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New Insights into the Neural Circuits that Support Pavlovian Fear Conditioning

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New Insights into the Neural Circuits that Support Pavlovian Fear Conditioning

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology

by

Lauren Elizabeth DiFazio

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ABSTRACT OF THE DISSERTATION

New Insights into the Neural Circuits that Support Pavlovian Fear Conditioning

by

Lauren Elizabeth DiFazio

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2024

Professor Avishek Adhikari, Chair

Pavlovian fear conditioning is the predominant procedure used to study fear learning in rodents. Decades of research has shown that disrupting activity in the basolateral amygdala (BLA) attenuates the acquisition and storage of fear memories (Cousens & Otto, 1998; Maren, Aharonov & Fanselow, 1996; Phillips & LeDoux, 1992). As a result, current models of fear learning posit that information about the stimuli and aversive event converge and are stored in the BLA (Maren & Quirk, 2004; Pitkänen, Savander & LeDoux, 1997). Per this theory, the BLA should be necessary during *both* the tone and the shock to form this association. To test this theory, **experiment 1** took advantage of the temporal specificity of optogenetics and inhibited the BLA during *only* the tone or *only* the shock of Pavlovian fear conditioning. The results show that BLA

inhibition during the tone, but not the shock, disrupted fear learning and memory, calling into question the necessity of the BLA for processing the shock. Prior experience has a marked effect on future fear learning, but this research primarily focuses on the detrimental effect of stressful experience. The Sharpe lab recently began exploring how prior positive experiences influence fear learning. They found that the lateral hypothalamus (LH) becomes necessary for fear conditioning after reward learning experience (Sharpe et al., 2021). **Experiment 2** investigated whether the BLA remain necessary to support fear learning and memory after LH is implicated in fear learning following reward experience. The BLA was optogenetically inhibited during the cue of fear conditioning in rats with or without reward experience. As expected, BLA inhibition disrupted fear memories in naïve rats. However, BLA inhibition did not affect fear memories in rats with reward learning experience. **Experiment 3** investigated if brief optogenetic inhibition of the BLA in **experiment 2** failed to disrupt the BLA at the crucial time to disrupt fear conditioning because the timescale of the BLA's activity shifted. Using chemogenetics, the BLA was inhibited across acquisition of a tone-shock association in rats with or without reward experience. The results of **experiment 3** were inconclusive due to insufficient expression of hm4di. Lastly, **experiment 4** inhibited the BLA during fear conditioning in reward experienced rats with or without exposure to chronic unpredictable stress (CUS). Rats that received CUS treatment showed enhanced fear learning, but there was no difference in the effect of BLA inhibition during fear learning. Overall, these experiments demonstrate a new perspective on the canonical theory of fear learning and the BLA and expand on how the newfound role of LH in fear learning affects the importance of the BLA for this process and the importance of considering prior experience in our models of the neural circuits that drive learning and memory.

The dissertation of Lauren Elizabeth DiFazio is approved.

Hugh T. Blair

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University of California, Los Angeles

2024

To Elijah – thank you for giving me a home to come back to after a long day of work.

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This last chapter of my life has been filled with highs, lows and many life lessons. I feel incredibly lucky to have so many wonderful people in my circle that continue to celebrate my successes and support me through the tough times. I look forward to discovering what the next chapter holds!

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Selected Publications

I. Hoang, J. Munier, A. Verghese, Z. Greer, S. Millard, **L. DiFazio**, C. Sercander, K. Wassum, A. Izquierdo, M. Sharpe (2023). "A novel hypothalamic-midbrain circuit for model-based learning." *BioRxiv.* https://doi.org/10.1101/2023.03.02.530856

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L. DiFazio, Z. Greer, R. Jia, M.J. Sharpe. "The temporal dynamics of encoding fear memories in the BLA and how the role of the BLA changes with reward learning experience." Poster presentation at Pavlovian Society, Milwaukee, WI (2022).

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CHAPTER 1:

The effect of stress and reward on encoding future fear memories.

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Prior experience changes the way we learn about our environment. Stress predisposes individuals to developing psychological disorders, just as positive experiences protect from this eventuality (Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Pechtel & Pizzagalli, 2011). Yet current models of how the brain processes information often do not consider a role for prior experience. The considerable literature that examines how stress impacts the brain is an exception to this. This research demonstrates that stress can bias learning about ambiguous events as aversive in future via changes in amygdala physiology (Holmes et al., 2013; Perusini et al., 2016; Rau et al., 2005; Shors et al., 1992). This is thought to be an important model for how people develop anxiety disorders like post-traumatic stress disorder (PTSD; Rau et al., 2005). However, more recent evidence suggests that experience with reward learning can also change the neural circuits that are involved in learning about fear (Sharpe et al., 2021). Specifically, the lateral hypothalamus, a region typically restricted to modulating feeding and reward behavior, can be recruited to encode fear memories after experience with reward learning. This review discusses the literature on how

stress and reward change the way we acquire and encode memories for aversive events, offering a testable model of how these regions may interact to promote either adaptive or maladaptive fear memories.

Keywords: fear conditioning, amygdala, lateral hypothalamus, reward learning, prior experience

Introduction

Throughout our lifetime we have many unique experiences that change the way we conceptualize our world. This is part of an adaptive strategy designed to promote survival. We need to encode information about the predictors of reward and danger to guide our future behavior. Remembering a particularly tasty taco truck will allow us to find it again in future, just as we need to remember to avoid food trucks that make us feel ill. These experiences do not just allow us to respond in a more efficient manner when encountering these scenarios again, they can also change the way our brains encode similar experiences in the future (Birn, 2017; Butler et al. 1990; Heim & Nemeroff, 2002; Rau & Fanselow, 2009; Sharpe et al., 2021). For example, having adverse experiences in early in life can increase the likelihood of developing an anxiety disorder, owed to a bias toward interpreting ambiguous events that occur in the future in a threatening manner (Holmes et al., 2013; Kim et al., 2013; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Pechtel & Pizzagalli, 2011 – for review). Similarly, positive relationships help promote adaptive behaviors that allow individuals to cope well with ambiguous circumstance in the future (Brown & Harris, 1993). Despite this, variability in demographics of research study participants (which relate to their prior and everyday experiences) is considered a confound in human research. In conducting experiments with human participants, we try to sample from homogenous groups and carefully control for varying factors when interpreting and analyzing data.

Indeed, one of the advantages of working with rodent models in research is the opportunity to use experimentally naïve subjects. This provides the benefit of carefully controlled experiments by removing the variability in prior experience that complicates human research. However, a

realistic model for how our brains process information requires an understanding of how these prior experiences influence learning. The few lines of research that have manipulated experience as an experimental variable in rodent studies have found dramatic effects on the way the brain processes information in the future (Conrad et al., 1999; Cordero et al., 2003; Knox et al., 2011; Kosten et al., 2006; Rau & Fanselow, 2009; Sharpe et al., 2020; Toledo-Rodriguez & Sandi, 2007). This research has generally focused on how stressful events alter fear processing in the future and is consistent with the findings in human literature that trauma predisposes individuals to developing psychological disorders (Enoch, 2011; Heim & Nemeroff, 2002; Pechtel & Pizzagalli, 2011; Taylor, 2010). For example, rodents exposed to an extremely stressful event will learn about a future aversive event so mild it would not support learning under normal conditions (Rau et al., 2005; Rau & Fanselow, 2009; Shors et al., 1992; Shors & Servatius, 1997). This is accompanied by significant changes in the neural circuits surrounding the amygdala, which houses fearful memories (Pennington et al., 2020; Perusini et al., 2015; Ponomarev et al., 2010; Rosenkranz et al., 2010). Consequently, it is generally thought that adverse experiences produce changes in fear circuitry that "primes" future processing of aversive events.

The finding that traumatic experiences can prime the processing of aversive events in future may be evidence of a more general model for how experience changes the way neural circuits encode learning. If this is the case, positive experiences should change the way we encode information too. Indeed, we have recently demonstrated that prior experience with reward learning recruits the lateral hypothalamus, which is restricted to encoding memories of rewarding events in experimentally naïve rats, to learn about fearful events in the future (Sharpe et al., 2021). These data suggest two things: 1) the phenomenon of priming neural circuits to learn in the future is not

restricted to experience with stressful events, and 2) once a particular neural circuit (e.g. a reward circuit) is primed by a specific experience, it may contribute to learning about information that is outside its traditional specialization (e.g. fear learning). These data are important because rodent studies are usually conducted with experimentally naïve rats, and as a result we may have drawn overly specific boundaries as to which neural circuits encode which types of memories. These data suggest we may need to reopen these neural boundaries.

The finding that prior experience can influence how and where fear memories are encoded also has implications for psychiatric disorders in humans. Could the balance of fearful and rewarding experiences in an individual's past influence how and where fearful events in the future are encoded, making them more or less likely to develop a disorder that is driven by aberrant fear processing? Here, we will review the literature that has examined a role for prior experience in changing how we encode memories for aversive events. We will focus on the basolateral amygdala (BLA) and lateral hypothalamus (LH) in the context of Pavlovian fear conditioning using rodents, where much of the research examining the impact of prior experience on fear conditioning lies (Rau et al., 2005; Rau & Fanselow, 2009). In doing so, we hope to encourage new directions of research that employ prior experience as an experimental variable and provide some potential mechanisms that could account for the findings within this literature.

Studying fear memories in the lab

Studying the neural circuits involved in fear conditioning is of particular interest to the field of behavioral neuroscience because it thought to provide a window into the way we learn and

store memories of aversive events (Tonegawa et al., 2015). Given that aberrant processing of stimuli associated with fearful events is thought to underlie anxiety disorders, it is thought that if we can understand how we encode such memories, we can understand what goes wrong when this process goes awry (Butler et al. 1990; Davis, 1992; Heim & Nemeroff, 2002; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009). Indeed, anxiety disorders are typically characterized by an excessive fear response that impedes day to day life. Further, the prevalence of these disorders is increasing, suggesting an urgent need to understand the cause for these maladaptive fear responses (Baxter et al., 2013; Kirkpatrick & Heller, 2014).

In the lab, we typically model fear learning using Pavlovian conditioning. This is a process where a stimulus, like a light or tone, is paired with delivery of a mildly aversive event, like a brief, mild shock to a rat's foot or human's hand (Davis, 1992; Fendt & Fanselow, 1999; Rescorla, 1973; Rescorla, 1974). Following conditioning, participants will acquire a memory of this association. This is indexed by demonstration of a robust fearful response to the shock-predictive stimuli. This fearful response persists when the stimulus is presented in absence of the shock itself, long after the initial encounter of the stimulus-shock pairing (Gale et al., 2004). This phenomenon is generally thought to parallel the process in our everyday lives, where we learn to fear stimuli that might lead to an aversive event in the future. For example, if you lived near a particularly aggressive dog as a child that was known to attack other dogs, or even required restraint around children, you might become conditioned to dislike or fear dogs for the rest of your life.

The Basolateral Amygdala (BLA)

The neural circuits involved in fear learning and anxious behaviors are widespread and involve complex interactions between many different neuronal populations and brain regions ((Berg, Schoenbaum & McDannald, 2014; Chaaya et al., 2019; Giustino & Maren, 2015; Jacobs & Moghaddam, 2021; Jacobs & Moghaddam, 2020; Miracle et al., 2006; Shin & Liberzon, 2010; Spannuth et al., 2011). However, for the purposes of this review, we will focus on the BLA, which is at the heart of nearly all models of Pavlovian fear learning. The BLA is thought to be the neural hub of Pavlovian fear associations as it is necessary for both the acquisition and storage of associations between stimuli and aversive outcomes (Cousens & Otto, 1998; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Koo et al., 2004; Maren et al., 1996; Maren & Fanselow, 1996; McDannald & Galarc, 2011; Muller et al., 1997; Phillips & LeDoux, 1992; Sparta et al., 2014; Sun et al., 2020 - for review). In fact, one of the most robust reports in the behavioral neuroscience literature is that lesions or inactivation of the BLA will significantly attenuate Pavlovian fear conditioning in rodents (Blanchard & Blanchard, 1972; Cousens & Otto, 1998; Kapp et al., 1979; Koo et al., 2004; Maren et al., 1996; McDannald & Galarc, 2011; Phillips & LeDoux, 1992). Early works established the importance of the amygdala for learning about, and responding towards, aversive stimuli by demonstrating that amygdala lesions attenuate the physiological response (heart rate changes) to stressors as well as cues that predict stressors (Blanchard & Blanchard, 1972; Hitchcock and Davis, 1986; Kapp et al., 1979). These motivated further investigation into the role of the amygdala in fear processing, which established that pre-training electrolytic amygdala lesions in rodents resulted in little or no behavioral responding to a shock-paired stimulus (Phillips & LeDoux, 1992). Since then, this has also been demonstrated with excitotoxic BLA lesions, which offer more specificity than electrolytic lesions and result in a similar decrease in freezing response (Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996). Importantly,

this result is achieved regardless of whether the BLA is lesioned before or after the initial stimulus-shock pairing (i.e., an effect on acquisition or memory expression) and in response to auditory, visual, olfactory or even contextual cues (Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996). Quite remarkably, deficits in freezing to a shock-paired stimulus resulting from lesions of the BLA can be found if the lesion or inactivation occurs up to 16 months after the initial stimulus-shock pairings (Gale et al., 2004; Maren et al., 1996). Work with pharmacological or optical inhibition has confirmed the causal relationship between BLA activity and fear conditioning (Helmstetter, 1992; Muller et al., 1997; Sparta et al., 2014). Pre-training infusions of muscimol, a GABA agonist, decreased fear responding to a shock-paired stimulus and associated contextual cues during subsequent testing, while pre-testing infusions of ammonium hydroxide attenuated expression of fear to shock paired cues (Helmstetter, 1992; Muller et al., 1997). Further, optogenetic inhibition of glutamatergic BLA neuron terminals in the entorhinal cortex during either acquisition or expression of contextual fear learning resulted in a decrease in freezing (Sparta et al., 2014). Together, these studies provide strong evidence for the fundamental role of the BLA as a likely site for acquisition and storage of aversive associative memories.

Importantly, the conclusion drawn from work with rodent models is supported by experiments with humans (Buchel et al., 1999; Cheng et al., 2004; Furmark et al., 1997; LaBar et al., 1998; Klumpers et al., 2015; Knight et al., 2004). Brain activity in human subjects cannot be manipulated with the level of specificity used in rodents or primates. Instead, neural activity is often measured using functional magnetic resonance imaging (fMRI), which is generally considered a proxy for neural activity (Cohen & Bookheimer, 1994). In addition, patients with bilateral amygdala damage can be tested on Pavlovian conditioning to investigate the impact of

such damage on the acquisition and expression of fear-related memories (Klumpers et al. 2015). Data collected using these approaches makes it clear that bilateral amygdala damage attenuates acquisition of conditioned fear responses in humans, without impacting the memory that participants have for the learning procedure itself (Bechara et al., 1995; Klumpers et al., 2015). In addition, there are now many studies that have shown that the extent of conditioned fear developed to a stimulus paired with an aversive outcome, like an air puff or shock in the laboratory, correlates tightly with neural activity measured in the amygdala during the learning episode (Cheng et al., 2004; Furmark et al., 1997; LaBar et al., 1998). That is, the greater the activity seen in the amygdala during learning as measured by fMRI, the greater the conditioned fear response in the subsequent test session. Additionally, fMRI scans of participants who underwent fear conditioning with a stimulus and shock found a spike in amygdala activity during the onset of the stimulus even when it no longer predicted a shock (i.e., extinction), possibly as a result of the amygdala's importance for updating the recently altered contingencies (Knight et al., 2004). These studies corroborate evidence from rodent research, suggesting that the amygdala is critical for the encoding of memories of aversive events in humans as well.

