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Processing of unpredictability in fear learning and memory

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PROCESSING OF UNPREDICTABILITY IN FEAR LEARNING AND MEMORY

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ABSTRACT

Unpredictability is one of the major drivers of associative learning. While unpredictability in the timing of events can enhance fear memory strength, the neural substrates that are involved in generating and processing these errors remain largely unknown. We first showed that unpredictability, generated by the varied timing of the aversive event following the predictive cue, greatly enhanced fear memory strength (Chapter 3). The unpredictability-processing neural network in basal and lateral amygdala (BLA) was then studied using time-lapse microendoscopy to monitor neuronal calcium response across fear conditioning and recall (Chapter 4). We identified four distinct functional classes of neurons based on the neuronal activity patterns during fear conditioning and long-term recall. “Memory Winner” neurons outcompeted the “Memory Loser” neurons to encode the fear memories; nonetheless, both classes of neurons exhibited learning-related plasticity during the fear conditioning. In contrast, Fear Expression neurons did not display learning-related plasticity during fear conditioning but did respond to the tone presentation during auditory fear recall. The introduction of temporal unpredictability during the fear conditioning increased the percentage of both the Memory Winner neurons and Fear Expression neurons, and decreased the percentage of Memory Loser neurons. Furthermore (Chapter 5), pharmacological inhibition of dorsal

hippocampus and optogenetic silencing of CA1 revealed the essential involvement of dorsal hippocampus in the processing of negative prediction errors, which is generated by unpredictability in their timing. Collectively, our data suggest that the processing of temporal unpredictability of aversive events requires the dorsal hippocampal activation to process the negative prediction errors; and the rearrangement of the BLA neural representation of fear learning and memory. Taken together, these processes underlie the mechanism of the unpredictability-enhanced fear memory strength.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAV ..... Adeno-Associated Virus

ANOVA ..... Analyses Of Variance

ArchT ..... Archaerhodopsin Halorubrum strain TP009

BLA ..... Basal and Lateral Amygdala

BOLD ..... Blood-Oxygenation-Level-Dependent

CA1 ..... Cornu Ammonis 1

$CaCl_2$ ..... Calcium Chloride

CS ..... Conditioned Stimulus

fMRI ..... Functional Magnetic Resonance Imaging

GABA ..... Gamma-Amino Butyric Acid

GCaMP6s ..... GFP-Calmodulin-M13 peptide-6 slow

GFP ..... Green Fluorescent Protein

H_20 ..... Dihydrogen Monoxide

ITI ..... Intertrial Interval

ISI ..... Interstimulus Interval

KCl ..... Potassium Chloride

KH_2PO_4 ..... Potassium Dihydrogen Phosphate

LTP ..... Long Term Potentiation

$MgCl_2$ ..... Magnesium Chloride

NaCl ..... Sodium Chloride

Na_2HPO_4 ..... Disodium Phosphate

PBS..... Phosphate Buffer Saline

PRED..... Predictable

PTSD .....Post-traumatic stress disorder

UNPRED..... Unpredictable

US..... Unconditioned Stimulus

CHAPTER ONE: INTRODUCTION AND SPECIFIC AIMS

In real life settings, fear is at its strongest when danger happens unpredictably. Traumatic events that cause fear often occur in the presence of cues that provide little to no information on the timing of the occurrence of subsequent aversive stimuli. For instance, soldiers on the battlefield often hear gunshots. Yet the sound of a gunshot does not enable soldiers to predict when they or their partners will be shot. These traumatic events often lead to the development of PTSD. This complex and chronic disorder is characterized, in part, by pathologically strong associations between the innocuous cues and the aversive events that are difficult to eradicate. To understand the formation of these strong associations, Pavlovian fear conditioning is often used as a behavioral model to study the neurological alterations and impact of the aversive stimuli in the brain (Goswami et al., 2013; Borghans and Homberg, 2015). Most of these studies, however, fail to incorporate the element of temporal unpredictability into the behavioral paradigm, making this behavioral model a poor representation of what occurs in the real world setting; this may also hinder the attempts to understand the underlying mechanisms of PTSD. Understanding the neural computations that occur in the brain during real-world fear learning directly informs hypotheses about the basis of dysfunction in pathological fear. Here, we sought to determine whether temporal unpredictability alters the fear memory strength, reveal the BLA neuronal networks that underlie the change in fear memory strength, and identify the role of prediction errors in this process.

Incorporation of Negative Prediction Error into Fear Conditioning

The concept of unpredictability in aversive associative fear learning and memory was first introduced by Rescorla-Wagner model in 1972 (Miller et al., 1995). Before this, it was commonly believed that the strength of associative memory was determined by the contiguity of the pairing between the cue and the aversive stimulus. In other words, fear conditioning across a greater number of trials (each trial consisting of a cue and an aversive stimulus) generates stronger fear memory than fear conditioning with a fewer number of trials. In contrary, the Rescorla-Wagner model features a more profound concept to describe the relationship between the cue and the aversive stimulus. It hypothesizes that the associative strength between the cue and the stimulus depends on how strongly the stimulus can be predicted based on the cue. The better the cue is able to predict the occurrence of the aversive stimulus, the stronger the associative memory is (Miller et al., 1995). In contrast, when the cue provides little to no information for predicting the occurrence of the stimulus, the strength of the memory decreases. With the ease of introducing predictability, fear conditioning serves as an ideal model for us to design a temporally unpredictable fear conditioning paradigm that accurately models real world fear learning by systematically varying the timing and occurrence of the aversive stimulus.

When temporal unpredictability is incorporated into fear conditioning, it is intriguing to ask how the brain processes this varied timing and occurrence of the aversive stimulus. In our studies, the temporal unpredictability is defined as the variability in the timing of aversive stimuli onset relative to the onset of predictive cues. When aversive stimuli are presented at variable times following a cue, subjects cannot

accurately predict the timing of the occurrence of the aversive stimuli. The brain acknowledges the mismatch between the expected outcome and actual outcome and processes this mismatch as a prediction error. This mismatch can lead to two opposing types of prediction errors: (1) Positive prediction error, when a stimulus occurs unexpectedly and (2) Negative prediction error, when a stimulus is absent from its predicted timing. Pavlovian fear conditioning is an ideal model to study negative prediction error. By varying the timing of the occurrence of the aversive stimuli, putative negative prediction errors can be generated in a pseudorandom manner when expected aversive stimuli fail to occur. Therefore, using this model of temporal unpredictability, we sought to reveal the behavioral effect and the underlying neural processing of negative prediction errors.

The Role of Basal and Lateral Amygdala (BLA) in Negative Prediction Errors

Numerous neural substrates supports fear learning, memory storage and fear expression. The amygdala is commonly known as one of the brain regions that is required for a wide range of fear learning and memory formation processes, including fear learning (Maren, 1999), fear memory formation (Wilensky et al., 1999) and storage (Campese et al., 2016), fear recall (Pare and Duvarci, 2012), fear extinction (Pare and Duvarci, 2012) and etc. The rodent amygdala is a complex brain structure that consists more than 12 distinct major nuclei (Ressler, 2010), of which basal, lateral and central are most well-defined in structure, connectivity and function (Janak and Tye, 2015). Central amygdala, consists of mainly GABAergic neurons, receives major inputs from the basal and lateral amygdala and other extra-amygdala sites (Ehrlich et al., 2009). By regulating

the fear expression through its projections to downstream structures like the bed nucleus of the stria terminalis, lateral hypothalamus and periaqueductal gray, the central amygdala is commonly considered to be the output center for the fear response (Duvarci et al., 2011). On the contrary, BLA is comprised of both glutamatergic and inhibitory neurons that are reciprocally connected with many other brain regions. In specific, the lateral amygdala receives strong inputs from sensory cortices like visual, olfactory and auditory cortex, as well as the sensory thalamus (McDonald, 1998). The association between the cue and the aversive stimulus is thought to be processed in the lateral amygdala (Blanchard and Blanchard, 1972; LeDoux et al., 1990; Nader et al., 2001). Basal amygdala then receives this processed information along with other external input and further processes the signal before reciprocally relaying it to cortical regions, and unidirectionally relaying it to nucleus accumbens, bed nucleus of the stria terminalis and central amygdala (Pitkanen et al., 2000). Therefore, BLA is also characterized as the center of fear learning and association processing. On that account, BLA is a rational target for studying the effect of temporal unpredictability on fear learning. While it is known that BLA is highly associated with fear learning and memory, additional studies have shown BLA's ability to process unpredictability and negative prediction errors. Some human studies show that patients with fear and anxiety disorders are disproportionately affected by the temporal unpredictability surrounding the occurrence of aversive events. When aversive stimuli are administered temporal unpredictably, patients with panic disorder display enhanced startle reactivity relative to healthy comparison subjects (Grillon et al., 2008). In addition, patients with generalized anxiety

disorder are more likely than healthy control subjects to interpret ambiguous stimuli as threatening (Dugas et al., 1998). Intolerance of uncertainty (Buhr and Dugas, 2002), even when uncertainty is not specifically related to aversive outcomes, is greater in patients with anxiety disorders than in human subjects without such disorders (Holaway et al., 2006). While temporal unpredictability and ambiguity play important roles in fear and anxiety disorders, elevated amygdala activity has also been implicated in various fear and anxiety disorders (Martin et al., 2009; Shin and Liberzon, 2010). Therefore, it is reasonable to speculate that the heightened amygdala activation might be associated with the processing of prediction errors. A human functional magnetic resonance imaging (fMRI) study has shown that amygdala activity increased with increasingly negative prediction errors (Meder et al., 2016).

Animal studies further suggested that the prediction errors were being processed in BLA (McHugh et al., 2014). BLA lesion abolished the enhancement in cue processing in the unblocking behavioral experiments (Esber and Holland, 2014). Chemogenetic excitation of rat BLA glutamatergic neurons also disrupted the use of prediction errors to regulate fear learning (Sengupta et al., 2016). Even though human and animal studies have suggested that the amygdala is necessary to process prediction error, additional evidence is still needed to reveal how temporal unpredictability and negative prediction errors are being processed in BLA.

Neural Substrates of Negative Prediction Error Processing

It is known that the BLA processes the association between the cue and the aversive stimulus. However, rather than effectively processing an expected aversive

stimulus as shown in previous single unit recording studies at the neuronal level, BLA neurons respond stronger to unexpected aversive stimuli (Belova et al., 2007; Johansen et al., 2010). This suggests that BLA might receive input from external neural substrates to facilitate preferential processing of unexpected aversive stimuli. To identify these additional neural substrates, we need to look into the structural and functional connectivity of the BLA. BLA strongly and reciprocally projects to perirhinal, entorhinal, dorsal agranular insular cortex and subiculum (Pitkanen et al., 2000); while basal amygdala has additional reciprocal connection with hippocampus (Pikkarainen et al., 1999; Pitkanen et al., 2000). While using classical fear conditioning to study associative fear learning and memory, subjects can be conditioned to either the cue or the context. The amygdala contributes to both cue and context fear conditioning. Amygdalar neural firing patterns are altered by the association of cues and stimuli (Applegate et al., 1982; Henke, 1983; Pascoe and Kapp, 1985), suggesting the encoding of the association occurs in amygdala. Furthermore, lesions of the rat amygdala lead to a failure to establish the cue-stimulus association (Goddard, 1964; Nader et al., 2001). These findings in animal models are consistent with the studies of human amygdalar damage. Patients with Urbach-Wiethe disease, a rare genetic disorder with bilateral calcification of amygdala, fail to be fear conditioned to either visual or auditory cues paired with loud tones (Bechara et al., 1995). Furthermore, patients who received amygdalectomy show impairment in auditory fear conditioning (LaBar et al., 1995). These studies reveal that the amygdala is required for associative learning and memory formation that are motivated by fear.

Although the hippocampus is not the center of fear learning and memory, it still plays an important role in encoding the representation of the contextual cue for the association with aversive stimulus (Wang et al., 2013). The ventral and dorsal hippocampus is involved in two distinct roles in fear conditioning. Ventral hippocampal lesions have been shown to impair cue-conditioned freezing (Ballesteros et al., 2014), associative learning between contextual cues and footshock (Wiltgen et al., 2006) and context-specific fear memory retrieval (Hobin et al., 2006). These findings might be due to the fact that the ventral hippocampus and amygdala are directly and strongly connected by glutamatergic projections (Wyss, 1981; Ottersen, 1982; Swanson and Kohler, 1986; Mello et al., 1992). In contrast, the dorsal hippocampus does not have direct connection with the amygdala. Studies have suggested that the dorsal hippocampus and amygdala interact through relay points in parahippocampal cortices like the entorhinal and perirhinal cortex (Room and Groenewegen, 1986; Witter et al., 1989), which have robust projections to BLA (McDonald and Mott, 2017). Even though the dorsal hippocampus might not be directly connected with amygdala, it plays an important role in contextual fear conditioning. Dorsal hippocampal lesions have been shown to block fear conditioning to contextual stimuli (Phillips and LeDoux, 1992; Maren and Holt, 2004). Optogenetic studies further support these findings, suggesting that the dorsal hippocampus is needed to project contextual information to the BLA for contextual fear conditioning (Goshen et al., 2011; Ramirez et al., 2013). Despite the fact that dorsal hippocampus is required for contextual fear conditioning, numerous fear-conditioning studies have argued that the hippocampus is not involved in fear conditioning in response

to discrete cues. Dorsal hippocampal infusion of muscimol, a GABAA agonist (Maren and Holt, 2004), and electrolytic lesion of the dorsal hippocampus (Kim and Fanselow, 1992) both failed to affect the auditory fear conditioning. Yet, many studies might shed light on the involvement of the dorsal hippocampus in fear conditioning under a unique circumstance. A human fMRI study showed that the hippocampus displayed elevated blood-oxygenation-level-dependent (BOLD) response when human subjects encountered an unexpected aversive stimulus (Ploghaus et al., 2000). The ability of dorsal hippocampal time cells to process temporal information further supports the hypothesis of dorsal hippocampus processes temporal-based negative prediction errors (MacDonald et al., 2011).

Amygdala Neuronal Network of Associative Learning and Prediction Error

To understand how the amygdala neuronal network processes negative prediction error in fear conditioning, we must first look at the neuronal processing underlying the pairing of conditioned stimulus (CS) and unconditioned stimulus (US) to induce long term potentiation (LTP) which facilitates associative learning in fear conditioning. “Cells that fire together wire together”, is one of the most prominent synaptic plasticity theories; Hebb (1961) hypothesized that the simultaneous activation of cells leads to an activity dependent, prolonged and significant increase in synaptic strength, mostly referred to as LTP. This theory has been commonly used to explain the associative fear learning in amygdala. When the cue is successfully paired with the aversive stimulus in fear conditioning, the neurons that are activated by the auditory cue are also activated by the aversive stimulus at about the same time. According to Hebb’s theory, the synapses that

process both the cue and aversive stimulus should be strengthened. After repetitive associative strengthening of the cue and stimulus inputs, the cue can acquire the ability to activate the fear response in the absence of stimulus input during the memory recall (Blair et al., 2001). Several studies have provided supporting evidence for the involvement of Hebbian plasticity at the cellular and synaptic level. First, single-unit recording studies have shown that the auditory cue and aversive stimulus converge in lateral amygdala at a single neuron level, in which the aversive stimulus could strengthen the processing of the cue (Romanski et al., 1993; Blair et al., 2001). Second, cue-evoked lateral amygdala neural responses were enhanced when the cue was temporally closely paired with the aversive stimulus (Quirk et al., 1995; McKernan and Shinnick-Gallagher, 1997; Quirk et al., 1997; Collins and Pare, 2000; Repa et al., 2001). Taken together, these data suggest that convergence occurs when the cue and stimulus were successfully paired, and convergence leads to the strengthening of the synaptic plasticity. However, evidence from single neuron studies might not provide us the full picture of this process. In addition, the Hebbian model might not entirely represent the dynamics of the BLA neural circuit in fear learning, as most of these studies were conducted on a single neuron level. The mechanism of fear learning and memory formation, as well as the effect of negative prediction error on them, are not fully understood on the circuitry level. Thus, using a neuronal calcium response imaging monitoring microendoscope on mice, we first investigated how BLA neurons encoded fear learning and memory in temporally predictable fear conditioning, then compared these results to those from the temporally

unpredictable fear conditioning to understand the impact of negative prediction error on fear learning and memory.

