent of two CS trials). (e) Quantification of cells tagged during conditioning (mCherry+) and activated following CS retrieval (endogenous c-Fos). (f) Although there were no differences between groups in the number of cells labeled during conditioning (mCherry+) or retrieval (c-Fos+), presentation of the BW CS resulted in significant increases in the proportion of double labeled cells (NoRet vs. Ret, $p = 0.012$). Groups: [NoRet ($n = 7$); Ret ($n = 7$)]. (g) Behavioral schematic. (h) Representative images from each group showing expression of hM3Dq-mCherry. Removal of DOX prior to retrieval resulted in robust labeling relative to animals that remained on DOX throughout the duration of the experiment. (i) Freezing behavior for exposure, conditioning, retrieval, and engram activation sessions. For conditioning, the panel depicts freezing during the 5 min baseline (BL) and across conditioning blocks. For retrieval, panel depicts average freezing during the 3 min baseline (BL) and average freezing across the first two retrieval trials (each trial consists of 10 s CS and 60 s ISI). During the test session (engram activation) CNO administration increased freezing in rats presented with the BW CS while off DOX ('Ret-CNO') relative to all other groups. Right panel shows average freezing across the activation session for each group (Ret-CNO vs. Ret-VEH, $p = 0.004$; Ret-CNO vs. NoRet-CNO, $p < 0.0001$; Ret-CNO vs. Ret-CNO-OnDOX, $p = 0.004$). Groups: [Ret-CNO ($n= 9$); Ret-VEH ($n = 11$); Ret-CNO-OnDOX ($n = 8$); NoRet-CNO ($n = 12$)]. All data are represented as means ± s.e.m.

3.2.5 Inhibition of protein synthesis in the dHPC following retrieval of a forward or backward CS

These experiments support the hypothesis that a backward CS evokes fear by retrieving a hippocampal-dependent contextual fear engram. This suggests the backward CS could serve as an indirect retrieval cue to covertly access a contextual fear memory in the HPC. Although directly reactivated contextual fear memories undergo a period of reconsolidation in which they are sensitive to protein synthesis inhibition, it is not known whether this is true for clinically relevant indirect retrieval procedures. To explore this, rats were implanted with bilateral cannula targeting the dorsal DG and, after recovery, were subject to forward or backward fear conditioning (Fig. 8a-c). During conditioning, freezing was low prior to the first trial and increased throughout the

duration of the session [main effect of trial: $F(4, 196) = 212.96$, $p < 0.0001$; no other significant main effects or interactions, F's < 1.9, p's > 0.17]. Next, rats underwent a retrieval session in which they were presented with the forward or backward CS to reactivate the fear memory and immediately thereafter received an intra-HPC infusion of the protein synthesis inhibitor rapamycin (1.5 μg/side) or VEH and were returned to their homecages. During the reactivation session ('reactivation'; Fig. 8d), FW and BW groups differed in their levels of conditioned freezing. A repeated measures ANOVA revealed a main effect of trial [$F(1, 49) = 115.5$, $p < 0.0001$], a main effect of conditioning procedure [$F(1, 49) = 8.36$, $p = 0.006$] and a significant trial × conditioning procedure interaction [$F(1, 49) = 23.2$, $p < 0.0001$]. Post hoc analyses revealed that although there were no differences within FW or BW groups (p's > 0.31), rats that were conditioned to a forward CS showed increased average levels of freezing during retrieval trials relative to groups conditioned to a BW CS ($p = 0.0003$).

Forty-eight hours later, freezing to the conditioning context was assessed in a 20-minute test session. As shown in Fig. 8d, rapamycin infusions into the HPC impaired contextual freezing in backward-, but not forward-conditioned rats relative to VEH-treated controls. A repeated measures ANOVA revealed a significant main effect of time [$F(19, 931) = 2.72$, $p = 0.0001$], a significant time × conditioning procedure interaction [$F(19, 931) = 2.42$, $p = 0.0006$] and, importantly, a significant conditioning procedure × drug group interaction [$F(1, 49) = 6.44$, $p = 0.01$]. Post hoc analyses indicated that while there were no differences between drug and VEH treated rats conditioned to a FW CS ($p = 0.52$), rats that were conditioned to a BW CS and received rapamycin following reactivation exhibited significantly less freezing than BW VEH-treated rats ($p = 0.006$). Thus, presentation of the backward CS yielded a covertly reactivated contextual fear memory that was sensitive to hippocampal protein synthesis inhibition. Importantly, this

experiment demonstrates that contextual fear memory could be indirectly reactivated and attenuated without exposing the animals to the conditioning context. This suggests that therapeutic strategies that rely on indirect retrieval in a clinical setting may be viable therapeutic options for inhibiting pathological fear.

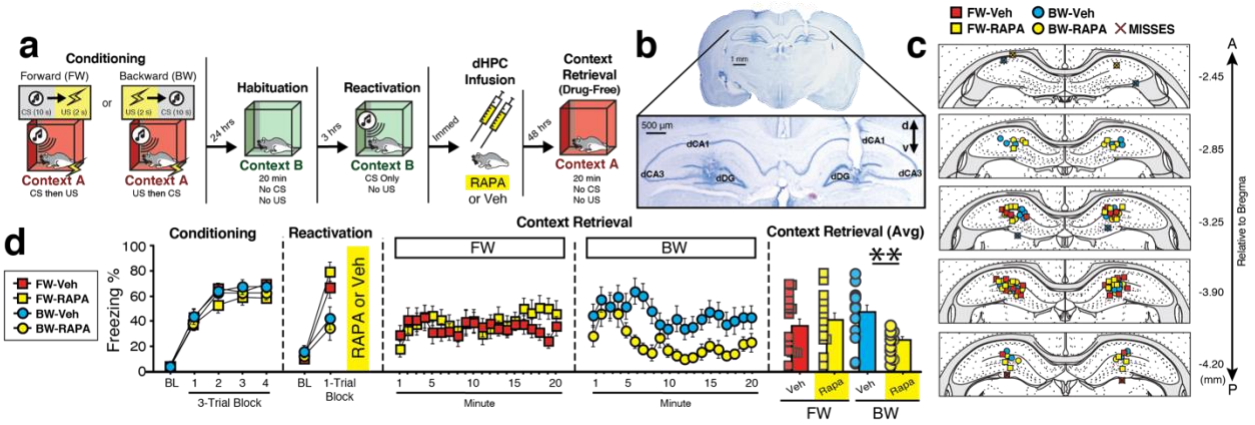

Figure 8. The covert retrieval of a contextual fear memory results in labile memory trace that is vulnerable to disruption by protein synthesis inhibition (Ressler et al., 2020a). (a) Behavioral schematic. (b) Representative photomicrograph depicting bilateral dHPC cannula placements. (c) Documentation of cannula placements in the dorsal hippocampus (corresponding to Figure 3). Symbols denote the location of the injector tips of the cannula tracts for each animal for each group. (d) Freezing behavior during conditioning, reactivation, and the context test. For conditioning, the left panel depicts mean percentage freezing for each group during the 5 min baseline period (BL) and across each conditioning block. For reactivation, the panel depicts freezing during 3 min baseline (BL) period and across one retrieval trial (the trial consists of one 10 s CS and the 60 s post-tone interval). Administration of rapamycin into the dHPC immediately following retrieval of a BW -but not FW- CS impaired freezing behavior during the subsequent drug-free context test. Right panel depicts average freezing across the entire 20 min context test for each group (BW-VEH vs. BW-Rapa, p = 0.006). Groups: [FW-VEH (n = 15); FW-Rapa (n = 13); BW-VEH (n = 14); BW-Rapa (n = 11)]. All data are represented as means ± s.e.m.

