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Contextual Fear Learning Circuitry and Contributions of Acetylcholine

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in Neuroscience

by

Sarah Jan Hersman

2016

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ABSTRACT OF THE DISSERTATION

Contextual Fear Learning Circuitry and Contributions of Acetylcholine

by

Sarah Jan Hersman

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2016

Professor Michael S. Fanselow, Chair

Learning about context is essential for appropriate behavioral strategies, though pathologically strong contextual memories may form the basis for anxiety disorders such as PTSD. Though the general network of brain regions involved in this learning has been characterized, how neural representations in each region contribute to memory formation and recall, as well as how cholinergic signaling affects normal and maladaptive contextual learning, is not well understood. I used catFISH to compare short-term contextual fear memory reactivation across hippocampus (DH), basolateral amygdala (BLA), prelimbic and infralimbic cortices (mPFC). I found that while DH reactivation is related to contextual processing, BLA reactivation is related to recall of contextual fear memory; mPFC reactivation tracked both contextual and fear information (Ch2). To follow up, I used Fos-Cre mice to tag a contextual fear memory representation in BLA with the inhibitory proton pump ArchT. I then inactivated these memory neurons during a return to the original context, and found that it impaired recall, but that if the memory was first allowed to be recalled, ongoing fear behavior was not disrupted.

This effect was similar at both recent and remote time points (Ch2). In order to probe how acetylcholine (ACh) in DH affects contextual processing, I used ChAT-Ai32 mice to selectively activate medial-septal cholinergic inputs to DH with ChR2 during contextual exposure. After pairing the context with shock, mice with enhanced ACh during contextual encoding showed higher levels of fear, suggesting stronger contextual memory formation. I also tested and confirmed the effects of light stimulation using anesthetized recording with choline biosensors (Ch3). As ACh increased the strength of contextual memories, I tested whether it was involved in maladaptive contextual fear in Stress-enhanced fear learning (SEFL), our rat model of sensitization in PTSD. I blocked muscarinic cholinergic signaling in DH or BLA using scopolamine before a traumatic event: 15 unsignaled shocks. I found that this blockade in either brain region not only disrupted fear to the trauma context, but blocked sensitization to a new context normally observed in the model. In DH, though sensitization to new contexts was blocked, sensitization to new tones was intact, suggesting a new role for ACh in DH in controlling contextual sensitization after trauma (Ch4).

The dissertation of Sarah Jan Hersman is approved.

Alcino Jose Silva

Kelsey C. Martin

Frank Krasne

Michael S. Fanselow, Committee Chair

University of California, Los Angeles

2016

For my parents,

Who never doubted.

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showed similar levels of fear to the trauma context one day later ($p>0.05$) and the one-shock context two days later ($p>0.05$). Error bars indicate S.E.M.

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Chapter 2 also includes unpublished data collected in collaboration with Jennifer Tribble.

Tribble J and Hersman S performed all Fos-Cre surgeries and behavior with equal contribution. Analysis and write-up was performed by Hersman S.

Chapter 3 is a version of:

Hersman S, Cushman J, Wassum K, Fanselow MS, and Lotfipour S. Optogenetic activation of medial septum cholinergic neurons improves contextual fear learning and alters choline levels in hippocampus. (In prep.)

Chapter 4 is a version of:

Hersman S*, Hoffman A*, Hodgins L, Shieh S, Lam J, and Fanselow MS. Dissociation of cholinergic modulation in dorsal hippocampus and basolateral amygdala on stress-enhanced fear learning. (In prep.)

*Asterisk represents equal contribution. In this case Hoffman A performed all BLA surgeries and the majority of BLA behavior, while Hersman S performed all DH surgeries and the majority of DH behavior. Analysis and write-up was performed by Hersman S.

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Chapter One: General Introduction

Humans have a nearly endless behavioral repertoire. This begs the question: how do we learn how to produce the correct behavior for a given situation? The short answer is that we learn about contexts, and this contextual learning guides behavioral responses. A *context* is a place or situation, which may be composed of sensory details, internal states such as emotions, or even abstract rules of contingency or duration.

The brain takes these varied inputs and crafts from the physical context a neural representation, known as a *contextual representation*. As a human or rodent samples stimuli in a new environment (Peterson et al., 1954), those inputs converge on the dorsal hippocampus (DH). The DH builds a unified contextual representation, stores it as a memory (along with any important events that have occurred there), and recalls the memory at a later date (Fanselow, 1990; Smith and Bulkin, 2014; Urcelay and Miller, 2014).

Contextual fear learning is a type of contextual learning that is essential for survival. If a context leads to a dangerous or painful situation even once, experiencing that context again should lead to rapid and complete expression of fear behavior (Fanselow and Lester, 1988). In this case of Pavlovian conditioning, the context representation acts as an initially neutral conditional stimulus (CS), which is then paired with a fear-inducing unconditional stimulus (US). This conditional pairing then leads to fear behavior upon re-experience of the contextual CS. This learning is modeled in the lab using rodents. During fear acquisition, a novel box, known as the *conditioning context*¸ is paired with one or more mild foot shocks. Returning the rodent to the box the following day, known as a *context test*, will result in levels of fear related to the shock magnitude, the number of shocks, and the strength of the contextual memory formed the day before.

During fear learning, information about the context must be stored and combined with the shock information so that the context takes on a negative valence. Then, when that context is re-experienced, the negative valence will be recalled, and the context will be fear-inducing even in the absence of further shock. The brain regions involved in both fear acquisition and context test fear expression are well characterized. As mentioned, the context representation, in this case the CS, is formed and stored in DH; this information is sent to the basolateral amygdala (BLA). While the route this information takes to reach the BLA is still the subject of research, one potential route includes ventral hippocampus, which has a direct bi-directional projection to the BLA (Fanselow and Dong, 2010). Shock information, in this case the US, enters the dorsal horn of the spinal cord, and takes both a cortical (insula) and subcortical (thalamic) route to the BLA (Shi and Davis, 1999). CS-US convergence, where the context takes on a negative valence, occurs in the BLA (Fanselow and LeDoux, 1999; Barot et al., 2009; Gore et al., 2015). The neurons involved in the context-shock association in the BLA then project to the central nucleus of the amygdala (CeA). This region then coordinates a fear response through projections to the periaqueductal gray (PAG) (Fanselow, 1991). Depending upon the nature and proximity of the threat, this may include cautious movement, freezing, or energetic escape or attack behavior (Fanselow and Lester, 1988).

Other brain regions contribute to learning and recall of contextual fear. The medial prefrontal cortex (mPFC), which is made up of prelimbic (PL), infralimbic (IL) and anterior cingulate (ACC) subregions, has been implicated in contextual fear (Morgan and LeDoux, 1999; Frankland et al., 2004; Quinn et al., 2008; Zelikowsky et al., 2013; Einarsson and Nader, 2012). The mPFC may affect the strength of fear acquisition (Arruda-Carvalho and Clem, 2014) as well as fear specificity during memory recall (Xu and Südhof, 2013; Cassel and Pereira de

Vasconcelos, 2015). However, the relationship between firing in this region and multiple aspects of fear acquisition and recall is still being characterized.

A host of neuromodulatory projection systems interact with these regions during contextual fear learning and recall. The focus for this paper will be the cholinergic projection system, particularly cholinergic inputs to DH and to BLA. Cholinergic inputs to DH from the medial septum (Mesulam et al., 1983), in particular, have been associated with both memory encoding and contextual processing (Easton et al., 2011). Nucleus basalis cholinergic inputs to BLA are important for fear learning and recall (McIntyre et al., 1998; Jiang et al., 2016).

Memory recall and neuronal reactivation

Though the aforementioned brain regions are important for contextual fear memory encoding and retrieval, there is still debate about where exactly the "memory" resides. Localization of individual memories captured the imagination of the first philosophers (Aristotle, 350AD), was thought to be impossible in the last century (Lashley, 1950), and is only now coming within reach (Mayford, 2014). Our ability to begin to answer this question is due to decades of detailed experimental lesions, pharmacology, and electrical recording, as well as the advent of new technologies based upon immediate early genes (IEGs).

IEGs, such as Arc and cFos, are proteins that are rapidly transcribed after significant cellular activity, including seizures, learning experience, and following induction of long-term potentiation (LTP). IEGs also play a role in multiple forms of protein synthesis-dependent synaptic plasticity (Dragunow and Faull, 1989; Guzowski et al., 2001; Bramham et al., 2008, 2010). These IEGs are integral to learning, as antisense administration of Arc in DH disrupts fear acquisition (Czerniawski et al., 2011), and of either Arc or cFos disrupts memory

consolidation processes (Guzowski, 2002). This relationship to learning processes has made these IEGs favored readouts of neural activity both in DH (Guzowski et al., 1999) and in BLA (Orsini et al., 2013). Though cFos is the more commonly used IEG, Arc is particularly useful because it timestamps neural activity at two behavioral time points, separated by 20 min, in a fluorescent in situ hybridization (FISH) technique called catFISH (Guzowski and Worley, 2001).

Extracellular and intracellular recording technologies, though unparalleled in temporal precision, have been difficult to use to find cellular correlates of particular memories (Eichenbaum et al., 1999; Robitsek et al., 2013). This is due to low neuron population sampling and an inability to manipulate particular subsets of neurons. IEGs and their promoters solve both of these issues. To counter low population sampling, mRNA and protein products of these genes allow read-outs of neural activity for, theoretically, an entire rodent brain; indeed, this complete sectioning and imaging technology is already in use (Kim et al., 2015). To target particular active neuronal populations for manipulation during behavior, IEG promoters can be coupled to genetic or viral delivery of light-sensitive opsins or DREADDs receptors (Ramirez et al., 2014; Liu et al., 2015). This coupling tags active populations with excitatory or inhibitory channels; these channels may then be activated later using certain wavelengths of light (opsins) or a drug injection (DREADDs).

This technology has had many early successes. Multiple studies have re-activated dentate gyrus cells that were active during contextual exploration or contextual fear acquisition using Tet-tagged mice, which allows control of transgene expression using an inducible cFos promoter (Reijmers et al., 2007). One study demonstrated that reactivating cells that were active during fear acquisition is sufficient for fear recall (Liu et al., 2012). Other studies showed that neutral reactivated cells can become part of another contiguous contextual representation (Garner

et al., 2012), go from neutral to aversive (Ramirez et al., 2013) or from aversive to appetitive and vice versa (Redondo et al., 2014), simply based on behavioral experience concurrent with their reactivation. The ease with which these cells can be used not only to evoke behavioral correlates of memory recall, but also to shape new learning about the physical context that they represent, suggests that a "sufficient" part of the contextual memory is stored in these neurons and their connections.

Functional manipulation of previously-active neural assemblies has so far been confined to "recent" memory; that is, memory that has not yet undergone systems consolidation. The hippocampus is not thought to be the permanent storage location of contextual memories. Rather, memory traces or their index are off-loaded to the neocortex between 10 and 28 days after learning in rodents. At this point, DH lesion or inactivation does not prevent memory recall, and memories are less specific (Kim and Fanselow, 1992; Wiltgen and Silva, 2007). Contextual fear memories are capable of being recalled throughout this process, and indeed remain dependent on the BLA for the lifetime of the rat (Gale et al., 2004). Only one study has addressed how memory reactivation changes across these time scales (Tayler et al., 2013). They demonstrated that reactivation of the population of BLA neurons active during learning decreased across time. However, how reactivation of this population is related to recall of contextual memory, at either recent or remote time scales, is currently unknown.

Acetylcholine and hippocampal processing

Contextual memory representations, and the ability to recall them, are dependent upon cholinergic inputs to DH from medial septum (MS), part of the basal forebrain cholinergic system. The disruption of these inputs in disease states can have disastrous effects on memory

(Coyle et al., 1983; Hasselmo and Sarter, 2011), and may be novel targets for memory impairments due to aging (Cansev et al., 2015) and disease (Kuhn et al., 2015).

The medial septum is a midline structure that is comprised of cholinergic, GABAergic and glutamatergic projection neurons (Kiss et al., 1990; Zaborszky, Laszlo et al., 2012) with extensive intraseptal connectivity within and between cell types (Leão et al., 2015). Lesion and inactivation studies report a wide range of effects (Baxter et al., 1997; Cai et al., 2012; Frick et al., 2004; Knox and Keller, 2015; Numan and Quaranta, 1990) including a loss of experience-induced Arc expression (Miyashita et al., 2009), though effects are often reduced by limiting the lesion to cholinergic neurons (Dashniani et al., 2015; Fletcher et al., 2007).

ACh has many functional effects in DH. These include initiation and promotion of hippocampal theta rhythm (Konopacki et al., 1987; Monmaur et al., 1997; Rowntree and Bland, 1986; Zhang et al., 2010, 2011), formation of spine head filopodia (Schätzle et al., 2011), suppression of sharp wave ripples (Vandecasteele et al., 2014), and promotion of LTP (Blitzer et al., 1990). These changes are also correlated with improved contextual processing and learning (Maren et al., 1994). In addition to enhancing LTP at CA1 synapses, ACh also inhibits recurrent collaterals in CA3. Both effects enhance feedforward information flow and storage of new representations in DH (Hasselmo et al., 1995). High levels of ACh promote encoding, while low levels promote consolidation and retrieval (Hasselmo and McGaughy, 2004; Rogers and Kesner, 2003; Thomas, 2015) and so this neuromodulatory system likely plays a dynamic role in encoding of new memories via multiple circuit mechanisms working in concert (Dannenberg et al., 2015; Hasselmo, 2006).

Many of these changes in DH are likely mediated primarily through muscarinic receptor activation, through a complex organization of both presynaptic and postsynaptic muscarinic

receptors (Levey et al., 1995; Nagode et al., 2011; Rio et al., 2010). This is supported by the requirement for signaling at muscarinic receptors in DH for normal contextual learning; no such requirement exists for tone fear learning, which is not dependent upon a functional DH (Anagnostaras et al., 1999; Gale et al., 2001). M1 receptors seem to be particularly involved in contextual learning (Dennis et al., 2015).

However, nicotinic inputs also modulate learning processes in the hippocampus (Tinsley et al., 2004; Leão et al., 2012; Jiang et al., 2014), and other reports demonstrate that cholinergic activity as a whole may not be explicitly necessary for some of these procedures (Fletcher et al., 2007; Frick et al., 2004; Parent and Baxter, 2004). Furthermore, memory-impairing effects of scopolamine may be complicated by alterations to nicotinic activation due to drug administration, as has been reported (Newman and Gold, 2015).

The recent development of ChAT-Cre mice (Madisen et al., 2012) and rats (Witten et al., 2011), which restricts Cre-recombinase expression to cholinergic neurons, has opened the door to functional studies of cholinergic influences on DH processing. Use of these rodents with genetic or viral optogenetic technologies has already begun to answer the question of how this neuromodulatory input to DH regulates spatial exploration and processing (Mamad et al., 2015), though functional effects of changes in cholinergic tone on contextual encoding and recall have not been tested.

Acetylcholine and BLA processing

Successful fear learning in the BLA is also dependent upon cholinergic signaling. This cholinergic signaling originates in the nucleus basalis of meynert (NBM), another sub-region of the basal forebrain cholinergic system (Woolf and Butcher, 1982). ACh into BLA both increases

excitability and elicits LTP, but it can also affect fear memory encoding to make the memory more durable (Jiang et al., 2016).

Pharmacological manipulations of ACh receptors have also shown effects in BLA. ACh improves consolidation through muscarinic receptors, as post-training infusion of scopolamine disrupted contextual fear consolidation (Passani et al., 2001; Baldi et al., 2007), while the muscarinic agonist oxotremorine enhanced consolidation (Cangioli et al., 2002). Deficits were also seen after scopolamine administration on performance of a conditioned place preference task, but post-training infusions were not performed, so blockade could have affected learning and/or consolidation of the memory (McIntyre et al., 1998). Interestingly, in the same study a correlation was observed between individual variations in BLA ACh release during training and memory recall the following day.

Research into the mechanism of ACh action in the BLA is still in its early stages, but there have been some promising developments. One study used optogenetic release of ACh to activate postsynaptic receptors in a more naturalistic manner than previous pharmacological manipulations targeting particular receptors had done. They found that the effect of ACh onto principle neurons in the BLA depended on their level of activity; ACh enhanced the firing of active neurons, and reduced the firing of lowly-active neurons (Unal et al., 2015). These findings suggest that ACh may be increasing the signal-to-noise ratio in BLA neuronal networks, a mechanism by which ACh might exert its effects on BLA-dependent learning and memory.

Acute stress: modeling human PTSD

Acute stress, which describes a short-term stressor of high intensity, induces widespread changes in contextual fear learning circuitry. Acute stress leads to activation of the

hypothalamic-pituitary-adrenal axis (HPA axis) (Herman et al., 2016). This activation causes widespread changes in neurotransmitter release in a highly organized manner (Joëls and Baram, 2009), likely through the release of stress hormones such as glucocorticoids (Roozendaal et al., 2009). These glucocorticoids also bind to specific receptors in the brain, and in a negative feedback loop shut down the stress response and promote resilience to stress (Paul et al., 2015). In particular, acute stress leads to enhanced release of ACh, though this effect is higher in males than females (Mitsushima et al., 2003). This increase in ACh is mimicked by application of corticosterone (Imperato et al., 1989), suggesting an interaction between these systems during stressful events.

In acutely dangerous situations, the stress response coupled with acute release of a host of neuromodulators can lead to rapid, adaptive responding. However, this response must be both titrated to the level of threat and specific for the threat-related stimulus. Both aspects of this responding are disrupted in anxiety disorders such as post-traumatic stress disorder (PTSD), where an individual's normal fear responses have been enhanced and disrupt normal functioning (Rosen and Schulkin, 1998).

Though PTSD in humans is thought to be heterogeneous, and a variety of underlying mechanisms have been hypothesized (Roozendaal et al., 2009; Mora et al., 2012; Bennett et al., 2015; Paul et al., 2015), most models of PTSD implicate the amygdala. The amygdala stores the traumatic memory, but may also mediate the influence of stress on emotional memory acquisition (Rosen and Schulkin, 1998; Waddell et al., 2008; Roozendaal et al., 2009). In these models, acute stress leads to a 'hyperactive' amygdala, which manifests with increased excitability of glutamatergic principle cells or reduced inhibitory drive from GABAergic inhibitory interneurons (Roozendaal et al., 2009); previous work from our lab indicates that an

upregulation of GluA1 in principal cells after the traumatic event may be responsible (Perusini et al., 2015). Another important component may be the enhanced release of ACh during stress, as stress may induce hyper-excitation of cholinergic circuits (Zimmerman and Soreq, 2006) that may contribute to the increased excitability of BLA principle cells after stress.