Specific Aims

The mechanisms underlying the processing of temporal unpredictability in fear conditioning are largely unexplored at both the circuitry and neuronal level. In this series of studies, we sought to understand how temporal unpredictability affects fear memory strength, identify the brain regions that process temporal unpredictability in fear conditioning and examine how negative prediction errors are processed in these brain regions. The hypothesis of our study is that *temporal unpredictability strengthens fear memory strength during classical fear conditioning via the recruitment of dorsal hippocampus and the rearrangement of the neural representation of fear learning and memory in BLA.* This hypothesis is tested by the following specific aims.

- In Chapter 3, we investigated whether temporal unpredictability that led to negative prediction errors altered the auditory fear memory strength and whether systematic increases in the degree of temporal unpredictability predicted auditory fear memory strength.
- In Chapter 4, we studied whether temporal unpredictability rearranged the neural representation of fear memory in BLA and the contribution of the rearrangement to the alteration in auditory fear memory strength.
- In Chapter 5, we explored the role of dorsal hippocampus in the processing of prediction errors by determining if the dorsal hippocampus processed the

temporally based negative prediction errors or processed the passage of time between the onset of the cues and the aversive stimuli.

CHAPTER TWO: GENERAL METHODS

Subjects

The subjects were either adult male Long-Evans rats (200–225 g) obtained from Taconic (Germantown, NY) or adult male mice (7-8 weeks of age) obtained from Charles River Laboratories (Wilmington, MA). Rodents were housed individually in plastic cages on a 12-hr light/12-hr dark cycle (lights on at 7:00 a.m.). Chow and water were provided ad libitum. All procedures were approved by the Committee on Animal Care at the Massachusetts Institute of Technology and the Animal Care and Use Review Office of the Army Research Office.

Training and testing were conducted in conditioning chambers (30 × 24 × 21 cm; MED-Associates, St. Albans, VT) with aluminum sides, a clear polycarbonate door and removable grid floors that delivered the foot-shock unconditioned stimuli (USs). Chambers were located in sound-attenuating cubicles containing speakers which auditory conditioned stimuli (CSs) were delivered. All training and testing was conducted between the hours of 10:00 a.m. and 5:00 p.m.

The interior of the chambers was manipulated to produce two distinct contexts. For Context A, the chambers were cleaned with 0.3% Pine-Sol (The Clorox Company, Oakland, CA), and chamber and room lights were on. Ventilation fans provided background noise. The animals were transported to and from the conditioning room in clear boxes. In Context B, a white plastic insert covered the chamber's grid floor, and a rounded white plastic insert was placed against the back wall. In this context, the chambers were cleaned with 1% acetic acid. Chamber and room lights were turned off,

and a red light provided room illumination. Animals were transported to and from the conditioning room in black boxes.

Histology and Immunohistochemistry

The animals were anaesthetized with isoflurane and perfused transcardially with 1× phosphate-buffered saline (PBS; 8,000 mg/L NaCl, 2,160 mg/L Na_2HPO_4, 200 mg/L KCl, 200 mg/L KH_2PO_4, 100 mg/L $MgCl_2 \cdot 6H_2O$, and 100 mg/L $CaCl_2$) followed by 4% paraformaldehyde. The brains were harvested and postfixed in 4% paraformaldehyde for 24 hrs, then cryoprotected by 30% sucrose in 1× PBS. For visualizing viral infusion, lens, cannula and fiber optics placement, the brains were cryosectioned into 30 to 50-μm slices, and alternating slices were mounted.

For cannula placement, sections were stained with 1% cresyl violet and examined by light microscopy. For viral infusion, lens, cannula and fiber optics placement, slices were mounted with coverslips and Vectashield-4,6-diamidino-2-phenylindole mounting medium (Vector Laboratories, Burlingame, CA). Sections were examined using an LSM 710 confocal scanning microscope equipped with a motorized *xy*-stage (Zeiss, Thornwood, NY). Image tiles (acquired in a 4 × 6 grid) were scanned using a 20× objective lens under optimal acquisition parameters (frame size = 1,024 × 1,024, pixel size = 0.42 μm, pinhole = 51.1 airy units, gain = 800, digital offset = 0, and digital gain = 1.0). Tiles were reassembled into a single image using Zeiss Zen Black software. All placement of lens, cannula and fiber optics was confirmed by comparing the images to a mouse (Franklin and Paxinos, 2008) and rat brain atlas (Paxinos and Watson, 2007).

CHAPTER THREE: TEMPORAL UNPREDICTABILITY ENHANCES FEAR MEMORY STRENGTH

Introduction

We aimed to determine whether variability in the timing of aversive stimulus altered the strength of auditory fear memory. Rats were fear conditioned using either standard conditions, in which a tone of fixed duration coterminated with foot-shock administration (predictable-shock groups), or unpredictable conditions, in which a foot-shock was delivered at a pseudorandom interval following each tone onset but during the tone presentation (unpredictable-shock groups). Contingency (defined as the probability of foot shock following the tone, held at 100%) and contiguity, factors that regulate associative memory strength (Rescorla, 1968; Bauer et al., 2001), were consistent across the predictable- and unpredictable-shock groups. Additional factors held constant across the two groups included the intertrial interval (ITI), cumulative exposure to the auditory stimulus, and the number of foot-shock presentations. The primary difference between the two conditions was whether the tone onset provided information about the specific timing of subsequent foot shock. Although some prior studies presented foot shock at variable times within a predictive cue during fear conditioning (Rescorla, 1968), the strength of the fear memory that resulted was not compared with the strength of fear memory after fear conditioning in which the foot shock follows cue onset at a fixed interval. In addition, studies using probabilistic timing of stimulus within predictive cues are unusual; the vast majority of contemporary studies using Pavlovian fear conditioning in rodents use fixed times relative to cue onset for stimulus.

Materials and Methods

Fear Conditioning

Rats were handled for at least 3 days before fear conditioning. Rats received fear conditioning in Context A with five pairings of tones (80 db, 2 kHz) and foot shocks (1 s, 0.7 mA); there was a 3-min period after the tone. For some rats, fear conditioning involved a 30-s tone (Fig. 1a); for other rats, fear conditioning involved a 42-s tone (Fig. 1b). Two groups of rats received predictable training, in which the foot shock was delivered at a consistent time after tone onset for every trial: 30 s after the onset of a 30-s tone (the predictable-shock/short-tone group) or 17 s after the onset of a 42-s tone (the predictable-shock/long-tone group). The other two groups of rats received one of two types of unpredictable training in which the timing of each foot shock varied after the onset of each tone. Rats in one unpredictable-training group (unpredictable-shock/short-tone) received fear conditioning with 30-s tones, but each foot shock was delivered at a pseudorandom time within the tone (6, 12, 18, 24, or 30 s after tone onset). Rats in the other unpredictable-training group (unpredictable-shock/long-tone) received fear conditioning with 42-s tones; foot shocks were also delivered at pseudorandom times within the tone (17, 23, 29, 35, or 41 s after tone onset). The day after fear conditioning, all rats were returned to Context A for context extinction (20 min). The following day, an initial tone-extinction session was conducted; rats were placed in Context B and received 20 tone presentations. The day after that, rats in the predictable-shock/short-tone and unpredictable-shock/short-tone groups were returned to Context B for a second tone-extinction session.

To investigate whether systematic increases in temporal unpredictability predicted fear memory strength, rats were assigned to one of three groups (Fig. 3a). A lower foot-shock intensity (0.5 mA vs. 0.7 mA) was used to insure ample room for any enhancement in fear by temporal unpredictability of foot shock. Rats in the one-unpredictable-trial group received one foot shock 2 s after tone onset, and the five remaining foot shocks were given 20 s after tone onset. Rats in the three-unpredictable-trials group received three foot shocks 20 s after tone onset, and the timing of the three remaining foot shocks was variable (2, 12, or 28 s after tone onset). Rats in the six-unpredictable-trials group received six foot shocks, the timing of which varied (2, 9, 16, 20, 25, or 30 s after tone onset). Thus, the average interval from CS onset to US onset was held constant across all groups (17 s). The day after fear conditioning, all rats were returned to Context A for context extinction (20 min). The day after that, rats were placed in Context B and received 15 tone presentations.

Statistics

Freezing behavior (i.e., lack of motion) in rats was used as a measure of the strength of fear memory. Behavior was recorded throughout all sessions (digitized at 30 Hz), and freezing was detected offline using commercial software (Video Freeze; Med Associates, Fairfax, VT). Using a proprietary formula, the software computes a motion index throughout the recorded session; this value increases in proportion to the amount of movement in the test box. The threshold of freezing (i.e., the value of the motion index below which no movement is detectable) was determined, and the percentage of observations below this threshold was calculated for the times of interest (interval before

the tone, tone presentation, and interval after the tone). Thus, freezing is reported as the percentage of time that rats displayed the freezing behavior within each period of interest. Motion had to be below the threshold for at least 1 s to be scored as freezing. Rats were excluded from all data analysis if, during the test of auditory fear-memory recall, they displayed high levels of freezing (> 80%) before the first tone presentation. Such behavior reflects inappropriate contextual generalization and interferes with the ability to attribute freezing specifically to the tone. Two rats were excluded from all analyses on the basis of this criterion. Conditioned freezing was compared using analyses of variance (ANOVAs), and planned comparisons were performed when the results of the analyses showed a significant omnibus *F* ratio. The data were analyzed using an ANOVA with factors of group (predictable-shock/short-tone vs. unpredictable-shock/short-tone, or predictable-shock/long-tone vs. unpredictable-shock/long-tone) or time (Bins 1–18 in the interval after the second tone).

Results

Despite the decrease in the information content of the tone onset under unpredictably timed foot-shock delivery, rats trained under these conditions displayed significantly stronger associative fear memories during auditory recall conducted in a novel context. There was a significant main effect of group, $F(1, 18) = 6.31$, $p = 0.022$ (Fig. 1c, right). In contrast, rats in the predictable-shock/short-tone and unpredictable-shock/short-tone groups displayed similar levels of freezing for contextual fear recall, $F(1, 18) = 0.001$, $p = 0.98$, which suggests that rats trained with unpredictably timed foot-shock delivery did not simply have nonspecific elevations in fear. The difference in

contextual versus auditory fear memory might arise because the context provided no reliable information about timing of the aversive foot shock and was thus a comparably unpredictable cue for rats in the predictable-shock/short-tone and unpredictable-shock/short-tone groups, whereas the auditory stimulus was significantly less informative for the unpredictable-shock/short-tone group than for the predictable-shock/short-tone group.

A straightforward interpretation of these results suggests that unpredictability in the timing of aversive outcomes can enhance fear. Alternatively, multiple theories suggest that timing plays an important role in regulating the formation of associative memories (Kirkpatrick and Balsam, 2016). In particular, these theories suggest that differences in the time between the onset of the predictive cue and the onset of stimuli (the ISI; sometimes called the trial time) are critical for determining the rate and asymptote of learning. When the timing of the foot shock was altered from trial to trial (Fig. 1a), the ISI value and other related measures also varied across the predictable-shock/short-tone and unpredictable-shock/short-tone groups (Table 1). Thus, differences in memory strength across the two groups could have arisen from group differences in any of these conditioning parameters.

To test whether fear-memory strength was enhanced by temporal unpredictability per se or was determined simply by the temporal duration of conditioning parameters, we ran a second set of rats for which the temporal differences between the predictable and unpredictable groups were systematically reversed from those used for the previous groups (Table 1). The rats in the unpredictable-shock/long-tone group displayed

significantly greater auditory fear memory recall than rats in the predictable-shock/long-tone group; there was a significant main effect of group, $F(1, 24) = 7.84$, $p = .0099$ (Fig. 1d). As before, contextual fear recall was unaffected, $F(1, 24) = 0.004$, $p = .95$ (Fig. 1d).

Thus, conditioned freezing in the unpredictable groups relative to that in the predictable groups was determined by the unpredictability surrounding the timing of the foot shock and not by temporal parameters that varied across the groups (Table 1). These findings should not be used to discount the important role of timing in the acquisition of associative memory. However, it does suggest that the restricted differences in ISIs that we used in this experiment (e.g., 12-s maximum difference in mean ISI) were not sufficient to drive differences in learning, at least for amygdala-dependent fear behaviors. These results reveal that fear learning, as measured by the magnitude of conditioned freezing, is exquisitely sensitive to temporal variability in the occurrence of negative events following predictive stimuli. This finding is surprising because rapidly acquired Pavlovian fear (as used in this experiment) evokes many behavioral and endocrine responses that are not regulated in a temporally precise manner. For example, the expression of most fear responses, including conditioned freezing, release of stress hormones, and changes in blood pressure and heart rate, is not limited to the CS. Instrumental avoidance responses, by contrast, must occur within a specific period relative to the CS to produce successful avoidance of aversive stimuli.

To further determine when information about the timing of an aversive event is acquired during learning, we examined freezing on the conditioning day (Fig. 2). The percentage of time that the rats in the predictable-shock and unpredictable-shock groups

displayed freezing behavior was statistically indistinguishable until the period after the second pairing of tone and foot shock; a 2 (group: predictable shock/short tone vs. unpredictable shock/short tone) × 18 (Time: Bins 1–18 in the interval after the second tone) ANOVA revealed a significant interaction, $F(17, 306) = 1.6$, $p = .050$; at this point, rats in the unpredictable-shock group exhibited higher levels of freezing than rats in the predictable-shock group. Thus, fear was heightened after the first trial in which the timing of the foot shock became unpredictable. This result strongly suggests that information about the timing between the onset of a cue and the occurrence of a subsequent aversive event is acquired during the first pairing of the cue and aversive event, which is surprising because novel cues do not necessarily have predictive value. Thus, one might expect that the need to encode the passage of time after the onset of a novel cue is minimal until repeated presentations of a cue indicate that the cue is associated with the occurrence of a significant event. However, the current finding is consistent with findings from other studies showing that, within aversive learning, temporal control of conditioned responding can sometimes emerge after very few conditioning trials (Davis et al., 1989; Drew et al., 2005).