3.3 Discussion

Here we combined an innovative behavioral paradigm with activity-dependent neuronal tagging and pharmacology to investigate whether memories reactivated using indirect retrieval methods are sensitive to protein synthesis inhibition within the HPC. We show that fear to a backward CS is mediated through the conditioning context and recruits hippocampal neurons to a greater degree

than a forward CS. We also found that exposure to the indirect CS reinstated conditioning-related activity in a hippocampal ensemble; this activity could be captured using activity-dependent expression of DREADDs and pharmacologically reactivated to drive freezing in a context never paired with shock. Moreover, we observed that intra-hippocampal protein synthesis inhibition disrupted the reconsolidation of a contextual fear memory retrieved covertly by the backward CS. In total, our work describes for the first time HPC representations for covertly retrieved memories and provides novel evidence that HPC engrams reactivated by covert retrieval cues are sensitive to protein synthesis inhibition.

Previous studies employing activity-dependent labeling strategies have shown that the reactivation of contextual fear engrams within the HPC is both necessary and sufficient for the expression of contextual fear (Liu et al., 2012; Tanaka et al., 2014). Our results are consistent with this insofar as we found that the reactivation of a BW-tagged ensemble (which is associated with a contextual representation of the conditioning context) was sufficient to drive conditional freezing in a neutral context. However, in contrast to the current study, most HPC memory engram studies have labeled HPC ensembles during conditioning. Although this work has been fundamental to our understanding of processes underlying memory encoding and retrieval (Josselyn and Tonegawa, 2020), it's not typically the case that clinicians have the opportunity to disrupt a traumatic memory while it's being formed; rather they are dealing with individuals that have prior histories of trauma. Accordingly, a critical question is whether retrieval methods used to facilitate episodic recollection of trauma in a clinical setting would result in the reactivation of neuronal populations that encoded the initial trauma. This is particularly relevant to studies of reconsolidation, in which manipulations are suggested to target the physical memory trace. Here we show that covert retrieval of a contextual fear memory results in the reactivation of a contextual

fear engram and that the chemogenetic activation of this BW-tagged ensemble supports conditioning-related behavior in a neutral context. Thus, a critical finding from the current study is that indirect retrieval of a context-associated memory permits the reactivation a contextual fear engram.

Although our results suggest that clinical interventions that rely on indirect retrieval methods (such as imaginal exposure) may be effective for opening a window to modify, edit or erase neural representations of unwanted traumatic fear memories, an important question is whether indirectly reactivated memories are sensitive to amnesic agents during reconsolidation (Alfei et al., 2020). For instance, given that memories may integrate into complex associative structures (including outside the hippocampus), it is unclear if the reactivation of one element of the associative network results in the reactivation of other parts of the associative network in a way that renders them sensitive to reconsolidation manipulations. A previous study using second-order conditioning procedures with discrete CSs found that directly—but not indirectly—reactivated fear memories undergo reconsolidation within the amygdala (Debiec et al., 2006). Although we did not explore whether amygdala protein synthesis is necessary for reconsolidation of fear to a backward CS, these results suggest that the HPC may have a privileged role in this process, which is consistent with its proposed role in episodic memory.

Lastly, although the ultimate goal of reconsolidation-based therapies is erasure of the memory trace, several studies have demonstrated that retrograde amnesia produced by protein synthesis inhibitors following learning or retrieval results in a retrieval deficit, rather than memory erasure per se (Lattal and Abel, 2004; Ryan et al., 2015; Roy et al., 2017). For instance, a recent study found that systemic administration of a protein synthesis inhibitor after a contextual fear conditioning session resulted in robust impairments in the expression of that memory that could

be recovered by artificial (e.g., optical) stimulation of the contextual fear engram within the HPC (Ryan et al., 2015; Roy et al., 2017). Based on these results the authors suggest that while the time-limited protein synthesis following learning is dispensable for memory storage, it may be required for effective memory retrieval processes. Thus, although intra-hippocampal rapamycin impaired reconsolidation of a covertly retrieved context memory in the current study, it is possible that this reflects a retrieval deficit, as opposed to memory erasure. Indeed, we observed spared freezing in rapamycin-treated rats during the early portions of the context test in the current study (Fig. 8d), which may reflect an incomplete attenuation of protein synthesis within the dHPC or sparing of engram ensembles outside of the dHPC (including extra-hippocampal regions). Nevertheless, these studies reveal that administration of amnesic agents after indirect retrieval render memory traces that are inaccessible to natural recall cues. Whether this is true for older memories that are less dependent on the hippocampus (Tonegawa et al., 2018) is an important avenue for future work. In total, our results suggest that the indirect retrieval of a contextual fear memory results in a labile contextual memory trace that is vulnerable to disruption and suggests that therapeutic strategies that rely on indirect retrieval in a clinical setting may be viable therapeutic options for inhibiting pathological fear.

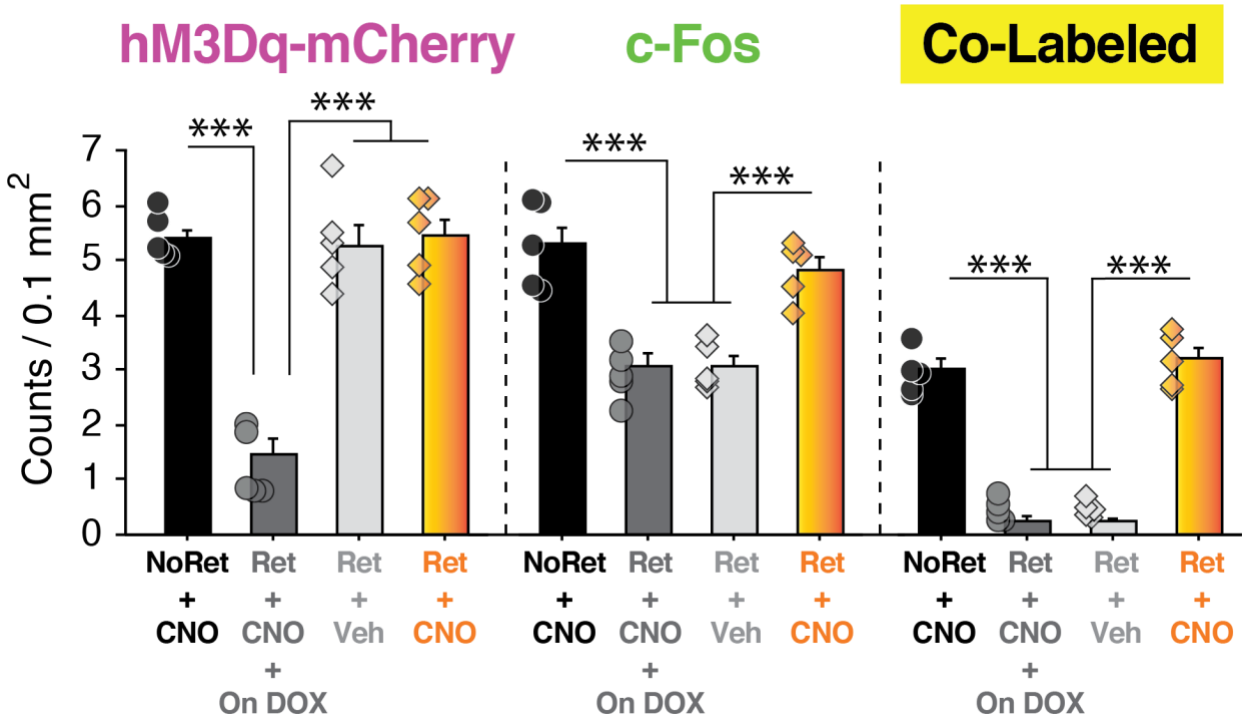

Figure 9. Quantification of hM3Dq-mCherry- and c-Fos-expressing cells in the DG following chemogenetic activation of a BW-tagged ensemble (Ressler et al., 2020a). Removal of DOX prior to CS retrieval resulted in robust expression of hM3Dq-mCherry relative to OnDOX controls [factorial ANOVA with Bonferroni multiple comparisons test: $F(3, 16) = 41.57$, $p < 0.0001$]. For animals that were taken off DOX, CNO administration prior to testing resulted in significant increases in c-Fos expression [factorial ANOVA with Bonferroni multiple comparisons test: $F(3, 16) = 22.65$, $p < 0.0001$]. Importantly, although both Ret-CNO and NoRet-CNO groups exhibited a significant increase in the number of cells expressing both hM3Dq-mCherry and c-Fos (co-labeled) relative to OnDOX and VEH controls [factorial ANOVA with Bonferroni multiple comparisons test: $F(3, 16) = 129.08$, $p < 0.0001$) only rats that received the BW CS during the tagging session (Ret-CNO) exhibited elevated levels of freezing behavior during the test. Groups: [NoRet-CNO (n = 5); Ret-CNO-OnDOX (n=5); Ret-VEH (n=5); Ret-CNO (n=5)]. All data are represented as means ± s.e.m. ***$p < 0.0001$)