Prior experience with an extremely stressful event appears to prime the amygdala to learn about stimuli that predict aversive outcomes in rats, which parallels the increased incidence of anxiety-based disorders after traumatic experiences in humans (Rau et al., 2005; Rau & Fanselow, 2009; Pechtel & Pizzagalli, 2011). When rats are exposed to these stressful events, it results in exaggerated fear responses that are dependent on physiological changes in the amygdala (Rau et al., 2005; Rau & Fanselow, 2009). Normally, a fear response is directly proportional to the intensity of the aversive stimulus, such as the number and amplitude of shocks (Fanselow & Bolles,

1979). However, when rodents are exposed to a significant stressor (4 or 15 shocks) they demonstrate a persistent and exaggerated freezing response to mildly aversive stimuli in the future (Rau et al., 2005; Rau & Fanselow, 2009). Specifically, they will show fearful behavior to a single pairing of a cue and a shock even when the shock is so mild that control animals do not learn about it (Poulos et al., 2015). This effect is known as stress enhanced fear learning (SEFL; Rajbhandari et al., 2018). This effect is robust; SEFL survives a change in context from the original stressor to the future aversive events and is not restricted to stressors of the same modality. For example, prior experience with low intensity tail shocks or restraint stress can both facilitate learning about a stimulus that predicts a shock to the eyelid (Shors et al., 1992; Shors & Servatius, 1997). This demonstrates that prior experience with highly stressful events enhances future learning about aversive events.

Exposure to stressful events is correlated with physiological changes in the amygdala, in addition to wider circuits that influence amygdala activity, like prefrontal cortex (Jacob & Moghaddam, 2020; Jacobs & Moghaddam, 2021 – for review; Miracle et al., 2006; Pennington et al., 2020; Perusini et al., 2015; Ponomarev et al., 2010; Rosenkranz et al., 2010). For example, *in-vivo* electrophysiological recordings in rats that experienced chronic restraint stress revealed hyperexcitability in lateral amygdala pyramidal neurons (Rosenkranz et al., 2010). Chronic restraint stress is also associated with changes in long-term potentiation and NMDA receptor expression in the lateral amygdala, linking stress exposure to increased plasticity and future fear learning (Suvrathan et al., 2014). Further, if rats receive a corticosterone blocker prior to a stressor, enhancement in learning about future aversive events is reduced (Perusini et al., 2015). This correlated with decreased expression of excitatory (Glu1A AMPA) receptors in the BLA, which

are implicated in fear learning (Walker & Davis, 2002). This suggests that the impact of stress on future fear learning relies on corticosterone-dependent changes to receptors in the BLA that are important for acquisition of conditioned fear. Finally, Ponomarev et al. (2010) identified clusters of genes from amygdala RNA which were overrepresented in neurons or astrocytes (indicating importance for the structure or function of these cells) and assessed how expression of these genes changed after rats were exposed to stress. Expression of the genes enriched in neurons negatively correlated with future fear learning, while expression of genes enriched in astrocytes positively correlated with future fear learning. This suggests there is a coordinated response to stress in the transcriptome, which may underlie the changes in function seen at a cellular and behavioral level. Thus, the stress-induced behavioral changes modeled with procedures like SEFL are accompanied by electrophysiological, cellular and genetic changes in rodents (Perusini et al., 2015; Ponomarev et al., 2010; Rosenkranz et al., 2010). Together, these help us to understand the mechanism by which prior stressful experiences change neural circuits and enhance the likelihood of pathological learning in the future.

The finding that changes in the amygdala can sensitize rodents to future fear learning bears resemblance to the human condition. Individuals that suffer from PTSD display overactive amygdala function, exaggerated fear responses, and difficulty regulating emotion and behavior (Butler et al. 1990; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009). Relative to healthy individuals, people with PTSD show enhanced amygdala activity during recall of personal trauma events (Shin et al., 2004), fear conditioning in the laboratory (Bremner et al., 2005), and presentation of fearful faces or trauma-related words (Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000). Indeed, patients with PTSD – ranging from combat-

exposed veterans to adult survivors of childhood abuse – presented with altered amygdala function (Bremner et al., 2005; Hendler et al., 2003). Importantly, increases in amygdala activity are correlated with symptom severity as diagnosed with a comprehensive clinician-administered PTSD symptom scale (including invasive thoughts, exaggerated startle, and paranoia) in patients recalling memories of traumatic events, undergoing fear conditioning, or being presented with fearful faces (Armony et al., 2005; Blake et al., 1998; Shin et al., 2004). These studies provide convincing evidence that traumatic events alter the amygdala and these stress-induced changes correlate with the exacerbation of PTSD.

The Lateral Hypothalamus (LH)

The lateral hypothalamus is a brain region typically thought of as a critical mediator of motivation, reward processing and feeding (Barbano et al., 2016; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Nieh et al., 2016; Stuber & Wise, 2016). Much of the evidence for its role in motivation and reward comes from studies demonstrating that rodents are willing to work to receive LH stimulation (Barbano et al., 2016; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962). Rats with electrodes implanted in LH will press a lever to receive stimulation, an effect that increases when the rats are food deprived (Margules & Olds, 1962). This rewarding effect is specific to the lateral region of the hypothalamus and does not occur when the medial hypothalamus is stimulated (Hoebel & Teitelbaum, 1962). More recent evidence shows that intracranial self-stimulation is also supported by optogenetic stimulation of the GABAergic neuron population in LH (Barbano et al., 2016; Nieh et al., 2016), which suggests GABA neurons contribute to the rewarding effects of LH stimulation. Indeed, optogenetic stimulation of the

glutamatergic neurons in LH does not support self-stimulation and instead produces behavioral aversion (Nieh et al., 2016). Thus, the LH appears to be involved in reward processing, which is likely mediated in part by the function of GABAergic neurons in this region.

The research showing that stimulation of LH can support intracranial self-stimulation was paralleled by investigation into its role in regulating feeding (Anand & Brobeck, 1951; Barbano et al., 2016; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Roberts, 1980; Stanley et al., 1993; Wise, 1974). Early work demonstrated that rats with bilateral lesions to LH cease to feed entirely until they starve (Anand & Brobeck, 1951). Further, the same electrical stimulation of LH that rats will press a lever for will induce voracious feeding behavior if food is available (Coons et al., 1965; Margules & Olds, 1962). This consummatory behavior occurs even in the absence of food deprivation and continues only for the duration of the stimulation (Margules & Olds, 1962). Jennings et al. (2015) also demonstrated that specific optogenetic or chemogenetic activation of LH GABAergic neurons in mice leads to increased consummatory behaviors. Here, rats demonstrated time-locked increases in food consumption and time spent in the food-associated context when LH GABA neurons were activated. Additionally, optogenetic inhibition of LH GABA neurons has the opposite effect on these behaviors, suggesting they can bidirectionally modulate consummatory and appetitive behaviors (Jennings et al., 2015). Like the effect on intracranial self-stimulation, this effect is specific to LH GABAergic stimulation– optogenetic stimulation of LH glutamate neurons does not produce increases in feeding, nor does electrical stimulation of the medial hypothalamus (Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Nieh et al., 2016; Roberts, 1980; Wise, 1974). Interestingly, stimulation of LH GABA neurons also increased appetitive behaviors and interactions with a social stimulus or novel object (Nieh et

al. 2016). Combined with the data showing rodents will work for LH stimulation, this firmly places LH as a critical node in driving motivated behavior to seek food and other rewards.

Despite the wealth of data on the role of LH stimulation-induced food consumption, there has been relatively less work examining the specific role of this nucleus in supporting appetitive or aversive associative learning. That is, LH is typically thought to mediate processing of reward or producing a tendency to approach reward, but it is not generally conceptualized as a region that facilitates the development of learned associations (Anand & Brobeck, 1951; Barbano et al., 2016; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Nieh et al., 2016; Roberts, 1980; Stanley et al., 1993; Stuber & Wise, 2016; Wise, 1974). There are, however, a few earlier studies that indicated it could also be involved in learning about reward-directed behaviors (Mendelson & Chorover, 1965; Coons et al., 1965). For example, Mendelson & Chorover (1965) found that electrical stimulation of the LH facilitated rats learning that one end of a T-maze task was food-baited and the other was not. Similarly, continuous electrical stimulation of LH helped rats to learn which of two available levers predicted food (Coons et al., 1965). Perhaps the best evidence from the early literature is that electrical stimulation of LH will not enhance feeding unless the food has been experienced before (Wise, 1974). That is, given an entirely novel food, stimulation of LH will not impact food consumption. However, once the rat experiences a specific food, LH stimulation promotes its consumption. Despite this older work suggesting that the LH might be involved in the more cognitive aspects of eating, a potential role for the LH in learning has not received the attention it deserves.

Part of the reason for the relative lack of focus on LH in learning, over and above a role in processing reward or reward approach, is because it is inherently difficult to manipulate LH function in ways that can impact learning about food while leaving food consumption itself intact. Indeed, while there were suggestions that the function of LH in reward processing could reflect learning, it was not possible to dissociate a role for learning over and above the consumption or approach response (Berridge & Valenstein, 1991; Petrovich et al., 2002; Petrovich et al., 2005). There are two studies that have been an exception to this. Firstly, Keefer et al. (2016) used an elegant design to implicate orexin/hypocretin within the LH in Pavlovian conditioning. Specifically, they trained rats to associate a cue with food. During this learning, the rats received a systemic injection containing either an orexin antagonist or a vehicle. Rats that received the orexin antagonist demonstrated decreased food seeking behavior and increased latency to approach the food cup relative to vehicle rats. Importantly, the differences between the groups only became evident during the second session of training. That is, the rats' consumption and behavior was normal during the initial session, suggesting that this is a learning deficit rather than a non-specific behavioral change. Secondly, Sharpe et al. (2017) used the temporal specificity of optogenetics to inhibit neuronal firing in LH GABA neurons during only the conditional stimulus (and not food presentation) of a stimulus-food associative learning task. Here, optogenetic inhibition of LH GABA neurons significantly reduced rats' food-port approach during the stimulus, indicating an inability to use the stimulus to predict food delivery. Importantly, all rats consumed the food from the port shortly after termination of the food-predictive stimulus, demonstrating that all rats experienced the food and stimulus in close succession. This reduction in responding to the food-predictive cue was maintained in an unrewarded test without inhibition of LH GABA neuronal function, which implicates this neuronal population as involved in *memory of* the stimulus-food

association and not temporary changes in motivation or attention. Sharpe et al. (2017) also trained rats on a stimulus-food association (without any inhibition of LH) and then inhibited LH GABAergic neurons during presentation of the stimulus alone. Again, this resulted in a reduction in food port approach during the food-predictive stimulus. This indicates that LH GABAergic neurons are also important for the expression of learnt food associations. Together, this establishes LH, and GABAergic and orexin-releasing neurons in particular, as important in both the learning and expression of memories about food-predictive cues.

Given the role of the LH in learning about predictors of reward, it becomes of interest to investigate whether the LH could also be involved in learning about the predictors of aversive events. To test this, Sharpe et al. (2021) presented rats with tone-shock pairings and examined the effect of inhibiting LH GABAergic neurons during the tone and not the shock. In experimentally naïve rats, LH GABAergic neurons were not necessary for associating the stimulus and shock. That is, all rats learned about the shock-predictive stimulus, regardless of whether LH GABAergic neurons were optogenetically inhibited or not. However, in rats that previously experienced reward learning, LH GABAergic neurons suddenly became important for learning about the shock-predictive stimulus. This was characterized by an almost complete block of learning about the shock-predictive stimulus, indexed by a lack of freezing to the stimulus during learning. These rats also demonstrated attenuated freezing to the shock-predictive stimulus in an extinction test when LH GABAergic neurons were no longer inhibited. Importantly, a number of control experiments verified that this was not due to generalization between the appetitive and aversive memories, extra handling, food restriction, or context exposure that was experienced during reward learning. These data suggest that reward learning primes the LH, specifically GABAergic neurons, to encode

memories of aversive events. This bears similarity to how stressful events prime the amygdala to learn about aversive events in the future and expands this phenomenon in two important ways. First, experience with rewards can also prime neural circuits for future learning and this effect is not restricted to stressful events. Second, if a neuronal population is primed by a particular experience (e.g. reward learning), it can be recruited to encode information it would not usually encode. Together, these results demonstrate that prior experience shapes the neural circuits that are involved in future learning and calls into question the strict neural boundaries we have drawn as to what regions contribute to particular learning phenomena.

The involvement of LH in appetitive and aversive learning procedures raises the question of whether it supports associative learning about sensory stimuli in the absence of food or shock. To investigate this, Sharpe et al. (2021) trained rats on second-order conditioning. Rats first learned to associate a stimulus and food reward (e.g., B→ food). Next, rats learned to associate a second stimulus with the original reward-predictive stimulus (i.e., A→B). Following training, A will motivate appetitive behavior due to its pairing with food-predictive B. Surprisingly, inhibition of LH GABA neurons during the A→B pairings led to an *increase* in appetitive responding to A, relative to control animals. That is, the A→B association was facilitated by inhibition of LH GABA neurons. This indicates that LH GABA neurons oppose learning about relationships between stimuli that are not paired with a motivationally significant outcome. As a result, inhibiting LH GABA removed the inhibitory influence and ultimately enhanced learning. To confirm that this effect was not contingent on the prior experience with reward that occurs in second-order conditioning, new rats received inhibition of LH GABAergic neurons during sensory preconditioning. Here, rats are trained that A leads to B, prior to either stimulus being paired with

food. Then, B is paired with food. Sensory preconditioning is indicated when rats presented with A demonstrate anticipation of food because they infer that A is likely to lead to food because of its association with food predictive B. Sharpe et al. (2021) found that inhibition of LH GABAergic neurons will still enhance the A→ B association under these conditions. Thus, the enhanced relationships between sensory stimuli seen after inhibition of LH GABAergic neurons establish a role for the LH in opposing learning about stimuli that are not motivationally significant. Taken together, these data demonstrate that LH biases learning towards stimuli that predict motivationally relevant outcomes (like food or pain), and away from information that does not predict anything that is currently relevant to the animal.

The BLA and LH: mediating a balance in encoding of adaptive fear memories?

Traditionally, a line is drawn between the BLA fear circuit on the one hand, and the LH reward circuit on the other. However, the discovery that LH can be recruited to learn about aversive events under particular circumstances challenges this conception. Further, we have known for a long time that the BLA is also involved in the encoding of appetitive memories and has a well-established role in motivation and reward learning (Balleine & Killcross, 2006; Balova et al., 2008; Blundell et al., 2001; Cador et al., 1989; Fuster & Uyeda, 1971; Malkova et al., 1997; Schwartzbaum, 1960; Tye et al., 2008; Weizkrantz, 1956; for review, see: Wassum & Izquierdo, 2015). As such, this work forces a more fluid model of how information is encoded within the brain. It is interesting to think about how prior experience influences involvement of these respective circuits in learning about aversive events. How might the BLA and LH form an integrative fear circuit? And what consequences could this have for the future processing of

aversive events? That is, could a shift in the balance of where the fear memory is encoded reduce the likelihood of developing pathological fear in the future?

Prior work examining the role for BLA and LH in appetitive behaviors could provide some useful information as to how these regions might interact during fear conditioning. Such research has demonstrated that the BLA projects both direct and indirectly (through the nucleus accumbens) to LH (Kirouac & Ganguly, 1995; Petrovich et al., 2002; Petrovich et al., 2005; Repucci & Petrovich, 2015). There is strong evidence the projections from BLA to LH are active during appetitive learning tasks (Petrovich et al., 2002; Petrovich et al., 2005). For example, the BLA-LH circuit has been implicated in the cue-potentiated feeding phenomenon, which is characterized by increased feeding behavior in sated rats when a food-predictive cue is presented. Specifically, when the connections between BLA and LH are severed with neurotoxic lesions this cue-potentiated feeding effect is abolished (Petrovich et al., 2002). Further, activity in BLA to LH projections increases to a food-paired stimulus (Petrovich et al., 2005). That is, expression of mRNA markers (Arc and H1A) that appear following neuronal activation increase in BLA→LH circuitry following presentations of the food-predictive stimulus. This indicates that projections from the BLA transmit information relevant to stimulus-food relationships to LH, which allow these food-predictive stimuli to regulate learned appetitive behavior.

Research illustrating the excitatory role the BLA plays in relaying information to LH during appetitive learning suggests that a similar mechanism could be engaged during aversive learning after subjects have had experience with reward learning. However, it is unlikely that BLA is acting to simply increase LH-dependent behavior. This is because we now know that the LH is

itself important for *learning* associations between cues and food or shock. That is, optogenetic inactivation of LH during presentation of a stimulus *prior* to food or shock delivery reduces responding to the predictive stimulus, and this reduction in responding is maintained in a test session when the LH is no longer inhibited (Sharpe et al., 2017, Sharpe et al., 2021). This demonstrates that LH inactivation reduces *acquisition* of these associative memories, rather than just generally reducing appetitive responding. Further, optogenetic inhibition of LH GABAergic neurons while rats are associating two sensory stimuli produces the opposite effect. Specifically, optogenetic inhibition of LH GABAergic neurons enhances stimulus-stimulus associations. This suggests LH biases learning towards motivationally significant events, and actively opposes those that are irrelevant to current biological needs. This does not happen in the BLA. While inhibition of BLA neurons attenuates learning about motivationally significant information (Dwyer et al., 2006; Holmes et al., 2013), inhibition of BLA has no effect on learning associations between sensory stimuli if both stimuli are neutral at the time of learning (Cousens & Otto, 1998; Holmes et al., 2013; Wassum & Izquierdo, 2015). This work suggests that BLA likely relays information to influence learning occurring in LH, but that the LH appears to be adding something unique to this process, which allows LH to arbitrate between different types of learning.