To further investigate the relationship between temporal unpredictability and fear, we examined whether systematic increases in temporal unpredictability predicted long-term strength of fear memory. We found that increasing levels of temporal unpredictability also increased fear-memory strength; there was a significant main effect of group, $F(2, 16) = 6.76$, $p = .0070$ (Fig. 3b), which provides further support for a direct relationship between temporal unpredictability and the strength of long-term fear

memory. In addition, because the average interval between CS onset and US onset was held constant across the three conditions, this experiment provided further support for the idea that unpredictability in the timing of the aversive foot shock is the key factor in fear-memory enhancement observed after conditioning with an ambiguously timed aversive stimulus.

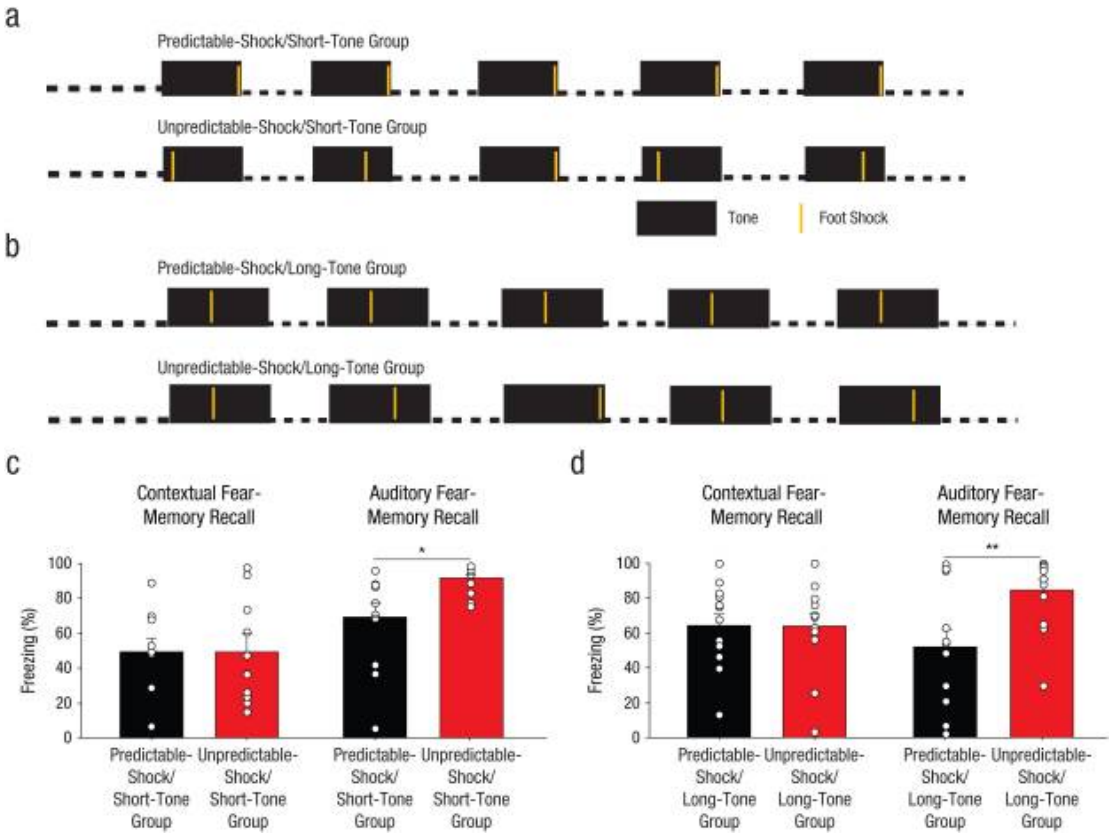

Figure 1. Enhancement of fear memory strength by temporal unpredictability

Rats (n = 10 per group) were fear conditioned with five pairings of a 30-s tone with a 1-s foot shock, as illustrated in (a); there was a 210-s intertrial interval (ITI). For the predictable-shock/short-tone group (which received predictable training and a 30-s tone), each tone coterminated with a foot shock. For the unpredictable-shock/short-tone group (which received unpredictable training and a 30-s tone), each tone was paired with a foot shock that occurred pseudorandomly during the tone. Thus, interstimulus intervals (ISIs) were shorter for this group than for the predictable-shock/short-tone group. The other two groups of rats, the predictable-shock/long-tone and unpredictable-shock/long-tone groups, were fear conditioned the same way, except that the tones were 42 s instead of 30

s, as illustrated in (b). For the unpredictable-shock/long-tone group, each tone was paired with a foot shock that occurred pseudorandomly during the tone, so the average ISI was longer for this group than for the predictable-shock/long-tone group. The bar graphs show the mean percentage of time the rats displayed freezing behavior in each (c) short-tone group and (d) long-tone group, separately for contextual fear-memory recall and auditory fear-memory recall. The small open circles represent the percentage of time that individual rats displayed freezing behavior. Error bars represent +1 *SEM*. Asterisks indicate significant differences between groups (*$p < .05$, **$p < .01$).

Conditioning Paradigm	Interstimulus Interval (ISI; from tone onset to footshock onset)				Cycle Time (C/T)
	Average (s)	Minimum (s)	Maximum (s)	Median (s)	
PRED	30	30	30	30	7
UNPRED-short	18	6	30	18	11.92
PRED	17	17	17	17	13.05
UNPRED-long	29	17	41	29	7.81

Table 1. Parameters for the auditory fear conditioning paradigms of temporally predictable and unpredictable footshock.

The values for different parameters used in Experiment 1 are depicted. Pink boxes indicate a value that is greater for the PRED condition compared to the UNPRED condition. Blue boxes indicate a value that is greater for the UNPRED condition compared to the PRED condition. White boxes indicate a value that is equivalent across the PRED and UNPRED conditions. Note that the relationship between the PRED and UNPRED groups is systematically reversed between the UNPRED-short and UNPRED-long groups.

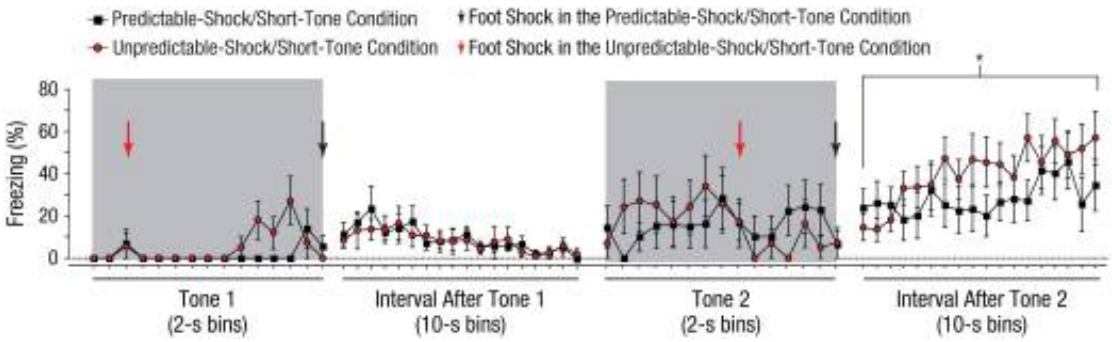

Figure 2. Rapid processing of temporal unpredictability enhanced freezing behavior

Freezing behavior during the first two conditioning trials is graphed as a function of time, separately for rats in the predictable-shock/short-tone group (n = 10) and rats in the unpredictable-shock/short-tone group (n = 10). Each data point during the tone presentations represents the mean percentage of time that groups of rats displayed freezing behavior during a 2-s period; each data point during the intervals after the tones represents the mean percentage of time that groups of rats displayed freezing behavior during a 10-s period. Error bars indicate ±1 *SEM*. The asterisk indicates a significant difference between the behavior of the two groups (*p < .05).

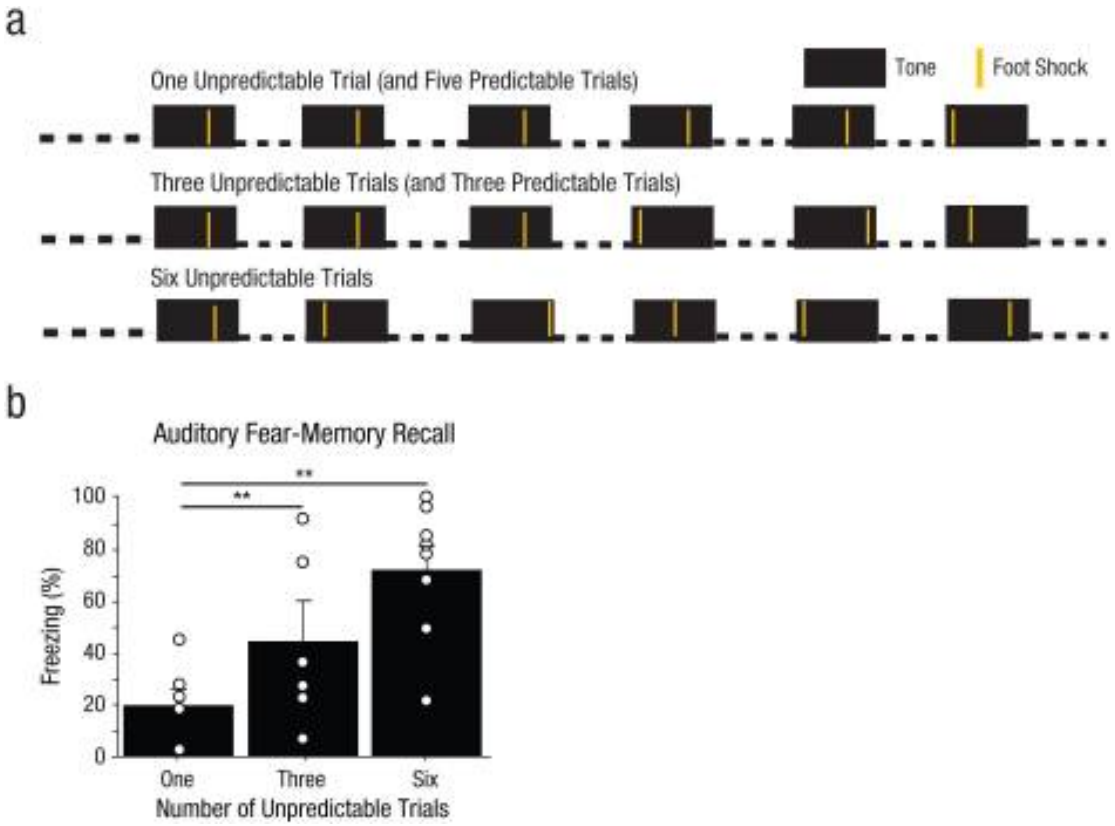

Figure 3. Level of temporal unpredictability affected the fear memory strength

Three groups of rats (n = 6–8 per group) were fear conditioned with pairings of a 30-s tone with a 1-s foot shock, as illustrated in (a); there was a 3-min interval after the tone. The three conditions had one, three, or six temporally unpredictable trials. The average interval between tone onset and foot shock onset was held constant across the three groups. The day after fear conditioning, all the rats were returned to the conditioning context for 20 min. The day after that, the rats were placed in a novel context, and auditory fear recall was measured as average freezing across the first 2 of 15 tone presentations. In (b), results are shown separately for the two groups. The bars show the mean percentage of time that groups of rats displayed freezing behavior, and the small open circles represent the percentage of time that individual rats displayed freezing

behavior. Error bars represent +1 *SEM*. Asterisks indicate significant differences between the group with one predictable trial and each of the other groups (**$p < .01$).

CHAPTER FOUR: NEURAL REPRESENTATION OF TEMPORAL UNPREDICTABILITY IN AMYGDALA

Introduction

Pavlovian fear conditioning consisting of predictive cues, such as tones followed by aversive stimuli, such as footshocks, is a behavioral model commonly used to study negative prediction error, due the ease in manipulating the predictive cues to create temporal unpredictability. Despite its prevalence in field of unpredictability studies, the fundamental question of how the negative prediction errors are being processed in amygdala has been omitted. A few studies have used chemogenetic manipulation (Sengupta et al., 2016), hemodynamic and local field potential recording (McHugh et al., 2014) to demonstrated the role of BLA in processing prediction errors, whereas most of the studies focus on the periaqueductal gray-based neural circuits that controls the fear memory strength by processing the prediction error signaling (Fanselow, 1998; McNally and Westbrook, 2006; Johansen et al., 2010; McNally et al., 2011; Herry and Johansen, 2014).

To study the neural representation of temporal unpredictability in amygdala and the mechanism to control the strength of fear memory, we must first find out how fear memory is being encoded in the amygdala. Decades of studies of associative memory have yielded a mechanistic model in which sensory inputs carrying information about a predictive cue and an aversive stimulus converge upon single neurons in the BLA and other brain regions (Campese et al., 2016). The sensory convergence of weak neural activation by a tone and strong neural activation by a footshock (Barot et al., 2009),

combined with “teaching signals” based on the predictability of the aversive stimulus (Johansen et al., 2010), is believed to be a critical driver of long-term synaptic strengthening within individual neurons that encode associative fear memories. Specifically, the strong activation by the footshock is thought to induce somal action potentials, which trigger both backfiring action potentials and transcriptional changes (Blair et al., 2001) that strengthen connections to a subset of auditory afferents. The BLA neurons that show synaptic strengthening during fear learning are believed to form sparse networks, arising from competition between the neurons that ultimately display long-term synaptic strengthening and their proximal neighbors (Han et al., 2007; Rashid et al., 2016). By enhancing the activation of a subset of BLA neurons in response to the predictive cue, synaptic plasticity in the BLA can promote fear behaviors via projections to other regions involved in the selection of such behaviors (Herry et al., 2008).

Although this model has come to dominate the field, many studies provide only indirect support for the ideas. To directly address the question of how activity in single neurons during learning influences participation in long-term memory recall, one must be able to assess the activity of neurons at both encoding and temporally distant recall. Until recently, techniques did not permit assessment of activity at multiple time points. Here, we first sought to confirm the hypothesis that long-term fear memories arise from competitive neural networks by expressing the calcium indicator GCaMP6s (Chen et al., 2013) in BLA neurons and using time-lapse microendoscopy (Ghosh et al., 2011) to monitor calcium dynamics in BLA neurons during both fear conditioning and subsequent long-term memory recall. Neurons were classified as tone-responsive or non-responsive

during fear recall, and calcium activity during fear conditioning was assessed to examine the hypothesis that neurons that participated in fear memory recall exhibit distinct activity patterns during fear conditioning. We then further investigated whether temporal unpredictability altered these calcium activity patterns and the ratio of these functionally classified neurons. By demonstrating the influence of temporal unpredictability on the BLA neural representation of fear learning and memory, it might shed lights on how fear memory strength might be regulated within amygdala.