3.4 Materials and methods

3.4.1 Subjects

Adult experimentally naïve male Long-Evans rats (200 – 240 g upon arrival) were obtained from a commercial supplier (Envigo) and used for all experiments. Rats were individually housed in clear plastic cages on rotating racks in a climate-controlled vivarium with a fixed 14/10 hour light/dark cycle and were given access to standard rodent chow (with exception of the reactivation

experiments, see below) and water ad libitum. All experiments were conducted in the daytime during the light phase. Upon arrival, all rats were handled by experiments (~30 sec/rat/day) for a minimum of 5 days prior to the start of any surgical or behavioral procedures. All experimental procedures were conducted in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Texas A&M University Institutional Animals Care and Use Committee (IACUC).

3.4.2 Viruses and drugs

Plasmids were a generous gift from the laboratory of Dr. Susumu Tonegawa and were packaged at the University of Pennsylvania Vector Core. From these plasmids, and only for the activity-dependent cell labeling experiments, rats received a 50:50 viral cocktail containing AAV9-TRE-hM3Dq-mCherry-rBG (titer: ≥5 × 10^13 GC/mL) and AAV9-cFos-tTA-bGH (titer: ≥5 × 10^13 GC/mL) as described below. Clozapine-N-oxide (CNO) was provided by the Chemical Synthesis and Drug Supply Program of the National Institute of Mental Health (NIMH). Rapamycin was obtained from LC Laboratories. Doxycycline-containing rodent food chow (DOX; 40 mg/kg) was obtained from Envigo. For the tagging experiments (described below), subjects were fed the DOX diet for at least 10 days prior to any surgical procedure.

3.4.3 Surgeries

For all surgeries, rats were anesthetized with isoflurane (5% for induction, 1-2% for maintenance) and placed into a stereotaxic frame (Kopf Instruments). The hair on the scalp was shaved, povidine-iodine was applied to the skin and a small incision was made in the scalp to expose the top of the skull. The skull was leveled by placing bregma and lambda in the same horizontal plane.

For experiments involving intracranial microinfusions of rapamycin, small holes were drilled into the skull for placement of two to three anchoring screws. Bilateral stainless-steel guide cannulas (5 mm; 26 gauge; Plastics One) were inserted into the dorsal hippocampus (dHPC) at the following coordinates (relative to bregma): anteroposterior (A/P), -3.5 mm; mediolateral (M/L), ±2.45 mm; dorsoventral (D/V), -3.0 mm (relative to dura). Dental cement was used to secure the guide cannulas to the skull and screws. Stainless-steel dummy guides (5 mm; 31 Gauge; Plastic One) were inserted into the guide cannulas. Topical antibiotic (Triple Antibiotic Plus; G&W Laboratories) was applied to the surgical site and one chewable carprofen tablet (2 mg; Bio-Serv) was provided for post-operative pain management. Rats were given a minimum of one week to recover prior to the beginning of behavioral testing.

For experiments involving activity-dependent cell labeling, rats received bilateral infusions of the viral cocktail (noted above) into the dHPC (same coordinates as above; 700 nL total infusion volume/hemisphere) using a microinfusion pump (KD Scientific). Specifically, 10-µl syringes (Hamilton) were mounted on the microinfusion pump; polyethylene tubing (PE-20; Brain Tree Scientific) connected the syringe to stainless steel injection needles (26 gauge) that were backfilled with the viral cocktail immediately prior to injection. Virus was infused at a rate of 100 nL/min and injector tips were left in the brain for five additional minutes to allow for virus diffusion. Following the infusion process the incision was closed with sutures and post-operative procedures were conducted as described above. Rats were given a two-week recovery period following surgery and prior to behavioral testing to allow for viral infection.

3.4.4 Drug injections

For post-reactivation dHPC microinfusions, rats were transported from the behavioral testing room to an adjacent infusion room and the dummy guides were removed from the guide cannula. Stainless steel injectors (5 mm, 33 gauge) were connected to polyethylene tubing (PE-20; Brain Tree Scientific); the other end of the tubing was connected to a 10 µl syringe (Hamilton) which was mounted on an infusion pump (KD Scientific). Rapamycin (LC Laboratories) was dissolved in 100% DMSO to a concentration of 5µg/µl and rats received bilateral infusions (0.3 µl/hemisphere) of rapamycin or vehicle (100% DMSO) at a rate of 0.275 µl/min. Injectors remained in the guide cannulas for 1 min after the infusion to allow for diffusion of drug and rats were immediately transported back to their home cages following the infusion process.

3.4.5 Behavioral apparatus

All behavioral experiments were conducted within two distinct rooms within the laboratory. Each room housed 8 identical rodent conditioning chambers (30 × 24 × 21 cm; Med-Associates). Each chamber was housed in a larger external sound-attenuating cabinet and consisted of two aluminum sidewalls and a rear wall, ceiling, and a hinged front door made from Plexiglas. The grid floor consisted of 19 stainless steel rods that were wired to a shock source and solid-state grid scrambler for delivery of the footshock US (Med-Associates). Each chamber contained a 15-W house light and ventilation fan to provide ambient background noise (~60 dB). Digital cameras were mounted above each chamber for visual recording and inspection of behavior. Cues were manipulated to generate three distinct contexts. For context A, the house light was turned off and the overhead white lights and ventilation fans were turned on. Cabinet doors remained open for the duration of each session. Chambers were wiped with 1.0% ammonium hydroxide prior to each behavioral

session. Rats were transported to context A in black plastic boxes. For context B, house lights were turned on, fans were turned off, and the room was dimly lit by overhead fluorescent red lights. Cabinet doors remained closed for the duration of each behavioral session. Black Plexiglas floors were placed over the grid and each chamber was wiped down with a 3.0% acetic acid solution prior to each behavioral session. Rats were transported to context B in white plastic boxes with a clean layer of bedding. For context C, both the house light and overhead white lights were turned on, fans were turned on, and cabinet doors remained open. Chambers were wiped with 70% ethanol prior to each behavioral session and rats were transported to context C in white plastic boxes with a clean layer of bedding.

For unbiased measurements of freezing behavior, each behavioral chamber rested on a load cell platform used to detect chamber displacement in response to each rat's motor activity. During behavioral testing, load-cell values (ranging from -10 to +10 V) were recorded and digitized at 5 Hz using Threshold Activity Software (Med-Associates). These values were then transformed to generate absolute values ranging from 0 to 100 with lower values indicating less cage displacement. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell values of 10 or less) for a minimum of 5 consecutive observations (1 s or more).

3.4.6 Histological procedures

Upon completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/mL, 0.5 mL, i.p.) and perfused transcardially with physiological saline followed by 10% formalin. Brains were extracted and stored overnight (at 4° C) in 10% formalin after which they were transferred to a 30% sucrose solution for a minimum of 3 days. After fixation and

cryoprotection, brains were flash frozen on dry ice and sections were collected using a cryostat (Leica Microsystems) at -20° C.

For behavioral experiments involving c-Fos quantification (but in the absence of surgical procedures), coronal section (40 μm) containing the dHPC were collected into well plates containing phosphate buffered saline (1× PBS) with 0.01% sodium azide and stored at 4° C until immunohistochemistry was performed. Identical procedures were used for experiments involving viral manipulations, however 30 μm coronal sections of the dHPC were collected.