There are many ways that the BLA and LH may interact to influence learning. We would advocate for a model that envisions prior experience with reward learning extending the fear circuit surrounding the BLA to include an "indirect" pathway that implicates the neurons projecting from BLA to LH (Kirouac & Ganguly, 1995; Petrovich et al., 2002; Petrovich et al., 2005; Repucci & Petrovich, 2015). Specifically, we would argue that after reward learning, this indirect pathway becomes primed to receive and evaluate information from BLA about shock-predictive cues. For

example, when a shock-predictive cue is presented, LH receives information from the BLA about the upcoming predicted shock, and arbitrates between whether it should devote more learning or responding towards the shock-predictive cue at the expense of pursuing or learning about other goals (e.g. foraging for food). That is, the LH could become integrated into the fear circuit in a manner that allows it to establish a balance between learning about shock-predictive stimuli, relative to learning about other stimuli, in light of which stimuli are most relevant to individual's current motivational goals. To this end, we might envision recruitment of this indirect pathway with LH as protective against pathological fear, which evaluates whether to learn or respond to fearful cues on the basis of other priorities that may be apparent in the environment.

Further research is needed to determine the specifics of the relationship with BLA and LH and how these regions may work together to achieve a balance of encoding information about aversive and appetitive stimuli. Currently, our best evidence for how stressful experiences might translate into physiological changes that alter learning circuits comes from physiological investigation of BLA (Pennington et al., 2020; Perusini et al., 2015; Ponomarev et al., 2010; Rosenkranz et al., 2010). Moving forward, it is essential to determine how rewarding and stressful experiences might differentially affect these properties in both LH and BLA. For example, in an environment where danger is pervasive, such as active combat, it is reasonable to expect that the neural circuits would adapt to prioritizing learning about fear cues. This might be biologically characterized by an upregulation of the "direct" BLA fear circuit where involvement of LH is limited. In this case, the associative information received in the BLA would activate projections to the central nucleus (Ce), which communicate with hypothalamic areas and the brainstem to trigger the behavioral fear response (Maren & Quirk, 2004; Pitkanen et al., 1997). In contrast, an

individual that has had many positive experiences in life may be more likely to recruit the indirect BLA-LH circuit to encode future aversive memories, where GABAergic neurons in LH would ensure that learning and behavioral resources are only devoted towards cues that warrant those resources in the current circumstances. Here, BLA projections to LH become involved in encoding memories of aversive events and LH projections could influence the degree to which BLA promotes pathological fear (Repucci & Petrovich, 2015; Weera et al., 2021). This would create a distinction where healthy individuals utilize the indirect circuit to prioritize learning about aversive events when it is motivationally necessary to focus on fear, but do not develop tendencies towards fear learning in situations where it is not adaptive. Future work is needed to test these speculative hypotheses and might also explore the wider nature of this potential indirect fear circuit, to investigate how it could influence fear learning and responding.

In summary, nearly all neurobiological research with experimental rodents comes from subjects that only have experience with either fear learning or reward learning. However, as we have discussed in this review, prior aversive or appetitive experience can profoundly change the way BLA and LH are recruited to encode fear memories in the future (Conrad et al., 1999; Cordero et al., 2003; Enoch, 2011; Heim & Nemeroff, 2002; Knox et al., 2011; Kosten et al., 2006; Pechtel & Pizzagalli, 2011; Pennington et al., 2020; Perusini et al., 2015; Ponomarev et al., 2010; Rau and Fanselow, 2009; Sharpe et al., 2017, Sharpe et al., 2021; Rosenkranz et al., 2010; Taylor, 2010; Toledo-Rodriguez & Sandi, 2007). Given humans have many and varied experiences across their lifespan, it becomes imperative that we investigate how fear memories are encoded after varied experiences. While there is a large literature that investigated how stress primes the amygdala to learn about aversive events in future, the research discussed here suggests we need to consider

how positive experiences could influence encoding of future fearful events. Further, an important and fruitful direction for research would be to understand how the wider circuits involved in fear learning might be changed with prior experience, and how the LH is recruited into this wider circuit. For example, recent work has shown that the prefrontal cortex regulates sensitivity to punishment in the context of reward learning, and this regulation changes after stressful experiences (Jacobs & Moghaddam, 2020). It could be that the recruitment of LH to learn about fear is influenced by mechanisms that drive a shift in the fear circuit. Finally, it may be the case that positive experiences protect against pathological fear, just as stressful experiences predispose individuals to developing pathological fear. This is good news. If we can establish the protective nature of reward learning and recruitment of the LH in encoding of fear memories, we could try to recruit these circuits in humans that are at risk of experiencing trauma, to reduce the likelihood of developing pathological fear.

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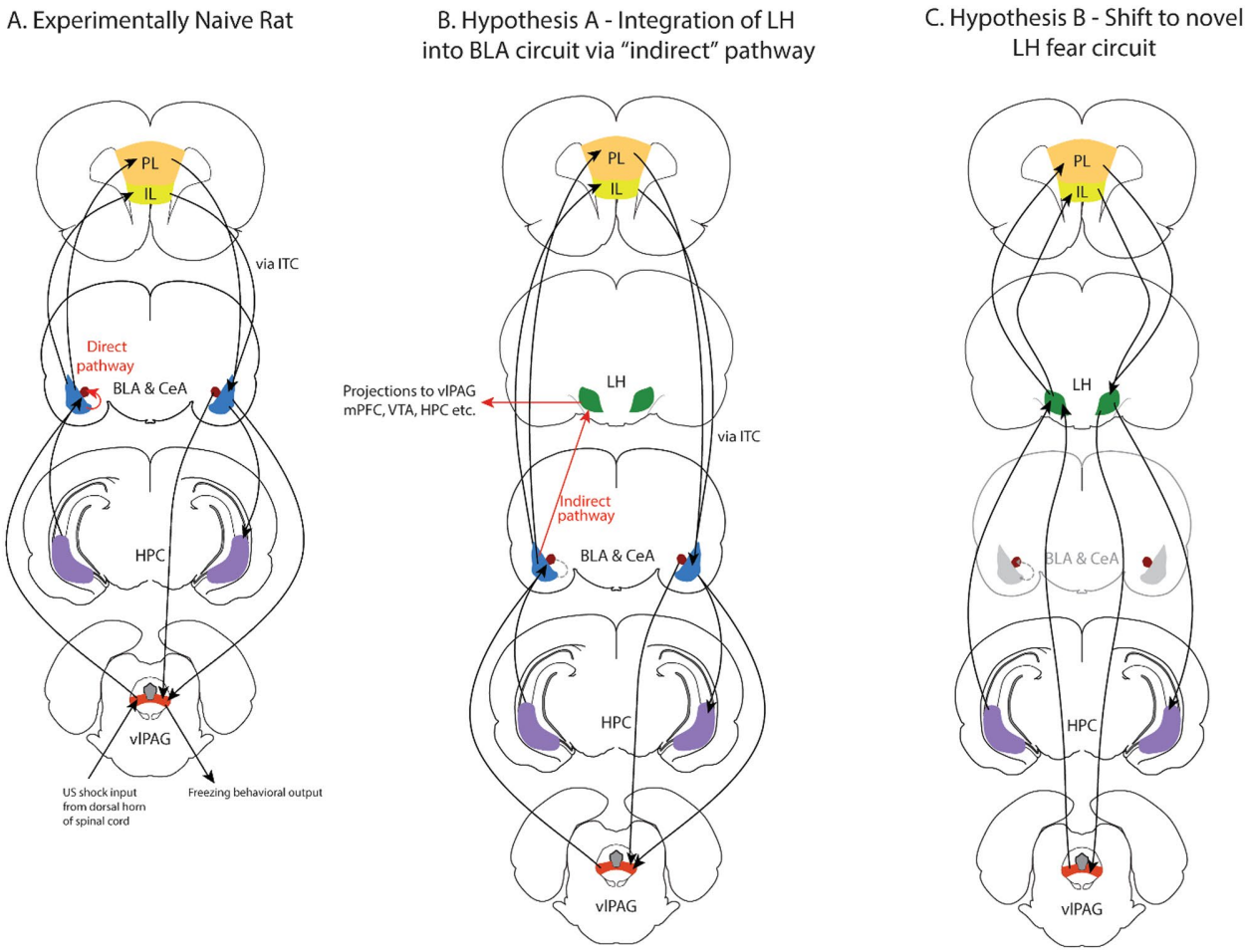

Figure 1. Possible ways that the lateral hypothalamus might be integrated into the Pavlovian fear circuit after experience with reward learning. The fear circuit is complex, where many different neural regions, populations, and projections contribute to the encoding of fear memories (Sharpe and Killcross, 2018; Marek et al., 2013; Amano et al., 2011; McNally et al., 2011; Wilensky et al., 2006; Maren and Quirk, 2004). A) The amygdala is generally conceptualized as the center of these models, implicated in the acquisition and storage of the fear memory. In experimentally-naïve subjects, the ventrolateral periaqueductal grey (vlPAG) sends the aversive prediction error to facilitate the linking together of information that forms the fear memories. The prelimbic (PL) and infralimbic (IL) cortices have reciprocal projections with BLA, where these connections are thought to facilitate the development of the context specificity of fear memories

following extinction, contributed to also by the hippocampus (HPC). B) After reward learning, it is possible that the lateral hypothalamus (LH) becomes integrated into the traditional amygdala fear circuit, where LH would receive information from BLA about upcoming predictions, which may help LH to bias learning and ongoing behavior towards or away from fear-related stimuli, depending on current circumstance. C) It is also possible that the recruitment of LH for the encoding of the fear memory constitutes a shift away from the amygdala circuit and towards a novel LH fear circuit. Given the LH has many comparable connections with the neural regions critical to Pavlovian fear learning in the amygdala fear circuit, there is physiologically plausibility to the existence of such a circuit (Jimenez et al., 2018; Repucci and Petrovich, 2016; Wayner et al., 1997; Behbehani et al., 1988).

CHAPTER 2:

How prior reward experience influences the neural circuits that encode fear learning

Introduction

Anxiety disorders are increasing in prevalence and demand better treatment options (Baxter et al., 2013; Kirkpatrick & Heller, 2014). To best treat fear-based pathologies, we need a fundamental understanding of the basic neural processes that drive fear learning and behavior. We model these learning processes with rodents in the lab through Pavlovian fear conditioning. Here, a neutral cue (e.g., tone) is repeatedly paired with an aversive outcome (e.g., foot shock) until rodents learn the cue-shock association. As rodents learn they will develop a freezing response to the tone – a fearful behavior where all movement ceases except breathing –indicating their anticipation for the shock (Davis, 1992; Fendt & Fanselow, 1999; Gale et al., 2004). The amount of freezing can be measured during training or cue-only extinction tests to evaluate learning and memory, respectively, for the tone-shock relationship.

Decades of research have established the basolateral amygdala (BLA) as the hub of acquisition and storage of associative fear memories (Cousens & Otto, 1998; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Koo et al., 2004; Maren et al., 1996; Maren, & Fanselow, 1996; McDannald & Galarce, 2011; Muller et al., 1997; Phillips & LeDoux, 1992; Sparta et al., 2014). The BLA receives input from thalamic regions and has bidirectional connections with cortical regions, positioning it well to facilitate associative learning (Maren & Quirk, 2004; Pitkanen et al., 1997; Romanski et al., 1993). The generally accepted model of how fear memories are formed is that the BLA receives information about the cue and the shock from thalamic and cortical afferents,

respectively, inducing plasticity to form an associative memory that links cue and outcome together (Duvarci & Pare, 2014). This theory is supported by ample evidence that disrupting BLA activity attenuates fear learning and memory (Cousens & Otto, 1998; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Koo et al., 2004; Maren et al., 1996; Maren, & Fanselow, 1996; McDannald & Galarce, 2011; Muller et al., 1997; Phillips & LeDoux, 1992; Sparta et al., 2014). These canonical behavioral studies were driven by early findings that amygdala lesions reduced the physiological response (fluctuating heart rate) to a stressor (Blanchard & Blanchard, 1972; Hitchcock and Davis, 1986; Kapp et al., 1979). This finding motivated investigations into the role of the amygdala in learning about cues that predict these stressors. Pavlovian fear conditioning was evaluated in rodents with electrolytic lesions, excitotoxic lesions and muscimol inhibition in the BLA (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). In all cases, freezing to the shock-paired cue was reduced indicating that conditioned fear responses are dependent on the BLA. These findings extended to conditioning with auditory, visual, olfactory and contextual cues. They included experiments that disrupted the BLA both before training, indicating an effect on learning and after training indicating an effect on memory retention during subsequent extinction tests. Evidence has shown lesions occurring up to 16 months after learning still disrupt freezing to the shock-paired cue – demonstrating how robust the relationship between the BLA and fear learning is (Gale et al., 2004; Maren et al., 1996). Together, this solidified the BLA as the site for association and storage of fear memories – driving the theory that the BLA must be intact during the tone and the shock to process and associate those stimuli. To best test this theory one would ideally inhibit the BLA during only the tone or only the shock, rather than across the whole training session. At the time of the original findings, this was not possible with the current technology. In **experiment 1,** we revisited this

question with the millisecond-level temporal resolution of optogenetics by selectively inhibiting the BLA during the cue or shock of fear conditioning.

We study fear learning in the laboratory to inform our research on human fear behavior and explore the underlying processes that go awry in anxiety disorders. To achieve this, we strive for the best possible translatability of experiments. Yet, research on fear learning is conducted almost exclusively on experimentally naïve animals. This means our models of fear learning are based on animals with very limited prior experience. This does not consider the major impact these experiences can have on neural circuits (Perusini et al., 2016; Ponomarev et al., 2010; Rosenkranz et al., 2010; Sharpe et al., 2021; Qin et al., 2021). For example, exposing rodents to a significant stressor results in exaggerated future fear responses (Rau et al., 2005; Rau & Fanselow, 2009). The exaggerated fear responses after a significant stressor are accompanied by local changes in the BLA to long-term potentiation, NMDA and AMPA receptor expression, gene expression and electrical excitability (Walker & Davis, 2002; Perusini et al., 2015; Rosenkranz et al., 2010; Shin et al., 2004; Bremner et al., 2005; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000; Hendler et al., 2003; Suvrathan et al., 2014; Jacob & Moghaddam, 2020; Miracle et al., 2006; Pennington et al., 2020; Ponomarev et al., 2010). This is paralleled in humans by increased risk for anxiety disorders following sustained stress or trauma, and enhanced amygdala activity in individuals with post-traumatic stress disorder during fear conditioning, recall of traumatic events and viewing of emotional stimuli in the lab (Butler et al. 1990; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Shin et al., 2004; Bremner et al., 2005; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000; Pechtel & Pizzagalli, 2011). This could be related to the cellular and genetic changes described that accompany stress-enhanced fear learning in rodent models (Rau et al., 2005; Rau & Fanselow, 2009; Perusini et al.,

2016; Ponomarev et al., 2010; Rosenkranz et al., 2010). While this poses promising evidence to understanding the mechanisms by which stress facilitates future fear learning, there is still progress to be made towards understanding and remedying pathological fear behavior.