Materials and Methods

Surgery

Mice received a stereotactic injection of 1000 nl AAV5.syn.GCaMP6s.WPRE. SV40 (Chen et al., 2013) (3.06x10^13 GC/ml; Penn Vector Core, Philadelphia, PA) into the right basolateral amygdale (BLA) at a rate of 100 nl/min. The mice were anesthetized with 1.75% isoflurane (inhaled) and head-fixed in a stereotaxic. A heating pad was used to maintain body temperature. Opthalmic lubricant was applied to the eyes to prevent dryness. Fur was removed from the scalp using clippers. The skin was cleaned with Betadine and alcohol swabs. An incision was made to expose the skull. A dental drill was used to make a 1 mm round craniotomy to allow the virus infusion and GRIN lens (0.6 mm in diameter; 7.3 mm length, Inscopix Inc, Palo Alto, CA) implantation. The surgical coordinates were (relative to bregma): anterior-posterior (AP) -1.6 mm; lateral-medial (ML) 3 mm in right hemisphere; dorsal-ventral (DV) -3.5 mm. The viral infusions were performed 4 weeks prior to the calcium imaging to allow optimal viral expression in the BLA. The stereotactic injection was performed with a 33-gauge metal needle and a 10μl

glass syringe that was controlled by a stereotaxic injector. The GRIN lens was implanted and secured with dental cement following the virus infusions. A lens probe protector was temporarily installed to keep the surface of GRIN lens clean and safe from impact.

Four weeks later, the mice were re-anesthetized. We removed the lens probe protector to inspect the expression of GCaMP6s with a microendoscope (nVistaHD, Inscopix Inc, Palo Alto, CA). A baseplate for the miniature microscope was then secured on the cured dental cement. A baseplate cover was attached to the baseplate to protect the lens probe imaging surface.

Imaging

The microendoscope was attached to the baseplate and imaging was performed throughout all behavioral sessions (described below). The nVistaHD system was connected to the microendoscope and the nVistaHD software was used to record the calcium activity in the BLA. With an exposure time of 50 ms per frame, the fluorescence Ca^{2+} imaging movies were acquired at a frame rate of 20 fps to yield 20 observations per second.

Fear Conditioning

Prior to fear conditioning, mice were handled daily for a week. Mice received fear conditioning that consisted of 6 tone (86 db, 7 kHz, 40 s) – footshock (0.6 mA, 2 s) pairings (2 min intertribal interval) in fear conditioning chambers (30 × 24 × 21 cm, MED-Associates, St. Albans, VT) with aluminum sides, a clear polycarbonate door and a speaker. Footshocks were delivered through grid floors which consisted of 36 rods (0.32 cm in diameter, 0.81 cm apart). For the PRED group, each footshock was delivered 20 s

after the onset of the tone. In contrast, each footshock was delivered at a pseudorandom time within the tone (30, 10, 23, 38, 15, 4 s after tone onset) for the UNPRED group. The chamber was cleaned with 0.3% Pine-Sol; room and chamber lights were on, and ventilation fans were on to provide background noise. The mice were transported to and from the behavioral room in clear boxes.

Two days following fear conditioning, all mice were returned to the identical fear conditioning boxes for contextual extinction (8 min). The chambers were cleaned with 0.3% Pine-Sol. Chamber and behavioral room lights were on; and ventilation fans were on to provide background noise. The mice were transported to and from the conditioning room in clear boxes. One day following contextual extinction, all mice were subjected to a long-term auditory fear memory test. Mice were placed in chambers with white plastic inserts against the back and side walls and on top of the grid floor. Chambers were cleaned with 1% acetic acid. Ten tone presentations (86 db, 7 kHz, 2 min intertrial interval) were played. Chamber and behavioral room lights were off and a dim red light (15 W) provided room illumination. The mice were transported to and from the conditioning room in black boxes.

Imaging Analysis

Mosaic software (Inscopix Inc, Palo Alto, CA) was used to perform all image analysis. Movies were not concatenated; rather, each movie was processed individually. All movie frames were preprocessed to fix defective pixels and isolated dropped frames. The movie size was reduced by 4-fold by using a spatial binning factor of 2. The motion correction tool was used to align the movie frames to a single target image to correct the

brain movement. A change in the pixel intensity over the baseline (ΔF/F0) was generated based on the average of the pixel intensity (F0) within a specific period (3 min before the onset of the first tone). Calcium responses from single neurons were isolated and identified with a Principle Component-Independent Component Analysis algorithm on ΔF/F0 movies. Single neuron images and traces representing the calcium response of individual neurons were selected for further analysis. Statistical analysis was performed on all trials from fear conditioning and the long-term auditory fear memory test. For each trial, the ΔF/F0 values were extracted from a period spanning the 5 sec before each tone presentation until 5 sec after the termination of each tone. The ΔF/F0 values for each trial were normalized (as z-scores) to the 5-sec period prior to each tone.

Statistics

Neurons were classified as Tone-Responsive or Non-Responsive during fear recall. For each neuron, the average calcium response across Trials 1-3 of the tone extinction session was calculated. The calcium response during tone onset (3 sec, or 60 observations) was compared to the calcium response during the period immediately prior to tone onset (also 3 sec) for each trial using the Wilcoxon Signed Rank Test with Bonferroni correction. The 3-sec window was set to include the complete phasic calcium response elicited by the tone. All neurons with significantly greater calcium during the tone onset were classified as Tone-Responsive. Two-tailed, unpaired t-tests with Welch's correction were used to test the hypotheses that Non-Responsive neurons were less tone-responsive at the start of fear recall than during late extinction, and that Non-Responsive

neurons were less tone-responsive at the start of fear recall than Tone-Responsive neurons.

The responsiveness of Tone-Responsive and Non-Responsive neurons to the tone and shock during fear conditioning was calculated. For each neuron, the z-score calcium response during tone onset (3 sec, or 60 observations) was compared to the z-score calcium response during the period immediately prior to tone onset (also 3 sec) for each trial using the Wilcoxon Signed Rank Test with Bonferroni correction. The same statistical tests were also used to compare the calcium response during the shock (4 sec, or 80 observations) to the period immediately prior to shock onset (also 4 sec) for each of the six conditioning trials. The 4-sec window was set to include the complete phasic calcium response elicited by the shock. The total number of Tone-Responsive and Non-Responsive neurons exhibiting a significant tone response, shock response, or tone and shock response, was calculated for each trial, and expressed as a percent of all Tone-Responsive or Non-Responsive neurons. Two-tailed Wilcoxon Signed Rank Tests were used to compare cue responsiveness and stimulus convergence during fear conditioning in individual neuron populations. An unpaired t-test with Welch's correction was used to compare the mean number of trials with stimulus convergence in the Tone-Responsive versus Non-Responsive populations and in the Memory Winner versus the Memory Loser populations.

A two-way ANOVA was used to compare the calcium-evoked responses either during the tone or during the shock within the fear conditioning session between all

neurons imaged during fear conditioning and the subset of neurons that was also imaged during the long-term memory test.

Results

We first identified the BLA neurons that displayed a significant tone-evoked increase in intracellular calcium during fear recall; for all analyses, "fear recall" measures were assessed during the first three trials of the fear recall test. BLA neurons that display tone-evoked firing during fear recall are thought to provide excitatory drive to downstream structures that determine and express an appropriate repertoire of fear behaviors, and thus exemplify some of the features believed to underlie putative long-term fear memory neurons (Han et al., 2007; Duvarci and Pare, 2014). Of the 334 BLA neurons that were imaged during long-term auditory fear recall of the PRED group, approximately 28% of all neurons were classified as Tone-Responsive during the recall test (Fig. 4b), a percentage consistent with studies using other methodologies such as immediate-early gene expression (Han et al., 2007) and single-unit recording (Quirk et al., 1995). Temporal unpredictability, which is known to increase associative long-term fear memory strength, had significant impact on the percentage of Tone-Responsive cells during recall, causing the percentage of Tone-Responsive cells increased more than two-fold from ~28% to ~61% (Fig. 4b).

As a population, the Tone-Responsive neurons of the PRED group displayed a pattern of tone-evoked calcium responses that mirror what has been reported for single-unit cells during fear recall and extinction (Quirk et al., 1995): the tone-evoked calcium response was greatest during fear recall, and decreased with continued non-reinforced

presentations of the auditory cue (Fig. 4c, first panel; main effect of extinction trial: $F(2,420) = 433.4$, $p < 0.0001$; Recall vs. Late Ext: $t(67.53) = 12.78$, $p < 0.0001$). Identical tone-evoked calcium responses pattern was observed from the Tone-Responsive neurons of the UNPRED group, but their calcium responses were evoked at a smaller magnitude (Fig. 4c, third panel; main effect of extinction trial: $F(1,240) = 658.5$, $p < 0.0001$; Recall vs. Late Ext: $F(1,240) = 1530$, $p < 0.0001$). Unlike single-unit recording, the calcium response displayed slow kinetics, with a peak response starting at ~450ms after tone onset and persisting for ~5 sec, a time course consistent with the properties of the calcium indicator used here (Chen et al., 2013).

While the population of Non-Responsive BLA neurons of the PRED group significantly altered its response across the fear memory test (Fig. 4c, second panel; main effect of trial: $F(2,420) = 117.0$, $p < 0.0001$), the tone-evoked calcium response was greatest at the end of the extinction session, rather than during fear recall (Fig. 4c, second panel; Recall vs. Late Ext: $t(83.91) = 12.09$, $p < 0.0001$). Additionally, Non-Responsive neurons exhibited a smaller tone-evoked calcium response during fear recall than the Tone-Responsive neurons (Fig. 4c, second panel, $t(24.53) = 25.53$, $p < 0.0001$). Comparing with the pattern of calcium responses of the PRED group, the population of Non-Responsive BLA neurons of the UNPRED group displayed a different pattern of calcium responses. The temporal unpredictability induced significant greater tone-evoked calcium response at both the extinction and late extinction sessions, while comparing with the recall session (Fig. 4c, bottom panel; main effect of extinction trial: $F(1,240) = 1876$, $p < 0.0001$; Recall vs. Late Ext: $F(1,240) = 2263$, $p < 0.0001$). However, the tone-

evoked calcium response of all neurons during the recall session was significant smaller in the UNPRED group while comparing with the PRED group (Fig. 4c, Fear recall of Tone-Responsive neurons PRED vs. UNPRED: $F(1,240) = 35.86$ $p < 0.0001$, Fear recall of Non-Responsive neurons PRED vs. UNPRED: $F(1,240) = 3516$ $p < 0.0001$).

A majority of BLA neurons can be activated by a tone (Quirk et al., 1995) or a shock (Johansen et al., 2010), indicating that a significant proportion of BLA neurons are eligible to encode associative fear memories. However, only approximately one-quarter typically display fear conditioning-related increases in tone-evoked activity, suggesting that neurons may compete to encode long-term fear memories (Han et al., 2007; Rashid et al., 2016). We sought to determine whether patterns of activity during fear conditioning could predict which individual neurons would subsequently participate in long-term auditory memory recall. Furthermore, we investigated whether the temporal unpredictability was able to alter these patterns during fear conditioning. Spatial neuron maps for the fear conditioning and auditory fear recall session for each mouse were manually aligned and neurons imaged during both sessions were identified. For the PRED, of the 334 BLA neurons imaged during the auditory fear recall test, 216 of these (64.7%) were also present during the fear conditioning session. As for the UNPRED, 426 BLA neurons were imaged during the auditory fear recall test and 196 (46%) of these were also present during the fear conditioning session.

Tone-Responsive neurons of the PRED group from fear recall displayed significant tone-evoked increases in intracellular calcium during fear conditioning, which increased with successive trials (Fig. 5a, main effect of Trial, $F(1,240) = 37.67$, $p <$

0.0001); these neurons also maintained the calcium response to the footshock reinforcer (Fig. 5a, main effect of Trial, $F(1,320) = 0.0074$, $p = 0.93$). This is consistent with classical associative learning theory, which posits that long-term associative memory neurons should be responsive to both the cue and reinforcer during fear conditioning (Barot et al., 2009). Similar with the pattern of activity of the PRED group, Tone-Responsive neurons of the UNPRED group also demonstrated significant tone-evoked increase in intracellular calcium across successive trials (Fig. 5e upper panel, Trial 1-3 vs. Trial 4-6, main effect of Trial, $F(1,240) = 24.80$, $p < 0.0001$). Unlike in the PRED group in which the calcium response to the footshock reinforcer was maintained across successive trials, temporal unpredictability increased the calcium response to the footshock reinforcer across trials (Fig. 5e lower panel, Trial 1-3 vs. Trial 4-6, main effect of Trial, $F(1,320) = 24.22$, $p < 0.0001$). This may strengthen the BLA neuronal plasticity and led to the temporal unpredictability-enhanced fear response.

In contrast, the Non-Responsive neurons from fear recall displayed a different pattern of calcium activity across fear conditioning. The tone-evoked calcium response of the population of Non-Responsive neurons did not increase with successive trials in both the PRED and UNPRED group (Fig. 5b and f). In the early trials of the PRED group, the population of Non-Responsive neurons was equally responsive to the tone as the population of Tone-Responsive neurons (Figure 5a gray vs. 5b gray, main effect of Population, $F(1,240) = .504$, $p = 0.48$). As for the UNPRED group, the population of Non-Responsive neurons counterintuitively displayed a significantly higher tone-evoked calcium response than the Tone-Responsive neurons did (Fig. 5e-f upper panel,

comparing first 3s after tone onset for Trials 1-3: $F(1,240) = 18.9$, $p < 0.0001$), and the higher tone-evoked calcium response was maintained across successive trials (Fig. 5f upper panel, Trial 1-3 vs. Trial 4-6, main effect of Trial, $F(1,240) = 1.028$, $p = 0.3106$). In the PRED group, in contrast to the Tone-Responsive neurons, the Non-Responsive neurons were significantly less shock-responsive at the start of fear conditioning than the Tone-Responsive neurons (Fig. 5a-b, comparing first 4s after shock onset for Trials 1-3: $F(1,320) = 26.17$, $p < 0.0001$) and decreased their sensitivity to the footshock reinforcer with successive fear conditioning trials (Fig.5b, main effect of Trial, $F(1,320) = 118.2$, $p < 0.0001$). Surprisingly, of the UNPRED group, the Non-Responsive neurons displayed significantly higher shock-responsive at the start of fear conditioning than the Tone-Responsive neurons (Fig. 5e-f lower panel, comparing first 4s after shock onset for Trials 1-3: $F(1,320) = 10.13$, $p = 0.0016$). Thus, Tone-Responsive neurons can be differentiated from Non-Responsive neurons based on calcium dynamics during fear conditioning. In PRED group, only Tone-Responsive neurons increase tone-evoked calcium and maintain shock-evoked calcium activity across fear conditioning trials; whereas in UNPRED group, only Tone-Responsive neurons increase both tone-evoked and shock-evoked calcium activity across fear conditioning trials, but the magnitude of tone-evoked and shock-evoked calcium activity are lower than those of the PRED group.

To further clarify whether Tone-Responsive and Non-Responsive BLA neurons from long term fear recall differ in terms of their responses to the tone and footshock during fear conditioning, we analyzed the tone- and footshock-evoked calcium response for each neuron for each of the six fear conditioning trials and computed the average

percentage of neurons that were tone-responsive, shock-responsive and responsive to both stimuli within single trials. It is surprising that the overall percentage of Tone-Responsive BLA neurons showing sensory convergence is low for any given predictable fear conditioning trial (14.7%), and that a similar, though statistically smaller, percentage of Non-Responsive BLA neurons also show sensory convergence (10.9%). One way in which neurons in the Tone-Responsive class could be more likely to exhibit associative plasticity than neurons in the Non-Responsive class is if individual Tone-Responsive neurons display sensory convergence across a greater number of the fear conditioning trials. In other words, while 10.9% of Non-Responsive neurons show sensory convergence on any given trial, perhaps it is a different population of neurons with convergence on each trial, whereas individual Tone-Responsive neurons might show sensory convergence across multiple trials. By determining the total number of fear conditioning trials in which each individual BLA neuron exhibited an increased calcium response to both the tone and shock, we found that Tone-Responsive neurons did display statistically greater convergence of the tone and shock during fear conditioning trials, compared to Non-Responsive neurons (Fig. 6; Unpaired student's t test, $p = 0.033$). In contrast, temporal unpredictability increased the percentage of Tone-Responsive BLA neurons and Non-Responsive BLA neurons that showed sensory convergence for any given fear conditioning trial from 14.7% to 49.6% and 10.9% to 44% respectively. While looking at the total number of fear conditioning trials in which each individual BLA neuron exhibited an increased calcium response to both the tone and shock, we found that

temporal unpredictability diminished the difference between Tone-Responsive neurons and the Non-Responsive neurons observed in the PRED group (Fig. 6).