For cannula experiments, coronal sections (40 μm) were dry mounted on subbed microscope slides and stained with thionin (0.25 %) for cannula tract visualization. Glass coverslips were mounted on slides using Permount mounting medium (Fisher Scientific) and coronal sections were imaged at 10× using a Leica Microscope (MZFLIII) with Leica Firecam software.

3.4.7 Immunohistochemistry

For immunohistochemistry to detect c-Fos, slices were first rinsed three times in tris buffered saline (TBS; 1×, 7.4 pH). All rinses were ~30 sec each; each step was done at room temperature and on a plate shaker. Tissue was transferred across wells using mesh well inserts. The tissue was then placed in 0.3% H2O2 (in TBS) for 15 min followed by three rinses in TBS. Sections were then incubated overnight in primary antibody (rabbit anti-c-Fos, 1:10,000; Millipore) in TBS containing Tween-20 (TBST). The next day, sections were rinsed three times in TBS and then transferred to secondary antibody for 1 hr (biotinylated goat anti-rabbit, 1:1000 in TBST; Jackson Laboratories). Following three more rinses in TBS, the tissue was incubated in avidin biotin complex (ABC, 1:1000 in TBST; Vector Labs) for 45 min. After three washes in TBS, sections

were then transferred to wells containing 3, 3' diaminobenzidine [(DAB) 5% stock, 1:200], nickel ammonium sulfate (5% stock, 1:10), and 30% H2O2 (1:2,000) in TBS for 10 minutes to generate chromophore products. Finally, tissue was rinsed three more times in TBS, mounted on subbed slices and coverslipped with Permount mounting medium (Fisher Scientific).

For fluorescent immunostaining, slices were first rinsed three times (10 min/wash) in 1× PBST (PBS with 0.1% Triton-X; pH 7.4) and then placed in 10% normal donkey serum (NDS in PBST) for one hour. All steps occurred at room temperature and on a plate shaker, unless stated otherwise. Tissue was transferred using mesh well inserts. Slices were then incubated with one or more primary antibodies (1:500 dilution in PBS) at room temperature for 24 hrs (guinea pig anti-c-Fos, Synaptic Systems; rabbit anti-RFP, Rockland). The next day, slices were again rinsed in PBS-T three times and then incubated with one or more secondary antibodies (1:500 dilution in PBS) for two hours at room temperature in 1% NDS in PBST (Alexa Fluor 488 donkey anti-guinea pig, Jackson ImmunoResearch; Cy3 donkey anti-rabbit, Jackson ImmunoResearch). After a final rinse in PBS, stained brain sections were then wet-mounted on gel subbed slides and coverslipped with DAPI-containing fluoromount mounting medium (Invitrogen).

3.4.8 Image analyses

All imaging and cell counting was conducted with experimenters' blind to group assignments. For c-Fos DAB quantifications, four to six brightfield images (20×) of the bilateral dHPC were taken at different A/P levels (ranging from approximately -2.85 mm to -4.6 mm relative to bregma) using a Zeiss microscope and Axio Imager software (Zen Pro 2012). Counts were confined to the following areas of interest: (1) dorsal DG 'dDG' (area of 619µm × 247µm, positioned at the middle of the upper blade of the dDG), (2) dorsal CA3 'dCA3' (an area of 247µm × 371µm, positioned

with its middlepoint at the center of dCA3), and (3) dorsal CA1 ‘dCA1’ (an area of 774μm × 247μm, positioned in the middle of dCA1). The number of c-Fos+ cells within each area for each image were counted, averaged and divided by the surface area (standardized to 0.1 mm2). ImageJ software was used for c-Fos counting (Schneider et al., 2012). For fluorescent viral expression and c-Fos quantification, four to six fluorescent images were taken at different A/P levels (ranging approximately from -2.85 to -4.60 mm relative to bregma) at 20× magnification [Fig. 2b, dDG: 676 μm × 307 μm; Extended Fig. 1, dDG: 845 μm × 404 μm] using a Zeiss microscope and Axio Imager software (Zen Pro 2012). ImageJ software was used to count cells. The number of c-Fos+, mCherry+, and co-labeled cells for each image were averaged and divided by the surface area (standardized to 0.1 mm2), unless stated otherwise.

3.4.9 Statistics

All data were analyzed using conventional parametric statistics (Statview; SAS Institute). Two-way ANOVA and repeated-measures ANOVA were used to assess main effects and interactions ($\alpha = 0.05$). For post hoc group comparisons involving three means, Fisher’s protected least significant differences (PLSD) was used; for group comparisons involving four or more means, Bonferroni’s post hoc was used. No statistical methods were used to pre-determine group sizes (group sizes were selected based on prior work and what is common in the field). All data are represented as means ± s.e.m.

3.5 Behavioral procedures

Overviews of each behavioral experiment are provided in the figures. In all experiments the conditioned stimulus (CS) was an auditory tone (80 dB, 2 kHz, 10 sec) and the unconditioned stimulus (US) was footshock (2 sec, 1 mA).

3.5.1 Effects of context extinction on fear to a forward or backward CS

In a 2 × 2 design, rats (n = 32, no exclusions) were randomly assigned to receive forward (FW) or backward (BW) conditioning procedures (day 1). After conditioning, rats were either returned to the conditioning context ('Ext') or were simply exposed to a novel context alone ('NoExt') for an equivalent amount of time (days 2 and 3) prior to a CS retrieval test (day 4). This design resulted in the following group numbers [BW-NoExt (n = 8); BW-Ext (n = 8); FW-NoExt (n = 8); FW-Ext (n = 8)]. For conditioning, FW and BW conditioned rats were run in alternating squads; extinction assignments were counterbalanced for chamber position in all sessions. For forward conditioning, rats were placed into the conditioning context (A) and following a 5-minute baseline period were presented with twelve CS-then-US trials, (CS offset immediately preceded US) each separated by a 58 sec inter-stimulus interval (ISI). Rats remained in the chamber for one minute following the last trial at which point they were returned to their homecages. Backward conditioning was conducted in identical fashion with the exception that the arrangement of the CS and US were switched such that CS presentation immediately followed the delivery of the US (i.e., US-then-CS).

For context extinction or novel context exposure, rats in both BW and FW groups were exposed to either the conditioning context (A; 'Ext') or a novel context (C; 'NoExt') for thirty

minutes/day for two consecutive days. No stimuli were presented during these sessions and rats were immediately transported back to their home cages following each session.

Twenty-four hours following the last extinction session all rats underwent a CS retrieval test. Rats were transported from the vivarium to context B and following a five min baseline received five presentations of the CS (in the absence of the US); each CS presentation was separated by a 1 min ISI. Rats remained in the chamber for 1 min following the last CS presentation, at which point they were removed and returned to their home cages.

3.5.2 Effects of CS exposure on c-Fos activity in the dHPC

Rats (n = 24, before exclusions) were randomly assigned to receive a forward (FW)-or backward (BW)-trained CS at testing, or no CS retrieval at test (NoTest). The NoTest group was divided such that half of the rats in that group received FW conditioning, while the other half received BW conditioning. One rat was excluded from the analysis due to poor tissue quality. This resulted in the following group numbers: FW (n = 7); BW (n = 8); NoTest (FW-conditioned: n = 4; BW-conditioned: n = 4).

One day prior to conditioning all rats were given a 15 min exposure session to what would be the retrieval context (context B). For conditioning, all rats (in squads of eight, groups intermixed) were transported to context A and received either forward or backward conditioning as described above. Twenty-four hours following conditioning, rats in the FW and BW groups were transported to the vivarium to a neutral context B and following a 3-min baseline period were presented with four CS-alone trials. Each CS presentation was separated by a one-minute ISI and rats remained in chamber for one minute following last CS presentation before being transported back the vivarium. Rats were perfused ninety minutes following the first CS of the test. Rats in the

NoTest group (with FW- and BW-conditioned animals intermixed) were not given a CS retrieval session but were perfused alongside groups of rats in the FW and BW groups.