Though the amygdala is an essential component of the stress response leading to PTSD, the DH has also been shown to play a role. MRI studies of PTSD patients frequently report substantial loss of gray matter in the hippocampus, though there is debate as to whether this is caused by the trauma or a predisposition for diagnosis. In rodent studies, both circumstances have been observed (Bennett et al., 2015), suggesting it may be both a risk factor and result of intense trauma. The primary target of glucocorticoid stress hormones is the DH, which has very high basal expression levels of glucocorticoid receptors that are regulated by stress (Mifsud et al., 2016).

Acute stress has variable effects on hippocampal-dependent memory. In some cases, memory is enhanced during and after stressful situations (Vogel and Schwabe, 2016), while in other cases it is impaired (Dorey et al., 2012). These differences may be related to stress intensity or time course, as corticosterone has a variable time course between different brain regions, many of which may contribute to the memory task being performed.

Stress is known to induce hyperexcitation of cholinergic inputs to DH (Finkelstein et al., 1988; Mitsushima et al., 2008; Pavlovsky et al., 2012; Stillman et al., 1997), and acetylcholinesterase inhibitors in some cases induce psychopathologies very similar to PTSD (Kaufer et al., 1998). ACh signaling interacts with the stress response in DH, as selective removal of cholinergic input to DH leads to HPA axis hyperfunction and decreased DH expression of glucocorticoid receptors (Han et al., 2002; Paul et al., 2015). These converging

lines of evidence suggest that signaling, and particularly cholinergic signaling, within DH during stress may be important for the effects of stress that are often observed.

Animal models of aspects of PTSD such as stress-enhanced fear learning (SEFL) have recapitulated many aspects of human PTSD symptomology. These include resistance to extinction therapy (Long and Fanselow, 2012) and sensitization to future mild stressors (Rau et al., 2005), 2005). These models also suggest molecular and cellular targets for future studies on acute traumatic stress (Ponomarev et al., 2010).

The SEFL model demonstrates that exposure to 15 inescapable foot shocks in one environment not only leads to high levels of fear to that environment, but to heightened levels of fear after a single shock in a novel environment (Rau et al., 2005), compared with animals that did not receive the 15 shocks. This sensitization to future mild stressors was not disrupted by extinction of the original trauma context, nor by blockade of N-methyl-D-aspartate receptors (NMDARs) during learning by intracerebroventricular (icv) infusion of APV before the trauma. These manipulations that alter the valence of the traumatic contextual representation or disrupt storage of the traumatic contextual representation, respectively, do not disrupt sensitization to novel contexts. This suggests that exaggerated fear observed after SEFL is mediated by circuit changes related to non-associative sensitization, in addition to formation of an associative memory. This is also supported by the lack of requirement for memory of the trauma in juveniles for expression of the phenotype as adults (Poulos et al., 2014). Preliminary work on the model has focused on changes in BLA during and after acute traumatic stress (Perusini et al., 2015). The contribution of DH to the development of sensitized responding has not yet been examined. The relationship between glucocorticoid action during stress, the release of ACh, and the establishment of a traumatic memory has likewise not been studied.

Objectives

The objectives in the current series of studies are threefold. The first objective is to characterize cellular reactivation of contextual fear memory in involved regions, and test whether reactivation in the BLA is necessary for recall in recent and remote memory. The second objective is to determine the functional importance of cholinergic input to the DH for fear memory strength. The third objective is to determine the functional importance of cholinergic input to the DH and BLA during acute stress, for both the strength of the memory and the sensitization of future learning. Taken as a whole, these studies will advance our understanding of fear circuitry across time, as well as suggest a novel role for ACh on memory strength in normal and pathological fear.

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Chapter Two: Contextual fear memory recall and neuronal reactivation: an update

Abstract

Memory for contexts, and events occurring in them, form the basis for human episodic memory. In rodents, this type of memory is often modeled using contextual fear conditioning. Much progress has been made using immediate early genes as proxies for neurons involved in storing and recalling a given memory, and more recently as a basis for selectively activating or inactivating portions of this "memory network" in order to see the behavioral consequences. This review will focus on recent work concerning cellular ensembles underlying contextual fear, as well as present data suggesting new temporal properties for basolateral amygdala involvement in short-term, recent, and remote recall.

Human episodic memories vary in degree of detail, emotional content, and even duration, but events typically exist within some sort of temporal and spatial context. Though it is impossible to directly probe the subjective experience of a rodent, contextual fear conditioning recapitulates this aspect of human memory; that of a salient event taking place within some context. This similarity, and the ability for read-out and direct manipulation of cells and circuits in the rodent brain, has enabled the beginnings of answers to some fundamental questions:

- What brain regions are involved in storage and recall of contextual memory?
- How does a given neuron become part of a memory trace?
- How do these functions change with the age of the memory between short-term, recent, and remote time scales?

Contextual fear learning and recall in rodents requires a well-characterized neural circuit; regional contributions to this learning and recall will be discussed. Novel methods for observation and manipulation of activity-defined populations will be described, and recent advances characterized. Finally, new data concerning activation of particular cellular populations in the larger fear circuit will be reported. The importance of populations activated by learning for recall at short-term, recent, and remote time points will be discussed.

Regional contributions to context fear acquisition and recall

A context is built up of a collection of sensory impressions and internal state; these are combined by the dorsal hippocampus (DH) into a configural representation (Fanselow, 1990; Urcelay and Miller, 2014). This representation takes time to develop (Fanselow, 1986; Landeira-Fernandez et al., 2006), but once formed, it becomes a conditional stimulus (CS) that may be associated with positive or negative events that occur nearby in time to its formation or reactivation. The DH is also essential for recalling this contextual memory at short term (minutes to hours) and recent (one day to a few days) time points (Kim and Fanselow, 1992; Smith and Bulkin, 2014; Sakaguchi et al., 2015). This notion of a DH context is flexible, and may instead relate to distinct sets of rules within the same spatial and sensory environment (Smith and Mizumori, 2006). This contextual representation is sent to the basolateral amygdala (BLA); though the path of this information transfer is still under study, one potential route includes ventral hippocampus, which has a direct bi-directional projection to the BLA (Fanselow and Dong, 2010). Feedback from the BLA is also critical for learning, though not for recall (Sparta et al., 2014).

The other input for contextual fear conditioning is the aversive event; in this case, often a foot shock. This sensory information enters the dorsal horn of the spinal cord, and takes both a cortical (insula) and subcortical (thalamic) route to the basolateral amygdala (BLA) (Shi and Davis, 1999). CS-US convergence, including context-shock, occurs in the BLA (Fanselow and LeDoux, 1999; Barot et al., 2009; Hashikawa et al., 2013; Gore et al., 2015). This plasticity is such that after learning, the CS input, in this case a context, is a strong enough input to re-activate these BLA neurons without a shock input. These BLA neurons project to the central nucleus of the amygdala (CeA), where plasticity also occurs during learning (Namburi et al., 2015). The CeA then coordinates a fear response through projections to the periaqueductal gray (PAG). Depending upon the nature and proximity of the threat, this may include cautious movement, freezing, or energetic escape or attack behavior (Fanselow and Lester, 1988).

Other regions also contribute to storage and recall of contextual fear memory. Primary and secondary sensory cortex are important for fear to simple stimuli (Letzkus et al., 2011; Kass et al., 2013), though the requirement for signaling in these regions for contextual cue processing has not been documented. The medial prefrontal cortex (mPFC), which is made up of prelimbic (PL), infralimbic (IL) and sometimes anterior cingulate (ACC) subregions, has been implicated in contextual fear (Morgan and LeDoux, 1999; Frankland et al., 2004; Quinn et al., 2008; Zelikowsky et al., 2013). In addition, the retrosplenial cortex (RSC), which receives projections from DH, is important for recall of contextual fear (Corcoran et al., 2011, 2016), suggesting at least some distinction between storage and recall circuitry.

Though our initial understanding of regions contributing to storage and recall of contextual fear memories came from lesions or pharmacological inactivation studies, to be able to make statements about the memories themselves we need to track them at a cellular level, as

many interconnected neurons across diverse brain regions. Immediate early genes (IEGs), and use of their promotors, have provided the necessary tools to do just that.

Immediate Early Genes Arc and cFos in Context Fear

The neural substrate of memory remains one of the important outstanding questions in neuroscience. From Aristotle's earliest musings on memory in humans and animals (Aristotle, 350 BCE) to Karl Lashley's search for the memory engram (Lashley, 1950) to now (Mayford, 2014), both the philosophical considerations of what characteristics a "memory" in the brain must possess, as well as the methods used to probe these ideas, have matured. Some characteristics that a memory engram must possess are, that a memory engram must contain a learning-induced change in a subset of neurons, and that prevention of this change must prevent learning. Immediate early genes fulfill these requirements, and have been useful tools for probing memory circuitry. However, more research is needed to determine their relationship to the "memory engram". Though this analysis will focus on cFos and Arc (Lyford et al., 1995), other IEGs such as Zif268 have also been used to characterize circuits involved in learning and memory.

The IEG cFos has been used as a proxy for neuronal activity since it was discovered that it was activated in response to a number of environmental stimuli in burst-firing neurons (Dragunow and Faull, 1989). The IEG Arc is a cytoskeletal protein, and was found to be produced after plasticity events, where it stabilizes f actin important for structural plasticity, and is important for memory consolidation (Lyford et al., 1995; Bramham et al., 2008, 2010; Shepherd and Bear, 2011). The evidence that these IEGs are not only markers of active neurons,

but important for learning, is extensive. Antisense administration in DH for either Arc or cFos disrupts long-term memory consolidation without affecting short-term memory (Guzowski, 2002). Arc knockout mice have intact short-term memories but cannot form long-term memories (Plath et al., 2006), and inhibition of Arc impairs LTP maintenance and memory consolidation (Guzowski et al., 2000). In the DH, both cFos and Arc have a ~50% increase in expression from baseline after a spatial task (Guzowski et al., 2001), and this expression persists after learning, presumably related to supporting ongoing memory consolidation (Hashikawa et al., 2011). After contextual fear conditioning, but not after immediate shock, both Arc and cFos levels in DH rise; inactivation of the BLA prevents this increase as well as preventing learning (Huff et al., 2006). NMDA antagonism in DH, which disrupts learning, also disrupts Arc expression; Arc antisense knockdown also impairs contextual fear conditioning (Czerniawski et al., 2011). These studies link the immediate early genes Arc and cFos with functional plasticity in neural circuits, as well as with behavioral outcomes of memory storage and consolidation.

Both cFos and Arc mRNA and protein production in neurons have been used as proxies of neuronal activity, and with sacrifice a defined time interval after a learning or recall experience, active populations can be identified across brain regions (Lonergan et al., 2010). Arc in particular has a unique time course of mRNA translocation from nucleus to cytoplasm that allows for two time points of behavioral experience, and neural activity corresponding to those time points to be sampled. This technique is called catFISH (**c**ellular compartment **a**nalysis of **t**emporal activity using **f**luorescence **i**n **s**itu **h**ybridization) (Guzowski et al., 1999; Guzowski and Worley, 2001). It has been used to demonstrate that DH neurons active in one context are likely to be reactivated in the same context, less likely to be reactivated in a similar context, and even less likely in a completely different context (Guzowski et al., 1999). Usefulness of this

technique is not confined to DH; in the BLA, reactivation of neurons was higher when recalling a fearful memory than an extinguished memory (Orsini et al., 2013).

In rodents, as in humans, contextual memories change over time. Within minutes of an experience, a process of cellular consolidation takes place. This involves a widespread mobilization of cellular machinery as de novo transcription, translation, and transport of important protein products move to stabilize changes that have occurred due to plasticity (Korte and Schmitz, 2016). This process takes from minutes to hours, but is generally agreed to be concluded by a day later. For contextual memories, this is followed by a period of systems consolidation. Between 14 days and a month later, memories are no longer dependent upon DH for recall (Kim and Fanselow, 1992; Wiltgen and Silva, 2007). These processes of consolidation provide three distinct phases of recall for studying reactivation of a contextual memory. Short-term recall is within the period of cellular consolidation. Recent recall is after the conclusion of cellular consolidation, but before systems consolidation. Remote recall occurs after systems consolidation is concluded. These three phases of recall will be discussed in succession, and differences and similarities between recall and reactivation at these time points will be discussed.

Context fear reactivation: short-term

Rodents that experience a fearful stimulus must be able to respond immediately. Despite the fact that cellular consolidation takes place from minutes to hours, and systems consolidation on the order of days or months, recall of the memory must be possible throughout that extended period (Schafe et al., 2001; Sutherland and Lehmann, 2011). A recent study (Zelikowsky et al., 2014) examined contextual fear memory reactivation at this short-term time point across several relevant brain regions: DH, BLA, PL, and IL.

In the study, rats were given either a context conditioning procedure or an immediate shock procedure, and returned to the context 20 min later to take advantage of the catFISH time course. Rats given a single delayed shock acquire fear of the context while immediate shocked rats do not (Figure 2.1). Using an immediate shock for the control group controls for the effect of both context exposure and shock, but does not lead to an association between the two. Therefore, reactivation that is similar between groups relates to contextual processing, but if reactivation is only seen in the delayed shock group, it corresponds to associative fear memory formation.

DH Arc expression, which was taken from CA1, did not differ between immediate and delay groups, and showed higher cellular reactivation than neurons only active during learning (cyto) or recall (nuc) (Figure 2.2). Despite the presence of shock, as well as behavioral freezing in the delay group, reactivation profiles are very similar to simple exposure to the same environment twice (Guzowski et al., 1999). As many of these neurons are likely canonical place cells from electrophysiology recordings of DH, this suggests that these neurons are capable of being activated just as readily during active exploration as during freezing. Furthermore, it suggests that pairing a DH representation with shock does not alter the reactivation likelihood of its constituent neurons, and that these neurons selectively track contextual rather than fear information. This is consistent with existing data showing that the DH is only sensitive to CS, rather than US, presentations (Barot et al., 2009).

Arc reactivation profiles in BLA were quite distinct from DH profiles. This region showed profound between-group differences. While immediate shocked rats showed close to 0% reactivation during the second exposure of neurons active during the first exposure, delayed shocked rats showed close to 100% reactivation of that original population during fear memory

recall (Figure 2.2). This difference strongly suggests that these neurons form the basis for the associative fear memory, and that stimulus convergence during learning led to plasticity of contextual information input, such that this input activated the same population during recall. This result is also consistent with existing catFISH data concerning reactivation in the BLA (Nonaka et al., 2014). There was a set of neurons only active at recall, which might correspond to neurons receiving sensory inputs that were not sampled during the first exposure, and hence did not become active at that time. Though the reactivation profile in the immediate shock group is basically at chance, this may be due to active mechanisms for suppressing the activity of BLA neurons that did not become part of a valenced memory. Otherwise, they would be likely to receive some complement of the same sensory input during both exposures, as well as have persistently elevated excitability after the first exposure (Han et al., 2007) and hence have some level of reactivation. This may be an adaptive process for the BLA to discard unimportant representations and remain receptive to forming new valenced memories.

Arc expression in the mPFC contained contextual as well as associative fear components (Figure 2.3). In the PL, immediate shocked rats showed a profile similar to the DH pattern, with a higher percentage of reactivation than were active only at one time point. Delay rats had a similar pattern, but nearly half of all neurons active at the first exposure were re-activated when the animals recalled the fear memory. This is consistent with a role for PL in processing contextual and emotional stimuli, for context-dependent responding to stimuli (Sharpe and Killcross, 2015), and for PL-BLA plasticity during fear learning leading to enhanced fear recall (Arruda-Carvalho and Clem, 2014). IL Arc expression showed no differences between groups or compartments, making the processing of this region difficult to interpret using this method. The IL is full of heterogeneous responses and cell types, which may complicate interpretation

(Ferreira et al., 2015). This may be due to a floor effect, based on the relatively small number of neurons recruited in this region during either fear acquisition or recall. However, this is consistent with the idea that the IL region is recruited specifically when contingencies are changed and representations need updating, such as during extinction (Quirk and Milad, 2010).

Though reactivation profiles in DH, BLA, and PL/IL were on the whole consistent with known contributions of these regions to contextual processing and contextual fear learning, these profiles were examined at a short-term time point. Twenty minutes is within the period of cellular consolidation; therefore, structural and synaptic changes may still be occurring that will alter reactivation of the original population when the system has reached a more stable configuration.

Context fear reactivation: recent

The recent time point has a variable meaning, but typically refers to a delay between learning and recall of 1 day to 7 days. After about a week, contextual memories begin to complete systems consolidation, and have different properties (Wiltgen and Silva, 2007). However, the time course of native Arc expression does not allow imaging learning and recall circuits in the same animal at these delays. In addition to using native mRNA and protein expression to image recently-active neuronal assemblies, use of IEG promotors coupled with longer-term proteins such as GFP with inducible expression (Hayashi and McMahon, 2002) have enabled long-term labeling of active populations, to great effect (Guzowski et al., 2005; Reijmers and Mayford, 2009). From there, it was not a great leap to couple this technology with viral vectors to selectively target active neurons for later manipulation (Ramirez et al., 2014; Liu et

al., 2015; Tonegawa et al., 2015). Optogenetics has been particularly useful for tight temporal control while testing these hypotheses (Nieh et al., 2013; Belzung et al., 2014; Riga et al., 2014).

This technology was the first to enable a test of whether particular groups of neurons, active during learning, are necessary and/or sufficient for recall of the memory. The majority of studies testing these hypotheses used Tet-tagged mice (Reijmers et al., 2007), a tool that puts a stop on transgene expression until Dox is removed from the diet. At that point, particular transgenes may be expressed under the control of the Fos promoter. The first use of this technology to manipulate neural activity tested whether reactivation of dentate gyrus cells active during context fear conditioning was sufficient to recall the fear memory; this reactivation led to recall of the memory in a familiar but unpaired context (Liu et al., 2012). This demonstrated that plasticity during learning likely led these particular neurons to become sufficient to activate a defensive fear network, and presumably to lead to cognitive recall of the context fear memory.

The next two studies addressed the question from a different angle, with some interesting results. In these studies, a "tagged" DH representation of one context was reactivated while conditioning occurred in a second context. In the first study, which used excitatory DREADDs, the reactivated context representation becomes an essential part of the new fear representation, and must be artificially reactivated to induce fear (Garner et al., 2012). In the second study, which used channel rhodopsin, natural recall of the actual conditioning context is possible, but is enhanced with concurrent reactivation of the unpaired context representation. Surprisingly, this reactivation alone, of a context that was not technically paired with shock, also leads to fear behavior (Ramirez et al., 2013). This difference between the outcomes of the two studies may be due to the differences between DREADDs and optogenetics, where the former merely depolarizes neurons, and the later may lead to action potentials or bursting. In this case, the

latter could lead to a stronger association, and hence a greater likelihood of controlling fear behavior at a later date. Interestingly, a separate study supported that these neurons were not only sufficient for recall, but necessary, as inactivation of DG cells with tetanus toxin greatly attenuated fear recall without preventing memory consolidation (Matsuo, 2015).