These analyses highlighted several unexpected findings. First, sensory convergence in BLA neurons is highly probabilistic during fear learning, and there is remarkable heterogeneity across individual neurons as to the degree of sensory convergence across trials. Despite this, there is a population of BLA neurons that has sensory convergence during fear conditioning that also displays significant tone-evoked activity during fear recall (~17% of the imaged neuron population in the PRED group); this population most likely corresponds to long-term "memory" neurons that encode and express fearful associations, termed as Memory "Winner" neurons (Fig. 7a,b). A second population completely lacks sensory convergence during fear conditioning and also fails to display significant tone-evoked activity during fear recall (~37% of the imaged neuron population in the PRED group; Fig. 7a,b); we refer to these neurons as "Other" because they may regulate non-fear functions of the amygdala, or contribute to fear-related processes that we did not investigate here. However, two additional neuron populations were identified: 1) Memory "Loser" neurons that exhibit sensory convergence during fear conditioning, but which fail to display tone-evoked activity during fear recall (~35% of the imaged neuron population, Fig. 7a,b), and 2) "Fear Expression" neurons that do not exhibit sensory convergence on any fear conditioning trial but which nevertheless display tone-evoked activity during fear recall (~11% of the imaged neuron population in the PRED group, Fig. 6a,b). The former population may correspond to the "Loser" neurons resulting from a process by which eligible BLA neurons (neurons with the appropriate

sensory inputs) compete to encode associative fear memories (Han et al., 2007; Rashid et al., 2016). The latter may correspond to a previously undiscovered class of BLA neurons that contributes to fear expression, per se. When temporal unpredictability was introduced into the fear learning, it dramatically shifted the ratio of these four classifications of neurons. Temporal unpredictability increased the proportion of Tone-Responsive neurons (Fig. 4b), in which the percentage of Memory "Winner" and "Fear Expression" neurons were approximately tripled (from ~10% to ~31%) and doubled (from ~17% to ~31%) (Fig. 7b). In response to this sharp increase, the proportion of Memory "Loser" and "Other" neurons were decreased. The percentage of Memory "Loser" neurons was halved from ~35% to ~17%; the percentage of "Other" neurons also dropped from 37% to 21% (Fig. 7b). The ratio of Memory "Winner" neurons/Memory "Loser" neurons increased from 0.49 to 1.82.

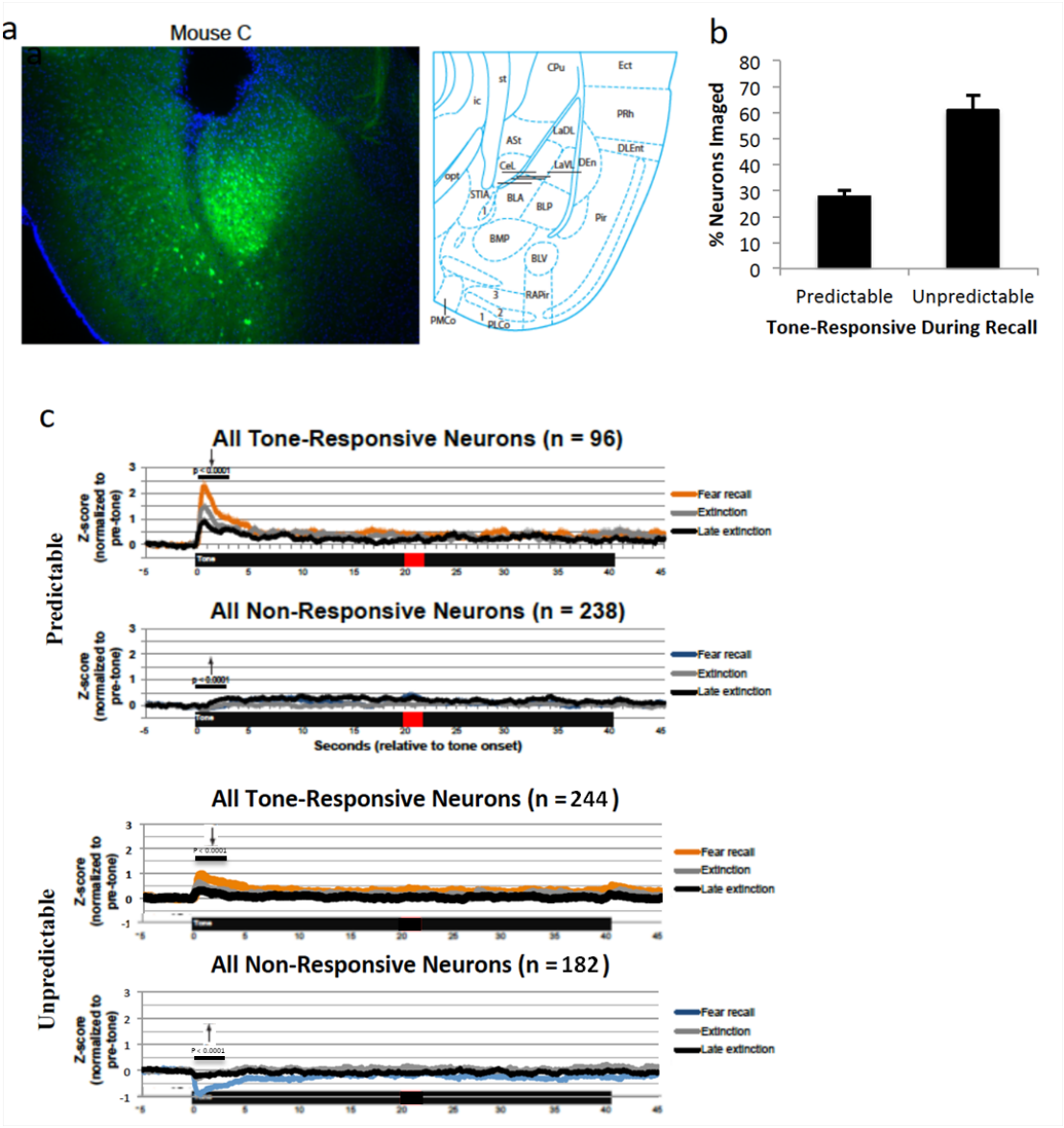

Figure 4. Calcium activity of Tone-Responsive and Non-Responsive neurons during fear recall

A photomicrograph (5X) showing representative infection in the BLA and the GRIN lens implanted above the infection site. (a) Green signal indicates GFP expression in infected cells. Blue signal indicates DAPI staining of the nuclei. The face of the GRIN lens for the

five mice with implantation successfully targeting the BLA is depicted. All neurons (334 neurons from Predictable and 426 neurons from Unpredictable group, each group consists of 5 mice) imaged during the memory test were classified as Tone-Responsive or Non-Responsive during fear recall. The percentages of Tone-Responsive cells from the Predictable and Unpredictable groups are depicted (b). Tone-evoked calcium responses during the long-term memory test of the Predictable and Unpredictable groups are depicted (c), averaged across all Tone-Responsive (upper panel) or Non-Responsive (lower panel) cells. The black bar indicates the presence of a tone, and the red bar in the Predictable groups indicates the time of expected footshock (footshock was not administered during the memory test). The black line below p-values indicates the period being tested (3-s for tone; 4-s for shock).

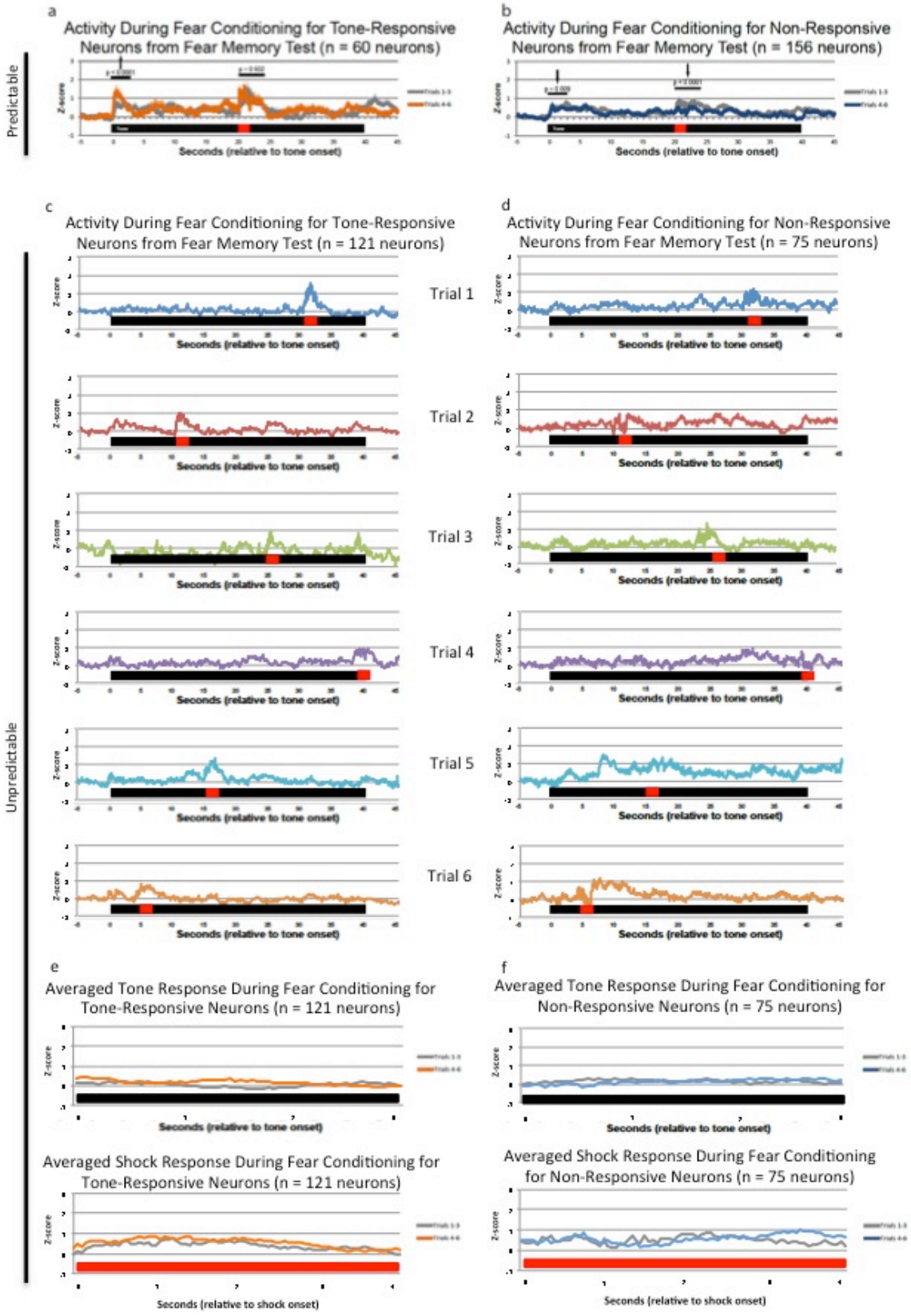
a
Activity During Fear Conditioning for Tone-Responsive Neurons from Fear Memory Test (n = 60 neurons)
b
Activity During Fear Conditioning for Non-Responsive Neurons from Fear Memory Test (n = 156 neurons)
Predictable
Z-score
Seconds (relative to tone onset)
c
Activity During Fear Conditioning for Tone-Responsive Neurons from Fear Memory Test (n = 121 neurons)
d
Activity During Fear Conditioning for Non-Responsive Neurons from Fear Memory Test (n = 75 neurons)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Unpredictable
e
Averaged Tone Response During Fear Conditioning for Tone-Responsive Neurons (n = 121 neurons)
f
Averaged Tone Response During Fear Conditioning for Non-Responsive Neurons (n = 75 neurons)
Averaged Shock Response During Fear Conditioning for Tone-Responsive Neurons (n = 121 neurons)
Averaged Shock Response During Fear Conditioning for Non-Responsive Neurons (n = 75 neurons)
Seconds (relative to shock onset)

Figure 5. Calcium activity of Tone-Responsive and Non-Responsive neurons during fear conditioning

A subset of the neurons (classified as Tone-Responsive or Non-Responsive based on tone-evoked calcium activity during auditory fear recall) imaged during the long-term memory test, were also imaged during the predictable fear conditioning (216/334 neurons) or the unpredictable fear conditioning (196/426 neurons). The tone-evoked calcium response during predictable fear conditioning is depicted for these Tone-Responsive (a) and Non-Responsive (b) neurons. The tone- and shock-evoked calcium responses of Tone-Responsive (c) and Non-Responsive (d) neurons during unpredictable fear conditioning are depicted individually for each trial. The averaged tone- (upper panels of e and f) and shock-evoked (lower panels of e and f) calcium responses of Trial 1-3 (gray) and Trial 4-6 (orange and blue for Tone-Responsive (e) and Non-Responsive (f) respectively) are demonstrated. The black bar indicates the duration of the tone; the red bar indicates the time of expected footshock. The black line below p-values indicates the period being tested (3-s for tone; 4-s for shock).