3.5.3 Impact of CS exposure on c-Fos activity in a dHPC fear engram

All rats (n = 14, before exclusions) were given a 20-min exposure session to what would be the retrieval context (B). Following the exposure, all rats were taken off DOX and 48 hours later received BW conditioning procedures in context A, identical to as described above. Immediately following conditioning, animals were placed back on the DOX diet to prevent further labeling. 24 hours later, half of the rats were randomly assigned to received five CS-only presentations, while the other half of rats were simply exposed to the same context for an identical amount of time. Note that groups were run in different (alternating) squads. Ninety min after the first CS presentation of the retrieval session, rats were sacrificed for c-Fos/mCherry immunohistochemistry. Although NoRet rats did not receive CS presentations, they were perfused at an equivalent time point as rats in the Ret group. Lastly, two rats were excluded due to poor viral infection and expression resulting in the following group numbers: Ret (n = 6); NoRet (n = 6).

3.5.4 Chemogenetic activation of a backward-tagged fear ensemble

After an exposure session (day 1), all rats (n = 64, prior to exclusions) were conditioned to a BW CS (day 2) and 48 hrs later were given a retrieval session in which they were presented with the BW CS to label putative engram cells in the dHPC (day 5). The next day we examined the impact of engram cell activation on freezing responses in a novel context during a 10-min test session (day 6).

For the exposure, rats were transported from the vivarium and placed into context B for 20 min. No additional stimuli were presented during this session and it was conducted in an effort bias cell labeling during the subsequent tagging session to the backward CS presentation, rather than context B itself. The next day, rats were conditioned to a backward CS using identical procedures to those described above. Immediately after conditioning, rats were taken off DOX (replaced with standard chow) for 48 hours to open a labeling window for cell tagging. In addition, we included a control group that remained on DOX throughout the duration of the experiment ('OnDOX'). For cell tagging, groups of rats were placed into context B and after a 3-minute baseline period received five CS presentations each separated by a 60 second ISI ('Ret'). Rats remained in the chamber for 1-minute following the last CS presentation at which point they were returned to their homecages. A control group was included that was exposed to context B for an equivalent amount of time however did not receive any CS presentations ('NoRet'). Upon being returned to their homecages, all rats were immediately placed back on DOX to prevent further cell labeling. Twenty-four hrs after cell labeling, rats were injected with CNO (3 mg/kg, i.p.) or VEH and were placed into a novel context C to assess whether reactivation of the tagged BW CS cell ensemble was sufficient to drive conditioned freezing. Lastly, 90 min after testing, a random subset of rats from each group was sacrificed for quantification of c-Fos and mCherry expression [Ret-CNO (n = 5); Ret-CNO-OnDOX (n = 5); Ret-VEH (n = 5); NoRet-CNO (n=5)].

During the experiment, one rat became ill and was immediately euthanized [Ret-CNO (n = 1)], and any animal (aside from OnDOX animals) that did not have bilateral expression of virus in the dHPC was excluded from the analysis [Ret-CNO (n = 4); Ret-VEH (n = 4); NoRet-CNO (n=2)]. Lastly, several rats that were placed into the tagging context without tones [NoRet-CNO (n = 4)] exhibited high levels of freezing behavior during the tagging session (> 25%), suggesting

that fear of the conditioning context had generalized to the tagging context, at least in these animals. These animals were excluded from the analyses to ensure that the behavior observed during the reactivation session under CNO was specific to reactivation of the backward CS neuronal ensemble, rather than a neuronal ensemble representing a generalized fear memory. This resulted in the following final group numbers for the behavioral experiment: NoRet-CNO (n = 12); Ret-VEH (n = 11); Ret-CNO (n = 9); Ret-CNO-OnDOX (n = 8).

3.5.5 Inhibition of protein synthesis in the dHPC following retrieval of a forward or backward CS.

In a 2 × 2 design rats (n = 64, prior to exclusions) were randomly assigned to receive either forward or backward fear conditioning procedures (day1); infusion of the protein synthesis inhibitor rapamycin ('RAPA') or it's vehicle ('VEH') were given immediately following a single CS retrieval session (day 2) and contextual fear responses were subsequently examined in a drug-free test session (day 4). During the experiment, two rats had their headcaps come loose; they were sacrificed and were excluded from the experiment (n = 1, FW-RAPA; n = 1, BW-RAPA). Three additional rats did not complete the study due to illness (n = 1, FW-RAPA; n = 1, BW-RAPA; n = 1, BW-VEH). Lastly, technical errors during the infusion process (n = 1, FW–RAPA; n = 1, BW-RAPA) and off-target cannula placements outside of the dHPC (n = 1, FW–VEH; n = 1, BW–RAPA; n = 1, BW–VEH) resulted in the following group numbers: FW-VEH (n = 15); FW-RAPA (n = 13); BW-VEH (n = 14); BW-RAPA (n = 11). Note that one additional rat in the BW-RAPA group was marked as an outlier (± 2 standard deviations from the group mean) during the context test and was removed from analysis (the above group sizes reflect this).

For conditioning, rats were transported from the vivarium to context A and received either forward or backward conditioning in alternating squads; chambers were counterbalanced for drug assignments in all sessions. Twenty-four hrs hours after conditioning (day 2), rats were given a 20 min exposure session to the retrieval context (B) in the absence of the CS or the US. This exposure session was conducted to reduce any fear that may have generalized across contexts and to ensure that drug manipulations following the subsequent retrieval session were molecular events associated with the reconsolidation of the CS-evoked memory. After exposure (later that same day), FW and BW rats (intermixed in each squad) were returned to the retrieval context (B) and after a 3-min baseline period were presented with a single CS. The rats remained in the chamber for 1 min (4 min and 10 sec for entire session) after which they were immediately transported to an adjacent room and received intra-DG infusions of either RAPA or VEH. Rats were returned to their homecages immediately following the infusion process.

Forty-eight hours after drug infusion, rats were returned to the conditioning context (A) for a 20-min context test. No additional stimuli were presented during this session and rats were transported to the vivarium following the conclusion of the test.

4.0 DISCUSSION

4.1 General discussion

Overall, my doctoral work has focused on the neurobiological and associative mechanisms underlying the *acquisition* and *expression* of fear to an unpredictable BW CS. From a clinical perspective there is great utility in understanding the brain processes mediating uncertainty, as it considered to be a key factor contributing to anxiety (Grupe and Nitschke, 2013). Despite this, very little is known about the brain processes that mediate such behavior. Pavlovian conditioning is a powerful experimental model to study these processes, and recent work has identified the BNST and the HPC as important structures for fear learning under situations of ambiguity (Amadi et al., 2017; Goode and Maren, 2017; Goode et al., 2019). Despite this, the precise contributions of these regions to different stages of learning in these instances remains unclear.

In the first part of my doctoral work, I examined a role for the BNST in the acquisition of fear to a BW CS. Specifically, I hypothesized that while NMDA receptors in the BNST would be dispensable for the acquisition of fear to a predictable FW CS, they would be required the acquisition of fear to an unpredictable BW CS. Consistent with my hypothesis, NMDA receptor antagonism in the BNST prior to fear conditioning significantly reduced freezing to an unpredictable BW CS, but not a predictable FW CS. NMDA receptor antagonism in the BNST also reduced contextual freezing across the entire test period. In contrast, APV administration into the CeA disrupted freezing to both the FW and BW CS, and although it also impaired contextual fear, this effect was restricted to the early portion of the test session. To our knowledge, this is the first study that has implicated NMDA-receptor signaling within the BNST and provides novel evidence indicating that NMDA receptors in the CeA and BNST may play dissociable roles in the acquisition of fear to predictable and unpredictable threats.