Other studies demonstrated that different brain regions had distinct reactivation rules, as was suggested by the differing reactivation profiles of DH, BLA, PL and IL at the short term time point. Cells active during learning in retrosplenial cortex could support recall of fear, even after DH inactivation (Cowansage et al., 2014). Dentate gyrus cells involved in an aversive memory could be made to switch their valence with concurrent appetitive experience, and vice versa (Redondo et al., 2014). In the latter case, the BLA representation was unable to switch valence, either due to permanent assignment to outcomes of a particular valence, or due to competition between populations controlling two valences; during light stimulation of one cohort, the other cohort would necessarily be inhibited.

However, the majority of these studies have focused on the contextual aspect of the learning, rather than the association in the BLA, though optogenetics is not a stranger to the BLA complex (Wolff et al., 2014; Gafford and Ressler, 2015; Janak and Tye, 2015). There is one notable exception. One group tagged two separate events, context exposure and an immediate shock, and then reactivated the representations concurrently using optogenetics (Ohkawa et al., 2015). They found that concurrent activation of a contextual representation with an immediate shock representation was sufficient for formation of a contextual fear memory, removing the necessity for particular timing information as well as a need for the concurrent sensation of shock.

An outstanding question in the field of memory reactivation is whether BLA neurons active during learning are required for recall, and what the temporal dynamics of that requirement may be. We set out to test this hypothesis using TRAP mice (Guenthner et al., 2013), rather than Tet-tagged mice. The TRAP construct allows expression of functional Cre-recombinase under the cFos promoter only after an injection of tamoxifen or 4-OHT, a metabolite of tamoxifen. This tightens the temporal window for tagging from a few days to 6 hours, allowing greater precision in targeting neurons related to a particular learning episode. This mouse has the same caveat as the Tet-Tag mice, and may not perfectly replicate natural Fos expression, due to the absence of multiple enhancers clustered around the natural Fos promoter region important for plasticity (Joo et al., 2015).

The study design for our experiment is shown in Figure 2.4. Briefly, we targeted ArchT-GFP to BLA neurons active during fear acquisition (6 context-shock pairings, shock 0.85mA) by injecting 4-OHT to induce transgene expression. A week later, we tested whether these neurons were required for recent memory recall. BLA fluorescence is visible in both principle cells and interneurons 1 week after learning (Fig 2.5, n=1 mouse).

Fos-Cre mice (n=9) acquired fear to the context, as indexed by post-shock freezing. They were broken into two groups to determine test order based on freezing during acquisition. Mice were tested in four 2-min bins, alternating between Light ON first and Light OFF first order, and were re-tested in the opposite group the following day, so that each individual received both test orders once (Figure 2.6). There was a significant three-way interaction between testing order (ON or OFF first), block (first or second repetition of a particular trial type), and trial (ON or OFF trial). When separating by block, there was also a significant two-way interaction of order

and trial, and a significant main effect of trial, for the 1st block, with no significant main effects or interactions for the 2nd block (For full stats, see Figure 2.7).

These data support the requirement of these cells for recall of recent contextual memory, but with a particular temporal pattern not previously described. These cells are only required to begin the recall of the memory; once they have been allowed to become active, inhibiting them no longer has any effect. This is in conflict with previous modeling data (Vlachos et al., 2011; Krasne et al., 2015) that suggests that activation of BLA neurons that received potentiation during fear learning, is required for control of fear behavior. Though this activation was required for complete fear recall in the current study, this requirement was temporary, and once activation presumably occurred, these neurons could be inhibited with no effect on ongoing behavior (Figure 2.7)

BLA microcircuits are complex (Haubensak et al., 2010), and the Fos promoter used in this study tags inhibitory as well as excitatory neurons. One potential explanation for the disruption due to light stimulation is that temporal firing patterns of interneurons, many of which are known to be modulated by hippocampal theta, were disrupted in the current study (Bienvenu et al., 2012). However, the transient effect of inactivation suggests that an explanation for this effect may be found in downstream maintenance of fear-related activity, after temporally-controlled burst activation of BLA principle cells. This BLA bursting has been demonstrated for CS onset after conditioned-taste aversion (Kim et al., 2010) as well as during recall of tone fear (Cambiaghi et al., 2016). Notably in the latter, the only difference in BLA response to a paired and unpaired stimulus was a brief burst in the first 50ms after tone onset; after that time point, the neuronal firing was indistinguishable from unpaired animals. As recall of contextual fear requires sampling stimuli in order to reinstate the contextual representation in DH (Fanselow,

1986), one would expect a broader initial activation of BLA neurons as contextual details are sampled, if a similar burst-firing activation is initialized by contextual CS's.

In this study, plugging in the mice took 15-20 seconds, during which time the mice could have presumably sampled some contextual stimuli, before the green light, and cellular inactivation, could be begun. This initial sampling could explain the low, but not inconsequential, levels of fear observed during light-ON-first sessions (Figure 2.6).

There is some evidence that downstream regions such as CeL and CeA both participate in contextual fear learning, and are integral in its recall, as has been reviewed (Janak and Tye, 2015). In particular, experience-dependent potentiation in CeL may indicate that this region, in addition to the BLA, participates in formation of the CS-US association, while expression of fear behavior is controlled by the downstream CeM (Ciocchi et al., 2010). However, another study suggested that SOM+ interneurons in CeL could also participate in controlling fear expression, as CeL inhibition may disinhibit fear output from CeM (Li et al., 2013). SOM+ interneurons are activated by the CS during fear recall, their activation is required for fear recall, and activation of SOM+ interneurons causes freezing in naïve animals, suggesting that this circuit is important for expression of fear behavior. Indeed, in determining their requirement for fear recall, the pattern of freezing behavior is very similar to the current study (Figure 2.7, bottom). Future recording studies of the central nucleus of the amygdala will likely determine how downstream regions compensate for cessation of the ongoing BLA fear input.

BLA cells that are activated by fear acquisition (or more likely a subset) are required for complete fear memory recall at a recent (7 days) time point. How does the requirement for this population, as well as neural populations in other brain regions, change as the memory ages and undergoes systems consolidation?

Context fear reactivation: remote

For contextual fear memories, the first notion that remote memories had different properties came from human patients with hippocampal damage. Often, there would be a gradient for memories that were lost (e.g. (Beatty et al., 1987)), with very recent memories invariably damaged, but often with remote memories intact. This suggested that hippocampal dependence for memory storage might be related to the age of the memory.

Evidence for a temporal gradient in hippocampal-dependent memory was forthcoming (Kim and Fanselow, 1992), and suggested that recent memories (1 day) were destroyed with DH lesions, while remote memories (28 days) were intact. This gradient has been upheld by multiple studies, including with temporary inactivation rather than lesion (Varela et al., 2016), but see others (Broadbent and Clark, 2013; Goshen et al., 2011)). This relationship leads to two unknowns: where the remote contextual memory is stored, and how other circuitry involved in contextual fear learning changes to support fear memory recall at remote time points.

A critical component of remote contextual memory is likely stored in the ACC. IEG activation in this region increases selectively for remote memory recall, and inactivation of the region disrupts remote but not recent memory, in a mirror image for hippocampal inactivation (Frankland et al., 2004). The time course for DH-independence varies, but the quality of the memory is consistent; DH-dependent memories are specific and detailed, while DH-independent memories at intermediate time points become less specific, causing higher generalization of fear (Wiltgen and Silva, 2007). Therefore, as time passes after learning, memory becomes less specific as it is off-loaded to permanent cortical storage. For more in-depth reviews of this topic, see (Frankland and Bontempi, 2005; Tayler and Wiltgen, 2013).

Though the circuit contributing to contextual fear acquisition and recall shifts considerably between short-term, recent, and remote recall, the BLA is the hub. It is required for learning about fearful stimuli, and the associative memory is stored there for the lifetime of the rat (Gale et al., 2004). However, the ways in which the circuits within the BLA may restructure across time is unknown. One study observed reactivation of BLA neurons at recent and remote time points using Tet-tagged mice coupled to a stable form of GFP (Tayler et al., 2013). Approximately 4% of BLA neurons were activated by context fear conditioning and labeled with GFP, which corresponds with short-term IEG data shown above. However, reactivation of these neurons changes across time. While reactivation at short-term time points was 100%, reactivation at recent time points had dropped to 20%. At remote time points reactivation was 10%, which was not above chance, and similar to reactivation observed in a distinct context.

Mice in the above study were able to recall the fear at both recent and remote time points, despite the change in percentage reactivation of the original learning BLA population. An observational study, however, cannot distinguish between the following cases: at recent and remote time points, whichever cells were reactivated form the core of the memory; at recent time points, the core of the memory is within the reactivated population, but at remote time points, memory recall is supported by an alternate population, and reactivation of the original population is epiphenomenal and irrelevant.

We differentiate between these two possibilities with the same cohort of Fos-Cre mice used for the recent experiments previously described. Our question was simple: as memory recall was disrupted by silencing of the original population at a recent time point, are some proportion of those cells still required for remote memory recall?

Mice from the Fos-BLA study reported above were tested for remote memory recall in the same manner as for recent fear recall. Viral expression at this time point was stronger than at the recent time point (Figure 2.8), a pattern common for AAV5 serotypes (Reimsnider et al., 2007). Since this was a repeated test, and mice had already undergone two 8-min context tests at the recent time point during which time they underwent extinction of fear, many of the cohort (6/9) showed no fear (0%) at the remote time point. These mice were not re-tested and were excluded from analysis. Of the remaining 3 mice, all three showed the same pattern as at the recent time point. Though overall levels differed between the three mice, all mice increased from light ON to OFF when that order began the session, and maintained steady freezing behavior during the reverse trial order (Figure 2.9).

Though this is very preliminary data, the within-subjects nature of the comparison is enough to suggest that, for BLA cells previously active during fear learning, the requirement for recall is not so different at the recent and remote time points. Though this is consistent with the requirement for the BLA for recall at all time points (Gale et al., 2004), it is inconsistent with many ideas about how memory recall may shift as contextual DH-dependent memories undergo systems consolidation. One model suggests that while recent contextual fear memory is dependent upon DH-basolateral synapses that have undergone potentiation during learning, remote fear memory shifts to cortical-lateral amygdala (LA) pathways for recall. This is supported by the high innervation of LA by sensory association areas. However, high level association cortex also innervates BL (Pitkänen et al., 2000); this may support recall, using the same BLA neural representation, at remote time points. During remote recall of tone fear, BLA neurons are synchronized in the theta band with secondary sensory cortex, which drives BLA activity (Cambiaghi et al., 2016).

Both of these studies, and any study that suggests that the same BLA neurons are required for recall at recent and remote time points, have to contend with the challenges inherent in an entirely separate input population of cells managing to activate the same downstream population, using separate synapses that likely weren't activated during the initial learning. However, one study suggests a mechanism for such reactivation (Nonaka et al., 2014). They found that contextual fear conditioning caused presynaptic potentiation of cortical (but not thalamic) inputs to BLA neurons that were activated by that fear learning. The same study showed that BLA neurons active during fear conditioning are preferentially reactivated during fear recall. It remains possible that this cortical input, which underwent potentiation during fear conditioning, contributes to recall of contextual fear at both recent and remote time points, but is strengthened during systems consolidation. In this fashion, DH input would be required to supplement this cortical input at recent time points, but not at remote time points. This would fit existing data on the subject, and would neatly abrogate the need to establish, long after contextual fear learning, de novo cortical connections for activating the same population of BLA cells.

These new data supplement the previous observational studies demonstrating decreasing patterns of BLA reactivation, from short-term (100%, Zelikowsky et al., 2014) to recent (20% reactivation) to remote (10% or chance reactivation) of contextual fear (Tayler et al., 2013). Before functional inhibition of these populations, it was difficult to say conclusively whether or not the memory is in the process of transferring to another BLA population at the recent time point, and is fully transferred at the remote time point. However, the results of the Fos-Cre study reported above demonstrate that this initial population is important for recall of fear at both recent and remote time points. This suggests that one function of systems consolidation may be

to streamline the BLA representation, strengthening inputs to those portions of the representation sufficient for recall of fear and reducing the contribution of other parts of the representation. This would lead to the observed results, where the 10% "chance" portion of the complete representation disrupts overall recall of the fear memory 4 weeks after learning.

Future Directions

Clearly, the BLA representation is not the only important component of short-term, recent, or remote contextual fear memory. Activation of the DH representation (or at least the dentate component) is sufficient for recall of fear at recent time points. While the BLA representation has a fixed valence, the valence of the DH representation has been shown to be flexible, dependent upon pairing during recall (Redondo et al., 2014). The mPFC, and particularly the prelimbic cortex, track both contextual and shock information (Zelikowsky et al., 2014). Whether or not cells active during learning in PL must be reactivated for retrieval of contextual fear, or whether activation of these cells can control particular behavioral states, has not yet been tested. However, the importance of this region for behavioral control, as well as for using higher-order cues to execute this control (Sharpe and Killcross, 2015), implies that this direction of research might become useful for human therapeutics in disorders of cognitive or behavioral control.

It seems likely that human memories similarly reactivate the same populations during learning and recall, at least in certain circumstances. Understanding the time course, mechanism, and principles behind this reactivation could potentially open the door to memory modification of unwanted memories. Behaviorally, therapists could, and often do, use the principles of

pairing aversive contextual recall with positive valence events in order to shift the valence of the contextual memory. Recent memories may be vulnerable to reconsolidation blockers, though traumatic memories may sometimes be resistant to such manipulations (Hoffman et al., 2015). Yet there has been some success with combining reconsolidation blockade with HDAC-inhibitors, which have disrupted both recent and remote memories through reconsolidation (Gräff et al., 2014; Zovkic et al., 2014). Future research into both the mechanisms of contextual fear memory acquisition, as well as the methods of memory maintenance at recent and remote time points, will lead to useful human therapeutics both for erasing unwanted memories and for strengthening weak memories.

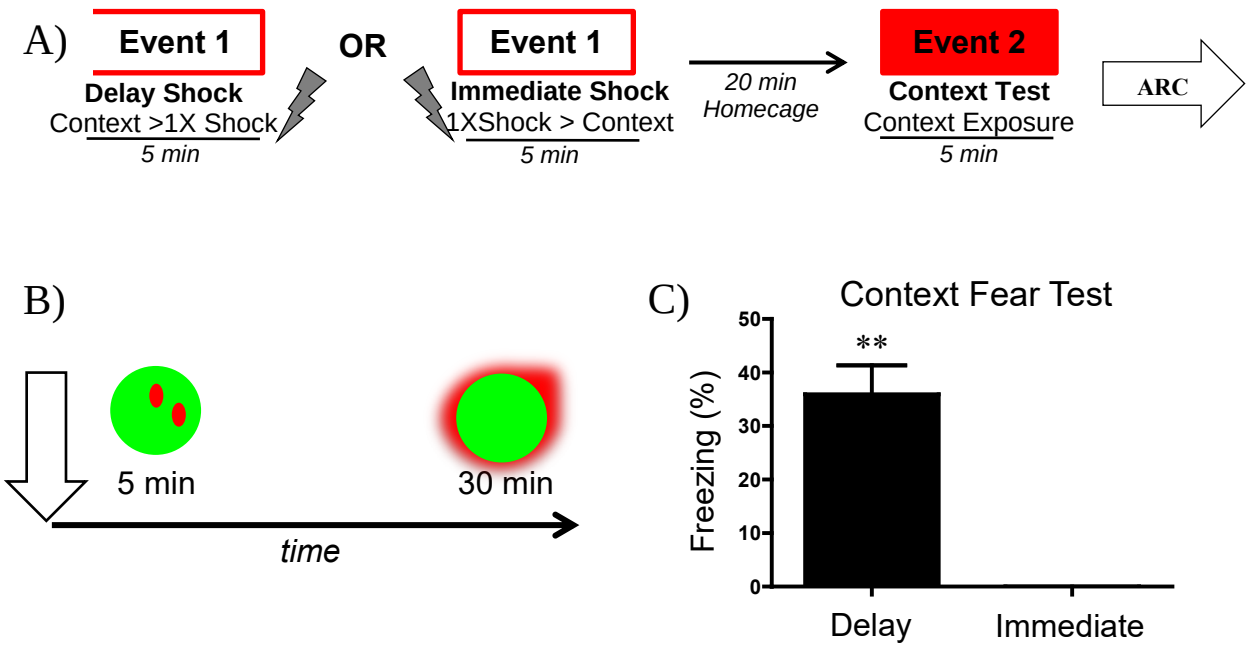
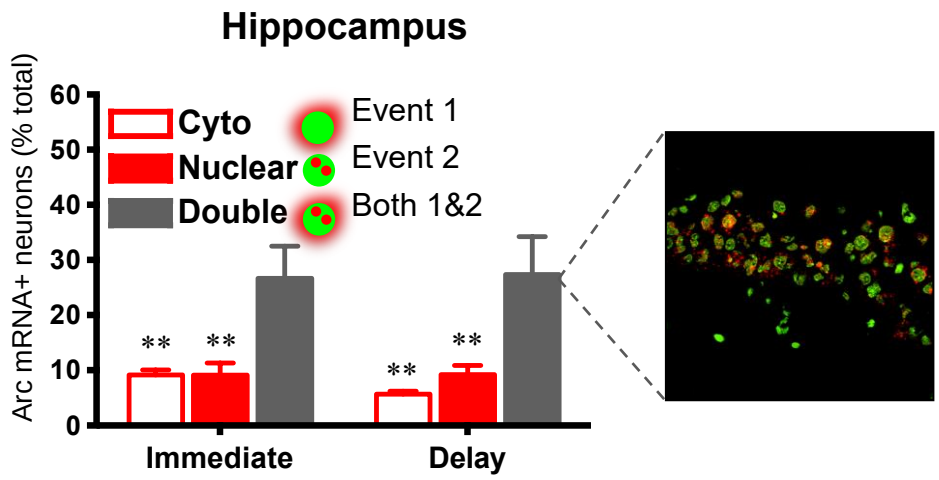


Figure 2.1. **Behavioral design and behavioral results for catFISH reactivation**. A) Rats were fear conditioned or immediately shocked (Event 1) and 20 min later were tested for context fear (Event 2). B) Time course of Arc mRNA transcription and translocation from the nucleus of a cell to the cytoplasm. Mean (error is SEM) percentage freezing during context test (Event 2). C) Delay-conditioned rats show significant freezing to the context compared with immediately shocked rats displaying the immediate shock deficit. **$p < 0.01$.