There is a wealth of research on how prior stress affects future behavior, but a marked lack of investigation on the potentially beneficial effects of prior rewarding experiences. If prior positive experiences influence the same circuits that stress do, this could provide a therapeutic avenue to correct some of the pathological changes induced by stress. The Sharpe lab recently found that prior Pavlovian reward learning experience does in fact change the neural circuits that encode fear (Sharpe et al., 2021). This work focused on the lateral hypothalamus (LH), a region known for its role in feeding, reward processing and motivational drives, but not in fear learning (Anand & Brobeck, 1951; Barbano et al., 2016; Hoebel & Teitelbaum, 1962; Margules & Olds, 1962; Roberts, 1980; Stanley et al., 1993; Wise, 1974; Nieh et al., 2016; Stuber & Wise, 2016). Recent studies have found that glutamatergic and GABA neurons in LH (LH_{GABA}) drive avoidance and defensive behaviors through projections to the paraventricular nucleus and periaqueductal gray, but historically the majority of LH research focused on feeding and motivated behaviors. For example, rats will work to self-stimulate LH_{GABA} and stimulating this same neuron population will lead to increased feeding and food seeking behavior (Margules & Olds, 1962; Hoebel & Teitelbaum, 1962; Nieh et al., 2016; Jennings et al., 2015). This work led to exploration into whether LH_{GABA}'s role expands from feeding into learning about food. Sharpe et al. (2017) in fact found that LH_{GABA} neurons are essential for learning a cue that leads to food. Following those results, Sharpe et al. (2021) discovered that while LH_{GABA} are not necessary for fear conditioning in experimentally naïve rats, they become necessary for this process after reward learning experience. This finding indicates that rewarding experiences do influence how neural circuits

support future learning. **Experiment 2** explored how the recruitment of LH_{GABA} to encode fear affects the canonical fear circuits surrounding the BLA. Because LH_{GABA} encodes fear memories after reward experience, the BLA may not be as important for acquiring and storing these same memories. **Experiment 2** combined Pavlovian reward and fear conditioning with optogenetic inhibition in rats to investigate if inhibiting neural activity in the BLA still disrupts fear conditioning after reward experience. Rats received reward learning followed by fear conditioning with either BLA or LH_{GABA} inhibition during the cue to replicate Sharpe et al.'s (2021) results and reveal how the reward intervention affects the importance of the BLA.

Methods

Subjects: A total of 62 male and female Long Evans rats were used in these experiments. The rats were individually housed and kept on a 12-hour light-dark cycle. All behavioral testing took place during the light cycle. Rats weighed a minimum of 280g and were randomly allocated to groups prior to surgical procedures. For **Experiment 1** rats remained on ab libitum food access and for **Experiment 1B** and **2** rats were food restricted and maintained at 85% of their free-feeding body weight 1 week prior to testing. Wildtype rats were used for optogenetic BLA inhibition. For cell-type specific manipulation of LH_{GABA} neurons transgenic GAD-Cre rats that expressed Cre-recombinase under the control of the glutamate decarboxylase-1 (GAD) promoter were used. All experimental procedures were approved by the University of California Institutional Animal Care and Use Committee.

Surgical Procedures: To target BLA neurons, rats were prepared surgically with bilateral infusions of 0.6 µL of the inhibitory opsin (Fig 2B/Fig2B; Addgene; AAV9-CamKIIa-eNpHR3.0-eYFP; 0.6µL) into the BLA (-2.7AP, +/-5.0ML, -8.8DV). To target LH_{GABA} neurons, transgenic GADCre rats received bilateral infusions of 1 µL of the Cre-dependent inhibitory opsin (Fig 3B; Addgene; AAV5-Ef1a-DIOeNpHR3.0-eYFP) in LH (AP -2.4, ML ±3.5, DV -8.4F/9.0M at a 10° angle towards midline). Controls were prepared with a virus without halorhopdopsin (Addgene; AAV8-CaMKIIa-eGFP for BLA and AAV5-Ef1a-DIO-eYFP for LH_{GABA}). All viruses were infused at a rate of 0.1 µL per minute with needles left at the infusion site for 10 mins to allow diffusion. Optic fibers were implanted 0.5mm above infusion site.

Behavioral Procedures:

Experiment 1: Rats were surgically prepared for optogenetic inhibition of the BLA and counterbalanced into "tone" (n=11) and "shock" (n=12) groups. For the "tone" group, laser was delivered to inhibit the BLA during only the tone. For the "shock" group laser was delivered to inhibit the BLA during only tone. Rats were food restricted 1 week prior to testing. The procedure was adapted from Sharpe et al. (2021)[4]. All behavioral testing took place in MedPC modular behavioral chambers. For fear conditioning, the chambers contained only a house light and grate flooring and were cleaned between sessions with 20% ethanol. For optogenetic manipulation DPSS lasers (532 nm; Shanghai Laser and Optics Century Co., Shanghai, China) connected to a dual-connection rotary joint commutator were attached to rats via two armored fiber-optic patch cords (Doric Lenses, Quebec, Canada). Light leakage was obscured by 5-cm black shrink tube shielding on the patch cord and cannula ferrules. Fear conditioning consisted of 4 days of training: habituation, acquisition, context extinction and extinction testing (Fig 2A). On day 1 rats were

habituated to the behavioral chamber for 30 minutes. On day 2, fear acquisition consisted of three tone-shock pairings with a 10 s, 77 dB tone and 1 s, 0.55mA foot shock with 7-minute variable intertrial intervals (ITIs). For optogenetic inhibition, continuous laser was delivered at 18mW beginning 500ms before onset of the shock or tone and concluding 500ms after tone or shock offset. A one second gap was left between the tone and shock to account for any effect after discontinuing optogenetic inhibition, although evidence suggests there is no rebound effect after 10 s of inhibition with halorhodopsin (Sharpe et al. 2017; Mahn et al., 2016). On day 3, rats were reintroduced to the chamber without stimuli for 30 minutes to extinguish context-related freezing. On day 4, rats received extinction testing with 5s, 10 s tone presentations (without shocks) and 7-minute variable ITIs.

Experiment 1B – Pilot: After data collection for **Experiment 1** concluded the same cohort of rats were counterbalanced into "reward" and "naïve" groups to pilot test how reward learning experience might affect the importance of the BLA for fear conditioning (Fig 2F-G). Rats were food restricted one week prior to training. "Reward" rats were pre-exposed to sucrose pellets in their homecage and received appetitive Pavlovian conditioning with an auditory cue and sucrose pellets. "Naïve" subjects received the cue alone as a control. Procedures for **experiment 1B** and **experiment 2** are adapted from Sharpe et al. (2021)[4]. Throughout testing, care was taken to deliver food to naïve subjects in a way that prevented any cue from predicting mealtime (to avoid cue-food learning naïve controls). Reward training consisted of 5 days of 12 pairings of a 10 s auditory cue (click or white noise, counterbalanced with fear cue) and two units of 45mg sucrose pellets (TestDiet, MA) with 4-minute variable ITIs. Naïve controls were exposed to the auditory cue alone without the sucrose pellets (Fig 5B). Reward/naïve training was conducted in context A: chambers

equipped with a pellet receptacle, plexiglass flooring and sandpaper walls that were wiped down with water between sessions (context A). The following week, rats began fear conditioning in context B: a unique set of chambers equipped with a house light and grate flooring that were cleaned with 20% ethanol between sessions. Fear conditioning involved three days of training. Day 1 was fear acquisition with three presentations of 10 s auditory cues (click or white noise, counterbalanced with reward cue) paired with a 1 s 0.55-mA footshock with 7-minute variable ITIs to test acquisition of associative fear memories. As in **experiment 1**, a 1 s gap was left between the tone and shock. Laser delivery for optogenetic inhibition of the BLA began 500ms before tone onset and discontinued 500ms after tone offset. On day 2, rats returned to the fear chamber for 30 minutes to extinguish contextual fear. On day 3 rats received extinction testing with 5 presentations of the tone with 7-minute ITIs to assess memory encoding.

Experiment 2: A new cohort of rats were surgically prepared for either optogenetic inhibition of the BLA (wildtype; n=21) or LH_{GABA} (GADCre; n=19) to test if prior reward experience changes the importance of the BLA or LH_{GABA} in supporting fear conditioning (Fig 3B). Rats received reward training or naïve control training followed by fear conditioning with optogenetic inhibition of BLA or LH_{GABA} during the (Fig 3A). Behavioral procedure was adapted from Sharpe et al. (2021) and **experiment 1B**. Rats were food restricted one week prior to testing and counterbalanced into "reward" and "naïve" groups (Fig 3B). "Reward" rats were pre-exposed to sucrose pellets in their homecage and received appetitive Pavlovian conditioning to learn an association between a light and sucrose pellets, while "naïve" subjects received the light alone as a control. A 10 s house light and a 10 s, 77dB tone were used as the reward and fear cues, respectively, to further encourage discrimination between reward and fear learning. This differs

from **experiment 1B** where two distinct auditory cues were used for reward and fear training. Reward learning was conducted concurrently with fear context exposure. Rats were pre-exposed to context B during the 5 days of reward training to facilitate context discrimination. The reward training – pre-exposure schedules were counterbalanced for time of day and training order. For example, a rat might receive pre-exposure in the morning and reward training in the afternoon on day 1 and then the opposite on day 2. The 4-day fear conditioning procedure began the following week. Days 1 and 2 were acquisition training with three pairings of the 10 s, 77dB tone with the 0.55mA shock. The second day of training was added due to insufficient freezing after 1 day of training. Optogenetic inhibition to the BLA or LH_{GABA} was delivered during the 10 s tone on days 1 and 2. Day 3 consisted of 30 minutes of context extinction and on day 4 rats received extinction testing with 5 tone presentations.

Histology: Rats were euthanized with an overdose of carbon dioxide and perfused with phosphate buffered saline followed by a 4% paraformaldehyde (Sigma-Aldrich) solution. Extracted brains were kept in 4% paraformaldehyde for 24 hours and then stored in 18% sucrose. After a minimum of 48 hours brains were cryostat sectioned at 40um and imaged on a confocal microscope (Zeiss) to determine spread and accuracy of virus and fiber tips. Images were processed in Fiji (ImageJ) and Adobe Photoshop and Illustrator.

Data and statistical analysis: Fear conditioning sessions were video recorded and hand scored for freezing behavior during the 10 s before, during and after the tone during acquisition and extinction sessions. Each 10-s period was divided into five, 2-s bins which were evaluated for displays of freezing. For acquisition, freezing on each trial was analyzed for effects of trial

(**experiment 1** & **1B**) or day (**experiment 2**) and between-subject differences for rats with NpHR vs eYFP/eGFP viruses. For extinction, average freezing across all cue presentations was analyzed for between-subject differences (NpHR vs eYFP/eGFP). Reward training data was collected by MedPC-V software. Reward learning was evaluated by calculating average magazine entries made during the 10 s cue minus entries made during the 10 s pre-CS baseline period for each rat. Statistical analyses were conducted using SPSS 28 IBM statistics package. Mixed factor ANOVAs were used to assess performance across learning and between groups for reward and fear learning and between-subjects ANOVAs were used to evaluate group differences during extinction testing. The sample sizes were determined from previous publications with similar methods and findings (Sharpe et al., 2017; Sharpe et al. 2021).

Results

Optogenetically inhibiting the BLA during the tone, but NOT the shock of fear conditioning disrupted learning and memory.

Decades of research showing attenuated fear learning and memories after the BLA was disrupted with lesions, muscimol and prolonged optogenetic inhibition designated this as the likely site of acquisition and storage of associative fear memories. The current theory suggests that information about the cue and shock coming from thalamic and cortical regions converge and are stored in the BLA. **Experiment 1** took advantage of the temporal specificity of optogenetics to test whether inhibiting the BLA during only the tone or only the shock would disrupt fear conditioning. Rats were surgically prepared for optogenetic inhibition of the BLA with bilateral

infusions of halorhopdopsin (AAV9-CamKIIa-eNpHR3.0-eYFP) or an eGFP control (AAV8-CamKIIa-eGFP) and optic fiber implants (Fig 2B). After four weeks, rats received fear conditioning with three tone-shock presentations and optogenetic BLA inhibition delivered during either only the tone or only the shock (Fig 2A). Two days later, extinction tests were conducted with five presentations of the tone (no optogenetic manipulation). Freezing behavior during the tone on the acquisition and extinction days was hand scored to evaluated learning and memory. Rats with inhibition during both the tone and shock displayed a significant increase in freezing across trials (Tone: $F_{2,18}$=7.774, p=0.004, η^2=0.463; Shock: $F_{2,20}$=12.104, p<0.001, η^2=0.548). Rats with inhibition during the tone showed a significant between-subjects effect during both acquisition (Fig 2D; NpHR vs eGFP: $F_{1,9}$=9.254, p=0.014, η^2=0.507) and extinction (NpHR vs eGFP: $F_{1,9}$=5.634, p=0.042, η^2=0.385). Rats with inhibition during the shock showed no between-subject differences during acquisition (Fig 2E; NpHR vs eGFP: $F_{1,9}$=.021, p=0.887) or extinction (NpHR vs eGFP: $F_{1,10}$=0.300, p=0.596). No interactions were present between trial and group for either treatment (Tone: $F_{2,18}$=2.965, p=0.077; Shock: $F_{2,20}$=1.358, p=0.280).

Inhibiting the BLA during the tone of fear conditioning in rats with Pavlovian reward learning experience did not disrupt fear memories.

The Sharpe lab previously found that if rats have Pavlovian reward learning experience, LH_{GABA} neurons become necessary to support fear conditioning (Sharpe et al. 2021). There is no prior evidence that LH supports fear conditioning – fear memories are traditionally stored in the BLA (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). This experiment began to explore how the reward

experience induced shift to LH_{GABA}-dependent memories influence the importance of the BLA for storing these same memories. **Experiment 1B** repurposed the rats from **experiment 1** that were prepared for optogenetic inhibition of the BLA. These rats were counterbalanced into "reward" and "naïve" groups (Fig 2F-G). The reward group received Pavlovian reward training (five days with 12 auditory cue-sucrose pairings) while the naïve group received the auditory cue alone as a control. Magazine entries during 10 s cue minus the 10 s baseline period before the cue were calculated and analyzed to evaluate reward learning. Reward rats showed a significant increase in magazine entries across days (Fig 2F; Reward: $F_{4,36}$=22.780, p<.001, partial η^2=0.717) and naïve rats did not show an increase in magazine entries (Fig 2G; Naïve: $F_{4,32}$=0.927; p=0.461). Neither reward nor naïve rats showed a significant group difference (Fig 2F-G; Naïve – NpHR vs eYFP: $F_{(1,8)}$=0.773; p=0.405; Reward – NpHR vs eYFP: $F_{1,9}$= 0.069; p=0.798). Naïve rats showed a significant group x day interaction, while reward rats did not (Naïve: $F_{4,32}$= 2.953, p=0.035, partial η^2=0.270; Reward: $F_{4,36}$= 0.261; p=0.901). After the reward phase, all rats received fear conditioning with a 10 s auditory cue (click or white noise, counterbalanced) followed by a 1 s, 0.55mA shock. The auditory cues were distinct from the tone fear cue in **experiment 1** and were counterbalanced such that if a rat had a click reward cue they has a white noise fear cue, and vice versa. Both the reward and naïve rats showed a significant increase in freezing across trials on the acquisition day (Fig 2F-G**;** Reward: $F_{2,18}$=7.026, p=0.006, partial η^2=0.438; Naïve: $F_{2,16}$=3.822, p=0.044, partial η^2=0.323). There was no significant group difference during acquisition for reward or naïve rats (NpHR vs eYFP: Reward – $F_{1,9}$=2.167; p=0.175; Naïve – $F_{1,8}$=0.513; p=0.494). There was also no trial x group interaction for reward or naïve rats (Reward: $F_{2,18}$=1.346; p=0.285; Naïve: $F_{2,16}$=0.622; p=0.549). For extinction testing, rats received 5 tone presentations with no shock or optogenetic

manipulation – freezing was averaged across all presentations and analyzed for between-subject differences. For the extinction test, there was a trend towards group difference in freezing for naïve rats, but not reward rats (Fig 2F-G; NpHR vs eYFP: Reward – $F_{1,9}$=.017, p=0.898; Naïve: $F_{1,8}$=3,514; p=0.098; partial η^2=0.305). Although the NpHR vs eYFP naïve group difference did not reach statistical significance, the effect size was of similar magnitude to the effect of BLA inhibition on freezing observed in **experiment 1**.

In naïve rats, the BLA was necessary for encoding fear memories and LH_{GABA} neurons were not. After Pavlovian reward learning, the BLA was no longer necessary for encoding fear memories but LH_{GABA} neurons were.