Although understanding the brain structures underlying the acquisition of fear to unpredictable threats is important for the development of targeted therapies, another important question is how fear to an unpredictable CS is expressed. That is, when presented with an unpredictable threat cue, what memory is retrieved such that it supports the expression of fear. Previous work suggests that contextual cues may be critical to the associative structure underlying the expression of fear to a BW CS. Based on these findings we hypothesized that the expression of fear to a BW CS would be mediated by an HPC-dependent contextual fear memory. Consistent with our hypothesis, we show that fear to a BW CS is mediated through a memory of the conditioning context and recruits HPC neurons to a greater degree than a FW CS. In addition, we found that HPC ensembles activated by the BW CS could be captured using activity-dependent expression of DREADDs, and that reactivation of this ensemble yielded freezing in a context never paired with shock. Finally, from a translational perspective and important finding was that intra-HPC protein synthesis inhibition impaired the reconsolidation of an indirectly retrieved contextual fear memory.

4.2 What does plasticity in the BNST encode?

If plasticity within the BNST does serve to store BNST-dependent fear memories, an important question concerns what that plasticity encodes. Perhaps the most obvious interpretation would be that this plasticity supports the formation of context-US associations. From an anatomical perspective this makes intuitive sense; the BNST receives massive projections from the HPC (which could serve to relay contextual information), and is connected to areas of the brain such as the periaqueductal grey (PAG) and parabrachial nucleus (PBN), both which could provide pain information related to the shock US (Weller and Smith, 1982; Fulwiler and Saper, 1984; Eberhart

et al., 1985; Holstege et al., 1985; Canteras and Swanson, 1992; Dong et al., 2001a). Moreover, overtraining studies have indicated a role for the BNST in the acquisition and consolidation of contextual fear, suggesting that the BNST has the capability to serve in this capacity (Poulos et al., 2010). Given that fear to a BW CS relies on a contextual fear memory and NMDA antagonism in the BNST disrupts the context-US association, then it would follow that fear to the BW CS would also be impaired by GluR antagonists. In line with this idea, a recent study demonstrated that infusions of an α-amino-3-hydroxy-5methyl-4-isoxazoleproprionic acid (AMPA) receptor antagonist into the BNST prior to conditioning impaired the acquisition of contextual fear (Davis and Walker, 2014). However, this same manipulation also affected non-associative sensitization that was induced by shock exposure alone. Based on these findings the authors argued that although it is possible that AMPA receptor antagonism interfered with synaptic plasticity underlying a context-US association, a more parsimonious explanation is that the effect was related to a role for BNST AMPA receptors in processing the emotional consequences of the shock. In line with this, a recent study found that exposure to motivationally salient stimuli (in this case appetitive and aversive odors) resulted in rapid changes in physiological arousal that were encoding by genetically distinct neurons within the BNST (Rodriguez-Romaguera et al., 2020). Considering these two studies together, one alternate possibility is that the BNST does not encode context-US associations, but rather provides a valence-like signal related to the emotional consequence of a particular experience, which in turn is important the formation of the fear memory (i.e., context-US) (Lebow and Chen, 2016). From an anatomical perspective the BNST is well positioned to do this; it sends dense projections to regions of the brain such as the ventral tegmental area (VTA) and locus coeruleus (LC) both which provide neuromodulatory input to areas of the HPC and have been implicated in valence signaling (Georges and Aston-Jones, 2001;

Geisler and Zahm, 2005; Wagatsuma et al., 2018; Han et al., 2020). In line with this, exposure to unpredictable shocks has been shown to recruit a BNST to VTA circuit which was also recruited when the animal was exposed to contextual cues in the absence of the US (Jennings et al., 2013). Taking this into consideration with a recent study that demonstrated causal role for a VTA to HPC projections in carrying a negative valence signal related to contextual information (Han et al., 2020), it is possible that the current results are not a reflection of impaired plasticity within the BNST *per se*, but rather impaired communication between the BNST and VTA, which in turn affects memory processing in other regions (e.g., HPC). In line with this idea, the BNST regulates input to the hypothalamic-pituitary-adrenal (HPA) axis, which controls the release of stress hormones (Cullinan et al., 1993; Herman et al., 1994). These stress hormones (e.g., glucocorticoids) have been shown to have significant effects on processes of memory consolidation and there is evidence that HPC-dependent memories are particularly susceptible (Pavlides et al., 1993; Roozendaal, 2000, 2003). Thus, it is also possible that NMDA receptor antagonism within the BNST altered levels of circulating hormones which in turn affected cellular processes within the HPC that were important for the consolidation of the contextual fear memory (and in turn the BW CS).

4.3 Stress and plasticity in the BNST

Although the current results suggest that plasticity within the BNST may serve a mnemonic function, it is also important to consider that exposure to stress may lead to aberrant synaptic plasticity within the BNST that may contribute to circuit dysfunction during future stressors or tasks (McElligott et al., 2010; Conrad et al., 2011; Dabrowska et al., 2013; Harris and Winder, 2018; Daniel and Rainnie, 2016). Glangetas and colleagues (Glangetas et al., 2013) demonstrated

that acute restraint stress leads to alterations in plasticity within neurons in the BNST in response to input from the medial prefrontal cortex (mPFC). Similarly, exposure to stress has been shown to lead to overall increases in BNST volume (Pêgo et al., 2008) as well as increases in dendritic branching (Vyas et al., 2003). With respect to how these changes may affect future behavior, previous work has shown stress may lead to enhancements in associative learning (Rau et al., 2005). For example, exposure to stress has been shown to result in enhancements in trace eyeblink conditioning in rats; this enhancement is eliminated when the BNST is inactivated during conditioning (Bangasser et al., 2005). Given that several theories of PTSD suggest that symptoms may reflect hyper-conditioning of learned associations between cues and traumatic events (Grillon and Morgan, 1999; Orr et al., 2000; Rubin et al., 2008; VanElzakker et al., 2014; Zuj and Norrholm, 2019), an understanding of how exposure to stress alone affects BNST circuit function, and how this may lead to aberrant associative learning will be an important avenue for future work.

Despite evidence implicating the BNST in stress-induced enhancements in associative learning the molecular mechanisms that contribute to these enhancements are not well understood. Indeed, considering the heterogenous nature of the BNST, from a therapeutic perspective it will be important to understand how exposure to stress differentially affects unique components of BNST circuitry. For instance, chronic stress in the form unpredictable shock exposure has been shown to generally increase serotonin (5-HT) release in the BNST and alters serotonin receptor function (Hazra et al., 2012), and other studies have shown that acute intra-BNST infusion (but not intra-CeA) of a selective serotonin reuptake inhibitor (SSRIs) results in enhancements in fear learning (Ravinder et al., 2013; Pelrine et al., 2016). Interestingly, these enhancements in fear learning are apparent in paradigms that are known to be BNST-independent (delay conditioning) (Burghardt et al., 2004, 2007; Ravinder et al., 2013; Marcinkiewcz et al., 2016) which suggests

that alterations in serotonergic signaling may result in the aberrant recruitment of BNST circuitry that may ultimately contributes to augmented associative learning. Given that SSRIs are among the most commonly prescribed medications for fear and anxiety-related psychiatric disorders, a better understanding of how stress alters neurochemical signaling and receptor expression patterns within the BNST may lead to the development of novel pharmacological approaches to treat and/or reverse aberrant stress-induced plasticity within the BNST that may contribute to emotional dysregulation.