A)



B)

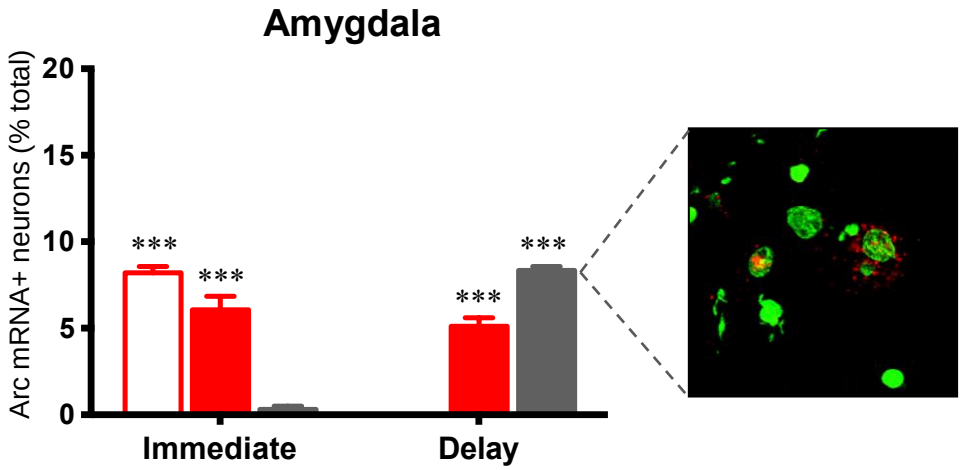


Figure 2.2. **DH and BLA reactivation**. Mean (and SEM) percentage total neurons (sytox; green) that were positive for Arc mRNA (red) in a given cellular compartment, and representative fluorescent images. A) CA1 region of DH showed a significant increase in double-labeled cells (gray) regardless of whether or not the environment was fear inducing (Immediate v. Delay). B) BLA region only showed reactivation during fear, with a complete absence of reactivation if no fear memory was formed. ***p <0.001, **p < 0.01.

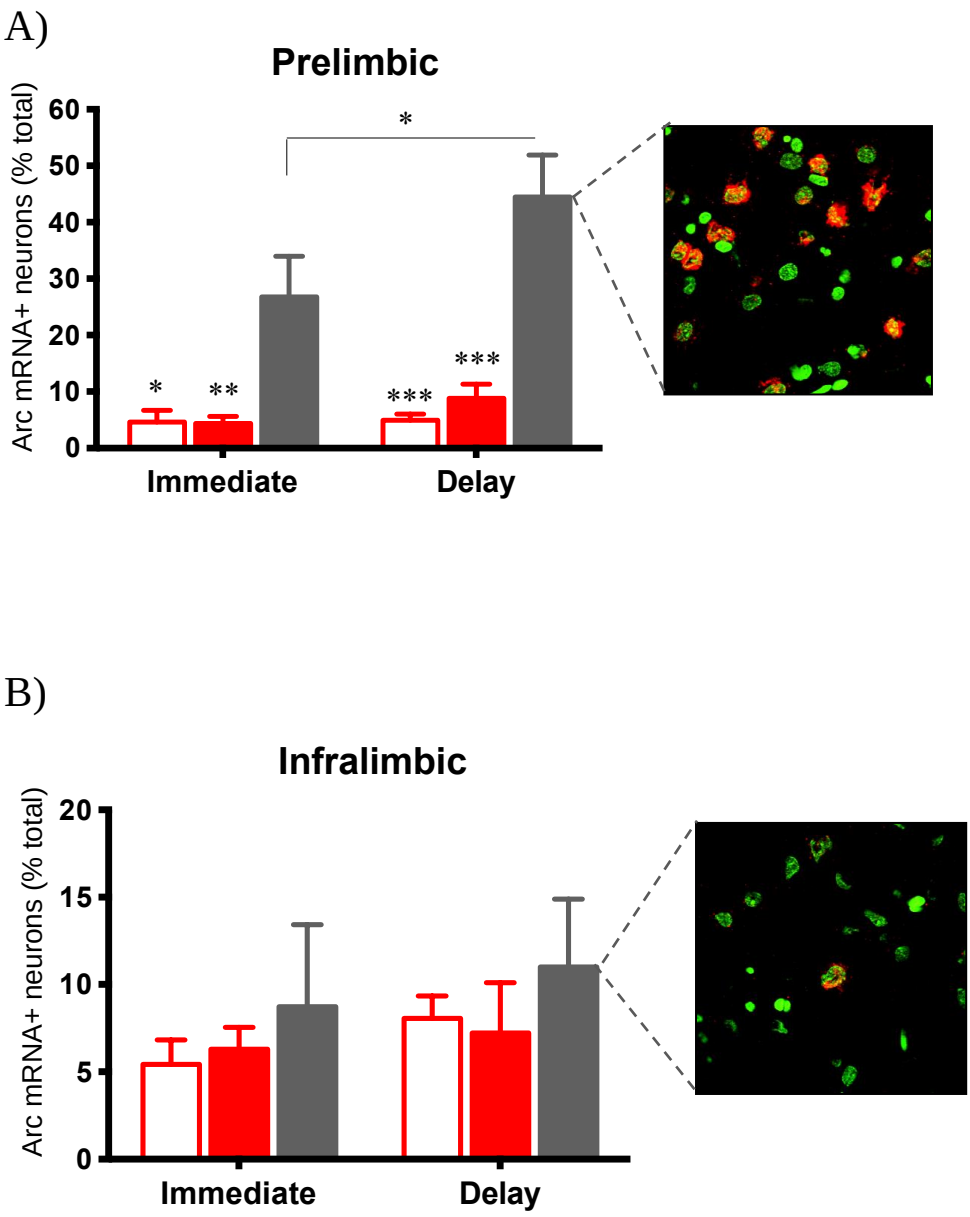
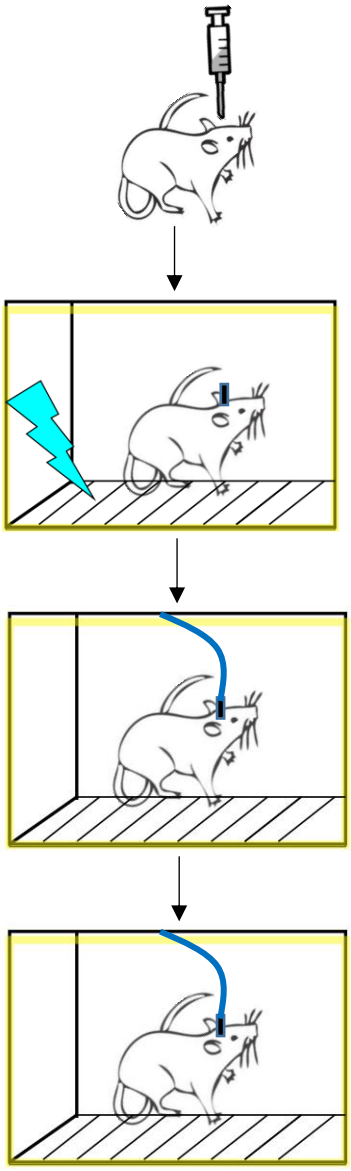


Figure 2.3. **PL and IL reactivation**. Mean (and SEM) percentage total neurons (sytox; green) that were positive for Arc mRNA (red) in a given cellular compartment, and representative fluorescent images. A) PL expression revealed overlap in neuronal ensembles involved in both events, which was further enhanced in fear-conditioned rats. B) IL expression showed no compartmental differences. *$p < 0.05$.



2 weeks before: Surgery in BLA
AAV5-FLEX-ArchT-GFP
Ferrules

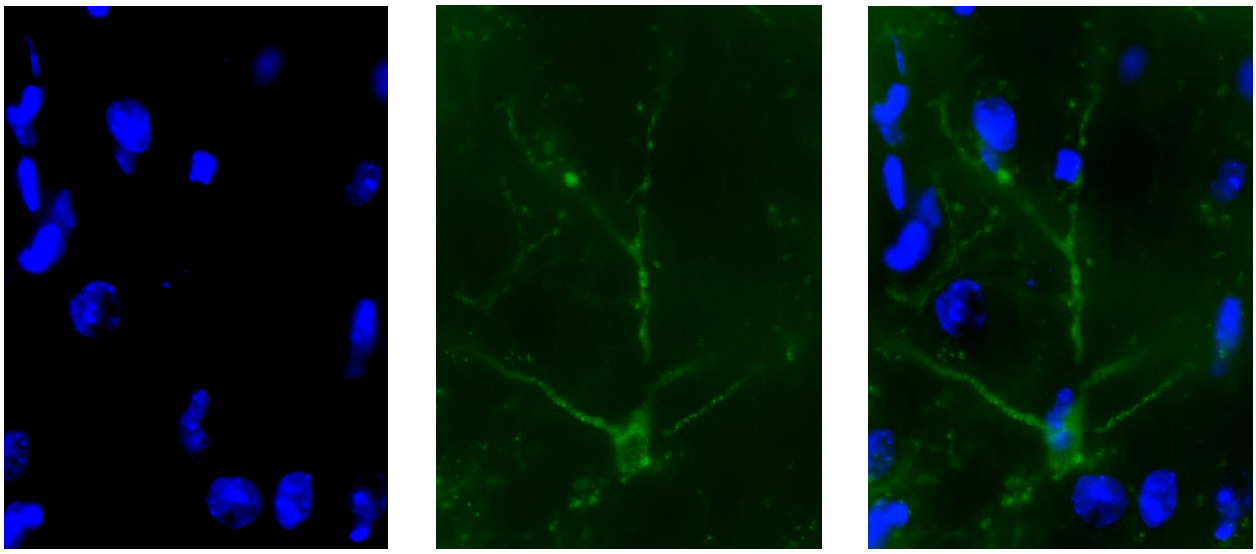
Day 0: Fear Acquisition after 4-OHT injection
to tag Fos+ BLA cells with ArchT

1 week after: Recent Tests (8 min)
Group swap between days
2 min bins, Light ON and OFF

4 weeks after: Remote Test (8 min)
Group swap between days
2 min bins, Light ON and OFF

Figure 2.4. **Fos-Cre Reactivation Design**. Heterozygous Fos-cre mice (n=9) were infused with AAV and ferrules were implanted to bilaterally target the BLA. Mice then underwent fear acquisition after an injection of 4-OHT and received 6 foot shocks (0.85mA, 2 s). A week later, mice were returned to the conditioning context and tested for recall of fear in the presence and absence of green light stimulation in 2 min bins (10-12mW, continuous). The following day, mice were moved to the other group (e.g. light ON to light OFF first) and were re-tested for fear. The same procedure occurred at a remote time point (4 weeks after conditioning).

A)



B)

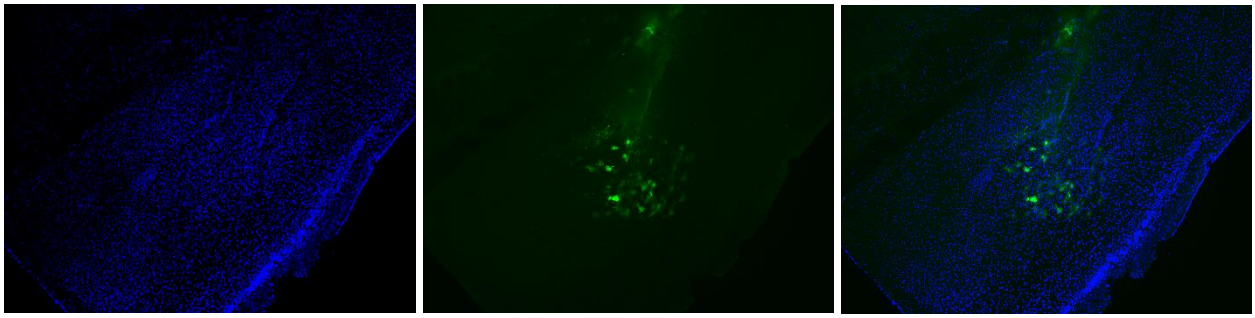


Figure 2.5: **Viral Expression, Recent**. A) Viral expression of ArchT-GFP in BLA pyramidal cell (40x, DAPI nuclear stain). B) Unilateral expression of ArchT-GFP in BLA (4x, DAPI nuclear stain). Slices taken from Mouse 6.

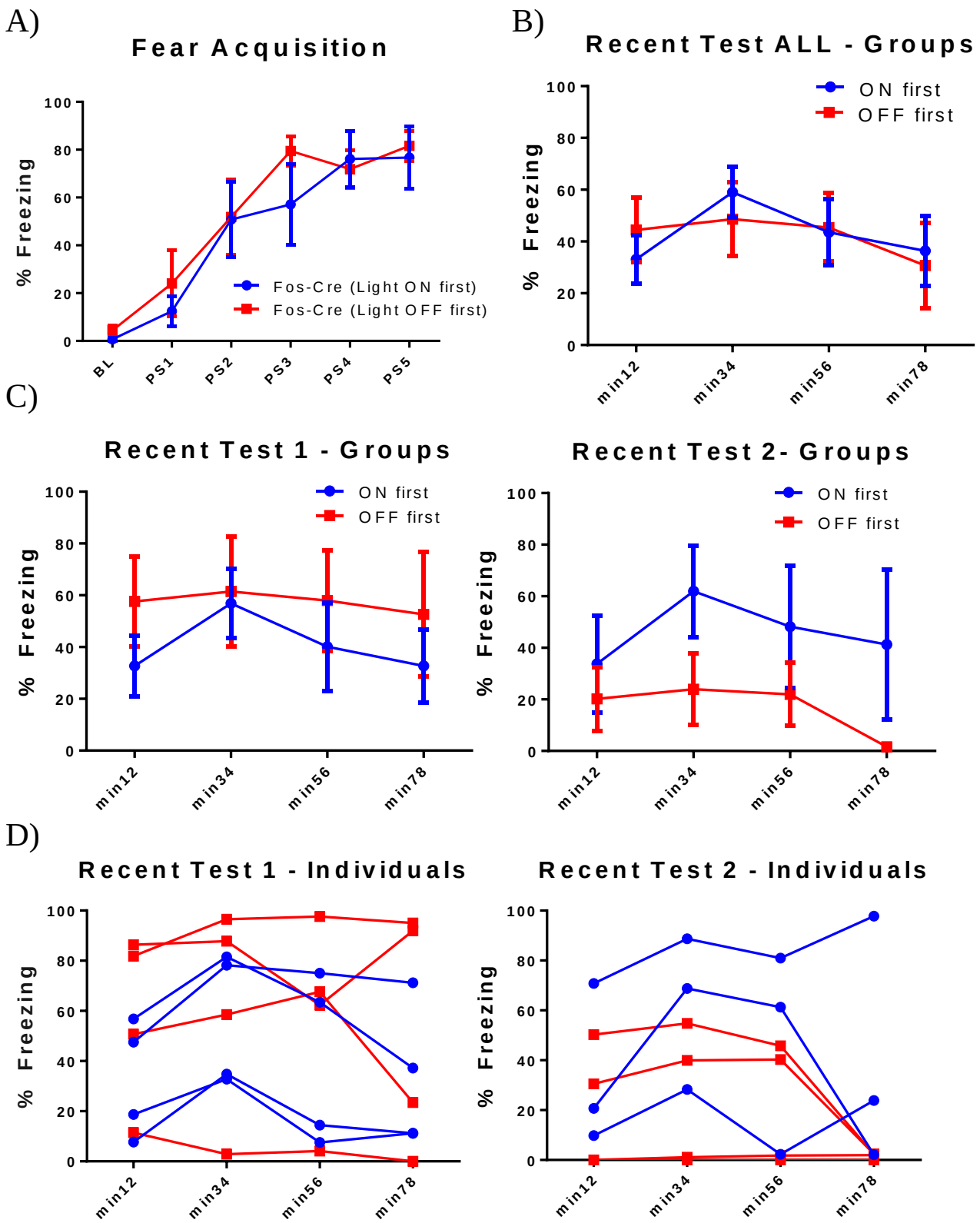


Figure 2.6. **Fos-Cre Recent Test**. A) Mice were divided into two groups based upon post-shock freezing during acquisition. Mice then received alternating 2-min bins of light ON and OFF throughout the 8 min test, with the opposite order during a retest the following day. Group data (C) and individual data (D) are separated out for visualization, but the combined data with two tests for each subject are shown in the upper right (with SEM). Statistics are on the following page.