Previous evidence from Sharpe et al. (2021) and **experiment 1B** found that after reward learning experience, fear conditioning is no longer dependent on the BLA, but becomes dependent on LH_{GABA} neurons (Fig 2). **Experiment 2** demonstrated that the same five-day Pavlovian reward learning treatment recruits LH_{GABA} neurons to support fear conditioning while rendering the BLA unnecessary for this same memory process (Fig 3). Rats received viral infusion and fiber optic cannula implant surgery to prepare them for optogenetic inhibition of BLA or LH_{GABA} neurons (Fig 3B-C). A halorhopdopsin virus (AAV9-CamKIIa-eNpHR3.0-eYFP) or eGFP control (AAV8-CamKIIa-eGFP) was used in wildtype rats for BLA inhibition. A Cre-dependent halorhodopsin virus (AAV5-Ef1a-DIOeNpHR3.0-eYFP) or eYFP control (AAV5-Ef1a-DIO-eYFP) was used in GAD-Cre rats for LH_{GABA} inhibition. Rats were assigned as "reward" or "naïve" and began training a minimum of 4 weeks after surgery (Fig 3A). This created four groups: LH_{GABA}-reward, LH_{GABA}-naïve, BLA-reward and BLA-naïve. Reward rats received five

days of 12 light-sucrose pellet pairings while naïve rats received an equal number of light-alone presentations as a control. This was changed from the auditory cue used for reward training in **experiment 1B** to differentiate the modalities of the reward and fear cues. Magazine entries during 10 s cue minus the 10 s baseline period before the cue were calculated and analyzed to evaluate reward learning. The LH_{GABA} and BLA reward groups of both genotypes showed a significant increase in magazine entries across days (Fig 3E & G; LH_{GABA}: $F_{4,32}$=19.686, $p<0.001$, partial η^2=0.711; BLA: $F_{4,40}$=6.727, $p<0.001$, partial η^2=0.402) but the naïve group did not (Fig 3D & F; LH_{GABA}: $F_{4,28}$=1.901; p=0.138; BLA: $F_{4,32}$=0.947; p=0.450). Neither reward nor naïve rats for LH_{GABA} or BLA showed a significant effect of group (NpHR vs eYFP: LH_{GABA}-reward – $F_{1,8}$=1.117; p=0.321**;** LH_{GABA}-naïve – $F_{1,7}$=0.006; p=0.941; BLA-reward: $F_{1,10}$=0.345; p=0.570; BLA-naïve: $F_{1,8}$=1.168; p=0.311). Neither reward nor naïve rats for LH_{GABA} or BLA showed a significant group x day interaction (LH_{GABA}-reward: $F_{4,32}$= 2.236; p=0.087; LH_{GABA}-naïve: $F_{4,28}$=1.501; p=0.229; BLA-reward: $F_{4,40}$=.291; p=0.882; BLA-naïve: $F_{4,32}$=1.066; p=0.389). The following week, all rats began fear conditioning with inhibition of either LH_{GABA} or the BLA during the tone (Fig 3A). Rats received two days of acquisition training with three tone-shock presentations on each day. Percent freezing was hand scored during the 10 s tone and average freezing across the three tone presentations was calculated for each day and analyzed for an effect of training day on freezing levels. Both BLA-reward and BLA-naïve rats showed a significant increase in freezing across days (Fig 3F-G; BLA-reward: $F_{1,9}$=5.581, p=0.042, partial η^2=0.383; BLA-naïve: $F_{1,7}$=10.234, p=0.015, partial η^2=0.594). Neither BLA-reward nor BLA-naïve rats showed a significant effect of group during acquisition (NpHR vs eYFP: BLA-reward – $F_{1,9}$=.009; p=0.926; BLA-naïve – $F_{1,7}$=1.049; p=0.340). Neither BLA-reward nor BLA-naïve rats showed a significant group x day interaction during acquisition

(BLA-reward: $F_{1,9}$=.079; p=785; BLA-naïve: $F_{1,7}$=0.433; p=0.532). LH_{GABA}-reward rats showed a significant increase in freezing across days during acquisition (Fig 3E; LH_{GABA}-reward**:** $F_{1,8}$=5.837, p=0.042, partial η^2=0.422**),** but LH_{GABA}-naïve rats did not (Fig 3D; LH_{GABA}-naïve: $F_{1,7}$=2.925, p=0.131). Neither reward nor naïve rats for LH_{GABA} showed an effect of group (NpHR vs eYFP: LH_{GABA}-reward – $F_{1,8}$=2.445; p=0.157; LH_{GABA}-naïve – $F_{1,7}$=.037; p=0.853). Neither LH_{GABA}-reward nor LH_{GABA}-naive groups showed a significant group x day interaction (LH_{GABA}-reward: $F_{1,8}$=0.434; p=0.528; LH_{GABA}-naïve: $F_{1,7}$=.008; p=0.931). For extinction testing, rats received five tone presentations with no shock or optogenetic manipulation. Freezing was averaged across all five tone presentations and analyzed for between-subject differences. The BLA-naïve group showed a significant between-subjects difference but the BLA-reward group did not (Fig 3F-G; NpHR vs eGFP: BLA-reward – $F_{1,9}$=.120; p=0.737; BLA-naïve – $F_{1,7}$=14.939, p=0.005, partial η^2=0.651). The LH_{GABA}-reward group showed a significant between-subject difference, but the LH_{GABA}-naïve group did not (Fig 3D-E; NpHR vs eYFP: LH_{GABA}-reward – $F_{1,8}$= 40.143, p<0.001, partial η^2=0.843; naïve – $F_{1,7}$=1.397; p=0.276).

Discussion

These experiments sought to expand on our existing knowledge about the role of the BLA in supporting fear learning. **Experiment 1** demonstrated that optogenetic inhibition of the BLA during tone-shock fear conditioning disrupted acquisition and expression of a freezing response if the BLA was inhibited during the tone, but freezing responses remain intact if the BLA was inhibited during the shock (Fig 2). This finding reiterates that the BLA is necessary for

processing and learning about the tone, which aligns with the findings of many prior studies (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). However, the evidence that the BLA is not necessary for processing the shock contradicts the currently accepted model of fear learning in the BLA. This model suggests that information about the tone and shock are relayed through thalamic and cortical projections to the BLA where an associative memory is formed and stored (Cousens & Otto, 1998; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Koo et al., 2004; Maren et al., 1996; Maren, & Fanselow, 1996; McDannald & Galarce, 2011; Muller et al., 1997; Phillips & LeDoux, 1992; Sparta et al., 2014). The results in Fig 2 demonstrate that rats acquired and expressed fear memories even though the BLA was inhibited during the shock. This would indicate that other brain regions can support associative fear learning without input from the BLA, and the BLA is more important for processing and learning about the tone than the shock.

The results of **experiment 1B** demonstrated that optogenetic BLA inhibition during the tone of fear conditioning had no effect on expression of conditioned freezing responses in rats with Pavlovian reward learning experience. Sharpe et al. (2021) found that after reward learning experience, LH_{GABA} neurons became necessary for acquisition and expression of fear memories. An important question following these results is how recruitment of LH_{GABA} neurons for supporting fear conditioning influences the importance of the BLA for this same process, since the BLA is the site of acquisition and storage of these memories in experimentally naïve rats (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). **Experiment 1B** provided compelling evidence that rats with reward learning experience can successfully encode fear memories while the BLA is

inhibited (Fig 2F-G). Rats with prior reward learning experience demonstrated intact fear memories during extinction testing, showing that BLA inhibition did not disrupt fear memory encoding (Fig 2E). However, naïve rats showed attenuated freezing during extinction testing of fear conditioning, indicating disrupted encoding of fear memories (Fig 2G). Although the effect of BLA inhibition in naïve rats only reached a statistical trend, given the similar effect size in **experiment 1** (Fig 2D)**,** the robust existing literature and our replication of these results in **experiment 2** (Fig 3F) it is reasonable to conclude that the behavioral irregularity in **experiment 1B** was a carryover effect from the rats' receiving two rounds of fear conditioning (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). The results of **experiment 1B** built upon the finding from Sharpe et al. (2021) and support our hypothesis that after reward learning, LH_{GABA} neurons encode fear memories instead of the BLA. To expand the results of this pilot study, we replicated this experiment in a new cohort of rats in **experiment 2**.

The results of **experiment 2** replicated the findings from Sharpe et al. (2021) and **experiment 1B** (Fig 2D-E). **Experiment 2** found in naïve rats, the BLA was necessary for fear conditioning and LH_{GABA} neurons were not (Fig D & F). But, in rats with reward learning experience the importance of these regions changed. In rats with reward learning experience, LH_{GABA} neurons became necessary for fear conditioning and the BLA was no longer necessary (Fig 3E & G). For this experiment rats were either trained on Pavlovian reward learning or remained naïve. Next, rats received tone-shock fear conditioning with optogenetic inhibition to the BLA or LH_{GABA} during the tone. All rats successfully acquired a freezing response to the tone during training indicating that they learned that the tone predicted a shock. There was no effect of optogenetic

inhibition of the BLA or LH_{GABA} on freezing levels during acquisition – the effect of the inhibition only became evident during extinction testing. In naïve rats, those with BLA inhibition froze significantly less to the tone than controls while LH_{GABA} inhibition had no effect on freezing levels (Fig D & F). These results were expected given the documented role of the BLA in supporting fear learning in naïve rats and the lack of evidence of involvement of LH_{GABA}. However, the opposite was true in rats with reward experience. BLA inhibition no longer had any effect on freezing levels (Fig G), but rats with LH_{GABA} inhibition froze significantly less than controls (Fig E). These results indicate that prior experience with reward learning fundamentally changed the neural circuits that support fear. Decades of research have established the BLA as the hub of associative fear learning, but introducing a simple experience with reward learning rendered the BLA unnecessary for this process. Instead, LH_{GABA}, a population of neurons only recently implicated in fear learning became responsible for encoding these memories.

While LH_{GABA} neurons have not previously been implicated in fear learning, other circuits within LH have (Chen et al., 2020; Li et al., 2018; Wu et al., 2015). Exposure to predator odors increases activity of glutamatergic activity in LH, and manipulation of this neuron population exerts bidirectional control over defensive behaviors such as avoidance and fleeing. This likely occurs via glutamatergic projections from the BLA to LH which synapse onto LH projections to the paraventricular nucleus (Chen et al, 2020). Glutamatergic LH neurons that project to the periaqueductal gray control evasion behavior, while LH_{GABA} neurons that project to the periaqueductal gray bidirectionally control predatory attack in mice (Li et al., 2018). Alternatively, stimulation and inhibition of LH_{GABA} and glutamate cells have the opposite effects on feeding behavior, suggesting these neuron populations exert opposing control over similar

behaviors (Nieh et al., 2016). While LH_{GABA} and glutamatergic neurons are both implicated in rewarding and fearful behaviors, there is no evidence that they communicate via local circuits in LH (Burkadov & Karnani, 2020). Instead, they may play a role in regulation and balance of rewarding and fear-driven behavior through distinct relationships with the overlapping regions they send and receive projections from (i.e., BLA, periaqueductal gray, paraventricular nucleus; Chen et al., 2020; Wu et al., 2015; Nieh et al., 2016).

The detrimental effect of prior stressful experiences on the neural circuits that support fear learning and memory has been well-documented (Walker & Davis, 2002; Perusini et al., 2015; Rosenkranz et al., 2010; Bremner et al., 2005; Rauch et al., 2000; Hendler et al., 2003; Suvrathan et al., 2014; Jacob & Moghaddam, 2020; Miracle et al., 2006; Pennington et al., 2020; Ponomarev et al., 2010; Butler et al. 1990; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Shin et al., 2004; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Pechtel & Pizzagalli, 2011). **Experiment 2** demonstrated that a positive associative learning experience fundamentally changed the neural circuits that support fear conditioning. Humans have robust variability in their prior experience, positive and negative associations, and it is crucial to take this into account in our rodent models of the neural circuits that drive learning and memory. This finding demands more research into the nature of LH_{GABA}-dependent fear memories.

DiFazio et al. (2022) posits the theory that skewing the neural circuits that drive fear and reward learning too far in either direction could beget pathological behavior. We know that stressful experiences facilitate future fear learning and increase the risk for anxiety disorders, maybe rewarding or positive experiences could tilt this balance back toward equilibrium and offer a

protective mechanism against the facilitation of fear learning that underlies anxiety-based pathologies. If this is the case, we must further explore the role of LH_{GABA} neurons and determine if they constitute a therapeutic target to reduce risk for problematic fear learning in the future.

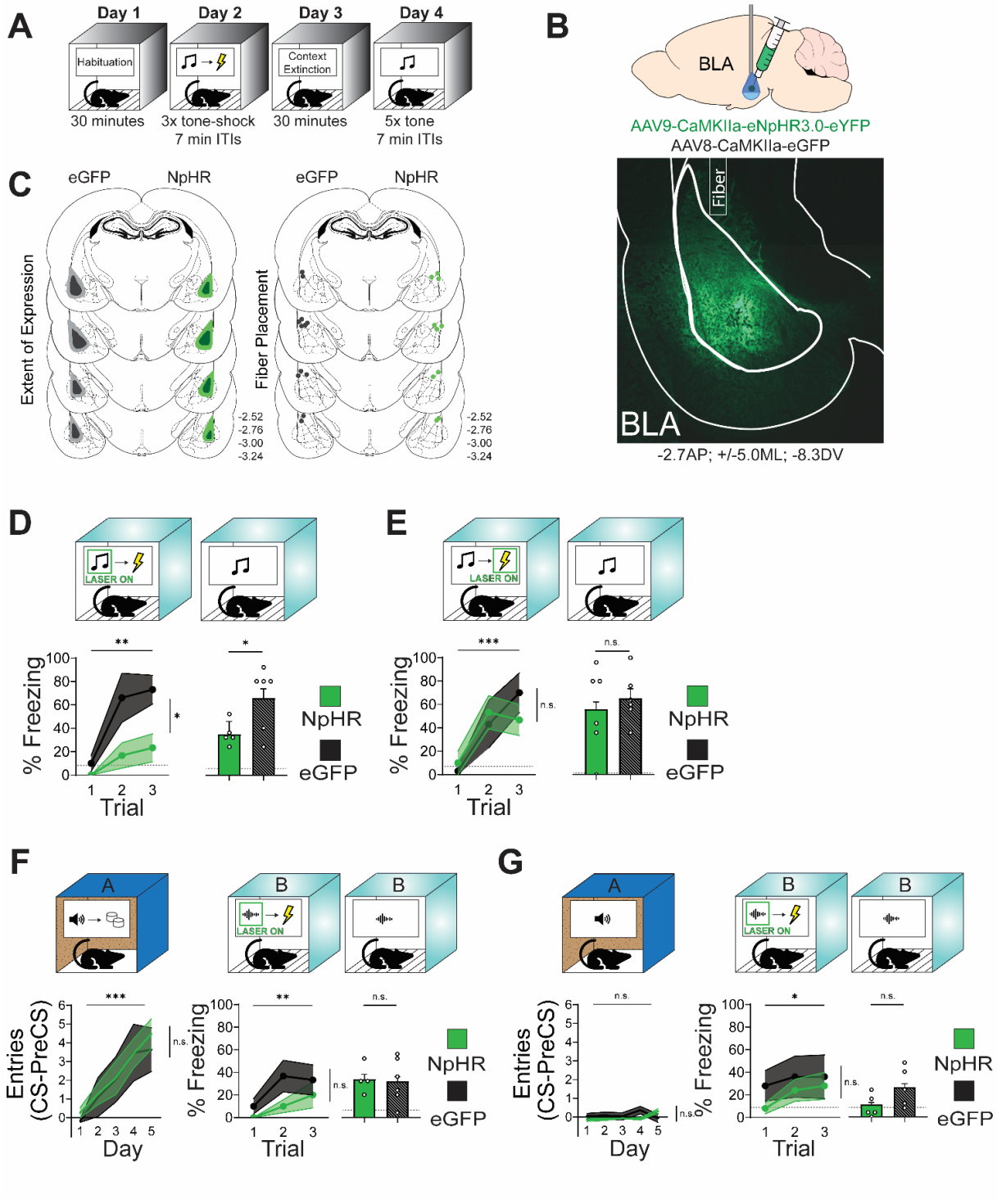
A
Day 1
Day 2
Day 3
Day 4
Habituation
Context Extinction
30 minutes
3x tone-shock 7 min ITIs
30 minutes
5x tone 7 min ITIs
B
BLA
AAV9-CaMKIIa-eNpHR3.0-eYFP
AAV8-CaMKIIa-eGFP
Fiber
BLA
-2.7AP; +/-5.0ML; -8.3DV
C
eGFP
NpHR
eGFP
NpHR
Extent of Expression
Fiber Placement
-2.52
-2.76
-3.00
-3.24
-2.52
-2.76
-3.00
-3.24
D
LASER ON
% Freezing
Trial
NpHR
eGFP
E
LASER ON
% Freezing
Trial
NpHR
eGFP
F
A
B
B
LASER ON
Entries (CS-PreCS)
Day
% Freezing
Trial
NpHR
eGFP
G
A
B
B
LASER ON
Entries (CS-PreCS)
Day
% Freezing
Trial
NpHR
eGFP

Figure 2: Inhibiting the BLA during tone, but not the shock, of Pavlovian fear conditioning disrupted learning and memory. (A) Schematic of fear conditioning procedure. (B) Schematic of optogenetic approach: wildtype rats were infused bilaterally with a halorhopdopsin (n=10) or control virus (n=11) along with optic fibers in the BLA to allow for inhibition of CaMKII+ neurons. (C) *Left:* Unilateral representation of viral expressions in cell bodies in the BLA; *Right:* Unilateral representation of approximate fiber tip placement in the BLA. (D-E) Rats learned that a tone leads to a shock and received tone only extinction without inhibition two days later; The dotted lines represent average pre-CS freezing for all subjects. (D) BLA neurons were inhibited in the during the tone, which led to significantly reduced freezing during acquisition and extinction relative to eGFP controls. (E) BLA neurons were inhibited during the shock, which did not affect freezing levels during acquisition or extinction. (F-G) The same rats were counterbalanced into new groups and received cue-sucrose training (F) or cue-only presentations as a control (G) before receiving a second round of fear conditioning with a new auditory cue (click or white noise, counterbalanced with reward training). (F) In reward experienced rats, inhibition of BLA neurons during the cue of fear conditioning had no effect on freezing levels during acquisition or extinction. (G) In naïve rats, BLA inhibition resulted in a trend towards disrupted freezing levels during extinction.