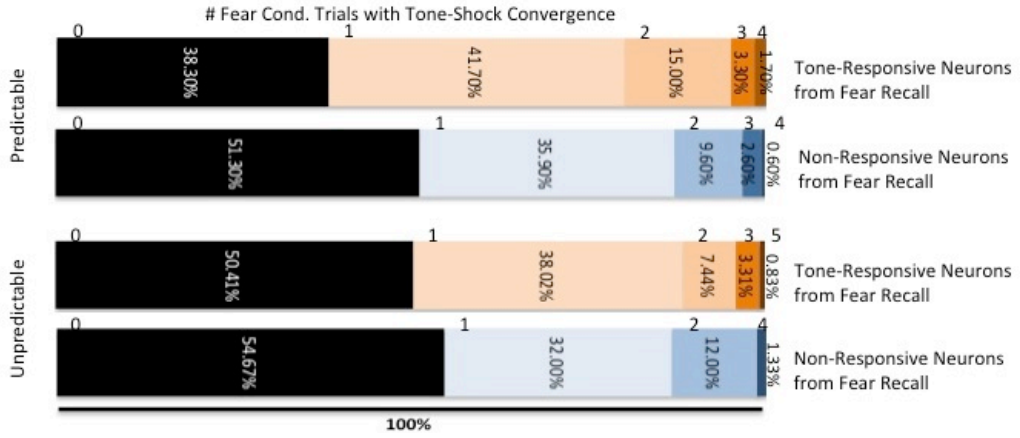

Figure 6. Number of tone-shock convergence of Tone-Responsive and Non-Responsive neurons

The number of fear conditioning trials with tone-shock convergence was computed for all neurons of the Predictable and Unpredictable groups. The percent of neurons with convergence across the range of possible values (0-6, labeled on top of each bar) is shown.

a

Eligible to encode memory? Tone-Shock convergence during fear conditioning?	Active during fear recall? Tone-Responsive during fear recall?	Classification
Yes	Yes	Memory "Winner"
NO	Yes	Fear Expression
Yes	NO	Memory "Loser"
NO	NO	Other

b

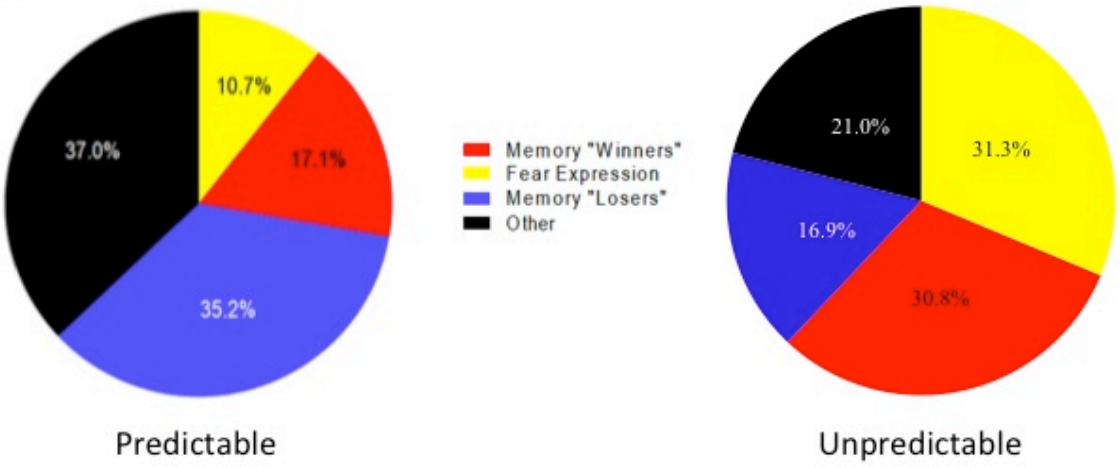

Figure 7. Four functional classifications of BLA neurons

a) Criteria used to classify all neurons imaged across both fear conditioning and the long-term memory test. b) Percentage of imaged neurons in each of the four classifications for the Predictable group and Unpredictable groups.

CHAPTER FIVE: DORSAL HIPPOCAMPAL PROCESSING OF NEGATIVE PREDICTION ERRORS

Introduction

As we have shown that fear memories and temporal unpredictability are being processed in BLA, BLA neurons are also known to preferentially respond to an unexpected stimulus comparing to an expected one (Belova et al., 2007; Johansen et al., 2010; McNally et al., 2011). This suggests that there may be a neural substrate that sends negative prediction error signal input to amygdala. Some neuroimaging studies on humans have shown that the hippocampus is one brain region that consistently exhibits an elevated blood-oxygenation-level-dependent response when an unexpected aversive stimulus is presented (Ploghaus et al., 2000). However, numerous fear-conditioning studies have argued that the hippocampus is not involved in fear conditioning in response to discrete cues (Kim and Fanselow, 1992; Maren and Holt, 2004).

Prediction errors, which are generated when outcomes and expectations do not match, are thought to drive associative learning; larger prediction errors lead to greater changes in learning (Rescorla, 1968; Pearce and Hall, 1980). Many factors may enhance the magnitude of a prediction error, including the size and the timing of reinforcement. The hippocampus has been implicated in aversive prediction errors (Ploghaus et al., 2000; Goosens, 2011). When aversive reinforcement follows predictive cues at variable intervals, this theoretically leads to two forms of prediction error: (a) when the reinforcer occurs at an unexpected time (positive prediction error) and (b) when the reinforcer is omitted at an expected time (negative prediction error). Because dorsal hippocampal

inactivation does not affect fear conditioning when foot shock is given at a consistent temporal interval after onset of the auditory cue, it is highly unlikely to be involved in generating positive prediction errors to drive learning. However, the selective recruitment of the dorsal hippocampus when foot shock is administered unpredictably suggests that the hippocampus may be involved in generating or processing aversive negative prediction errors. Thus, we explore the role of the dorsal hippocampus in temporal unpredictability by using muscimol, an agonist of the γ-aminobutyric acid type A receptor to transiently inactivate dorsal hippocampus during fear learning.

The results from dorsal hippocampal inhibition support the idea that the dorsal hippocampus generates temporally based aversive negative prediction errors to enhance fear-memory strength. However, they are also consistent with the idea that the dorsal hippocampus may function as a stopwatch, computing the passage of time between the start of a predictive cue and the occurrence of the reinforce (MacDonald et al., 2011). To distinguish between these two roles for the dorsal hippocampus, we performed an experiment in which we used optogenetics to selectively and briefly silence dorsal hippocampal cells in cornu ammonis 1 (CA1). This silencing took place in mice that received fear conditioning in which foot shock occurred pseudorandomly after predictive auditory cues. The cues occurred either during putative aversive negative prediction errors or during times in which foot shock was never presented. Mice were used because viral infusion and laser-light delivery could be targeted to a greater portion of the dorsal hippocampus than would be the case if rats were used. If silencing simply disrupts a stopwatch function, then silencing at any time within the CS presentations during fear

conditioning should produce an equivalent decrease in subsequent fear-memory strength. In contrast, if silencing blocks prediction errors, then the specific timing of the inactivation should determine whether there is an effect on fear memory.

Materials and Methods

Cannula Implantation

One week after the rats' arrival, cannulae were implanted. Rats were anaesthetized using a cocktail of ketamine, xylazine, and acepromazine (75, 8, and 1.5 mg/kg, respectively, ip) and then mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Small burr holes were drilled in the skull for placement of the guide cannulae and three jeweler's screws. Bilateral stainless steel guide cannulae (23 gauge, 10 mm) were implanted, aimed at the dorsal hippocampus (3.8 mm posterior and 2.5 mm lateral to bregma and 1.8 mm ventral to dura). The cannulae and screws were affixed to the skull using dental acrylic, and a dummy cannula (11 mm) was inserted into each guide cannula to prevent obstruction. Each animal received a postoperative injection of buprenorphine (0.03 mg/kg sc). Animals recovered for at least 5 days before behavioral training commenced.

Intracranial Infusions

Rats received intrahippocampal infusions 20 min before fear conditioning. Injection cannulae (30 gauge, 11 mm) were attached to 10-µl Hamilton syringes (Hamilton, Reno, NV) via polyethylene tubing (PE-20; Intramedic, Sparks, MD), and the syringes were mounted in an infusion pump (Harvard Apparatus, South Natick, MA). Animals were transported to the infusion room, and the dummy cannulae were replaced

with injectors. The rats were placed in plastic buckets containing shredded wood chips. Rats were bilaterally infused with either 0.9% saline (vehicle) or 1 μg/μl muscimol (labeled with fluorescent boron-dipyrromethene; Thermo Fisher Scientific, Waltham, MA) at a rate of 0.15 μl/min for a total infusion volume of 0.5 μl per side (Fig 8a, Muscimol is an agonist of the γ-aminobutyric acid type A receptor that causes transient inactivation of targeted regions. After infusion, the injection cannulae were left in place for an additional minute to allow the drug to diffuse before injector removal.

Viruses

An expression cassette containing the gene coding for the light-driven outward proton pump archaerhodopsin from *Halorubrum sodomense* strain TP009, ArchT (Han et al., 2011) fused to green fluorescent protein (GFP) was placed in a plasmid under the control of the CAG promoter. A second plasmid without ArchT expressed GFP under the control of the CAG promoter. The two types of plasmids were each packaged in with adeno-associated virus (AAV) serotype 1 capsids. Viruses were obtained from the Vector Core at the University of North Carolina, Chapel Hill. Viral stocks were diluted to 1.25×10^{11} infectious particles per milliliter, and a total of 2 μl was injected into each hemisphere of a mouse's dorsal hippocampus. Because ArchT is a membrane-trafficked protein, it is preferentially expressed in the dendrites and axons, rather than the cell bodies, of transduced cells. The fused GFP can be readily observed in the dendritic branches and axon terminals of the CA1 field.

Viral Injections and Fiber Optic Cannula Implantation

Mice that had been assigned to receive implants were mounted in a stereotaxic apparatus under isoflurane anesthesia. Small burr holes were drilled on the skull for bilateral infusion (2 µl at 0.1 µl/min) of AAV expressing either GFP only (GFP groups) or GFP and the silencing opsin ArchT (ArchT groups) targeting the CA1 of dorsal hippocampus at 2.3 mm posterior and ±1.75 mm medial to bregma, and 1.5 mm ventral to dura. Anatomical specificity of the targeting was conferred by the stereotaxic coordinates, not the properties of the virus; any mice with viral infections extending beyond CA1 were excluded from all analyses. Injections were made using an UltraMicroPump 3 (World Precision Instruments, Sarasota, FL) containing a 10-µl Hamilton syringe with a 33-gauge needle. After the completion of the infusion, the needle remained in position for an additional 10 min. Two small jeweler's screws were placed in the skull. Zirconia ceramic ferrules (Kientec Systems, Palm City, FL) containing a multimode optical fiber (with a diameter of 200 µm) were then lowered into the site of injection and secured with dental acrylic. Mice recovered for at least 4 weeks before behavioral experiments.

Optical Silencing of Hippocampal CA1 Neurons

A 532-nm green laser diode (100 mW; Shanghai Laser & Optics Century Co., Shanghai, China) was coupled to a 200-µm multimode silica-core optical fiber through an FC/PC adapter. A fiber-optic rotary joint (Doric Lenses, Quebec, Canada) was used to release torsion in the fiber caused by the animal's rotation. Laser output was controlled via a transistor-transistor logic pulse generator (National Instruments, Austin, TX), with timing controlled by Python 2.6 software (Python Software Foundation, Beaverton, OR).

Before implantation, an optical power meter (815-C; Newport, Irvine, CA) was used to ensure that the fiber optics delivered 10 mW of constant laser light. Laser light was applied for 4-s periods to induce photoinhibition.

Fear Conditioning

In the muscimol inhibition study (Fig 8), the fear-conditioning procedure was similar to that in Chapter 1. Rats received auditory fear conditioning with a lower foot-shock intensity (0.5 mA) to insure ample room for any enhancement in fear the temporal unpredictability of foot shock.

For the optogenetics studies, mice without implants were fear conditioned in Context A with three pairings of a 30-s tone (85 db, 2.2 kHz) and a 2-s foot shock (0.6 mA). One cohort of mice received predictable conditioning, in which the offset of each tone triggered foot-shock delivery. Another cohort of mice received unpredictable conditioning, in which each foot shock was delivered at a pseudorandom time within the tone (6, 18, or 29 s after tone onset). The following day, mice were placed in Context B and received four tone presentations (85 dB, 30 s) with a 150-s ITI.

After several weeks of surgical recovery and expression of ArchT or GFP, mice with implants were subjected to three trials of auditory fear conditioning, as described for mice without implants. Fiber-optic cables equipped for the delivery of green laser light were attached to the cranial implants before fear conditioning. Light (12 s total) was delivered to mice with implants during three periods (4 s each) within the second and third tones of the fear-conditioning session.

Mice in the unpredictable-conditioning/light-during-negative-prediction-error groups and predictable-conditioning/random-light groups received light starting 5 s into the second tone presentation and 5 s and 17 s into the third tone presentation. Thus, on Trial 2, light was applied during the time at which the foot shock had occurred on Trial 1. On Trial 3, light was applied during the times at which foot shock had occurred on Trials 1 and 2. Mice in the unpredictable-conditioning/random-light group received light starting 24 s into the second tone presentation and at 10 and 24 s into the third tone presentation; these were not times at which foot shock was received on any trial. For all mice with implants, long-term auditory fear memory was assessed 2 days later in a novel context without a cable attached to the implant.

For mice in the ArchT groups, it was expected that application of laser light to CA1 cells infected with ArchT would produce robust photoinhibition of neuronal firing, as was shown in a previous study (Sakaguchi et al., 2015). For mice in the GFP groups, the application of the laser served as a control for light-induced thermal effects in brain tissue.

Statistics

Statistical analysis was conducted as described in Experiment 1. The data were analyzed using ANOVAs with factors of training type (predictable shock vs. unpredictable shock), infusion condition (vehicle vs. muscimol), or time (ten 3-s bins within tone presentation). Eight rats were excluded from all data analyses because of incorrect cannula placement ($n = 6$) or extensive gliosis at the cannula tip ($n = 2$).

The data were analyzed using an ANOVA with a factor of group. Three mice were excluded from all data analyses because an equipment problem omitted the foot shock on the fear-conditioning day. Mice were also excluded from all analyses because of inadequate or inappropriate viral spread ($n = 4$) or high pretone freezing (> 60%) when placed in the novel context for auditory fear extinction ($n = 2$).

Results

Rats received infusions of either muscimol or vehicle in the dorsal hippocampus before fear conditioning with either temporally predictable or unpredictable foot shocks (Figs. 8a and b). One indication of the effectiveness of our hippocampal-inactivation procedure is that contextual fear memory was abolished in all the rats that received intrahippocampal muscimol before fear conditioning (Fig. 8c); there was a significant main effect of infusion condition, $F(1, 35) = 18.72$, $p = .0001$, a finding consistent with results from other studies (Zhang et al., 2014).

In contrast, although we observed a significant effect of hippocampal inactivation on auditory fear memory, the effects of hippocampal inactivation were different in the PRED and UNPRED groups (Fig. 8c); the Training Type × Infusion Condition interaction was significant, $F(1, 35) = 6.70$, $p = .014$. Hippocampal inactivation before standard auditory fear conditioning had no effect on fear-memory strength (Fig. 8c, right, black vs. white bars; planned comparison, $p = .57$), which is a finding consistent with those from prior studies showing that hippocampal activity does not play a necessary role in the acquisition of learned fear (Kim and Fanselow, 1992). However, inactivation of the hippocampus during temporally unpredictable fear conditioning completely abolished the

memory-enhancing effects of temporal unpredictability of the aversive event (Fig. 8c, red vs. gray bars; planned comparison, $p < .0001$). Because muscimol reduced fear only in the unpredictable-shock group, it is clear that the auditory stimuli used in our temporally unpredictable conditioning paradigms (30 s in length) did not function as diffuse, contextual cues to be associated with foot shock. If our auditory stimuli functioned as contextual cues, hippocampal inactivation would have reduced fear memory in both the predictable- and unpredictable-shock groups.