4.4 Role of corticotropin releasing factor in BNST-dependent fears

A large body of preclinical literature implicates the actions of corticotropin releasing factor (CRF) in BNST-dependent fear behavior. Although CRF has largely been studied with regard to its role as a physiological regulator of the HPA axis, activity of CRF within the BNST appears to be sufficient, and in some cases necessary, to drive anxiety behavior in animal models. For instance, intracerebroventricular infusion of CRF increases startle responses in rats and this effect is blocked by infusion of CRF antagonists into the BNST (Lee and Davis, 1997). Along these same lines, intra-BNST infusion of CRF has been shown to increase anxiety-like responses and induce conditioned place aversion, both of which are eliminated by infusions of CRF antagonists into the BNST (Sahuque et al., 2006). Interestingly, infusions of CRF antagonists also block SSRI-induced enhancements in fear learning suggesting that these transmitter systems may interact to shape BNST activity for optimal learning (Marcinkiewcz et al., 2016). From a functional standpoint it has been shown that CRF acts presynaptically to enhance glutamatergic transmission within the BNST via a CRFR1-dependent mechanism, which may serve to tune the BNST to whatever inputs are carrying the receptor (Kash et al., 2008). Studies using knockout mice have shown CRF

receptors within the BNST to be necessary for the expression of contextual, but not discretely cued fear (Risbrough et al., 2009), which parallels a proposed role for the BNST in fear learning. Although there is a strong link between CRF and anxiety-like behavior in the BNST, the origin of CRF is not well understood. The lateral division of the CeA produces CRF, contributes to immunoreactive fibers in the BNST, and recent work has implicated these projections in the modulation of contextual fear learning (Pitts et al., 2009; Asok et al., 2016, 2018). In addition, CRF cells, particularly in the anterior division of the BNST (e.g., oval nucleus) are themselves activated by stressors and project to various brain stem nuclei that are known to mediate defensive responding (Kim et al., 2013b; Dabrowska et al., 2016; Giardino et al., 2018). With regard to the current work, there is evidence that NMDA receptors on CRF-positive cells within the BNST shape excitatory signaling within the BNST and modulate anxiety like behavior (Salimando et al., 2020) and other work has shown that dopamine (DA) and norepinephrine (NE) release in the BNST enhances excitatory transmission indirectly through actions at CRF1 receptors (Kash et al., 2008; Nobis et al., 2011). Accordingly, while our current results indicate a necessary role for NMDA receptors in the acquisition of fear to unpredictable threats, it's likely that plasticity within this region is highly regulated by a number of transmitter systems that may serve as viable pharmacological targets to treat fear and anxiety disorders.

4.5 Neural circuits for top-down control of BNST activity

Recently, there has been growing evidence for a regulatory role for mPFC connections to the BNST in stress and anxiety-related behavior. The rodent mPFC is a frontal midline region that consists of two major subsystems that have been argued to play opposing roles in fear behavior.

Studies have shown that while projections from the prelimbic (PL) division of the mPFC to the amygdala are hypothesized to play a privileged role in the expression of fear, projections from the infralimbic (IL) division are thought be involved in the suppression of fear responses (Sierra-Mercado et al., 2011; Milad and Quirk, 2002; Gilmartin and McEchron, 2005; Vidal-Gonzalez et al., 2006; Laurent and Westbrook, 2009; Bloodgood et al., 2018). Although there has been significant work conducted in an effort to understand the mechanisms by which interactions between the mPFC and amygdala contribute to different stages of learning (Giustino and Maren, 2015), very little is known about how mPFC projections to the BNST regulate fear behavior, although there are some studies that have directly examined this projection in other capacities (Glangetas et al., 2017; Johnson et al., 2018). Indeed, these regions are anatomically connected and the IL division of the mPFC sends particularly dense projections to anterior regions of the BNST that have been implicated in fear behavior (Hurley et al., 1991; McDonald et al., 1999). In line with this, recent work from our lab has demonstrated that the retrieval of an unpredictable BW CS results in increased activation of BNST-projecting IL neurons (Goode et al., 2019). Given that the mPFC is involved in experimental situations requiring the integration of higher-order cues or disambiguation between conflicting cues (Sharpe and Killcross, 2014; Badre and Nee, 2018), it is possible that interactions between the IL and the BNST mediate aspects of conditioned fear related to unpredictability. Consistent with this, a recent study demonstrated an important role for BNST projecting IL neurons in limiting the expression of fear to a CS that was partially reinforced; in this case the CS is rendered ambiguous because it only predicts the occurrence of the US on half of the training trials, which typically results in lower levels of fear responding to the CS than fully reinforced schedules (Rescorla, 1968). While chemogenetic inhibition of IL projections to the BNST had no effect on the expression of fear to a CS that was fully reinforced, it resulted in

increased the expression of fear in animals that were conditioned using a partial reinforcement schedule (Glover et al., 2020). This is consistent with a proposed role for the IL insofar as the IL is thought to be important for limiting the expression of fear when there is conflict between excitatory and inhibitory CS associations, such as in fear extinction. Accordingly, given that activity within the BNST is known to be important fear conditioning to ambiguous threats, one possibility is that the IL provides top-down control to limit BNST activity in these situations, which in turn reduces levels of fear.

4.6 Strategies to suppress fear

4.6.1 Enhancing extinction

As previously discussed, although extinction (or exposure therapy) has been fundamental to therapeutic interventions for fear and anxiety disorders in humans, its transient nature has led to significant interest in developing strategies that provide more long-term relief. Along these lines, several investigators have examined whether extinction learning can be facilitated, which theoretically would result in a stronger extinction memory that may mask the expression of the fear memory in some relapse situations (Fitzgerald et al., 2014). For instance, several investigators have shown that administration of D-cycloserine (DCS), an FDA approved positive allosteric modulator of the NMDA receptor, prior to extinction training can facilitate learning (Walker et al., 2002; Ledgerwood et al., 2003). Along these same lines, other research has shown that systemic administration of yohimbine (α-2-adrenergic agonist) prior to extinction can enhance the extinction memory in mice (Cain et al., 2004). Interestingly, there is evidence that extinction acquired under DCS is less likely to exhibit relapse (Ledgerwood et al., 2004) and other work has shown that administration of DCS prior to extinction training results in the reversal in synaptic

changes within the LA that accompany fear learning (Mao et al., 2008). Although these results are encouraging, other studies have failed to find that these manipulations render the extinction memory less susceptible to relapse (Woods and Bouton, 2006; Bouton et al., 2008) and in some instances have found that these manipulations produce impairments in extinction learning (Holmes and Quirk, 2010).

4.6.2 Disrupting reconsolidation: Translational issues

Although reconsolidation has been identified as a potential therapeutic that may hold advantages over extinction procedures, translating findings from basic research to clinical practice has been a challenge. There are several possible reasons for this. As previously discussed, one major procedural difference is that indirect retrieval methods often employed in a therapeutic setting may not necessarily foster memory retrieval in a manner that renders it sensitive to disruption. The current work took advantage of the associative structure underlying BW conditioning to address this issue and provides an important path for future work. Indeed, in life memories are not typically stored in isolation, but are weaved into complex associative structures. For example, in a patient suffering from PTSD exposure to a discrete cue (e.g., car backfiring) may result in a full-blown episodic recollection of the traumatic experience. This process has been hypothesized to involve the activation of distributed engram ensembles that represent different aspects of the learned experience [e.g., smells, sights, sounds, emotions; (Wheeler et al., 2013; Roy et al., 2019)] and whether the reactivation of one engram population results the reactivation of related elements such that they become susceptible to behavioral or pharmacological intervention is an important consideration.

Another translatable issue is disrupting reconsolidation in animal models often involves administration of compounds with considerable neurotoxicity (e.g., protein synthesis inhibitors, NMDA antagonists). Although informative, these approaches to not necessarily offer attractive novel intervention strategies for the large-scale treatment of emotional disorders in humans. In light of this, several labs have demonstrated that administration of propranolol, a β-adrenergic receptor antagonist used widely for the treatment of hypertension, following the retrieval of a fear memory results in deficits in long term storage in both rodents and humans (Debiec and Ledoux, 2004; Abrari et al., 2008; Brunet et al., 2008, 2018; Kindt et al., 2009; Poundja et al., 2012; Schwabe et al., 2012). Given that β-adrenergic receptors play an essential role in protein synthesis necessary for synaptic plasticity (Otis et al., 2015), the identification of human approved pharmacological agents that impinge on similar intracellular signaling cascades necessary for synaptic plasticity may pave that way for effective and safe therapeutic strategies in humans (Tronson and Taylor, 2007; Gamache et al., 2012; Maddox et al., 2013; Surís et al., 2013; Blake et al., 2014; Ratano et al., 2014).