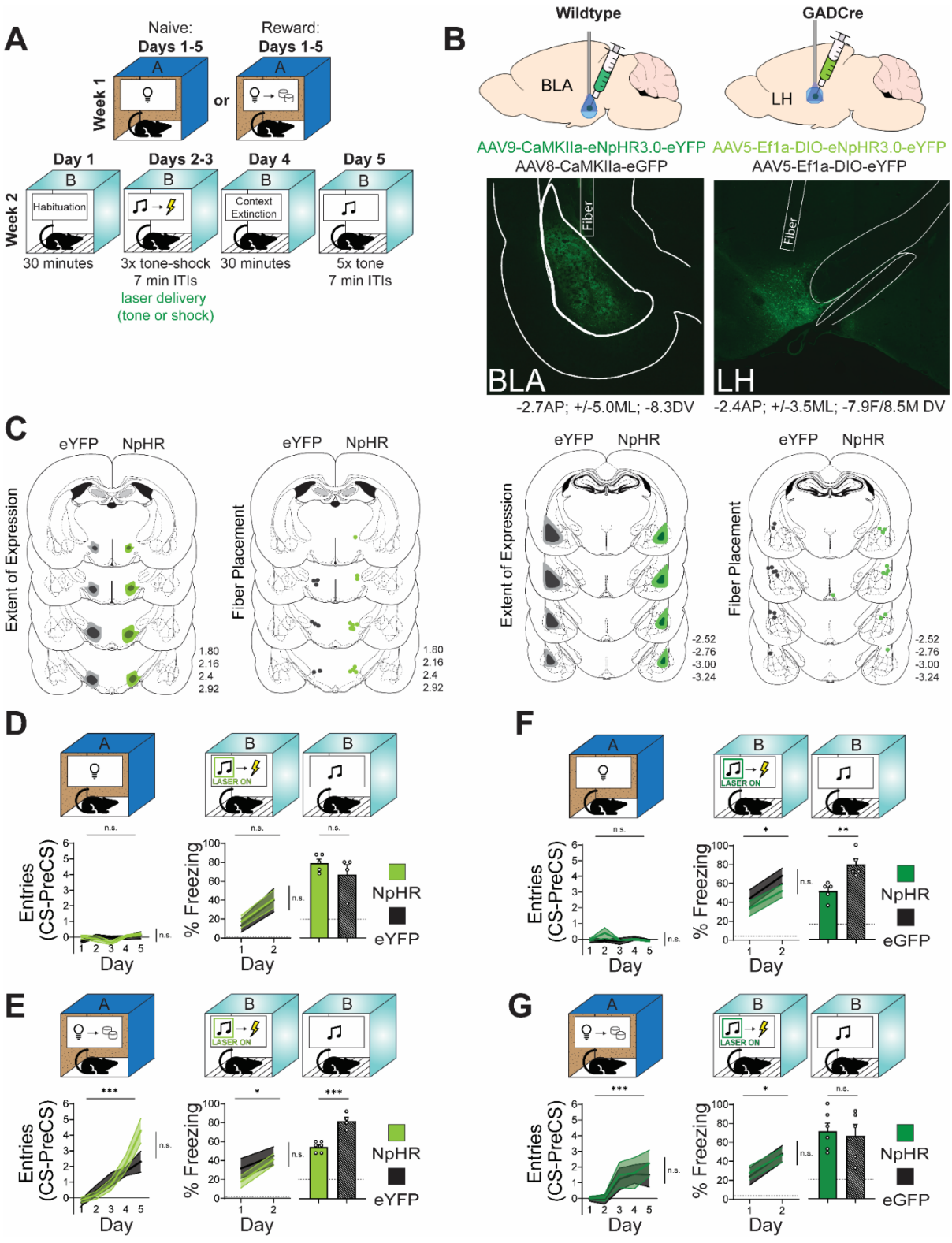
A
Naive:
Days 1-5
Reward:
Days 1-5
Week 1
or
Day 1
Days 2-3
Day 4
Day 5
Week 2
Habituation
Context Extinction
30 minutes
3x tone-shock
7 min ITIs
laser delivery
(tone or shock)
30 minutes
5x tone
7 min ITIs
B
Wildtype
GADCre
BLA
LH
AAV9-CaMKIIa-eNpHR3.0-eYFP
AAV8-CaMKIIa-eGFP
AAV5-Ef1a-DIO-eNpHR3.0-eYFP
AAV5-Ef1a-DIO-eYFP
Fiber
-2.7AP; +/-5.0ML; -8.3DV
-2.4AP; +/-3.5ML; -7.9F/8.5M DV
C
eYFP
NpHR
Extent of Expression
Fiber Placement
1.80
2.16
2.4
2.92
-2.52
-2.76
-3.00
-3.24
D
F
E
G
LASER ON
n.s.
Entries (CS-PreCS)
% Freezing
Day
NpHR
eYFP
eGFP

Figure 3: In naive rats the BLA was necessary for fear memory encoding, but LH_{GABA} neurons were not. In reward experienced rats, LH_{GABA} neurons were necessary for fear memory encoding, but the BLA was not. (A) Schematic of behavioral procedures. (B) Schematic of optogenetic approach: wildtype rats were infused bilaterally with a halorhopdopsin (n=11) or control virus (n=10) and implanted with optic fibers in the BLA to allow for inhibition of CaMKII+ neurons. GADCre rats were infused bilaterally with a halorhodopsin (n=11) or control virus (n=8) to allow for inhibition of LH_{GABA} neurons. (C) *Left:* Unilateral representation of viral expressions in cell bodies and approximate fiber tip placement in the BLA; *Right:* Unilateral representation of viral expression in GAD+ neurons and approximate fiber tip placement in LH. (D-G) Rats received either light-cue reward training or light only presentations as a control before receiving tone-shock fear conditioning with either LH_{GABA} neurons; The dotted lines represent average pre-CS freezing for all subjects (F-G) or BLA neurons (D-E) inhibited during the tone. (D) In naïve rats, LH_{GABA} inhibition during the tone does not effect freezing levels during acquisition or extinction. (E) In reward experienced rats, LH_{GABA} inhibition during the tone significant reduces freezing during extinction testing relative to eYFP controls. (F) In naïve rats, BLA inhibition during the tone of fear conditioning significantly reduced freezing levels during extinction relative to eGFP controls. (G) In reward experienced rats, inhibition of BLA neurons during the tone had no effect on freezing levels during acquisition or extinction.

CHAPTER 3:

The effect of prior reward learning and chronic unpredictable stress on the neural circuits that encode fear learning.

Introduction

Ample evidence suggests the BLA is necessary for associative fear conditioning to occur (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997). If an associative fear memory is acquired without intervention, the BLA is also necessary for recall and expression of that memory (Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996). This means BLA inhibition both before and after acquisition results in decreased freezing responses that indicate deficient fear memories. The results of **experiments 1b** and **2** in chapter 2 of this dissertation suggest that after reward learning experience the BLA is not necessary for encoding fear memories, but LH_{GABA} neurons are. Rats with reward learning experience can successfully encode fear memories while the BLA inhibited, while their naïve counterparts show deficits in fear memory. The opposite is true in rats with LH_{GABA} inhibition. LH_{GABA} neurons are not necessary for fear learning in naïve rats, but become necessary once rats experience reward learning. During these experiments the BLA and LH_{GABA} were only inhibited during acquisition sessions and led to deficits in freezing behavior during subsequent extinction testing. **Experiment 2b** explored whether the same reward-experienced or naïve rats would show deficits in fear memory expression if they were trained on a new fear association and received BLA or LH_{GABA} inhibition during extinction testing. This allows us to understand whether these regions are necessary for expression of normally encoded fear memories. Rats were re-trained on a new fear conditioning pairing (white noise-shock) with

either the BLA or LH_{GABA} optogenetically inhibited during extinction testing to pilot test if these regions are necessary for memory expression.

In chapter 2, I concluded that optogenetic inhibition to the BLA during the cue of Pavlovian fear conditioning disrupts fear memory encoding in naïve rats, but not rats with reward learning experience. Using optogenetics is beneficial because the tight temporal control allowed for the conclusion that the BLA is specifically necessary during the cue. However, the possibility remains that the reduced dependence on the BLA is due to a shift in the temporal dynamics of neural activity. The BLA may remain important for fear learning after reward experience, but not specifically during the cue. If the timescale of BLA activity shifted, inhibiting during only the 10 s cue could have missed the essential time window to disrupt fear conditioning. The canonical studies that established the role of the BLA in fear learning inhibited across the whole training session (Cousens & Otto, 1998; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Koo et al., 2004; Maren et al., 1996; Maren, & Fanselow, 1996; McDannald & Galarce, 2011; Muller et al., 1997; Phillips & LeDoux, 1992; Sparta et al., 2014). **Experiment 3** investigated if BLA inhibition still has no effect on encoding fear memories if the inhibition is delivered across the entire acquisition phase. Chemogenetics was used to inhibit glutamatergic neurons in the BLA during acquisition in rats with or without reward learning experience. This approach built upon the results from chapter 2 by ruling out the possibility that brief optogenetic stimulation wasn't sufficient to disrupt learning because the timing of BLA activity shifted. By closing replicating the methods of the benchmark findings we can verify that reward learning experience shifts fear memory encoding away from the canonical fear circuit and reinforce the finding that reward learning renders the BLA unnecessary for encoding associative fear memories.

Experiences with extreme stress or trauma increase the likelihood of pathological or enhanced future fear learning (Rau et al., 2005; Rau & Fanselow, 2009). Individuals with PTSD show increased activity in the amygdala that correlates with self-reported severity of symptoms (Butler et al. 1990; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Shin et al., 2004; Bremner et al., 2005; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000). Understanding the neural circuits that drive fear behavior is key to targeting and reducing problematic fear memories. To do this, we must understand the mechanism by which stress facilitates these changes. Research with rodent models also finds that extreme stress enhanced future fear learning (Rau et al., 2005; Rau & Fanselow, 2009). These behavioral changes are accompanied by findings that that same stressful experience induces cellular, genetic and functional changes in the BLA (Walker & Davis, 2002; Perusini et al., 2015; Rosenkranz et al., 2010; Shin et al., 2004; Bremner et al., 2005; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000; Hendler et al., 2003; Suvrathan et al., 2014; Jacob & Moghaddam, 2020; Miracle et al., 2006; Pennington et al., 2020; Ponomarev et al., 2010). Newfound evidence that fear memories are dependent on LH_{GABA} rather than the BLA after reward learning leads to the question of whether these memories are enhanced by prior stress (Sharpe et al., 2021). The reward-induced shift from BLA to LH_{GABA} dependent fear memories may be adaptive and decrease the risk for future pathologies. If the nature of these memories is different since they are encoded by a distinct fear circuit, they could be less likely to lead to maladaptive learning in the future – indicating a mechanism by which positive experiences can increase resilience to anxiety disorders (Geschwind et al., 2010). Evidence suggests that compared to predictable stressors, unpredictable stress is a stronger risk factor for anxiety disorders that is also correlated with changes in the BLA (Wang et al., 2010). **Experiment 4**

explores if rats with reward learning experience show enhanced fear learning following a chronic unpredictable stress (CUS) treatment and whether this CUS experience reverts memories to dependence on the BLA. Here, rodents experienced reward learning followed by 7-days of CUS treatment before receiving optogenetic inhibition of the BLA during fear conditioning.

Methods

Subjects: A total of 65 male and female Long Evans rats were used in these experiments. The rats were individually housed and kept on a 12-hour light-dark cycle. All behavioral testing took place during the light cycle. Rats weighed a minimum of 280g and were randomly allocated to groups prior to surgical procedures. For all experiments, rats were food restricted and maintained at 85% of their free-feeding body weight 1 week prior to testing. Wildtype rats were used for optogenetic BLA inhibition. For cell-type specific manipulation of LH_{GABA} neurons transgenic GAD-Cre rats that expressed Cre-recombinase under the control of the glutamate decarboxylase-1 (GAD) promoter were used. All experimental procedures were approved by the University of California Institutional Animal Care and Use Committee.

Surgical Procedures: To target BLA neurons for optogenetic inhibition, rats were prepared surgically with bilateral infusions of the inhibitory opsin (Addgene; AAV9-CamKIIa-eNpHR3.0-eYFP; 0.6uL) into the BLA (-2.7AP, +/-5.0ML, -8.8DV; Fig 4). To target LH_{GABA} neurons for optogenetic inhibition, transgenic GADCre rats received bilateral infusions of a Cre-dependent inhibitory opsin (Addgene; AAV5-Ef1a-DIOeNpHR3.0-eYFP) in LH (AP -2.4, ML ±3.5, DV -8.4F/9.0M at a 10° angle towards midline). Controls received a virus without halorhopdopsin (Addgene; AAV8-CaMKIIa-eGFP for BLA and AAV5-Ef1a-DIO-eYFP for LH_{GABA}). Optic

fibers were implanted 0.5mm above infusion site. To target BLA neurons for chemogenetic inhibition, rats were prepared surgically with bilateral infusions (0.1 µL/minute) of the inhibitory designer receptor hM4Di (Addgene; AAV8-hSyn-hM4Di-mCherry; 0.5uL) into the BLA (AP -3.0, ML ±5.1, 3 µL at DV -8.1mm and 2 µL at DV -7.8). Controls received a virus without hM4Di (Addgene; AAV8-hSyn-mCherry; 0.5uL).

Behavioral Procedures

Experiment 2b: Rats that were previously prepared for optogenetic inhibition of the BLA (wildtype; n=21) or LH_{GABA} (GADCre; n=19) in **experiment 2** (to explore if prior reward experience changes the importance of the BLA or LH_{GABA} for fear conditioning) were repurposed for this pilot experiment. These rats previously received reward or naïve training and tone-shock fear conditioning with optogenetic inhibition of BLA or LH_{GABA} during the tone (Fig 3). No additional reward training was administered, and rats were trained on a new fear association with a 10 s white noise cue and 1 s, 0.55mA shock (Fig 4A). The 4-day fear conditioning procedure included two days of acquisition training with three white noise-shock pairings with 7-minute ITIs, one day with 30 minutes of context extinction and a fourth day with cue-only extinction testing with five presentations of white noise. During the extinction testing optogenetic inhibition of the BLA or LH_{GABA} was delivered during the 10 s white noise cue.