A)

Comparison	F statistic	DF	P value
3-way Interaction, Order x Block x Trial	F=16.804	6	p = 0.006
2-way comparison, 1st Block: Order x Trial Interaction	F=60.500	6	p < 0.001
2-way comparison, 1st Block: Main Effect of Trial	F=13.953	6	p = 0.010
2-way comparison, 2nd Block: No Main Effects or Interactions			p > 0.05

B)

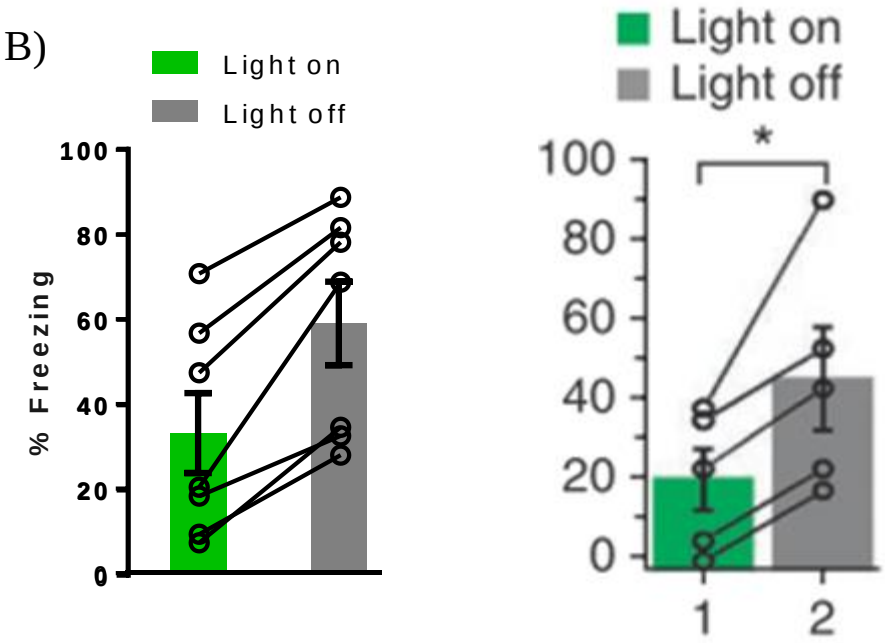
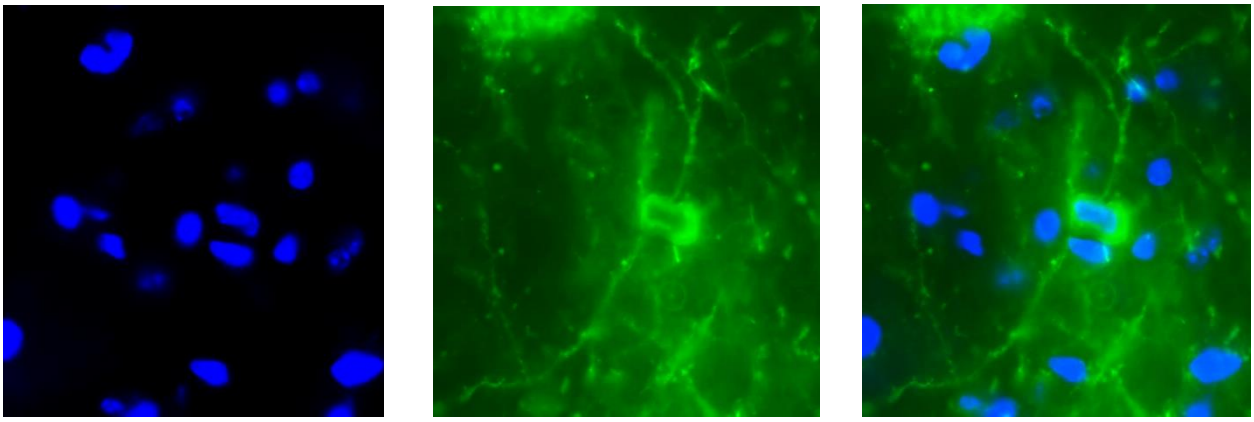


Figure 2.7. **Fos-Cre Recent Test Statistics**. A) A significant 3-way interaction (Order x Block x Trial) was found, and 2-way comparisons were done for the 1st and 2nd block of testing (first 4 min, second 4 min of 8 min test). An interaction between Order and Trial, and a main effect of Trial, were found for the 1st testing block, but these effects did not persist into the second block. This suggests that inhibiting BLA cells (light ON) only had an effect in the 1st testing block, and only when the ON session was the first session. B) During contextual fear recall, comparison between ArchT inhibition of BLA cells previously active during fear learning, and Arch inhibition of SOM+ cells in CeL. CeL is one potential pathway for maintenance of fear-expression information in the face of BLA inhibition.

A)



B)

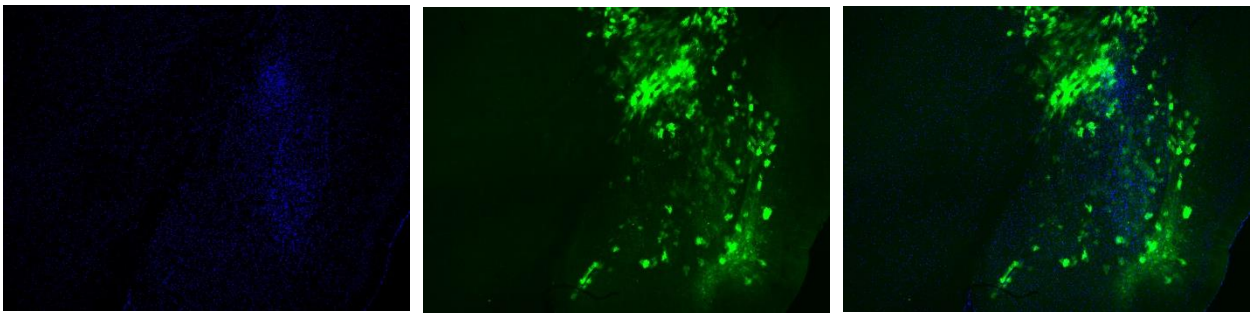


Figure 2.8: **Viral Expression, Remote**. A) Viral expression of ArchT-GFP in BLA pyramidal cell (40x, DAPI nuclear stain). B) Unilateral expression of ArchT-GFP in BLA (4x, DAPI nuclear stain). Slices taken from Mouse 1.

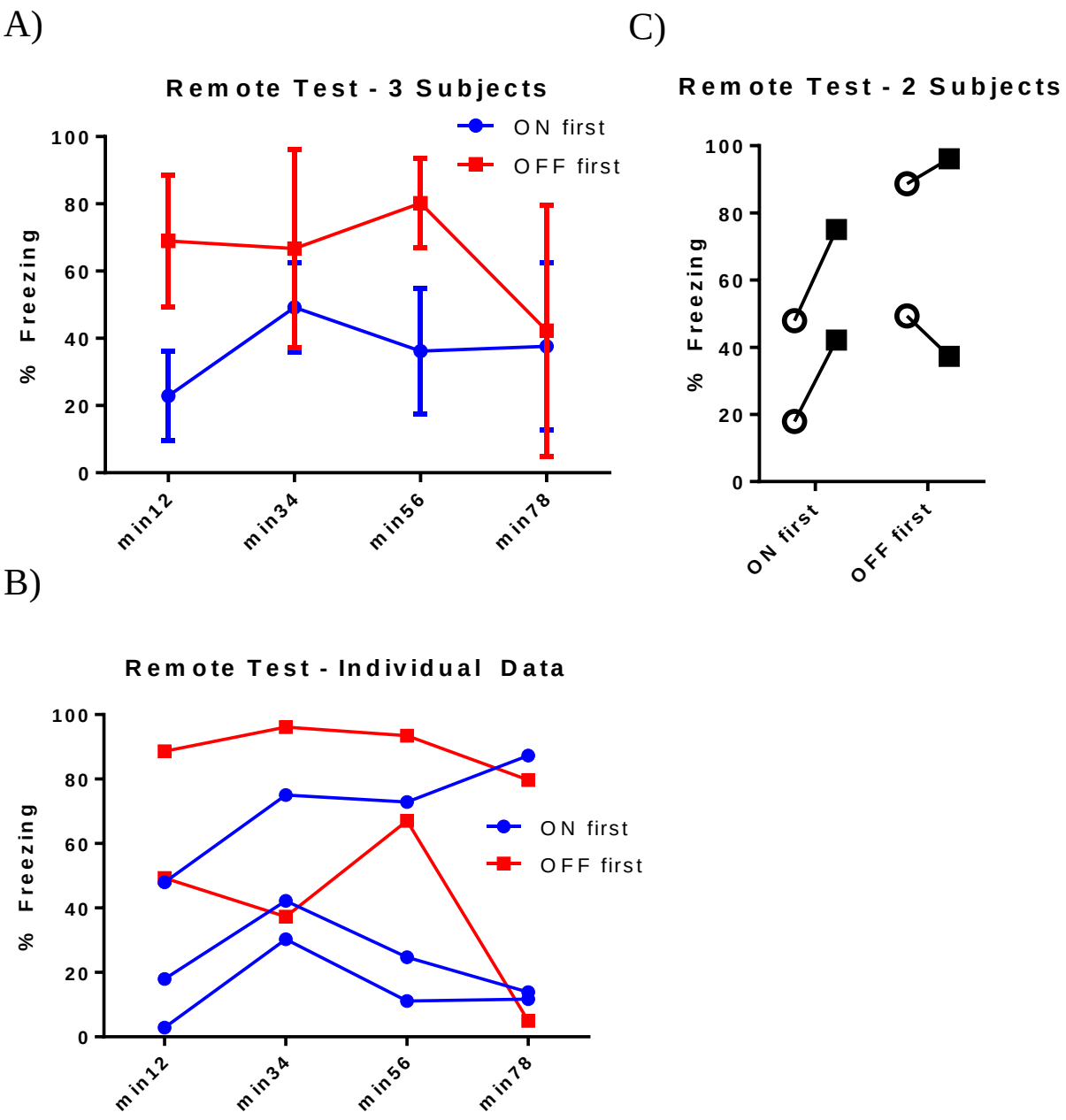


Figure 2.9. **Fos-Cre Remote Test**. Mice were divided into two groups for ON first or OFF first testing. The majority of mice (6/9) showed no fear (0% freezing across the session) at the remote time point, and so did not receive two tests; all remote data from these mice was excluded from further analysis. Of the remaining three mice, three mice showed fear during at least one test (A and B). Two mice showed fear during both tests, and demonstrated the same effect seen at the recent time point (C: circles represent minutes 1-2, while squares represent minutes 3-4). This provides preliminary evidence that these cells may still be necessary for recall of fear at this remote time point.

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Chapter Three: Optogenetic Excitation of Cholinergic Inputs to Hippocampus Alters Acetylcholine Release and Improves Contextual Fear Learning

Abstract

Learning about context is essential for appropriate behavioral strategies, but the strength of contextual learning varies with attention and length of exposure. We used a challenging contextual learning procedure coupled with in vivo optogenetics to test whether elevated acetylcholine (ACh) release could enhance contextual memory. Using ChAT-Ai32 mice, we stimulated release of ACh in hippocampus during exploration of a novel environment, and later paired that environment with shock. Though enhancing cholinergic release did not change degree or rate of exploration of the context, mice showed elevated levels of fear at test. This demonstrates that increased cholinergic tone improves cognitive processing and storage of contextual elements. This same stimulation protocol also led to detectable increases in ACh release in anesthetized recordings with choline biosensors; this release scaled with pulse width and laser power across a range of simulation parameters. These findings provide the first direct evidence for ACh as an enhancer of hippocampal contextual processing.

Introduction

The dorsal hippocampus (DH) is the primary structure involved in acquisition and storage of a contextual memory (Smith and Bulkin, 2014). In order to form these contextual representations, a rodent requires time to sample stimulus elements of the context and bind these together into a unified representation that can be associated with positive or negative valence (Fanselow, 1990; Krasne et al., 2011, 2015; Urcelay and Miller, 2014; Wiltgen et al., 2001). The DH is essential for both binding of the stimulus elements as well as recall of this configuration

(Landeira-Fernandez et al., 2006; Matus-Amat et al., 2004; Schiffino et al., 2011; Stote and Fanselow, 2004).

The DH receives a constant stream of highly processed sensory input, and an outstanding question is what neural signals might lead to the binding of these inputs together into a stable, configural contextual representation (O'Reilly and Rudy, 2001). Cholinergic inputs to hippocampus from the medial septum (MS) (Mesulam et al., 1983), in particular, have been associated with both memory encoding and contextual processing (Easton et al., 2011). Disruption of these inputs in disease states can have deleterious effects on memory (Coyle et al., 1983; Hasselmo and Sarter, 2011), and are potential targets for treatment of memory impairment (Cansev et al., 2015; Jeong et al., 2014; Kuhn et al., 2015; Morasch et al., 2015; Robinson et al., 2011).

Acetylcholine (ACh) levels in the DH increase with exploration of a novel environment (Day et al., 1991; Giovannini et al., 2001; Mitsushima et al., 1998), exposure to novel stimuli, and exposure to stimuli that have previously been paired with shock, though not after exposure to habituated stimuli (Acquas et al., 1996). These dynamics suggest that activation of these networks may be related to arousal and shifts in attentional processes (Baxter et al., 1997; Picciotto et al., 2012) in addition to direct relationships with memory formation.

Though pharmacological manipulations of nicotinic and muscarinic receptors in the hippocampus has provided valuable information of potential mechanisms of action of ACh (Changeux et al., 2015; Tinsley et al., 2004), particular subtypes of these receptors are not activated in isolation during behavior. Instead, overall cholinergic population firing changes in response to task demands, requiring newer techniques to alter or mimic these changes in order to understand circuit function. Optogenetics is a powerful tool for understanding the functional

consequences of higher or lower levels of ACh in defined neural circuits during ongoing behavior (Luchicchi et al., 2014; Nagode et al., 2011).

We used the CPFE procedure in order to test the effect of elevated ACh release on hippocampal encoding of context. We selectively increased ACh release by optogenetically stimulating cholinergic cell bodies in the MS using fiberoptic ferrules in ChAT-Ai32 mice (Madisen et al., 2012; Sauer, 1998) and Ai32 littermate controls. In addition, we validated and characterized our in vivo optogenetic manipulation using choline biosensors to measure light-evoked ACh release in dCA1 of anesthetized mice and rats. The combination of optogenetic manipulation of genetically-defined neuromodulatory populations with spatially and temporally precise measurement of evoked release enables real-time control of neurotransmitter systems and their direct outputs in behaving animals.

Materials and Methods:

Subjects. Twenty-three naïve male ChAT-Ai32 mice, weighing 25-30g were singly housed post-surgery and maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*. Animals were handled for five days leading up to behavioral experiments. 4 naïve female ChAT-Ai32 mice, weighing 25g, and 3 naïve female ChAT-Cre rats, weighing 350-400g, were used for non-survival anesthetized choline biosensor recordings. The behavioral and surgical procedures used in this study were in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Apparatus. Behavioral training used fear conditioning chambers (30 X 25 X 25 cm, Med-Associates, Inc St. Albans, VT), equipped with a Med-Associates VideoFreeze system. The boxes were enclosed in larger sound-attenuating chambers in an individual, dedicated

experimental room. The context was comprised of a chamber with aluminum sidewalls and a white Plexiglas rear wall. The grid floor consisted of 16 stainless steel rods (4.8 mm thick) spaced 1.6 cm apart (center to center). The ceiling was clear Plexiglas with a central hole allowing for passage of fiberoptic cables. Pans underlying each box were sprayed with a thin film of 50% Windex solution to provide the context with a scent. Chambers were individually lit from above by white lights and cleaned with 50% Windex in between trials. Fans mounted above each chamber provided background noise (60 dB). The experimental room was brightly lit with an overhead white light. Animals were kept in a holding room and individually transported to the experimental room in their home cage. On the first day of training, animals were transported to the habituation cart for cable attachment before conditioning, and returned to the cart for cable disconnection afterward. Chambers were cleaned with a Virkon solution following each day of behavioral testing.

Surgery for In Vivo Optogenetics. Mice were anesthetized with 3% isoflurane, and standard surgical procedures were used to implant a single fiberoptic ferrule cannula targeting the medial septum, with these coordinates (AP: +0.70, ML: 0.00, DV: -3.5). Cannula were fixed in place using one layer of Metabond (Parkell, Inc.), and a second layer of dental acrylic (The Bosworth Company). Mice were allowed to recover for at least one week before undergoing behavioral training.

Context Pre-exposure Procedure. Animals received two days of transport to holding room and attachment to the fiberoptic cable (5 min per animal, per day) prior to the experiment. On the second day, animals were pre-exposed to the LED light stimulation for use during behavior (470 nm, 5 mW, 10 Hz, 5 ms pulses) for 5 min. For context pre-exposure, animals were transported in home cage to the habituation cart, where they were briefly restrained and connected to the

fiberoptic cable. After a 2 min habituation period, animals were transported to the context chamber, where 10 min LED light stimulation and contextual exposure took place. Mice were then removed to habituation chamber, and after 2 min were disconnected and returned to their home cage. The following day, mice were transported in the home cage to the same context chamber, were after 10 seconds they received a foot shock (0.75 mA, 1 sec); 30 sec later they were removed and returned to home cage. The following day, mice were transported in the home cage to the same context chamber, where freezing was recorded for 8 min.

Acetylcholine Recording:

Summary: Briefly, these biosensors use choline oxidase as the biological recognition element and rely on electro-oxidation, via constant-potential amperometry (0.7 V versus a Ag/AgCl reference electrode), of enzymatically-generated hydrogen peroxide (reporter molecule) to provide a current signal. This current output is recorded and converted to choline concentration using a calibration factor determined *in vitro*. Choline sensing allows for an accurate extracellular measure of acetylcholine (ACh), which is rapidly hydrolyzed by endogenous acetylcholinesterase (Burmeister et al., 2000; Giuliano et al., 2008; Parikh and Sarter, 2006; Parikh et al., 2004). Indeed, adding acetylcholinesterase onto the sensing electrode does not enhance detection of cholinergic activity (Giuliano et al., 2008), suggesting that choline biosensors provide an accurate measurement of evoked ACh. Interference from both electroactive anions and cations is effectively excluded from the amperometric recordings, while still maintaining a <1 s response time, by application of polymer coatings to the electrode sites prior to enzyme immobilization (Wassum et al., 2008). Additionally, incorporation of two non-enzyme-coated sentinel electrodes on the MEA enabled removal of correlated noise, including

light artifact, from the choline sensing electrode output by signal subtraction (see Data Analysis). Completed sensors were sealed in a container with desiccant and stored at 4°C.

Reagents. Nafion (5 wt % solution in lower aliphatic alcohols/H_2O mix), bovine serum albumin (BSA, min 96%), glutaraldehyde (25% in water), pyrrole (98%), choline chloride (99%) L-ascorbic acid, 3-hydroxy-tyramine (dopamine) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). CholOx with a rated activity of 10 units per mg protein (U mg-1, Lowry's method) was purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Phosphate buffered saline (PBS) was composed of 50 mM Na 2 HPO 4 with 100 mM NaCl (pH 7.4). Ultrapure water generated using a Millipore Milli-Q Water System was used for preparation of all solutions used in this work.

Instrumentation. Microelectrode array (MEA) probes were fabricated in the Nanoelectronics Research Facility at UCLA and modified for choline detection. Electrochemical preparation of the sensors was performed using a Versatile Multichannel Potentiostat (model VMP3) equipped with the 'p' low current option and low current N' stat box (Bio-Logic USA, LLC, Knoxville, TN). In vitro and in vivo measurements were conducted using a low-noise multichannel Fast-16 mkIII potentiostat (Quanteon), with reference electrodes consisting of a glass-enclosed Ag/AgCl wire in 3 M NaCl solution (Bioanalytical Systems, Inc., West Lafayette, IN) or a 200 μ m diameter Ag/AgCl wire, respectively. All potentials are reported versus the Ag/AgCl reference electrode.