In addition to this concern, another issue involves the ability to selectively target the fear memory without risking the disruption of memories that are incidental to the targeted memory. In animal studies this is partially circumvented via delivery of drugs into different brain regions known to be important for certain components of associative memories (e.g., amygdala, HPC). In addition to not being a tractable approach for clinical populations, these manipulations are still relatively non-specific in that it is impossible to empirically determine that manipulations within discrete regions were specific to engram ensembles representing the fear memory. In clinical populations, drugs targeted at reconsolidation are typically administered orally making it difficult to directly target the fear structure without also affecting other neurobiological and physiological

processes. Although it is feasible to design drugs with minimal off target effects, the ability to target cell ensembles representing a fear memory would be a more selective therapeutic approach that in theory would minimalize unwanted side effects. With regard to the current work, although engram-based viral tagging methods may hold clinical value in the future, the invasive nature of these constructs as they are currently delivered (stereotaxic surgery) does not necessarily make it a feasible approach for the treatment of emotional disorders in a therapeutic setting. However, advances in our understanding of adeno-associated viruses may allow for noninvasive delivery methods (e.g., intravascular) to target the central nervous system, which may help advance these techniques in some capacity to human populations (Choudhury et al., 2016; Merkel et al., 2017; Hudry et al., 2018; Zhang et al., 2018; Challis et al., 2019).

Although the identification and/or development of effective pharmacological tools to disrupt reconsolidation may take some time, other behavioral approaches that would be amendable to human subjects have been utilized to capitalize of the lability of memory during the reconsolidation window. For example, (Monfils et al., 2009) demonstrated that administering extinction training within the reconsolidation window resulted in an attenuation of fear that was immune to relapse phenomena often observed following extinction training. Specifically, the authors first conditioned rats to an auditory CS. The next day the rats were given a single retrieval trial and groups of rats received extinction training either 1 or 6 hrs later. Although both groups of rats exhibited similar levels of freezing on a subsequent extinction retrieval test, rats that had been extinguished 1hr (but not 6hrs) following the retrieval session showed impairments in both renewal and spontaneous recovery. In this case, administering extinction within the reconsolidation window (at a time when the memory is destabilized) was hypothesized to update the original fear memory, rather than create a new inhibitory memory, which in turn resulted in a more enduring

suppression of fear (Monfils and Holmes, 2018). This retrieval-extinction approach has been successful in several rodent fear conditioning studies (Clem and Huganir, 2010; De Oliveira Alvares et al., 2013; Gräff et al., 2014; Liu et al., 2014; Johnson and Casey, 2015; Auchter et al., 2017) as well as in human subjects (Schiller et al., 2010; Telch et al., 2017), although other studies have failed replicate these effects (Chan et al., 2010; Ishii et al., 2012; Luyten and Beckers, 2017; Chalkia et al., 2020). To my knowledge there are no studies to date that have attempted to facilitate extinction (using procedures similar to described above) within this time period which may be an important avenue for future work.

4.6.3 Disrupting reconsolidation: Memory erasure or interference?

Another major goal of reconsolidation work is to understand the mechanisms that govern postretrieval amnesia. For example, the standard view is that reconsolidation results in a period of memory destabilization, which in turn invokes the need for a process of protein synthesis-dependent stabilization (Przybyslawski and Sara, 1997; Nader et al., 2000; Duvarci and Nader, 2004). Although earlier studies attributed impairments in memory following reconsolidation interference to memory erasure (i.e., storage deficit), other reports have challenged this idea (Lattal and Abel, 2004; Caffaro et al., 2012; Ryan et al., 2015; Trent et al., 2015; Roy et al., 2017; Alfei et al., 2020). For example, (Gisquet-Verrier et al., 2015) demonstrated that administration of a protein synthesis inhibitor (cycloheximide) following reactivation of the fear memory resulted in deficits that were ameliorated if the animal was subsequently tested in the presence of the protein synthesis inhibitor. The authors attributed these findings to a state-dependent effect such that postreactivation treatments induce an internal state which becomes encoded within the memory, which in turn should be present at the time of testing for successful retrieval (Gisquet-Verrier et

al., 2015; Gisquet-Verrier and Riccio, 2018). Importantly, this idea is consistent with the concept of reconsolidation as an adaptive and integrative process that allows for the incorporation of new information into existing memories and directly challenges the idea that impairments observed following reconsolidation interference can be attributed to memory erasure. In line with this idea, a recent study examined the effects of a systemically delivered protein synthesis inhibitor (anisomycin) on the reconsolidation of a contextual fear memory that had been functionally labeled during conditioning. Although administration of anisomycin impaired the retrieval of the contextual fear memory when animals were returned to the conditioning context, optical activation of the engram ensemble within the HPC was sufficient to recover the memory (Ryan et al., 2015). Follow up studies suggested that impairments following protein synthesis inhibition were the result of impaired communication between engram ensembles in the HPC and those downstream (e.g., amygdala). Indeed, not only did optogenetic activation of the HPC ensemble restore memory, but this also correlated with engram cell-specific connectivity. Based on these findings the authors argue that deficits following reconsolidation do not reflect a deficit in memory storage, but rather reflect a retrieval deficit stemming from a lack of communication between engram ensembles in distributed brain regions (Roy et al., 2017). Indeed, in our current work we did observe some spared freezing during the early portions of the context test and it's possible this reflects either incomplete inhibition of protein synthesis within the HPC or the sparing of engram ensembles outside of the HPC.

Lastly, while reconsolidation work in rodents has been fundamental to our understanding of cellular and molecular processes governing reconsolidation, from an experimental standpoint they are limited in that the effects of reconsolidation manipulations on memory are often interpreted through unimodal readouts of freezing behavior (at least in the case of most Pavlovian

fear conditioning preparations). For instance, in humans post-retrieval administration of propranolol reduces the expression of a startle response in humans, despite the fact that the participants declarative knowledge of the CS-US contingency remain intact (Kindt et al., 2009; Soeter and Kindt, 2010). This raises the possibility that emotional components may be particularly susceptible to interference, while the declarative or predictive aspects remain unaffected (Cogan et al., 2019). It's noteworthy that from a translational perspective this may be the best case scenario, as disrupting solely the emotional component of the memory may assuage ethical concerns about complete memory erasure in humans (Otis et al., 2015; Elsey and Kindt, 2016). However, it's also possible that during reconsolidation propranolol selectively affects areas of the brain (e.g., amygdala) that are important assigning emotional components to particular associations; whether other disruptors of reconsolidation produce similar effects of various aspects of memory will be an important avenue for future work.

4.7 General conclusions

Although it is appreciated that the BNST may mediate distinct aspects of conditioned fear, whether plasticity within the BNST serves to store BNST-dependent memories is not known. Although we cannot definitively determine that NMDA-antagonism within the BNST in our current results impaired learning-related plasticity *per se*, our results do provide an interesting avenue for future research. As mentioned previously, it will be particularly important to understand how NMDA-mediated signaling influences the activity of distinct subpopulations of neurochemically defined cell types within the BNST and how activity at each of these populations may contribute to different aspects of fear (e.g., associative versus non-associative). Indeed, a more integrative

understanding of BNST function may enable more targeted treatments in clinical populations that may minimalize unwanted side effects.

Lastly, from a translational perspective an important finding in the current work was that the indirect retrieval of an HPC-dependent fear memory resulted in a labile memory trace that was vulnerable to disruption. In clinical populations it is appreciated that re-exposure to trauma related cues can result in a full-blown episodic recollection of the trauma, however whether indirect retrieval methods used in a clinical setting reactivate a memory such that is it susceptible to reconsolidation interference is unknown. It is noteworthy that in the current work CNO-induced activation of the HPC-contextual fear engram (as signaled by a BW CS) was sufficient to drive freezing in a neutral context. Although our results suggest that presentation of the BW CS results in the reactivation of HPC-dependent contextual fear memory that is susceptible to disruption, whether the artificial activation of the engram ensemble (via CNO) would result in a similar protein synthesis-dependent phase of reconsolidation may be an interesting avenue for future work.

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