Experiment 3: Wildtype rats were surgically prepared for chemogenetic inhibition of the BLA to test if fear memory encoding remains intact in reward-experienced rats if BLA inhibition is prolonged (Fig 5B). Rats were assigned to "reward" or "naïve" groups and trained according to the following specifications, which are identical to those in **experiment 2** and adapted from Sharpe

et al. (2021). “Reward” rats were pre-exposed to sucrose pellets in their homecage and received appetitive Pavlovian conditioning to learn an association between a 10 s light and two sucrose pellets, while “naïve” subjects received the light alone as a control (Fig 5A). Reward learning consisted of five days of 12 light-sucrose pairings with 4-minute ITIs in context A. Rats were pre-exposed to the fear chamber (context B) during the five days of reward training to facilitate context discrimination. The reward and pre-exposure schedules were counterbalanced for time of day and training order. For example, a rat might receive fear pre-exposure in the morning and reward training in the afternoon on day 1 and then the opposite on day 2. The 4-day fear conditioning procedure began the following week. Days 1 and 2 were acquisition training with three pairings of the 10-s, 77dB tone with a 0.55mA shock. Intraperitoneal CNO (3mg/kg) was injected 25-30 minutes prior to acquisition training on days 1 and 2. Day 3 was 30 minutes of context extinction and day 4 was extinction testing with five tone-only presentations.

Experiment 4: Wildtype rats were surgically prepared for optogenetic inhibition of the BLA to test if exposure to CUS after reward learning will revert fear memories to dependence on the BLA. Rats were assigned to “CUS” or “no stress” groups. The behavioral procedure is modified from **experiments 2** & **3**, to add a 7-day CUS procedure between reward learning and fear conditioning (Fig 6A). All rats were pre-exposed to sucrose pellets in their homecage and received reward learning according to specifications in **experiment 2** & **3** (five days, 12 light-sucrose presentations, 4-minute ITIs). For the following 7 days, “CUS” rats received two stressors per day while “no stress” rats were handled for 2-minute per day as a control. Six different CUS treatments were chosen from the literature and conducted at variable times throughout the light cycle in a context distinct from the reward and fear rooms (Sequeira-Cordero, 2019; Haile et al., 2001; Cox et al.,

2011; Larsen et al., 2010; Banasr et al., 2007; Chaby et al., 2015; Gouirand & Matuszewich, 2005). The following stressors were used – restraint stress: 60 minutes in a restraint device (Kent Scientific, CT); Cage tilt: three hours in a cage with a 30-degree tilt; Wet bedding: 4 hours in a cage with 6 cups of corncob bedding soaked in 400mL of water; Continuous illumination: 24-hrs in an illuminated room (no dark cycle); Predator scent: exposure to 3mL of fox urine (Tink's Red Fox-P) on a cotton pad inside a 1"x1"x1" ventilated plastic box for 60 minutes; White noise: rats were exposed to white noise for three hours. The 4-day fear conditioning paradigm began the following day. Days 1 & 2 consisted of acquisition training with three pairings of the 10 s tone and 1 s, 0.55mA shock with BLA inhibition delivered during the tone. Day 3 was 30 minutes of context extinction and day 4 was extinction testing with five tone-only presentations.

Histology: Rats were euthanized with an overdose of carbon dioxide and perfused with phosphate buffered saline followed by a 4% paraformaldehyde (Sigma-Aldrich) solution. Extracted brains were kept in 4% paraformaldehyde for 24 hours and then stored in 18% sucrose. After a minimum of 48 hours brains were cryostat sectioned at 40um and imaged on a confocal microscope (Zeiss) to determine spread and accuracy of virus and fiber tips. Images were processed in Fiji (ImageJ) and Adobe Photoshop and Illustrator.

Data and statistical analysis: Fear conditioning sessions were video recorded and hand scored for freezing behavior during the 10 seconds before, during and after the tone during acquisition and extinction sessions. Each 10 s period was divided into five, 2 s bins which were evaluated for displays of freezing. For acquisition, freezing on each trial was analyzed for within-subject effects of day and between-subject differences for rats with NpHR vs eYFP/eGFP. For extinction, average

freezing across all cue presentations was analyzed for between-subject differences (NpHR vs eYFP). Reward training data was collected by MedPC-V software and exported using MedPCtoXL. Reward learning was evaluated by calculating average magazine entries made during the 10 s cue minus entries made during the 10 s pre-CS baseline period for each rat. Statistical analyses were conducted using SPSS 28 IBM statistics package. Mixed factor ANOVAs were used to assess performance across learning and between groups for reward and fear learning and between-subjects ANOVAs were used to evaluate group differences during extinction testing. The sample sizes were determined from previous publications with similar methods and findings (Sharpe et al., 2017; Sharpe et al. 2021).

Results

It remains inconclusive whether inhibiting LH_{GABA} or BLA neurons during the extinction phase of fear conditioning in reward experienced and naïve rats can disrupt memory expression.

Findings from Sharpe et al. (2021) and chapter 2 of this dissertation indicate that the BLA is necessary for fear memory encoding in naïve, but not reward experienced rats. LH_{GABA} neurons are not needed for fear memory encoding in naïve rats but become necessary following reward experience. These conclusions come from experiments where the BLA or LH_{GABA} were inhibited during the acquisition phase of fear conditioning. The question remains whether these neuron populations are necessary during extinction testing to recall memories that are acquired with no manipulation. **Experiment 2b** continued testing on the cohort of rats from **experiment 2** to

answer this question. These rats that were previously prepared for optogenetic inhibition of either LH_{GABA} (Fig 4B; AAV5-Ef1a-DIOeNpHR3.0-eYFP) or BLA neurons (AAV9-CamKIIa-eNpHR3.0-eYFP) or as controls (AAV8-CamKIIa-eGFP; AAV5-Ef1a-DIO-eYFP). The rats received two days of three white noise-shock pairings for fear conditioning. Freezing behavior during the tone on the acquisition and extinction days was hand scored to evaluate learning and memory. Both of the groups with BLA inhibition (BLA-reward, BLA-naïve) showed a significant increase in freezing across acquisition days, but neither of the groups with LH_{GABA} inhibition (LH_{GABA}-reward, LH_{GABA}-naïve) did (Fig 4C-F; LH_{GABA}-reward: $F_{1,8}$=2.309; p=0.167; LH_{GABA}-naïve: $F_{1,7}$=1.660; p=0.239; BLA-reward: $F_{1,9}$=5.432; p=0.045; BLA-naïve: $F_{1,8}$=27.225; p<0.001). Neither LH_{GABA} nor BLA rats in either treatment showed significant between-subject differences (NpHR vs eYFP/eGFP: LH_{GABA}-reward: $F_{1,8}$=1.693; p=0.229; LH_{GABA}-naïve: $F_{1,7}$=1.071; p=0.335; BLA-reward: $F_{1,9}$=0.155; p=0.703; BLA-naïve: $F_{1,8}$=0.536; p=0.485). Both groups with reward experience (BLA-reward and LH_{GABA}-reward) showed a significant group x day interaction during acquisition, but neither naïve group (BLA-naive and LH_{GABA}-naïve) did (BLA-reward: $F_{1,9}$=5.432; p=0.045; BLA-naïve: $F_{1,8}$=3.025; p=0.120; LH_{GABA}-reward: $F_{1,8}$=6.211; p=0.037; LH_{GABA}-naïve: $F_{1,7}$=0.058; p=0.817). Two days later all rats received 5 presentations of 10 s of white noise for extinction testing with LH_{GABA} or BLA inhibition during the white noise. Freezing was averaged across all five tone presentations and analyzed for between-subject differences. Of the four groups, only the LH_{GABA}-naïve rats showed a significant between-subject difference in freezing during extinction testing (NpHR vs eYFP/eGFP: BLA-reward: $F_{1,9}$=1.404; p=0.266; BLA-naïve: $F_{1,8}$=1.087; p=0.328; LH_{GABA}-reward: $F_{1,8}$= 0.008; p=0.929; LH_{GABA}-naïve: $F_{1,7}$=7.998; p=0.025)

It remains inconclusive whether inhibiting the BLA during acquisition with chemogenetics can disrupt fear learning in reward experienced or naïve rats.

Chapter 2 of this dissertation asserted that optogenetic inhibition of the BLA during the tone of fear learning is necessary for fear memory encoding in naïve rats, but not rats with reward experience. **Experiment 3** investigated if reward learning will still render the BLA unnecessary for fear learning if it is inhibited across the entire acquisition phase with chemogenetics. Rats were surgically prepared for chemogenetic inhibition of the BLA with infusions of the inhibitory designer receptor hM4Di (Fig 5B; AAV8-hSyn-hM4Di-mCherry) or a control virus without hM4Di (AAV8-hSyn-mCherry). Rats received five days of reward learning with 12 light-sucrose pairings or equal light-alone trials as a naïve control. Magazine entries during the 10 s cue minus the 10 s baseline period before the cue were calculated and analyzed to evaluate reward learning. Reward rats showed a significant increase in magazine entries across days (Fig 5D; reward: $F_{4,40}$=28.766; p=<.001) while naïve rats did not (Fig 5E; naïve: $F_{4,40}$=.566; p=0.689). Neither reward nor naïve rats showed a significant between-subject difference during reward learning (reward: $F_{1,10}$=.005; p=0.945; naïve: $F_{1,10}$=.395; p=0.544). Neither reward nor naïve rats showed a significant group x day interaction (reward: $F_{4,40}$=.310; p=0.870; naïve: $F_{4,40}$=.800; p=0.532). Fear conditioning began with two days of three tone-shock pairings with intraperitoneal CNO injections 25-30 minutes before training. Two days later rats received extinction testing with five tone-alone presentations. Freezing behavior during the tone on the acquisition and extinction days was hand scored to evaluated learning and memory expression. Reward rats did not show a significant increase in freezing across days (Fig 5D; reward: $F_{1,10}$=.558; p=0.472), but naïve rats did show a significant increase in freezing across days (Fig

5E; naïve: $F_{1,10}$=5.653; p=0.039). Neither reward (hm4Di vs mCherry – reward: $F_{1,10}$=2.593; p=0.138) nor naïve (hm4Di vs mCherry – naïve: $F_{1,10}$=.246; p=0.631) showed a significant between-subject effect in freezing levels during acquisition. Neither reward nor naïve rats showed a significant group x day interaction (reward: $F_{1,10}$=.115; p=0.741; naïve: $F_{1,10}$=.025; p=0.877). Two days later all rats received five presentations of 10 s of white noise for extinction testing. Freezing was averaged across all five tone presentations and analyzed for between-subject differences. Neither reward nor naïve rats showed a significant between-subjects effect on freezing levels during extinction either (hm4di vs mCherry – reward: $F_{1,10}$=1.754.; p=0.215; naïve: $F_{1,10}$=.700; p=0.422).

Exposure to CUS following reward learning enhanced fear responses during extinction testing in subsequent fear conditioning. The importance of the BLA in reward-experienced rats was not affected by experience with CUS.

After reward learning, fear memories became dependent on LH_{GABA} neurons rather than the BLA (Sharpe et al., 2021). The BLA is implicated in the pathological fear learning associated with anxiety-based disorders such as post-traumatic stress disorder (Butler et al. 1990; Kirkpatrick & Heller, 2014; Koenigs & Grafman, 2009; Shin et al., 2004; Bremner et al., 2005; Armony et al., 2005; Dunkley et al., 2016; Protopopetscu et al., 2005; Rauch et al., 2000). **Experiment 4** used a CUS procedure to determine if LH_{GABA} dependent fear memories in rats with reward experience reverted to being BLA-dependent after CUS. Rats were surgically prepared for optogenetic inhibition of the BLA with a halorhopdopsin or control virus (Fig 6B; AV9-CamKIIa-eNpHR3.0-eYFP; AAV8-CamKIIa-eGFP) and assigned to “no stress” or “CUS” groups. Both

groups began with 5 days of 15 light-sucrose pairings (Fig 6A). Magazine entries during 10 s cue minus the 10 s baseline period before the cue were calculated and analyzed to evaluate reward learning. Both CUS and no stress rats showed a significant increase in magazine entries across days (Fig 6C-D; CUS: $F_{4,20}$=4.445; p=0.010; no stress: $F_{4,24}$=4.195; p=0.010). Neither CUS nor no stress rats showed a significant between-subject effect of group (NpHR vs eGFP – CUS: $F_{1,5}$=2.222; p=0.196; no stress: $F_{1,6}$=.348; p=0.577). Neither CUS nor no stress rats showed a significant group x day interaction (CUS: $F_{4,20}$=0.426; p=0.788; no stress: $F_{4,24}$=2.004; p=0.192). After reward learning rats received two CUS treatments per day or were handled for two minutes as a control (Fig 6A). All rats received two days of fear acquisition training with three tone-shock presentations with optogenetic inhibition delivered during the 10 s tone. Both CUS and no stress rats showed a significant increase in freezing across acquisition days (Fig 6C-D) CUS: $F_{1,5}$=16.458; p=0.010; no stress: $F_{1,6}$=15.226; p=0.008). Neither CUS nor no stress rats showed a significant between-subject effect of virus (NpHR vs eGFP – CUS: $F_{1,5}$=.494; p=0.513; no stress: $F_{1,6}$=.615; p=0.463). Neither CUS nor no stress rats showed a group x day interaction (CUS: $F_{1,5}$=.959; p=0.372; no stress: $F_{1,6}$=.008; p=0.934). Following acquisition all rats received extinction testing with five tone presentations. Freezing was averaged across all five tone presentations and analyzed for between-subject differences. Neither CUS or no stress rats showed a between-subject effect during extinction testing (NpHR vs eGFP – CUS: $F_{1,5}$=2.060.; p=0.211; no stress: $F_{1,6}$=3.545; p=0.109). Lastly, given the null between-subject effects of the optogenetic manipulation all CUS and no stress rats were evaluated together to assess if the CUS treatment enhanced freezing responses during acquisition and extinction. Rats showed a significant increase in freezing across acquisition day (Fig 6E; $F_{1,13}$=34.704; p=<.001) with no between-subject effect (CUS vs no stress: $F_{1,13}$=0.007; p=0.936) or group x day interaction

($F_{1,13}$=.120; p=0.734). During extinction testing, there was a significant increase in average freezing in the CUS rats (Fig 6E; $F_{1,13}$=7.726; p=0.016).

Discussion

It remains inconclusive whether inhibiting LH_{GABA} or BLA neurons during the extinction phase of fear conditioning in reward experienced and naïve rats can disrupt memory expression.

The results of **experiment 2b** did not definitively determine if the role of LH_{GABA} or BLA neurons during extinction testing changed after experience with reward (Fig 4). A logical follow-up question to **experiment 2**'s findings – that reward learning induced a shift from BLA-dependent to LH_{GABA}-dependent fear memories – was to ask if activity in these regions is necessary to express normally acquired fear memories. The results of **experiment 2b** did not show any promising trend or effect of BLA or LH_{GABA} inhibition during extinction testing of a conditioned fear cue (Fig 4). This experiment was a pilot study meant to collect preliminary data to inform future directions of this line of research. Accordingly, this dataset had some limitations. Most apparently, these rats were previously used for another experiment and were experiencing their second round of fear conditioning. The carryover effects could explain the lack of a significant increase in freezing across acquisition in the GADCre rats. The conclusion was ultimately to go in a more promising direction with future work, but this question remains worth revisiting in the future.

It remains inconclusive whether inhibiting the BLA across the acquisition phase of fear conditioning can disrupt learning or memory in reward-experienced or naïve rats.

The results of **experiment 3** were also inconclusive. While rats did demonstrate successful acquisition and expression of reward and fear learning behavior, the chemogenetic manipulation had no effect on freezing levels during acquisition or extinction in naïve or reward-experienced rats (Fig 5). While the lack of effect in the reward-experienced rats is in line with previous findings, given the null result in the naïve control group this data cannot be interpreted. Based on ample prior findings both from the dissertation and existing literature (Phillips & LeDoux, 1992; Cousens & Otto, 1998; Koo et al., 2004; Maren et al., 1996; Helmstetter, 1992; Muller et al., 1997), inhibiting the BLA during fear acquisition training should disrupt freezing responses. The lack of attenuation of fear learning or memory in the naïve group suggests the chemogenetic inhibition failed. Indeed, histological analysis indicated insufficient expression and targeting of hm4di, providing an explanation for the null result. The question addressed in **experiment 3** remains unanswered – future experiments in the Sharpe Lab will seek to replicate this experiment using muscimol inhibition rather than chemogenetics.

Exposure to CUS following reward learning enhances conditioned fear responses during extinction testing. The importance of the BLA in reward-experienced rats is not affected by CUS exposure.