Sensor Preparation and Calibration: Biosensors prepared for choline detection were calibrated in vitro to test for sensitivity, selectivity and response time to choline. Coating layers on the sensors used in the study were as follows: one sensor with PPY and three sensors with PPD; all sensors also contained a Nafion coating layer. The final layer on all sensors was

BSA-glutaraldehyde; on the choline-sensitive electrode, this layer also contained choline oxidase. In vitro was carried out using constant potential amperometry with the FAST-16 electrochemistry system. A constant potential of 0.7 V was applied to the working electrodes against a Ag/AgCl reference electrode in 40 mL of stirred PBS at pH 7.4 and 37 ºC within a Faraday cage. Data were collected at 80 kHz and averaged over 1 s intervals. After the current detected at the electrodes equilibrated to baseline (approx. 30 min), an aliquot of the potential interferents, AA (250 µM final concentration; representative potential anionic interferent) and DA (5-10 µM final concentration; representative potential cationic interferent) were added to ensure selectivity for choline. For all sensors used in these experiments no current responses to these interferents were detected above the level of the noise. The sensors were calibrated against three 40 µL aliquots of choline (20 mM), which were added to the beaker to reach a final choline concentration of 20, 40 and 60 µM choline. Hydrogen peroxide (10 µM final concentration) was also added, to ensure similar sensitivity and response time on control and choline-sensitive channels. Average H2O2 sensitivity for the sensors used in the study varied no more than 10% between control and choline sensor electrodes and was not significantly different (ttest, $p>0.05$). A graphic depiction of the biosensor and a representative calibration are shown in Figure 3.4. A calibration factor based on analysis of these calibration data was calculated for each electrode on the biosensor to be used for in vivo anesthetized experiments. Data were output as current as a function of time and analyzed in Microsoft Excel.

In Vivo Validation of Optically-Evoked Acetylcholine Release. Briefly, a biosensor was implanted into the DH and a ferrule delivering blue light was inserted near the biosensor to activate cholinergic terminals and evoke ACh release. Standard stereotaxic surgical techniques under isoflurane anesthesia were used to unilaterally implant a biosensor, pre-calibrated to

choline (see above) into the DH (dCA1), following coordinates according to the atlas of Paxinos and Watson (4th ed.) (AP: -1.95, ML: +1.25, DV: -1.25 to -1.5). Additionally, a fiberoptic ferrule cannula (Doric Lenses) was implanted, angled at 20 degrees, into dCA1 (AP:-1.95, ML: +0.2, DV: -1.6 to -1.8). For rat biosensor surgeries, a primary survival surgery was performed to infuse a Cre-dependent ChR2 virus (AAV5-EF1a-DIO-hChR2(H134R)-EYFP) into MS (0.7 μl at 0.1 μl/min with 5 min wait for diffusion, coordinates: AP: +0.45, ML:0.00, DV: -6.80); incision was sealed with wound clips, which were later removed. After waiting 2-6 months for complete viral expression, rats underwent non-survival biosensor implant surgery and recording. For rats, the same procedure was followed as for mice, but with the following coordinates in dCA1 for the biosensor (AP: -3.80, ML: 2.5, DV: -2.5 to -3.8) and fiberoptic ferrule at 20 degrees at the edge of the biosensor hole (medial 0.5mm entry to biosensor). A Ag/AgCl reference electrode was implanted in contralateral prefrontal cortex. The entire experiment was conducted inside a Faraday cage. The probe was advanced to the pyramidal layer of dCA1 and the electrode signal was allowed to equilibrate to baseline for approximately 30 min prior to application of 3s light pulses at 10 mW. Successive pulses of light and advancing of the fiberoptic occurred by 0.1 to 0.2 mm each time until the maximal current response was evoked on the biosensor (pyramidal dCA1). When a response was consistently observed, all mice and rats received the following protocol with three repetitions of each. First, a power titration (1.5 s pulses at 1, 2, 3, 5, 10, 20 mW), followed by a pulse width titration (5 mW power, 1000 ms, 500 ms, 250 ms, 125 ms with continued halving until no visible response was seen) was administered. This was followed by a strong pulse train (5 mW, 1 s on 0.5 s off for 45 s). Finally, a test of 10 Hz pulse trains (5 mW, first 5 ms, then 10 ms, then 20 ms pulses, for 10 min) was administered. Stimulations were administered at least 30 s apart, with longer baseline

periods for longer pulse trains. All data were plotted as current versus time using GraphPad Prism (La Jolla, CA) and SPSS (IBM Corp, Chicago, IL).

Statistical Analysis. For contextual fear conditioning, statistical analyses on freezing behavior were performed using an automated near infrared (NIR) video tracking equipment and computer software (VideoFreeze, Med-Associates Inc.), as previously described (Zelikowsky et al., 2013). For biosensor recordings, currents are reported as calibrated changes in choline concentration; each stimulation was repeated at least three times. The current changes from baseline on the control electrode were subtracted from current changes on the choline biosensor electrode to remove noise correlated among the electrodes on the MEA; this also removed the light induced-electrical artifact, which was similar across channels. The baseline subtraction for shorter (< 30 s) stimulation protocols was taken from an averaged 10 s bin 20 s before the onset of light stimulation. For longer stimulation protocols (< 30 s), the baseline subtraction was taken from an averaged 10 s bin 1 min before the onset of light stimulation. The choline biosensor response then was converted to choline concentration using an electrode-specific calibration factor obtained in vitro, which averaged 54.95 μM/nA. For all hypothesis tests, the α level for significance was set to $p < 0.05$. The data were analyzed with paired t-tests, repeated-measures ANOVAs (with post hoc analysis correcting for multiple comparisons), correlation and regression, where appropriate.

Results

ChAT-Ai32 mice and virally-infused ChAT-Cre rats express ChR2-eYFP in cholinergic neurons of medial septum

In order to express channel rhodopsin conjugated to eYFP selectively in cholinergic cells, we crossed the ChAT-Cre driver mouse line (Madisen et al., 2010) with the Ai32 reporter line (Madisen et al., 2012) to produce ChAT-Ai32 mice which are heterozygous for Cre, and littermate controls negative for Cre recombinase. All subjects are homozygous for Ai32.

ChAT-Ai32 mice expressed eYFP in a subset of cells in MS, as well as in other forebrain regions (Figure 3.1). No fluorescence was visible in littermate controls negative for Cre.

In order to also examine optically-induced ACh release after viral administration of channel rhodopsin, we used the ChAT-Cre line of rats, previously validated for selective Cre expression in cholinergic neurons by others (Witten et al., 2011). Viral expression did not significantly vary between individual subjects (data not shown), though eYPF was consistently visible in cell bodies of MS as well as in cholinergic terminals in the DH (Figure 3.6).

Stimulation of medial septum cholinergic neurons during novel environment exploration selectively improves encoding strength

A typical context fear procedure consists of two distinct processing epochs during the course of acquisition: sampling contextual details in order to form a configural context representation (context encoding), and association of that representation with the aversive foot shock (associative learning). In order to determine the selective effect of increased ACh release in the DH on contextual encoding, we used the context pre-exposure procedure to separate these two learning epochs across two days. On the first day, ChAT-Ai32 mice and littermate controls (opto and control) received light stimulation during pre-exposure to a novel context. The

following day, mice were returned to the same context for ten seconds before receiving a foot shock, which we have found is sufficient time to recall a previously-formed contextual representation, but not sufficient for novel encoding (pilot data not shown). Thirty seconds after the shock, they were removed. Mice were tested for fear in an 8-min context exposure the following day (Figure 3.2).

We found that stimulating cholinergic release did not change overall exploratory activity ($F(1,11) = 0.2$, $p>0.5$) nor did exploration habituate throughout the ten minute exposure at the population level in either group (Greenhouse-Geisser corrected Repeated Measures ANOVA, $F(4.6,64.5) = 0.614$, $p>0.5$). There was also no interaction between session time and genotype ($p>0.5$). This demonstrates that cholinergic activation does not cause general activity enhancement, which may be indicative of increased exploration, but is a selective effect on encoding of the sampled stimuli. No difference was observed in freezing before or after shock on the second day ($p>0.05$), nor were there changes in responsivity to shock (activity burst, $p>0.05$), which rules out a change in sensitivity to shock as a result of cholinergic enhancement, otherwise an alternative explanation for group differences at test.

Increased cholinergic activity during contextual encoding led to a profound enhancement in fear after that context was paired with shock (Repeated Measures GLM, Main Effect of Genotype, $F(1,10) = 8.805$, $p<0.05$). This effect was greatest during the first three minutes of the context test, before both groups underwent within-session extinction of the fear response (Greenhouse-Geisser corrected, Main Effect of Time, $F(2.66,26.5) = 16.82$, $p<0.0001$, Time X Genotype Interaction, $F(2.66,26.5) = p<0.01$, Sidak-Bonferroni corrected ttests on minutes 1-3 ($p<0.005$)). This difference suggests that increased cholinergic tone during contextual encoding

increased the strength of that encoded memory, which manifests as increased fear after the context was paired with shock.

One alternative explanation for enhanced fear at test was that mice with higher levels of ACh in hippocampus have higher exploratory drive, leading to increased sampling of stimuli in the context across the ten minute session. These pre-exposure periods were hand-scored for crossings and rearings, which are specific behavioral actions required for complete contextual sampling (Figure 3.3). Neither crossings nor rearings differed between groups ($p>0.05$), suggesting that optogenetic release of ACh does not enhance exploratory behavior.

Another alternative explanation for enhanced fear at test is that enhanced ACh release might simply act as an aversive US, summing with the shock to lead to enhanced fear at test. We tested this idea by exposing mice to a novel context for ten minutes while undergoing light stimulation, and returning them to the context the following day in the absence of shock or light for a test of fear (Figure 3.3). No freezing was elicited, nor were there differences in overall activity between groups on either day ($p>0.05$), suggesting that the stimulation itself was not acting as an innately aversive US. Therefore, enhanced fear at test was due to pairing a shock US with a stronger or more salient contextual representation after enhanced encoding.

Optogenetic stimulation of medial septum cholinergic neurons leads to temporally-locked rise in choline levels in hippocampus

The functional consequence of MS cholinergic cell light stimulation was tested using choline biosensors (See Methods). A biosensor, pre-calibrated for response to choline and to ensure a lack of response to electroactive interferents, was acutely implanted unilaterally in the CA1 pyramidal region of the DH of ChAT-Ai32 mice or ChAT-Cre rats under isoflurane anesthesia. ChAT-Cre rats had previously received a survival surgery to infuse a Cre-dependent

AAV, in order to ensure selective ChR2 expression in cholinergic neurons. A fiberoptic ferrule connected to a blue laser was lowered near the coordinates of the biosensor in CA1. In one rat, spontaneous release events were observed (Figure 3.11), but in all other cases a steady baseline was achieved and no release that was not light-evoked was observed. Multiple depths for each implant were tested until a maximal light-evoked choline response was observed, at which point a range of powers, pulse widths, and stimulation frequencies of light were used and the biosensor response was recorded.

Evoked choline scaled with laser power, as was demonstrated using simple 1.5 s pulses of a range of light intensities with the fiber located in CA1 (Figure 3.5). The choline response began within a quarter of a second and decays back to baseline levels on the order of seconds. A similar profile was visible for ChAT-Cre rats as well (Figure 3.7). Evoked choline also scaled with pulse width in both mice and rats (Figure 3.5, 3.8), suggesting that the underlying relationship is actually a direct relationship between energy and evoked choline. Indeed, this relationship was evident when this response was directly compared (Figure 3.11). Further evidence that the signal measured was evoked ACh can be demonstrated with very intense stimulation protocols (ex. 1 sec pulses, 0.67 Hz, 45 sec train), which produce noticeable vesicle pool depletion, leading to lower peaks with both within the train and between subsequent pulse trains in both mice and rats (Figure 3.10).

A range of pulse trains were tested for use in the 10 min parameters that were employed in the behavioral study. 5 mW power and 5 ms pulse width were chosen as being representative of mild stimulation protocols used in other recent optogenetic experiments (Mamad et al., 2015) (ex: 10-20 mW, 5-10 ms, 8-10 or 40-50 Hz). The 10 Hz frequency was chosen to exceed slightly the highest recorded firing rate of putative MS cholinergic neurons in vivo, which was measured

at 4-6 Hz (Zhang et al., 2011), and which typically fired at much lower rates (Simon et al., 2006). As these cells will likely be active in vivo during the light stimulation period, we chose a light stimulation protocol intended to drive neuron firing slightly higher than the reported baseline firing rate. In anesthetized recordings, this pulse train generated elevated choline that persisted for the 10 min stimulation period and returned quickly to baseline (Figure 3.9).

For our behavioral experiments, the fiberoptic was implanted in the MS, while in our biosensor recording experiments, the fiberoptic was implanted near the biosensor in the DH. It is possible that cholinergic activation release profiles will differ between cell body and terminal stimulation, and indeed data from one mouse with the ferrule in the MS suggests that this is the case (Figure 3.12). Over a range of stimulation protocols tested, cell body stimulation consistently led to lower levels of ACh release as measured by the biosensor in dCA1, as well as to elevated levels of choline persisting for tens of seconds to minutes after the conclusion of the light stimulation. If this persistent elevation is indicative of prolonged ACh action in DH after the end of the behavioral exploration period, this profile suggests that a portion of the behavioral effect may be due to enhancements in contextual memory consolidation. Enhancements of this type have been demonstrated with cholinergic agonists administered after contextual exploration (Malin and McGaugh, 2006).

Discussion

These data provide evidence that cholinergic signaling controls processing of contextual information in the hippocampus. Specifically, increasing cholinergic inputs, above the release normally produced by exposure to a novel environment, leads to enhanced encoding of contextual information, and facilitates future fear responding to that context. Based on a long

history of pharmacological manipulations of the DH during learning (Tinsley et al., 2004), these effects on encoding are likely mediated by both muscarinic and nicotinic receptors. Low doses of scopolamine prior to the pre-exposure day impaired contextual processing and reduced fear at test in both juvenile rats (Robinson-Drummer et al., 2016) and adult mice (Brown et al., 2011). Systemic nicotine prior to pre-exposure enhances contextual processing and fear at test (Kenney and Gould, 2008).

Optogenetic manipulation of cholinergic neurons provides a novel method of probing the circuit, as particular receptors or subtypes are not activated in isolation during natural behavior. This study demonstrates that enhancing the activity of a homogeneous neural population enhances contextual processing in the hippocampus. Multiple receptor subtypes likely participate in this effect, as they participate in signaling under enhanced cholinergic tone during exposure to a novel environment in the absence of optogenetic manipulation.

Other studies that have used optogenetic manipulation in conjunction with electrophysiological recordings of MS and DH highlight potential circuit mechanisms. One study demonstrated that while mice actively explored an environment, activation of cholinergic inputs was insufficient to change firing of hippocampal principle cells, and only mildly enhanced theta band local field potential (LFP) (Mamad et al., 2015). However, the effect reported may be lower magnitude than our results due to hitting only ~ 45% of ChAT+ cells in MS using a viral targeting method, unlike the genetic targeting method employed by the present study. It should be noted that the most potent effect was observed after using relatively low-frequency stimulation (10 Hz), the same frequency employed in the present study. Another study reported greater magnitude effects, demonstrating that optogenetic activation of cholinergic neurons enhanced theta band oscillations and suppressed competing frequency bands such as sharp-wave

ripples (Vandecasteele et al., 2014), which may explain the profound enhancement on contextual encoding observed here. The relationship between LFP and firing frequency may even be altered by cholinergic signaling; though ACh enhances theta rhythm power in the DH, it actually leads to decoupling of firing from LFP in cortex (Kalmbach and Waters, 2014). It remains to be seen if similar mechanisms exist in the DH.

The mechanism of action of ACh in the DH is complex. In addition to medial septal cholinergic inputs to the DH, local cholinergic interneurons have been reported (Yi et al., 2015) that may contribute ACh to hippocampal circuits; however, due to placement of ferrule in MS for behavioral manipulation, activation of this population did not contribute to the effects observed here. ACh receptors are expressed both pre- and post-synaptically on a range of hippocampal cell types. In addition to principle cells, hilar cells and other interneurons, cholinergic effects may be mediated by astrocytes (Pabst et al., 2016) as well as by differential effects on mature and immature granule cells (Zhang et al., 2010). In addition, feedback from GABAergic neurons in hippocampus back to cholinergic cells in MS lead to complex effects in medial septal cells, including hyperpolarization of putative cholinergic neurons (Mattis et al., 2014). These feedback connections complicate interpretation of cholinergic activation. Furthermore, cholinergic neurons in MS have dense local connections with both GABAergic and glutamatergic septal projection neurons. Intraseptal circuit dynamics could play a role in the observed effects, as selective stimulation of these populations can entrain theta rhythm as well as enhance the magnitude of observed theta rhythm (Mamad et al., 2015; Robinson et al., 2016). To further complicate the picture, co-release of ACh and GABA has been reported from ChAT positive neurons in the basal forebrain (Granger et al., 2015). In order to tease apart these effects, future

studies might compare effects of terminal stimulation in the DH with cell body stimulation in MS, in combination with pharmacological blockade of selective receptor subtypes.

Rather than measuring the firing rate elicited by optogenetic stimulation, our study measured the cellular output of cholinergic septal neurons using choline biosensors. This study demonstrates that a pulse train that evokes electrochemically-measurable choline also evokes behavioral changes, suggesting that these measured elevations are at a behaviorally relevant scale. Anesthetized recording of optogenetically-evoked ACh in the hippocampus also approximates the magnitude of naturally evoked transients recorded in awake rats in the prefrontal cortex (Parikh et al., 2007; Sarter et al., 2014).

There may be some important differences between the biosensor results (n=1) with fiberoptic placement in medial septum, and behavioral results with fiberoptic placement in medial septum. The lower release observed, compared with illumination of the region near the biosensor, is likely due to placement. Compared with shining light directly onto terminals proximal to the biosensor, we are less likely to fully activate all cholinergic cells in the MS that project directly to the location of the biosensor. The second characteristic of persistent elevation of release has not been noted elsewhere. Terminal stimulation with ChR2 has sometimes been demonstrated to cause back-propagating action potentials (Sparta et al., 2013), though in this case it seems that the functional effect of the two stimulation sites is distinct. Perhaps with cell body activation, there are feedback mechanisms from local circuitry in the MS, whose activation may lead to sustained maintenance of elevated levels of ACh in the DH. Future studies will need to disentangle differential contributions of terminal and cell body stimulation to ACh signaling in the DH.

The relationship between cholinergic release in the hippocampus and contextual processing suggest multiple new avenues of inquiry. For example, males show stronger conditioning than females after a context pre-exposure facilitation procedure (Barker and Galea, 2010); this may be related to sex-dependent differences in overall cholinergic tone, as adult male rats have higher ACh levels than adult female rats as measured by microdialysis (Takase et al., 2014). This overall cholinergic difference may be found to underlie other sex differences in learning procedures, but has never been directly examined.