To determine whether the temporally unpredictable foot shock or muscimol infusions simply altered the time course over which conditioned freezing was expressed, we performed an analysis of tone-evoked conditioned freezing in 3-s bins during auditory fear extinction (Fig. 8d). Replicating our previous findings, we observed that there were significant changes in conditioned freezing over the course of the tone presentation, $F(9, 153) = 7.00$, $p < .0001$, and higher levels of conditioned freezing during the last 3 s relative to the first 3 s, $F(1, 20) = 12.3$, $p = .0020$. These findings suggest mild inhibition of delay across groups, as the conditioned freezing level was lower at the initial of tones than the end of the tones. However, the predictable- and unpredictable-shock groups did not differ in the rate at which conditioned freezing changed across the presentation of the tone. Neither the Training Type × Time interaction, $F(9, 153) = 1.37$, $p = .21$, nor the Training Type × Infusion × Time interaction, $F(9, 153) = 0.61$, $p = .79$, was significant. Thus, neither muscimol infusions nor temporally unpredictable fear conditioning altered conditioned freezing by reshaping the temporal window during which behavior was expressed.

The mice without implants received auditory fear conditioning with either temporally predictable or unpredictable foot shocks after auditory cue onset (Fig. 9a). As was found for rats in Experiments 1 through 3, the mice in the unpredictable-conditioning group exhibited higher conditioned freezing during auditory fear recall than the mice in the predictable-conditioning group (Fig. 9b), $F(1, 11) = 5.28$, $p = .04$.

The mice with implants received auditory fear conditioning with either temporally predictable or unpredictable foot shocks after auditory cue onset combined with the transient application of light (Fig. 9c). For these mice, viral infusions were aimed at CA1, and produced robust transduction of cells in this subregion of the hippocampus (Fig. 9d) consistent with results from other studies (Sakaguchi et al., 2015).

As we observed for rats in Chapter 3 and for mice without implants in this experiment, GFP-infused mice in either unpredictable-conditioning group exhibited higher conditioned freezing during auditory fear recall compared with GFP-infused mice in the predictable-conditioning groups; there was a significant main effect of training type, $F(1, 22) = 6.00$, $p = .02$. Photoinhibition of CA1 during putative aversive negative-prediction errors produced a significant decrease in long-term auditory fear-memory recall (Fig. 9e); there was a significant main effect of group, $F(1, 20) = 11.21$, $p = .003$, an effect that was not observed when photoinhibition was applied at the same times (relative to auditory cue onset) during temporally predictable fear conditioning (Fig. 9f); there was a significant main effect of group, $F(1, 14) = 0.44$, $p = .52$. Thus, these results were virtually identical to what we observed when the dorsal hippocampus was inactivated with muscimol during fear conditioning (Fig. 8c). This finding suggests that

the effects of the muscimol infusion were probably due to effects specifically within the dorsal hippocampus, rather than from any possible diffusion to nearby structures such as the subiculum. Brief photoactivation of CA1 cells at times when foot shock has never occurred was not sufficient to elevate fear-memory strength in mice that received temporally predictable auditory fear conditioning (Fig. 9h), which suggests that changes in structures outside of the dorsal hippocampus are also required to enhance fear-memory strength during temporally unpredictable fear conditioning.

We ran an additional set of mice with implants that received unpredictable auditory fear conditioning and received photoinhibition at random times within the auditory CS presentation. Thus, ArchT-infused mice in the unpredictable-conditioning/random-light group and ArchT-infused mice in the unpredictable-conditioning/light-during-negative-prediction-error group received an equivalent amount of dorsal hippocampal silencing. There was no difference in the GFP and ArchT groups under these conditions (Fig. 9g); there was no significant main effect of group, $F(1, 10) = 1.04$, $p = .33$. The observation that mice in the ArchT group exhibited conditioned freezing levels as high as those of mice in the GFP group suggests that negative prediction errors were present in both groups. This further suggests that, insofar as information about the passage of time since the CS onset is important for generating negative prediction errors, such information must be computed outside the dorsal hippocampus. Because silencing of CA1 reduced fear only when it was applied during aversive negative prediction errors, these data suggest that the dorsal hippocampus

enhances fear during temporally unpredictable fear conditioning by generating prediction errors.

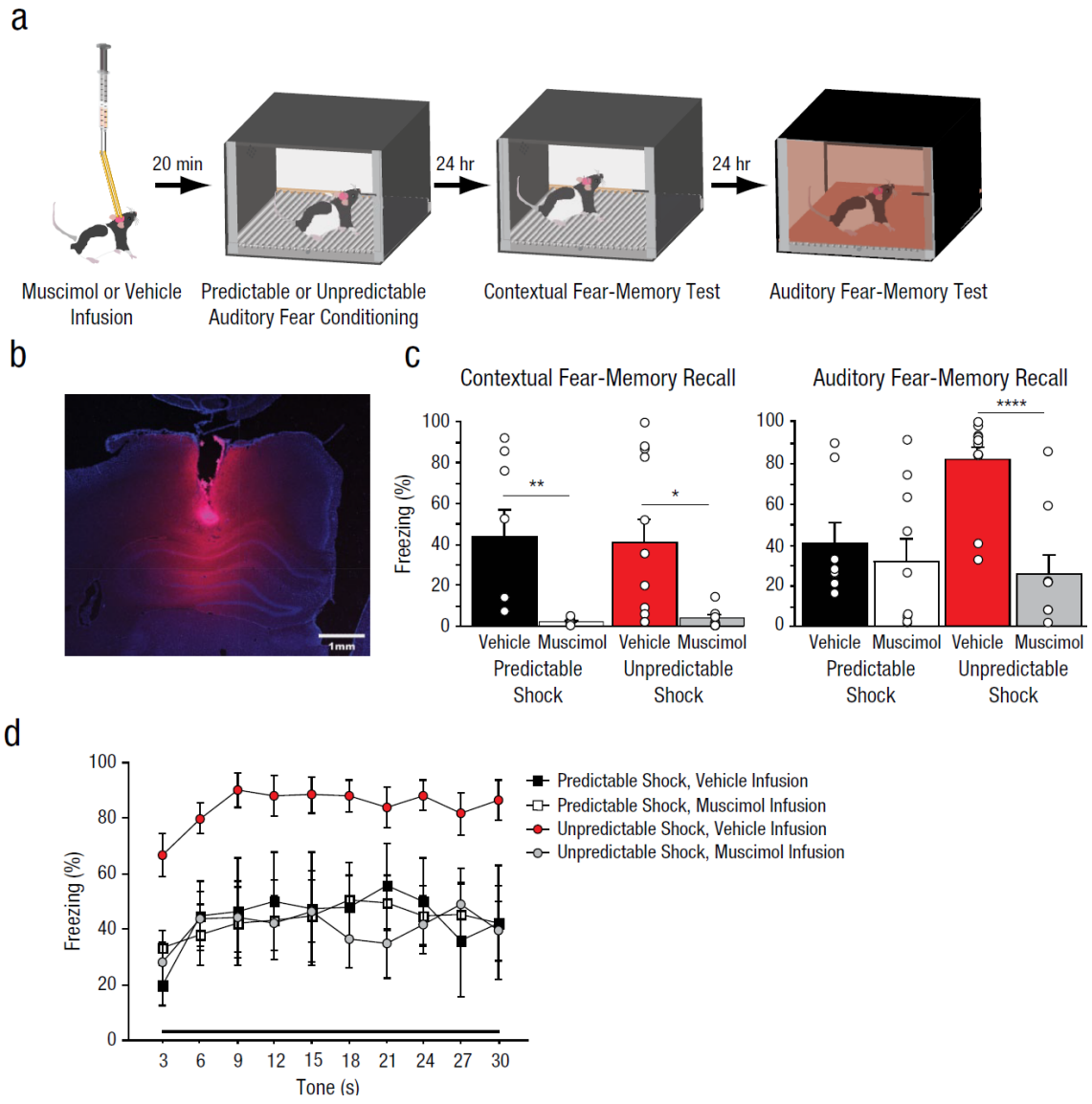

Figure 8. Hippocampal muscimol injection abolished temporal unpredictability-enhanced fear memory strength

As illustrated in (a), the rats (n = 8–12 per group) received intrahippocampal infusions of muscimol or vehicle before auditory fear conditioning (30-s tone, 2-s foot shock, and 210-s intertrial interval) with foot shocks delivered at either predictable or unpredictable intervals after tone onset. The next day, all the rats were returned to the conditioning

context for an 8-min contextual fear-memory test. The day after that, the rats were placed in a novel context, and eight tones were presented (auditory fear-memory test). A representative photomicrograph (b) shows one brain hemisphere after infusion with fluorescent muscimol. The pink fluorescent signal indicates the spread of the muscimol. The bar graphs (c) depict the mean percentage of time the rats displayed freezing behavior over the test sessions for contextual fear-memory recall (left) and auditory fear-memory recall (right). The bars show the mean for each group, and the small open circles represent the percentage of time that individual rats displayed freezing behavior. Asterisks represent significant differences between groups (*$p < .05$, **$p < .01$, ****$p < .0001$). Freezing behavior during the first two conditioning trials (d) is graphed as a function of time, separately for rats from each group. Each data point represents freezing during a 3-s period averaged over the first two trials of tone presentation during extinction. Error bars in (c) represent +1 *SEM*; error bars in (d) represent ±1 *SEM*.

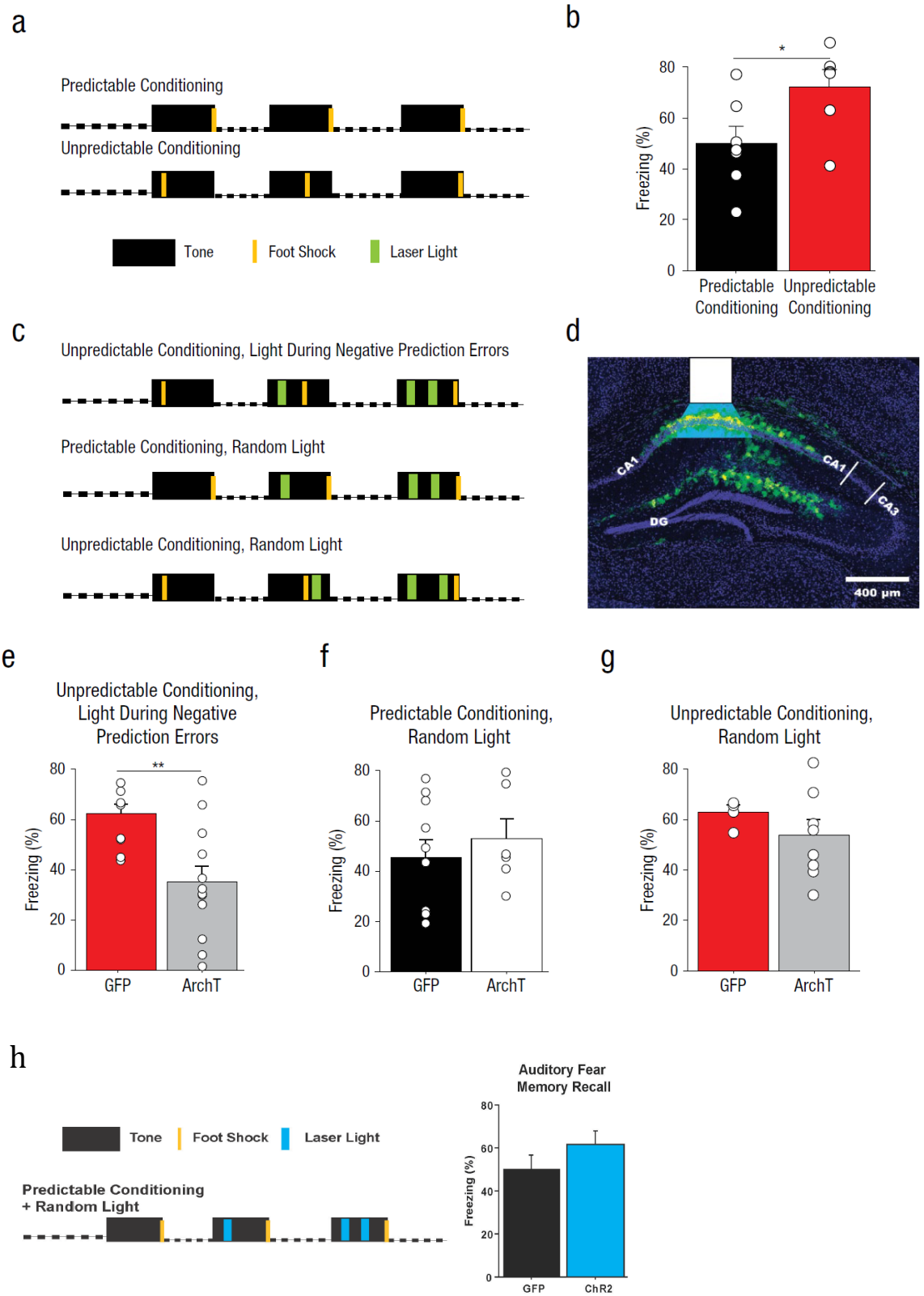
a
Predictable Conditioning
Unpredictable Conditioning
Tone
Foot Shock
Laser Light
b
*
Freezing (%)
80
60
40
20
0
Predictable Conditioning
Unpredictable Conditioning
c
Unpredictable Conditioning, Light During Negative Prediction Errors
Predictable Conditioning, Random Light
Unpredictable Conditioning, Random Light
d
CA1
CA1
CA3
DG
400 µm
e
Unpredictable Conditioning, Light During Negative Prediction Errors
**
Freezing (%)
GFP
ArchT
f
Predictable Conditioning, Random Light
Freezing (%)
GFP
ArchT
g
Unpredictable Conditioning, Random Light
Freezing (%)
GFP
ArchT
h
Tone
Foot Shock
Laser Light
Predictable Conditioning + Random Light
Auditory Fear Memory Recall
Freezing (%)
GFP
ChR2

Figure 9. Optogenetic manipulation of hippocampal CA1 demonstrated the mechanism of negative prediction error processing

Mice without implants (*n* = 6–7 per group) were fear conditioned with three pairings of a 30-s tone with a 2-s foot shock delivered with predictable or unpredictable timing, as illustrated in (a). Auditory fear recall was then tested in a novel context. The graph in (b) shows mean percentage of time the mice without implants displayed freezing behavior, separately for predictable- and unpredictable-conditioning groups. The small open circles represent the percentage of time that individual mice displayed freezing behavior. The mice in the ArchT groups received a bilateral infusion of an adeno-associated virus (AAV) expressing the silencing opsin ArchT fused to green fluorescent protein (GFP); the mice in the GFP groups received an AAV expressing GFP. Both viruses targeted cornu ammonis (CA) 1 of dorsal hippocampus. The mice that received brain implants were fear conditioned with three pairings of a 30-s tone with a 2-s foot shock, delivered along with 4-s applications of green laser light, under one of three conditions, as illustrated in (c). Mice in the unpredictable-conditioning/light-during negative-prediction-error group (*n* = 10–12) received intrahippocampal delivery of green light (λ = 575 nm) during 4-s periods (the 2 s in which foot shock was actually delivered, and an additional second of light delivery before and after the time of the previous foot shock) surrounding the times at which foot shock had been administered on previous trials. Thus, on Trial 2, light was applied during the time at which the foot shock had occurred on Trial 1. On Trial 3, light was applied during the times at which foot shock had

occurred on Trials 1 and 2. Mice in the predictable conditioning/random-light group received intrahippocampal green light at the same times (relative to conditioned stimulus, or CS, onset) as mice in the unpredictable-conditioning/light-during-negative-prediction-error group, but foot shock had never occurred at these times for the predictable conditioning/random-light groups. Mice in the predictable-conditioning/random-light group (n = 5–8) received intrahippocampal green light during 4-s periods in which foot shock had never been delivered. Expression of GFP after infection with ArchT is shown for a representative brain section in (d). The white overlay indicates the location of the fiber-optic tip and the estimated light spread. DG = dentate gyrus. Auditory fear-memory recall was measured across two tone presentations during a subsequent laser-free extinction session. The bar graphs show mean percentage of time that the mice displayed freezing behavior as a function of infusion type for (e) the unpredictable-conditioning/light-during-negative-prediction-error group, (f) the predictable-conditioning/random-light group, and (g) the unpredictable-conditioning/random-light group. The small open circles represent the percentage of time that individual mice displayed freezing behavior. (h) Mice were exposed to three trials of fear conditioning with predictably timed footshocks (Predictable Conditioning + Random Light + GFP, n = 10; Predictable Conditioning + Random Light + ChR2, n = 6). Mice received intra-hippocampal delivery of blue light (λ = 473 nm) during 4 s periods at times at which footshock had never been administered on previous trials, with an additional second of light delivery prior to and following the time of the previous footshock. Auditory fear recall during a subsequent laser-free extinction session assessing auditory fear memory

strength is shown. Each bar represents conditional freezing averaged over the first four auditory CS presentations. Error bars indicate +1 *SEM*. Asterisks represent significant differences between infusion conditions (*$p < .05$, **$p < .01$).