Experiment 4 sought to investigate if exposing reward-experienced rats to CUS prior to fear conditioning would revert fear memories back to dependence on the BLA (Fig 6). The results of this experiment indicated that compared to no stress controls, exposure to CUS did not affect the importance of the BLA for fear learning or memory (Fig 6C-D). While both CUS and no stress animals successfully acquired a conditioned freezing response to the tone, there was no effect of BLA inhibition on freezing levels during acquisition or extinction in either group (Fig 6C-D). An important consideration is that all rats in this experiment received reward learning, so there were no naïve counterparts to compare these results to. Based on my previous findings, a null effect was expected in the no stress reward group, but without a no stress naïve group for comparison it is not possible to conclude that the optogenetic manipulation was successful. There was also a null result in the CUS group, which is also difficult to interpret without appropriate naïve controls for comparison. Because the optogenetic manipulation did not affect freezing levels, the eGFP and NpHR subjects were combined for a comparison in freezing behavior between all the CUS and no stress subjects. Here, there was no difference in freezing levels during acquisition, but CUS rats displayed more freezing during extinction testing (Fig 6E). This supports previous literature showing that prior stressful experiences enhance future fear learning (Pechtel & Pizzagalli, 2011; Wang et al., 2010), and indicates that the CUS procedure used is sufficient to induce behavioral changes which can be further investigated in the future. Lastly, these results bore an important theory about the mechanism by which the reward-induced shift in the fear circuit may occur. Compared to **experiment 2**, there were two major changes introduced to the procedure. One was the obvious: introduction of the CUS manipulation. Along with that came a 7-day delay between the reward learning treatment and fear conditioning. While the no stress

control rats still demonstrated that BLA inhibition did not disrupt fear memories, the trend of the data suggests that the introduction of a time delay may have lessened the magnitude of this effect (the NpHR rats froze slightly less than controls). One potential explanation for the reward-induced shift in the fear circuit is that LH_{GABA} neurons, which are known to be necessary for Pavlovian reward learning (Sharpe et al., 2017) are "primed" during reward learning, making them more likely to support future learning epochs than the BLA (Lee & Levin, 2012). Although LH_{GABA} neurons do not traditionally encode fear memories, their recent activation during reward learning could cause their involvement in encoding the subsequent fear conditioning. If this is the case, this effect would likely be transient. Future experiments will explore if the reward-induced shift to LH_{GABA} neurons persists if a substantial time delay is introduced between reward learning and fear conditioning.

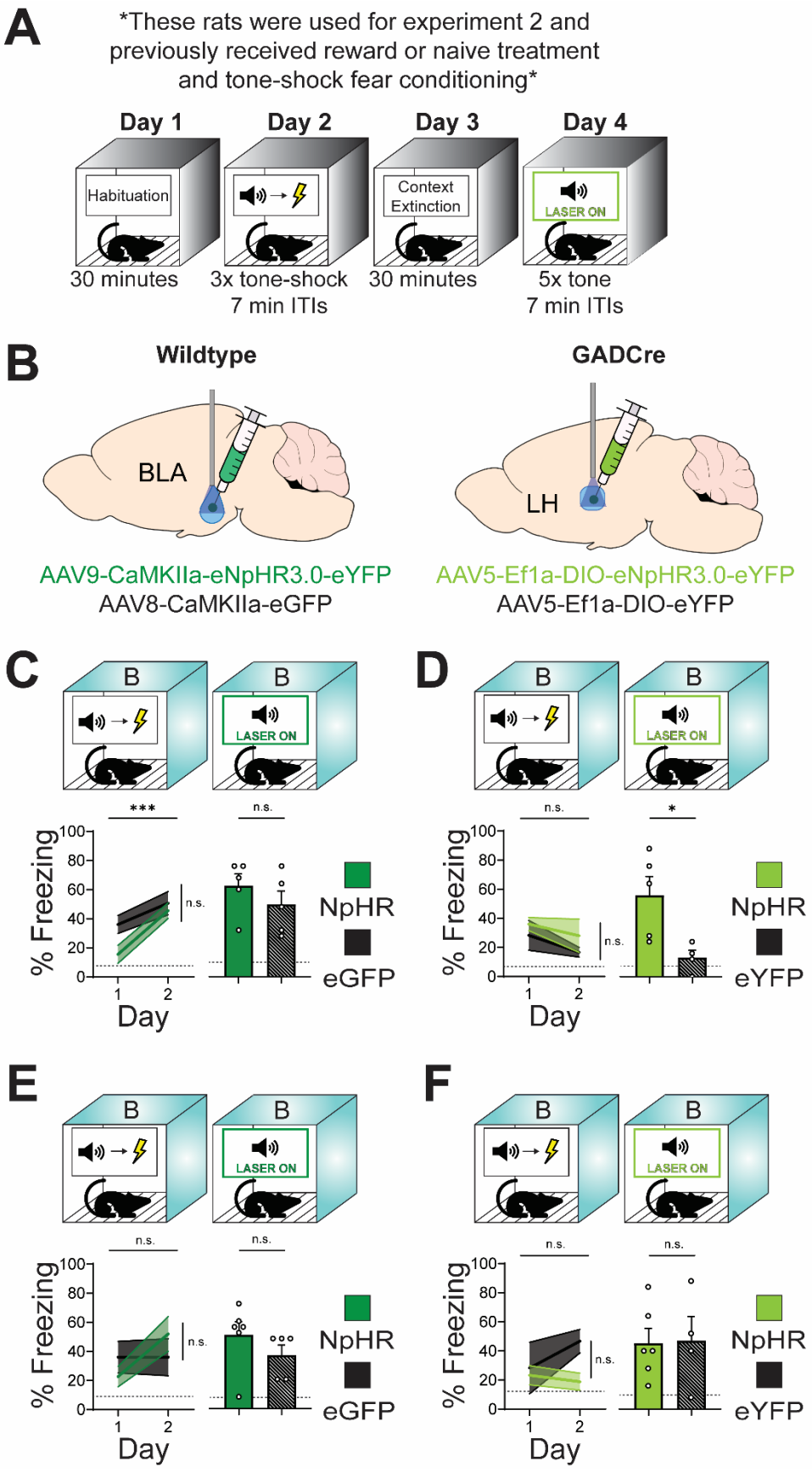
A
These rats were used for experiment 2 and previously received reward or naive treatment and tone-shock fear conditioning
Day 1
Day 2
Day 3
Day 4
Habituation
Context Extinction
LASER ON
30 minutes
3x tone-shock 7 min ITIs
30 minutes
5x tone 7 min ITIs
B
Wildtype
GADCre
BLA
LH
AAV9-CaMKIIa-eNpHR3.0-eYFP
AAV8-CaMKIIa-eGFP
AAV5-Ef1a-DIO-eNpHR3.0-eYFP
AAV5-Ef1a-DIO-eYFP
C
D
E
F
B
LASER ON
***
n.s.
*
% Freezing
Day
NpHR
eGFP
eYFP

Figure 4: Prior reward experience did not change the importance of the BLA or LH_{GABA} for recall of fear memories. (A) Schematic of behavioral procedures. (B) Schematic of optogenetic approach. Prior to **experiment 2**, wildtype rats were infused bilaterally with a halorhopdopsin (n=6) or control virus (n=4) and implanted with optic fibers in the BLA to allow for inhibition of CaMKII+ neurons. GADCre rats were infused bilaterally with a halorhodopsin (n=8) or control virus (n=8) to allow for inhibition of LH_{GABA} neurons. (C-F) All rats received a second round of fear conditioning with a white noise cue; (C) wildtype naïve, (D) GADCre naïve, (E) wildtype reward, (F) GADCre reward; Either LH_{GABA} neurons (D & F) or BLA neurons (C & E) were inhibited during the white noise; The dotted lines represent average pre-CS freezing for all subjects (C-D) In naïve rats, freezing levels were not significantly affected by LH_{GABA} inhibition or BLA inhibition. (E) The same was true in reward experienced rats: freezing levels were not significantly affected by LH_{GABA} inhibition or BLA inhibition during extinction.

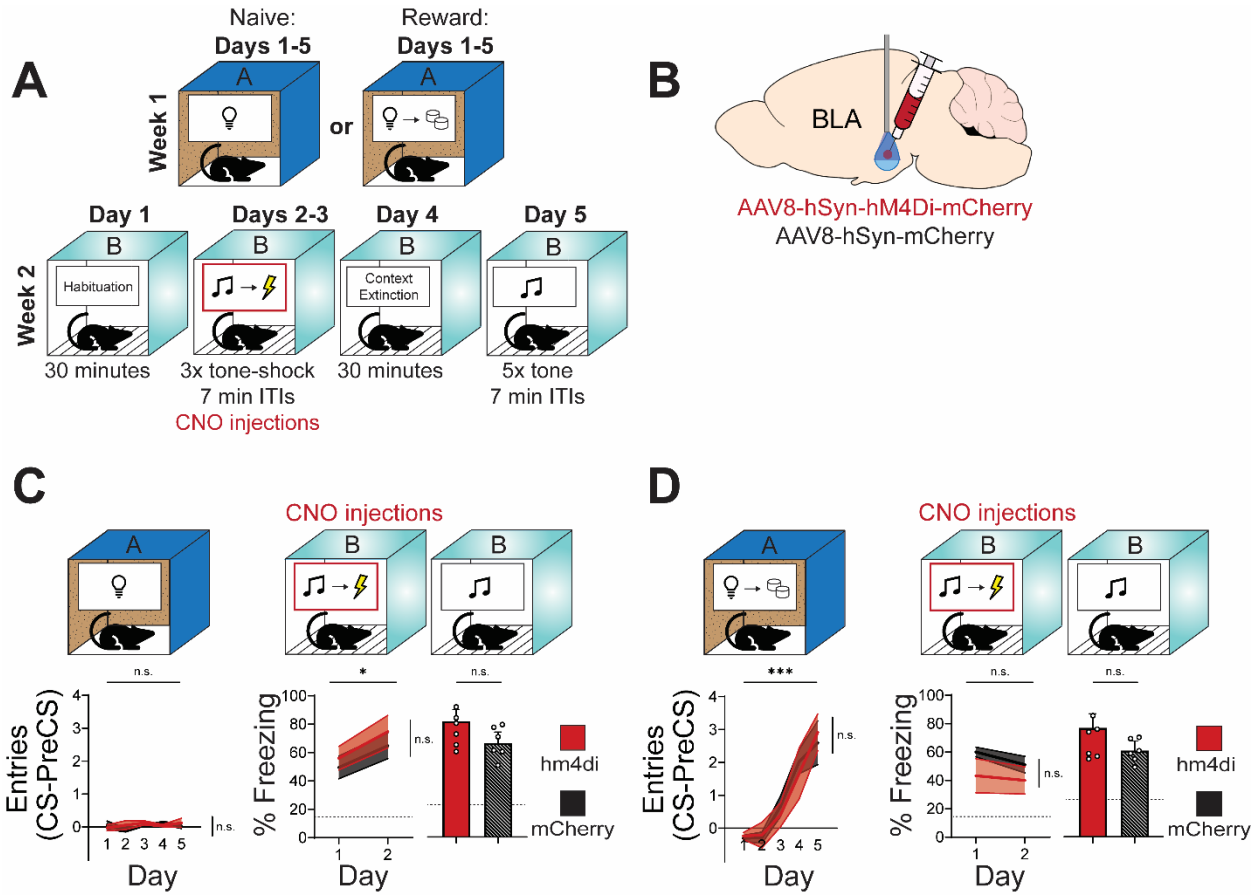

Figure 5: It remains inconclusive whether prolonged BLA inhibition across fear acquisition training had a different effect in reward experienced and naïve rats. (A) Schematic of behavioral procedures. (B) Schematic of chemogenetic approach: wildtype rats were infused bilaterally with a hm4Di (n=12) or control virus (n=12). (C-D) Rats received either light-sucrose reward training or light-only presentations as a control before receiving tone-shock fear conditioning with CNO injections prior to acquisition training to inhibit the BLA. (C) In naïve rats, there was no effect of chemogenetic BLA inhibition on freezing levels during acquisition or extinction. (D) In reward experienced rats, there was also no effect of chemogenetic BLA inhibition on freezing levels during acquisition or extinction.

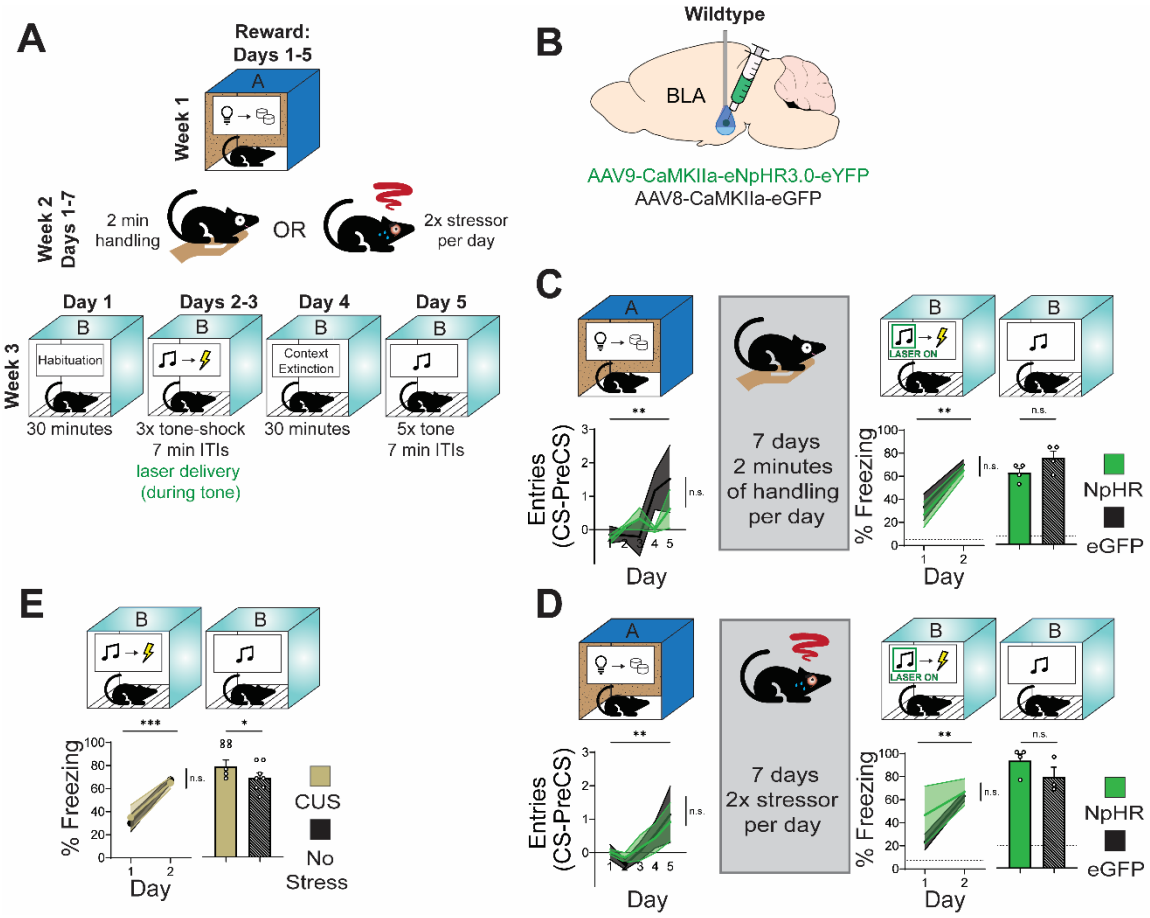

Figure 6: After reward experience, CUS sensitized rats to future fear learning, but did not affect the role of the BLA in supporting fear learning in reward-experienced rats. (A) Schematic of behavioral procedures. (B) Schematic of optogenetic approach: wildtype rats were infused bilaterally with a halorhopdopsin (n=8) or control virus (n=8) and implanted with optic fibers in the BLA to allow for inhibition of CaMKII+ neurons. (C-D) Rats received light-sucrose reward training followed by CUS or no stress treatment. Next, rats received tone-shock fear conditioning with BLA neurons inhibited during the tone. (C) BLA inhibition during the tone of fear conditioning in no stress rats did not affect freezing levels during acquisition or extinction. (D) BLA inhibition during the tone of fear conditioning in CUS rats did not affect freezing levels during acquisition or extinction either. (E) CUS treatment led to increased freezing levels during extinction testing.

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