In addition, the cognitive mechanism for the cholinergic effect observed here remains to be determined. Control experiments demonstrated that the enhancement of fear at test was not due to increasing exploratory behavior or to an innate aversion to increased ACh in the hippocampus. Effects might be mediated by reducing the working memory load to facilitate detail-finding and conjunctive binding (Numan and Quaranta, 1990). Potential effects on memory consolidation must also be considered (Atherton et al., 2015; Chang and Liang, 2012; Hasselmo, 1999). The current results are consistent with the idea that cholinergic activation in the DH leads to stronger encoding of more contextual details. This mechanism suggests a prediction that while cholinergic activation will lead to enhanced fear in the original context, there will actually be reduced generalization to a similar context. There is some evidence from cholinergic lesion studies that this might occur (Knox and Keller, 2015), with potential therapeutic implications for contextual generalization in anxiety disorders.

Enhanced ACh release in the DH improved contextual encoding despite the reported high levels of ACh already present in the DH during exposure to a novel environment. This begs the question of what processes set the cholinergic level during environmental exploration, and whether there is some "optimal" level for maximal encoding. Self-directed exploration may be

modulated by receptor subtypes in the hippocampus (Saab et al., 2009), and there may be feedback mechanisms to set cholinergic tone to promote learning during this exploration. Though our manipulation led to strong memory formation during a challenging procedure, it may also have altered processes fundamental to normal memory degradation, or reduced the cognitive resources available for other tasks. Future studies will need to address the mechanisms and potential tradeoffs of altering cholinergic signaling to modify contextual memory strength.

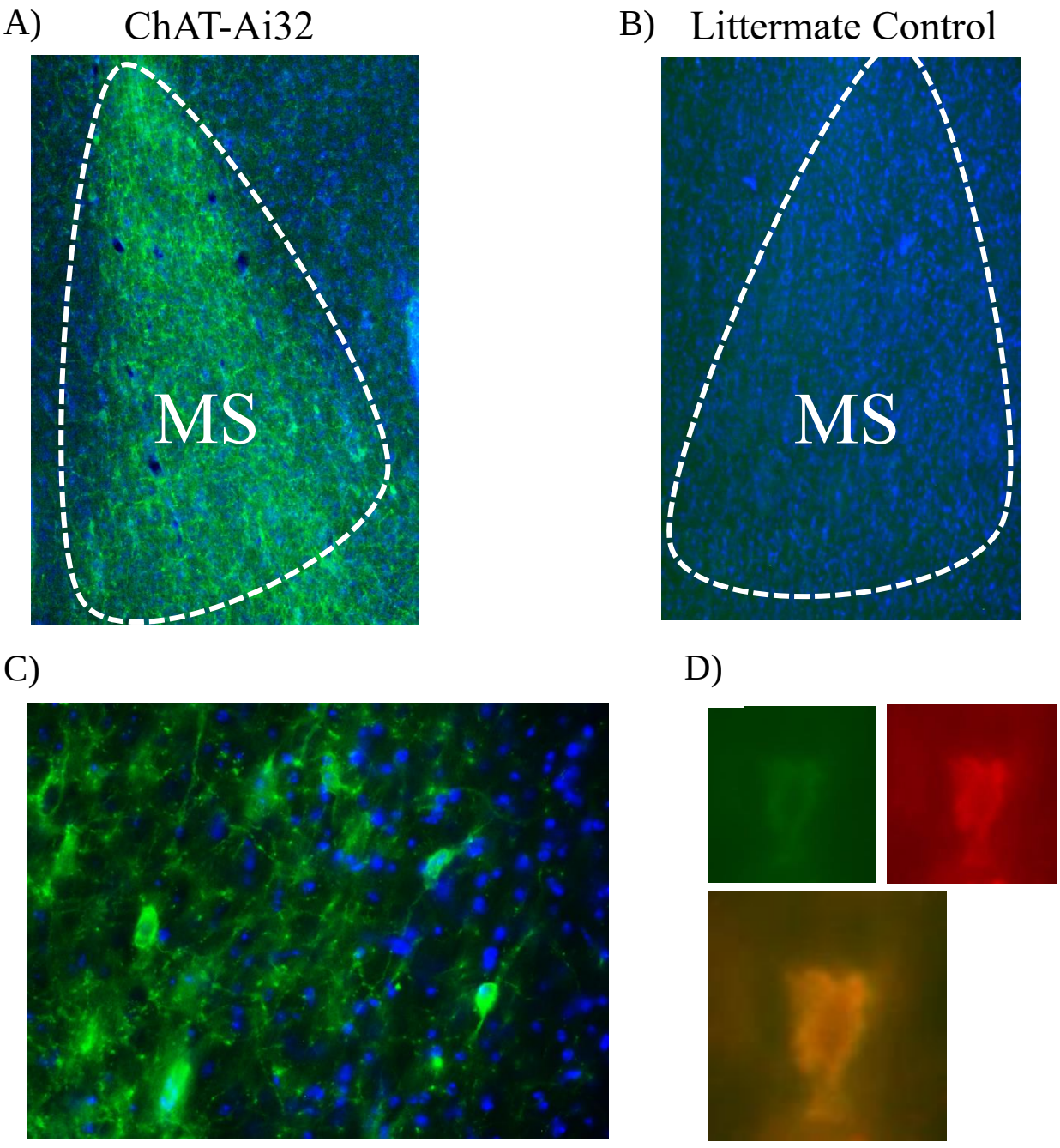
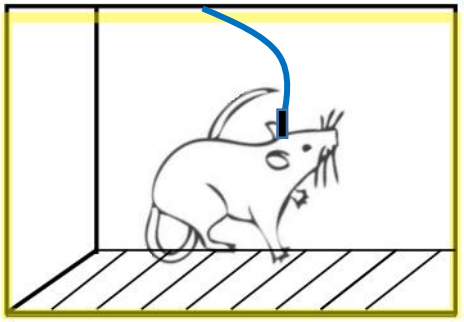


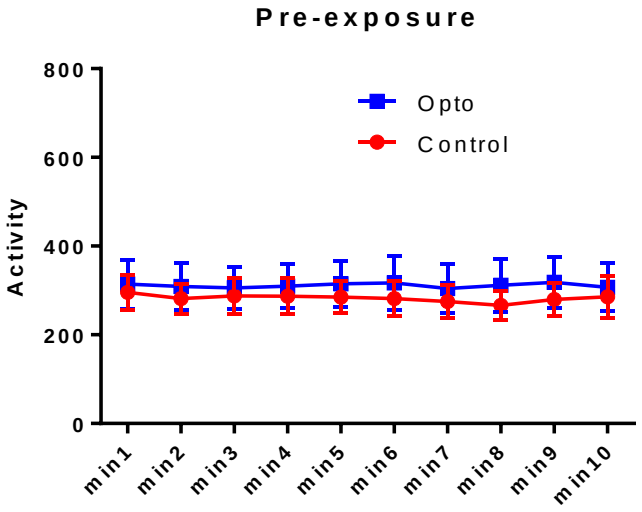
Figure 3.1: **Visualization of transgene expression.** Slices were taken from ChAT-Ai32 mice (A) and littermate controls (B) negative for Cre and imaged using light microscopy (A,B: 4x; C: 40x.). YFP demonstrates cell body and process expression (C). A cholinergic neuron is shown with native YFP fluorescence and ChAT counterstain (R). Cholinergic neurons and their processes are visible from native YFP fluorescence in ChAT-Ai32 mice, while no fluorescence was visible in controls. Nuclei are stained with DAPI.

A)

Day 1

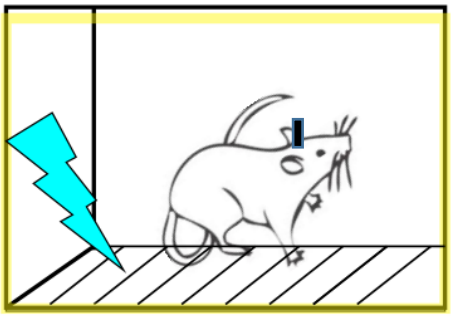


10 min PE with light

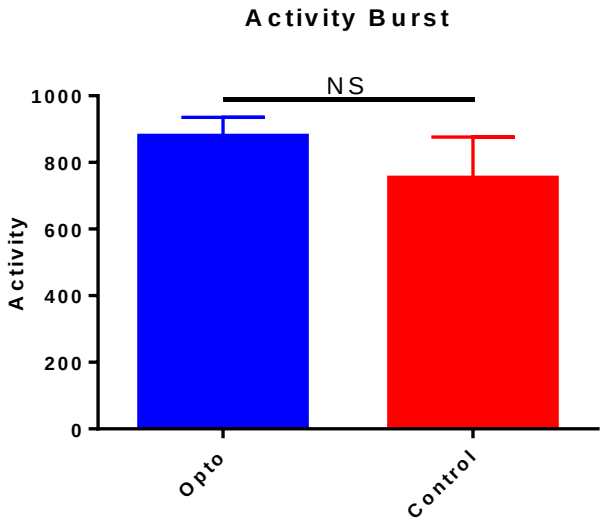


B)

Day 2

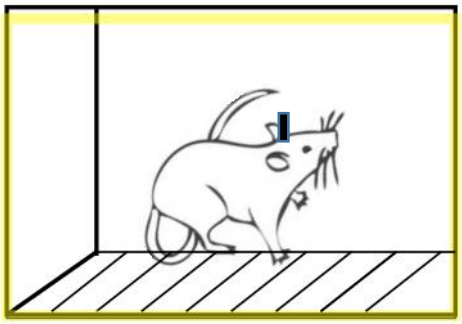


Immediate shock



C)

Day 3



8 min context test

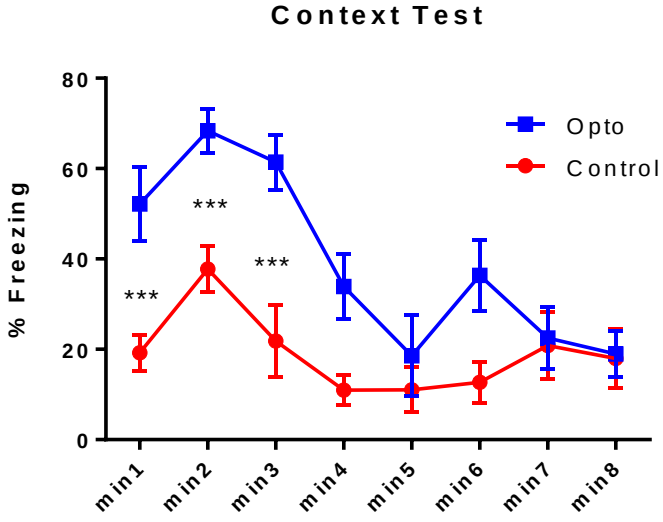
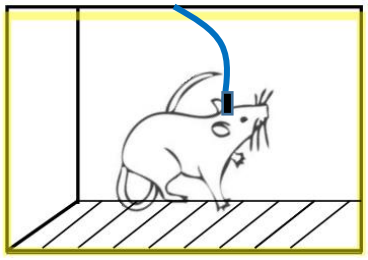


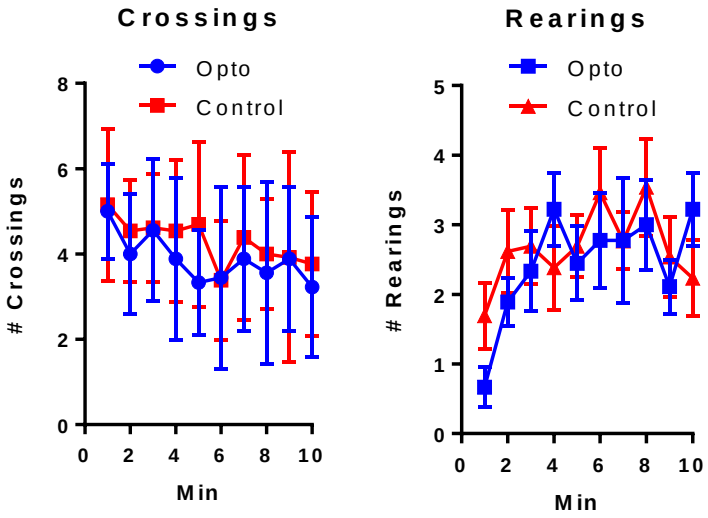
Figure 3.2: **Optogenetic enhancement of Ach and contextual encoding.** The behavioral protocol is shown above the results from each session. A) Mice with a single fiberoptic ferrule implanted above medial septum explored a novel context under laser light stimulation (n=6 ChAT-Ai32 and n=10 Littermate Controls). No activity difference was measured between ChAT-Ai32 (Opto) and littermate controls (Control) ($p>0.05$). B) The following day, all mice received a foot shock 10 sec after placement in the same context, and were removed 30 sec later (n=6 per group). No difference in freezing before or after shock (data not shown) nor in activity burst to the shock ($p>0.05$) was observed. C) A profound effect on freezing during the 8 min context test (n=6 per group) was observed during the first three minutes of exposure ($p<0.005$).

A)

Day 1

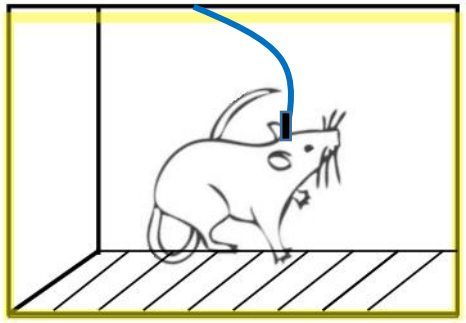


10 min PE with light

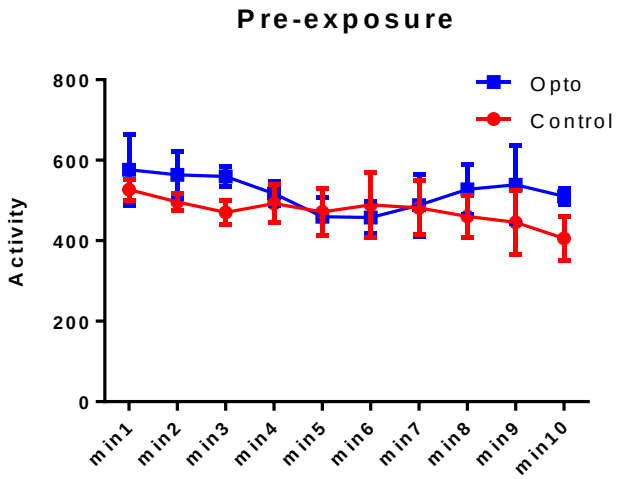


B)

Day 1

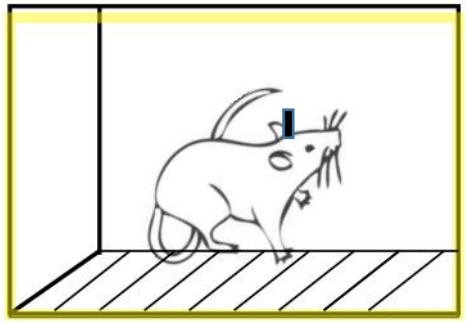


10 min PE with light



C)

Day 2



5 min context test

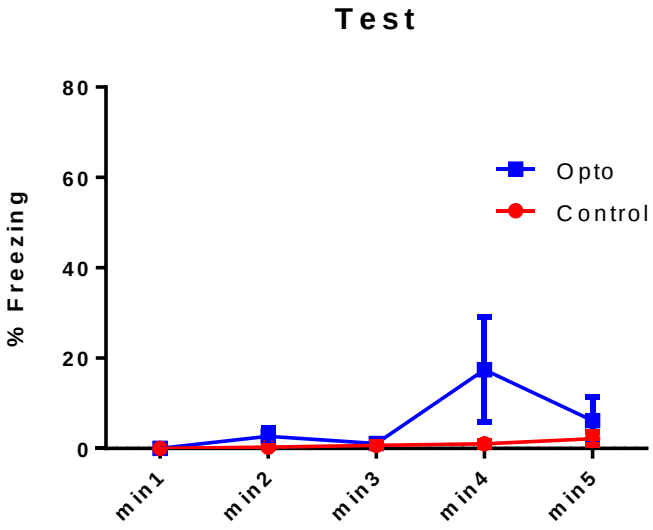


Figure 3.3: **Enhancement of freezing not due to enhanced exploratory behavior, nor an aversive nature of the optogenetic stimulation**. A) No effect of optogenetic enhancement of Ach release was observed on rearings or crossings during stimulation (n=9 opto, n=13 control). Pre-exposure sessions were hand-scored to quantify number of crossings and rearings. No difference was observed in either metric ($p>0.05$), suggesting that optogenetic release of Ach does not enhance exploratory behavior. A separate cohort was given optogenetic stimulation (n=3 opto, n=6 control) during pre-exposure and tested for fear behavior the following day. No difference was observed in activity during pre-exposure with light stimulation (B, $p<0.05$), and no difference was observed in freezing behavior during the second context exposure (C, $p<0.05$), suggesting that optogenetic release of Ach does not act as an aversive US and lead to fear in a paired context.