CHAPTER SIX: DISCUSSION

This series of studies advances the knowledge in our field in several ways. We have shown that associative fear memory strength is influenced by unpredictability surrounding the timing and occurrence of aversive reinforcement and that this information is rapidly acquired during fear learning. Temporal unpredictability also changes the neural representation of fear learning and memory in BLA and the calcium activity of the neurons that are involved in fear learning and memory coding. We have also demonstrated that the dorsal hippocampus plays a critical role in generating error signals (specifically, when a reinforcer is expected but does not occur) and the causal role of these signals in promoting fear memory. Thus, our results identify an important and novel role of the dorsal hippocampus in Pavlovian fear conditioning. These data place new emphasis on the relevance of timing to this form of learning and the ability of timing to drive prediction errors. Taken together, our results suggest that models of fear learning should embody the features of fear learning found in natural settings because these may lead to qualitative differences in fear memory strength.

Our results demonstrated that temporal unpredictability not only increased fear memory strength, but also altered the calcium activity of BLA neurons. These BLA neurons that receive information about both the tone and footshock within single conditioning trials, rendering them eligible to encode a fear memory, can be separated into two distinct populations: one that is activated by the tone during fear recall (Memory Winners) and one that is not responsive to the tone during fear recall (Memory Losers). It is interesting to note that the Memory Loser population substantially outnumbers the

Memory Winner population (at a 2:1 ratio) in the PRED group, an observation which might explain why studies typically report <10% overlap between neurons activated by learning and long-term memory recall (Reijmers et al., 2007; Liu et al., 2012; Tayler et al., 2013). Because disinhibition of principal BLA neurons exerts powerful control over footshock sensitivity during fear conditioning (Wolff et al., 2014), it is tempting to speculate that changes in disinhibition contribute to memory competition in the BLA. Indeed, computational models of associative fear learning in the BLA predict that regulation of local inhibition is critical for competition between neurons (Kim et al., 2013). As Rescorla-Wagner model posits that unpredictability drives fear learning, an alteration in BLA neuronal activity should be observed during the omission of an expected aversive stimulus. However, electrophysiological studies on amygdala neurons have failed to display excitatory or inhibitory firing pattern during the processing of prediction errors (Belova et al., 2007; Johansen et al., 2010). Our data are consistent with these previous studies, showing minimal alteration in neuronal calcium activity pattern under the influence of temporal unpredictability. Even though temporal unpredictability might not dramatically affect calcium activity pattern at the neuronal level, it does impact the recruitment of the neurons that are involved in processing the fear learning and/or memory. Rather than increases the intracellular calcium activity of Memory Winner neurons, temporal unpredictability surprisingly shifts the ratio of the Memory Loser : Memory Winner population from approx. 2:1 to approx. 1:2. While the negative prediction errors might not be processed by BLA neurons, it is possible that the BLA receives instructional signals from external neural substrates that process the negative

prediction error and lead to the changes in the neural representation of the fear learning and memory in amygdala.

In contrast to other studies which suggest that the sensory convergence of a weak stimulus and a strong stimulus upon single neurons is essential for synaptic strengthening during learning (Abrams et al., 1991; Blair et al., 2001; Barot et al., 2009; Chung et al., 2011; Hashikawa et al., 2013), we report here that sensory convergence on single BLA neurons within single conditioning trials is surprisingly low. On average, roughly 14.7% of neurons in the Tone-Responsive neurons, of which comprised just ~28% of the all neurons, exhibited sensory convergence within a trial (Fig. 6), and most neurons in this population displayed within-trial sensory convergence on only one of the six conditioning trials (Fig. 6). There are several unexplored mechanisms by which this could occur. First, it is possible that BLA neurons compute "convergence" on an across-trial basis, rather than within a narrow temporal window as suggested by studies of electrically-induced synaptic plasticity (Dan and Poo, 2006). This is might explain the reason that associative fear learning can still proceed when a predictive stimulus and aversive consequence are separated by lengthy (Raybuck and Lattal, 2014) or variable temporal windows (Fig. 1). A second possibility is that plasticity in BLA dendrites may be induced in the complete absence of action potentials (Golding et al., 2002; Dudman et al., 2007). By this logic, techniques such as calcium imaging and tetrode recording may dramatically underestimate sensory convergence within BLA neurons. Alternatively, it is possible that sensory convergence occurs in BLA efferents such as the central nucleus of the amygdala (Yu et al., 2017), and that replay (Johnson et al., 2009; Wu et al., 2017) or other

consolidation-related processes (Squire et al., 2015) may enable this convergence to influence plasticity within the BLA. Even though the overall tone- and shock-evoked calcium activity of the BLA neurons decreased during both fear learning and recall under the influence of temporal unpredictability, the percentage of BLA neurons that showed sensory convergence for any given fear conditioning trial increased around 3.5 to 4 times than that of the PRED group. This indicates that the temporal unpredictability-enhanced fear memory strength might be mediated by the number of neurons that display convergence properties rather than by the neuronal synaptic strength. While this contradicts the hypothesis that states the positive correlation between synaptic plasticity and memory strength (Song et al., 2012), yet it is supported by another study that demonstrates that the number of amygdala neurons in the memory ensemble was positively correlated with the fear memory strength (Nonaka et al., 2014).

Our observation shows that there are neurons that respond robustly to the tone during fear memory recall, but which receive no convergent tone and shock information during fear conditioning. We termed this population “Fear Expression neurons” to reflect the fact that they do not display plasticity during fear conditioning yet exhibit similar levels of neuronal activation as Memory Winner neurons during fear recall (Fig. 7a, b). Approximately one-third of neurons activated by the tone during fear recall of the PRED group (~10% of neurons imaged overall) were classified as Fear Expression neurons, a finding that might contribute to the low overlap between cells activated during learning and memory recall reported in other studies (Reijmers et al., 2007; Liu et al., 2012; Tayler et al., 2013). This finding adds to a growing body of evidence that new neurons

may be recruited during memory recall tests (Grewe et al., 2017), yet the purpose of this recruitment remains unclear. One possibility is that Fear Expression neurons are recruited by local Memory Winner neurons to facilitate fear behaviors during memory recall; that is, Fear Expression neurons could passively mirror long-term plasticity from their Memory Winner neighbors. Alternatively, it is possible that Fear Expression neurons are a specialized population of memory cells that acquire plasticity after fear conditioning. Such plasticity could be induced during replay or systems-level consolidation. The mechanisms by which Fear Expression neurons come to be activated during memory recall is both a complicated and fundamental question for the study of memory. Nevertheless, the fear memory strength might be affected by the sum of both Memory Winner and Fear Expression neuronal expression. Another interesting finding lies in the influence of temporal unpredictability on the percentage of Memory Winner and Fear Expression neurons among other subsets of neurons. The ability of temporal unpredictability to triple the percentage of Fear Expression neurons while increasing fear memory strength indicates that the expression of memory might not rely solely on the contribution of Memory Winner neurons, but also on Fear Expression neurons. Memory Winner neurons obeyed the Hebbian principle, whereas the Fear Expression neurons did not, suggesting that there might be other learning mechanisms involved other than Hebbian principle that is known to govern the fear learning process.

At least two distinct processes may engage the dorsal hippocampus during computations of temporal unpredictability in aversive outcomes. The dorsal hippocampus may calculate the passage of time; time cells within the hippocampus are thought to

facilitate associations between cues and reinforcers that are discontiguous in time (Modi et al., 2014; McDonald and Mott, 2017) and maintain memories across delay periods (Gill et al., 2011). Alternatively, the hippocampus may encode the ambiguity of predictive cues on either an ongoing basis (Harrison et al., 2006) or a trial-by-trial basis (Vanni-Mercier et al., 2009). Our results are most consistent with the latter idea and further suggest that the hippocampus uses the encoding of ambiguous outcomes to enhance fear memory.

Although an emerging body of literature suggests that the hippocampus plays a role in the prediction of future events (Goosens, 2011), it is not known how the brain uses these predictions. We show that the hippocampus is essential for the enhancement of associative fear by ambiguity in the timing or occurrence of aversive outcomes. The role of the hippocampus in prediction per se is not important for determining associative fear-memory strength, given that hippocampal inactivation (Maren and Holt, 2004) and overt hippocampal damage (Kim and Fanselow, 1992) do not affect associative fear memory to discrete cues when aversive outcomes occur at predictable times. Such hippocampal manipulations do not affect predictable auditory fear memory strength, which suggests that the dorsal hippocampus does not play an essential role in positive prediction errors (when reinforcement occurs unexpectedly) or that any such hippocampal prediction errors do not enhance fear-memory strength. In other words, the hippocampus plays an important role in associative fear-memory strength only when (a) cues have an ambiguous relationship with outcomes and (b) outcome-related predictions during

learning are sometimes incorrect. Our study is one of the first to identify a specific neural substrate and role for negative aversive prediction errors in fear memory.

There are two primary classes of theoretical models that account for how prediction errors are used to change learning. In one class, positive prediction errors directly enhance associative learning, whereas negative prediction errors weaken associative learning (Rescorla and Wagner, 1972). The result from our studies, that shown negative prediction errors strengthen associative fear learning (Fig. 9), seems to contradict the Rescorla-Wagner model. However, negative prediction error defined in this model is not identical to the negative prediction error that was generated by the temporal unpredictability in our studies. Negative prediction error described in the Rescorla-Wagner model is often used to explain the fear decrease in the process of fear extinction, which tones are administrated with the omission of the expected shocks (Li and McNally, 2014). In contrast, none of the shocks were omitted from the tones in our studies. These negative prediction errors were simply generated by the variability in the timing of the shocks, not by the omission of the shock. Therefore, it might inappropriate to directly compare the findings of our study with the negative prediction error studies that supports the Rescorla-Wagner model. In a second class, prediction errors influence the rate of learning by modulating attention to the predictive stimuli: Larger negative or positive prediction errors enhance learning (Pearce and Hall, 1980). We found that hippocampal signaling during times of putative negative prediction errors strengthens learning (Fig. 9), which suggests that the hippocampus contributes to attentional (e.g., Pearce-Hall) enhancement of fear. Our finding that the hippocampus plays a role in the enhancement

of fear by temporal unpredictability (Figs. 8 and 9), provides further support for this claim. Indeed, other groups have argued that the hippocampus may play a role in Pearce-Hall-like changes in attention (Han et al., 1995).

Based on these two classes of theoretical models, two types of prediction errors are derived to explain the differences in updating of the stimulus-outcome associations for effective adaption to current situation (Likhtik and Gordon, 2013). Based on the Rescorla-Wagner model, when the US is bigger than expected, there is a positive prediction error and it increases the associative strength. Whereas when the US is smaller than expected, there is a negative prediction error and it decreases the associative strength. Therefore, these prediction errors are named as signed prediction errors because they drive the strength of the association up or down based on the expectation. The second type of prediction error is named as unsigned prediction error (Pearce and Hall, 1980). Based on the Pearce-Hall model, when there is an unexpected outcome, regardless of its valence (bigger or smaller than expected), the associative strength is strengthened. Thus, the associative strength is driven in a manner independent of the sign of its prediction error, but dependent on the amount of surprise the mismatch generated to recruit attention. As previously mentioned, hippocampal signaling during times of putative negative prediction errors strengthens learning might suggest that the dorsal hippocampus processed these prediction errors as unsigned prediction errors. Growing evidence have supported that both signed and unsigned prediction errors coexist in a variety of brain regions (Schultz et al., 1997; Roesch et al., 2010; Roesch et al., 2012). In support of our results, previous studies have shown that both amygdala (Li et al., 2011;

Esber et al., 2012) and hippocampus (Han et al., 1995; Donnelly et al., 2015; Davidow et al., 2016) are capable to process unsigned prediction errors.

Because the amygdala is an important storage site of long-term fear memories (Han et al., 2009) and single amygdalar neurons show Pearce-Hall-like changes in associative plasticity across fear-conditioning trials (Roesch et al., 2012), it seems likely that the dorsal hippocampus enhances fear learning by sending an error signal to the amygdala, which then implements a Pearce-Hall algorithm to modify fear learning. Hippocampal error signals may enhance fear memory strength by increasing excitability or synchrony among neurons in the amygdala during fear learning (Whalen, 2007). In support of this, temporal unpredictability of a sensory stimulus, even in the absence of a reinforcer, is sufficient to increase spontaneous activity in the basolateral amygdala (Herry et al., 2007). The mechanism by which hippocampal error signals affect fear learning in the amygdala is an important topic for future studies.

It may seem surprising that the difference between the predictable- and unpredictable-conditioning groups was determined by the unpredictability of the time of foot-shock delivery (Fig. 1) and not the duration of conditioning parameters known to play an important role in regulating the speed and asymptote of associative learning. It is important to note that unpredictability surrounding the timing of aversive reinforcement provides a modulatory influence that enhances fear beyond a baseline level. This baseline level of freezing is shared across the temporal predictable and unpredictable conditions and is probably determined by a combination of fear-conditioning parameters, including the number of pairings of CS and US, intensity of US, and temporal values of specific

parameters. Our findings, therefore, do not discount the importance of timing theories of associative learning (Kirkpatrick and Balsam, 2016), and our findings are not inconsistent with these theories.

Together, our data highlight the sensitivity of fear to temporal unpredictability. These results show that temporal unpredictability can produce an unexpected enhancement of fear-memory strength and at the same time either have no effect on the contingency between predictive cues and aversive reinforcement. Although our studies reveal that temporal unpredictability regulates fear in normal rodent populations, temporal unpredictability is likely to exert an even more important influence on fear levels in pathological conditions. Mouse models of anxiety (Tsetsenis et al., 2007), patients with fear and anxiety disorders (Grillon et al., 2008) and humans who experience unexpected aversive events (Grillon et al., 2004) are disproportionately affected by temporal unpredictability during trauma. Thus, strategic interventions aimed at reversing changes in hippocampal circuits in patients with fear and anxiety disorders may help normalize fear learning.

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