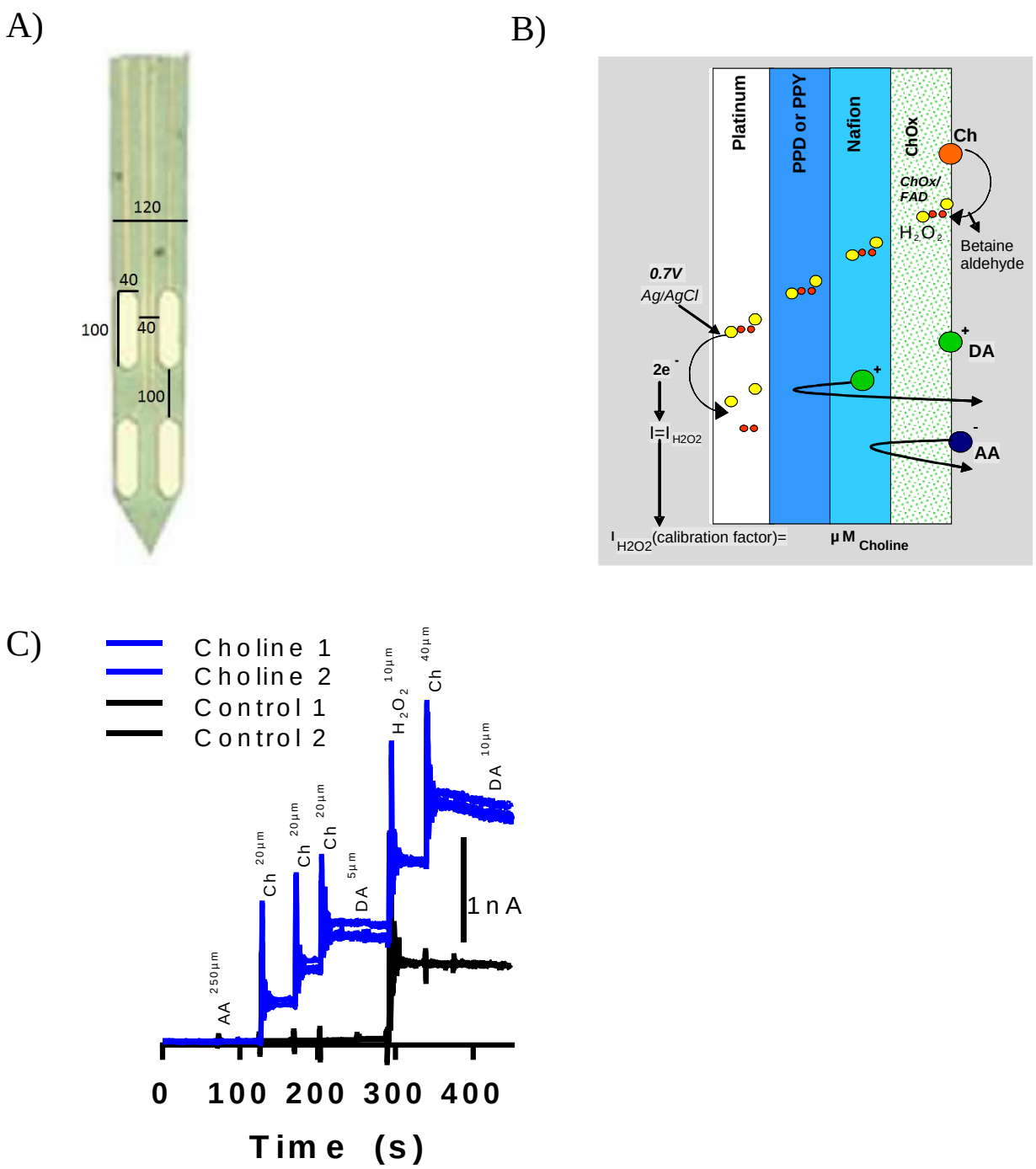
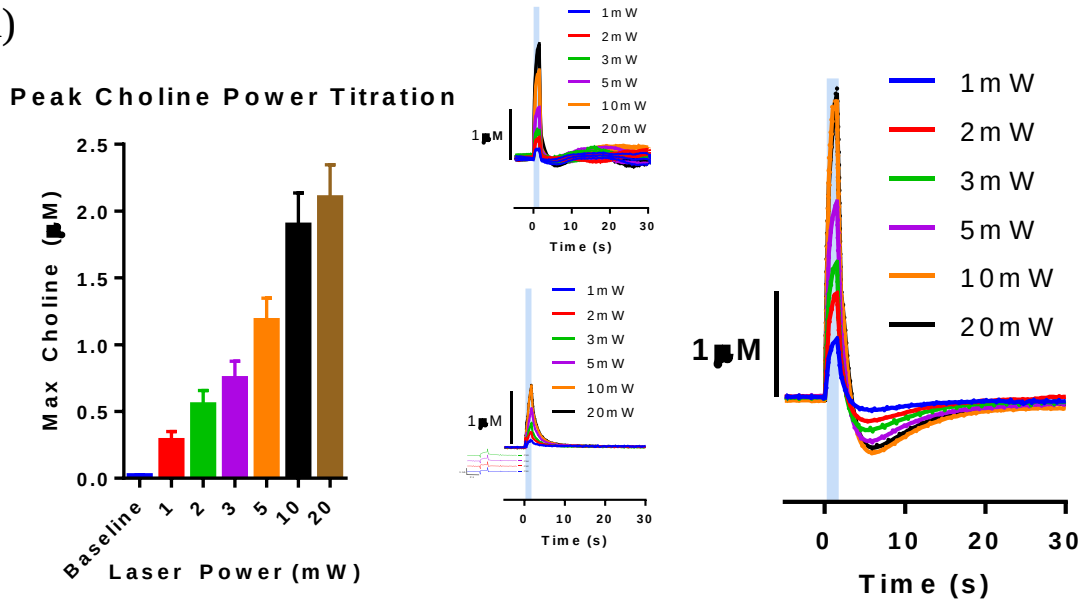


Figure 3.4. **Biosensor diagram and calibration.** A) Diagram of the silicon-platinum electrodes used in the study before succession of coating layers are applied, and shows a sensor tip with dimensions reported in micrometers. B) Diagram of a PPD or PPY//Nafion choline biosensor. Choline reacts with choline oxidase at the surface to form hydrogen peroxide, which can pass through subsequent layers to contact the platinum electrode and cause a change in current measured. Dopamine (DA) and Ascorbic Acid (AA) are repelled by the PPD and Nafion layers, respectively. C) Representative raw calibration current trace from a choline biosensor; control channels are in black, and only respond to the addition of peroxide. Choline channels respond to choline and peroxide but not to AA or DA. The response to choline administration is used to compute the calibration factor used to measure micromolar choline concentrations in vivo.

A)



B)

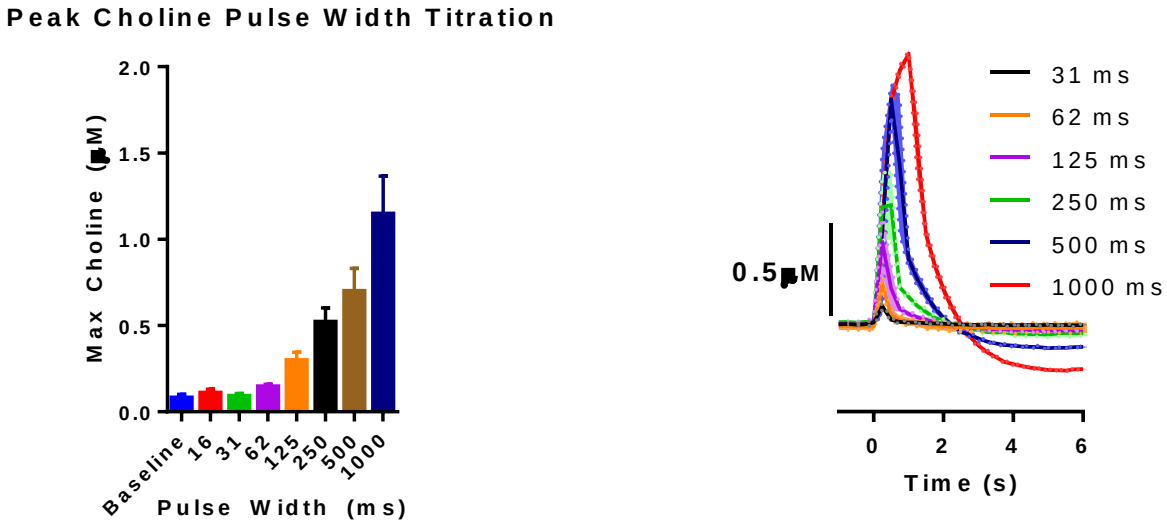
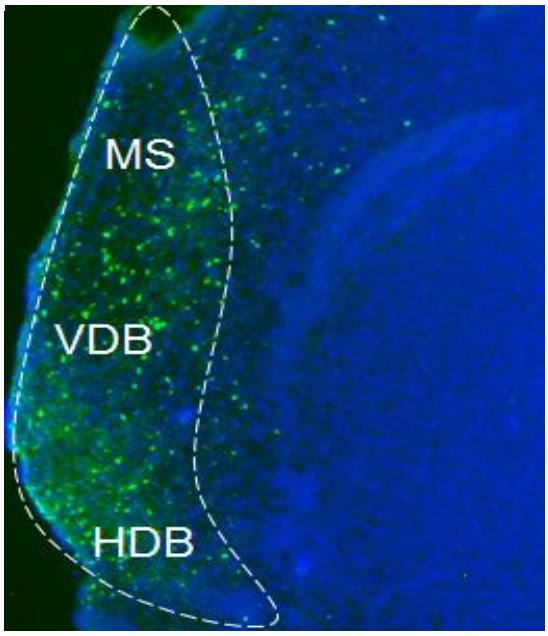


Figure 3.5: **Choline biosensor recording of optically-evoked choline from ChAT-Ai32 mice.** A) Summary of the maximal choline evoked at multiple powers of the laser in three mice after a 1.5 s continuous pulse (SEM shown). Individual traces of evoked choline for three female ChAT-Ai32 mice are shown at right; raw current data for 1 mW stimulation is shown for one figure (1 mW power). Though the absolute magnitude varies between the individuals, evoked choline scales with power in all subjects, saturating at about 10 mW. B) Summary of the maximal choline evoked at multiple pulse widths in three mice while keeping the power at 5 mW. Evoked choline also scales with pulse width in all subjects, and 62 ms pulses and above are significantly elevated above baseline ($p<0.05$, 3 repetitions for each pulse width). One individual trace is shown at right.

A)



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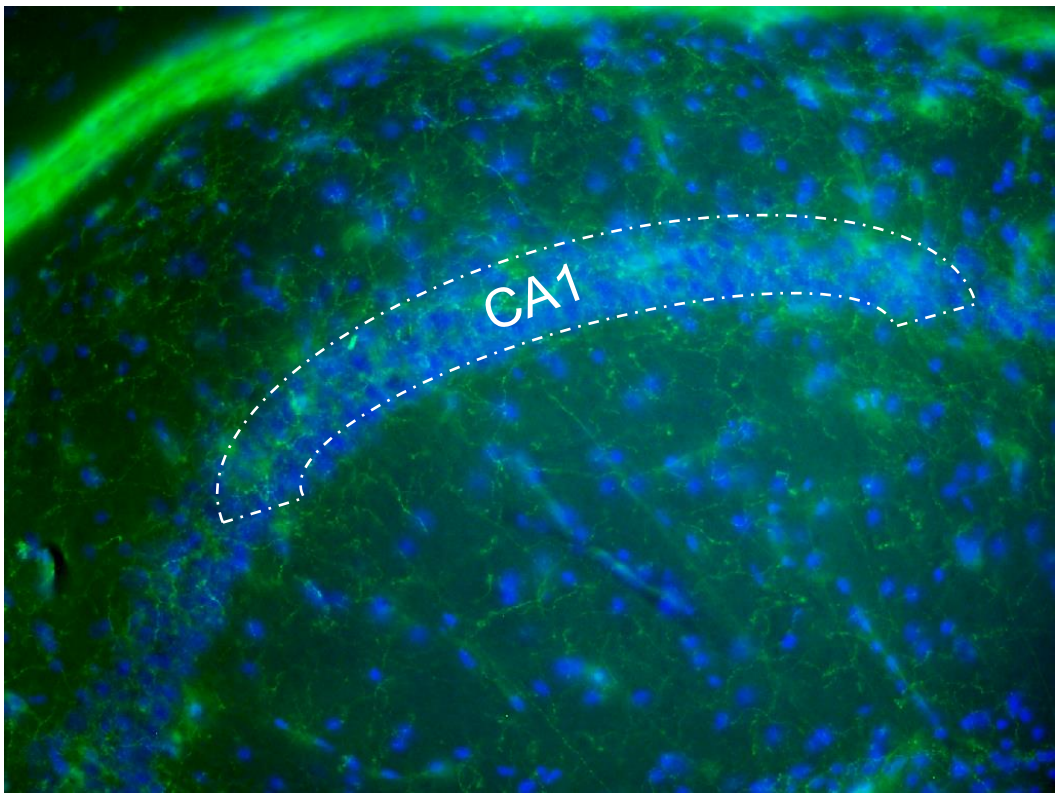
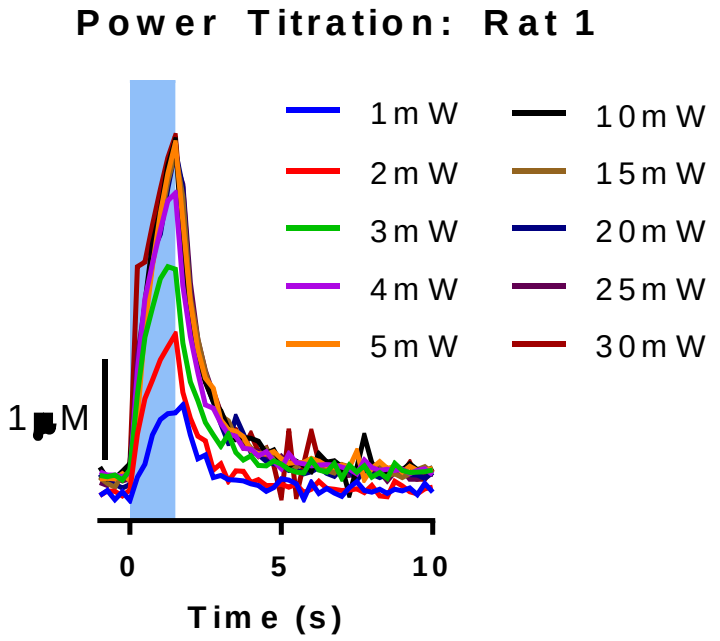
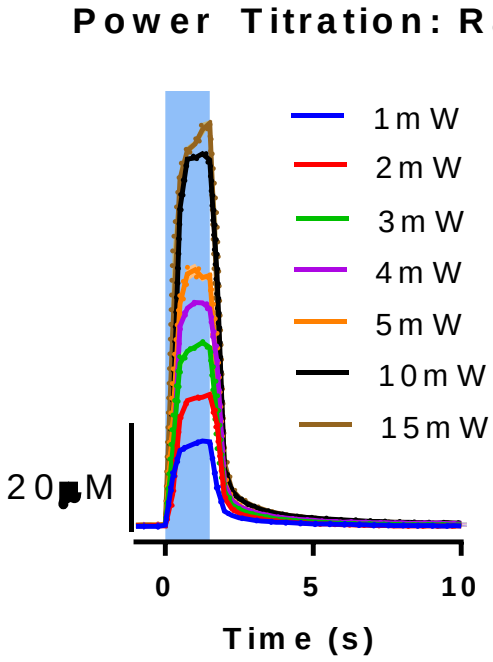


Figure 3.6: **Visualization of transgene expression in ChAT-Cre rats.** Slices were taken from ChAT-Cre rats and imaged using light microscopy (4x magnification). YFP demonstrates cell body and process expression (A) and cholinergic terminals are evident throughout dCA1 pyramidal layer (B). Nuclei are stained with DAPI.





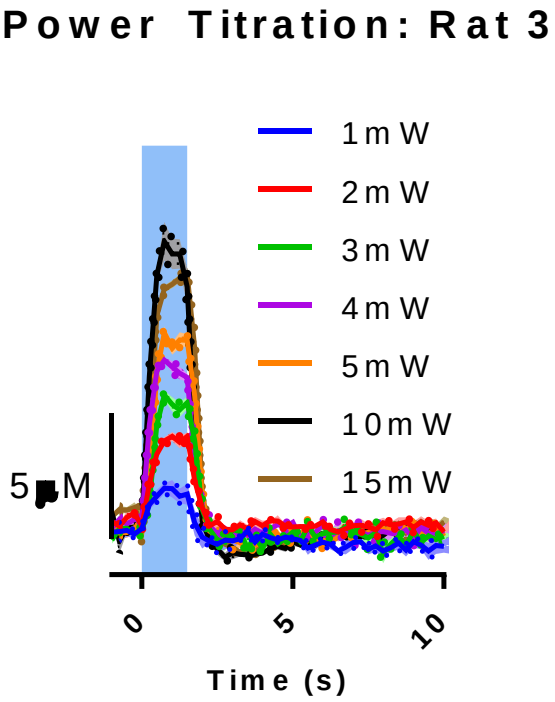
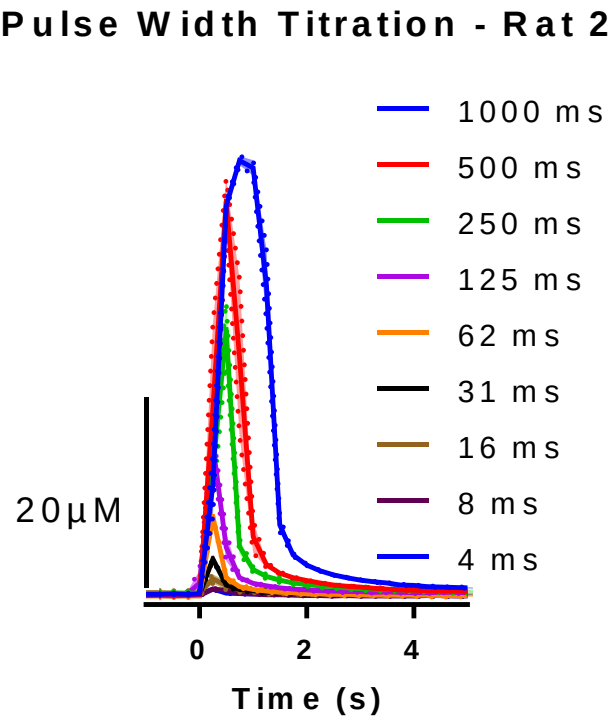


Figure 3.7**: Choline biosensor recording of optically-evoked choline from ChAT-Cre rats for 1.5 s pulses**. Three repetitions of each power are shown as mean and SEM of evoked acetylcholine for three female ChAT-Cre rats (though rat 1 had only a single trial of each). Though the absolute magnitude varies between individuals, evoked acetylcholine scales with power in all subjects, saturating at about 10 mW. Evoked acetylcholine is similar to values obtained in ChAT-Ai32 mice.



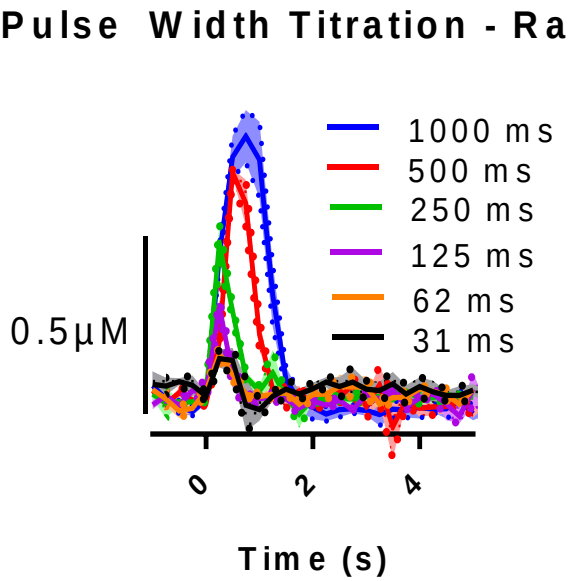


Figure 3.8: **Pulse Duration Titration for ChAT-Cre rats**. Mean and SEM for choline for two female ChAT-Cre rats are shown above (5 mW all pulses, 3 repetitions of each). Though the absolute magnitude varies between individuals, evoked acetylcholine scales with power in all subjects, with no saturation evident at 1000 ms. Evoked acetylcholine is similar to values obtained in ChAT-Ai32 mice.

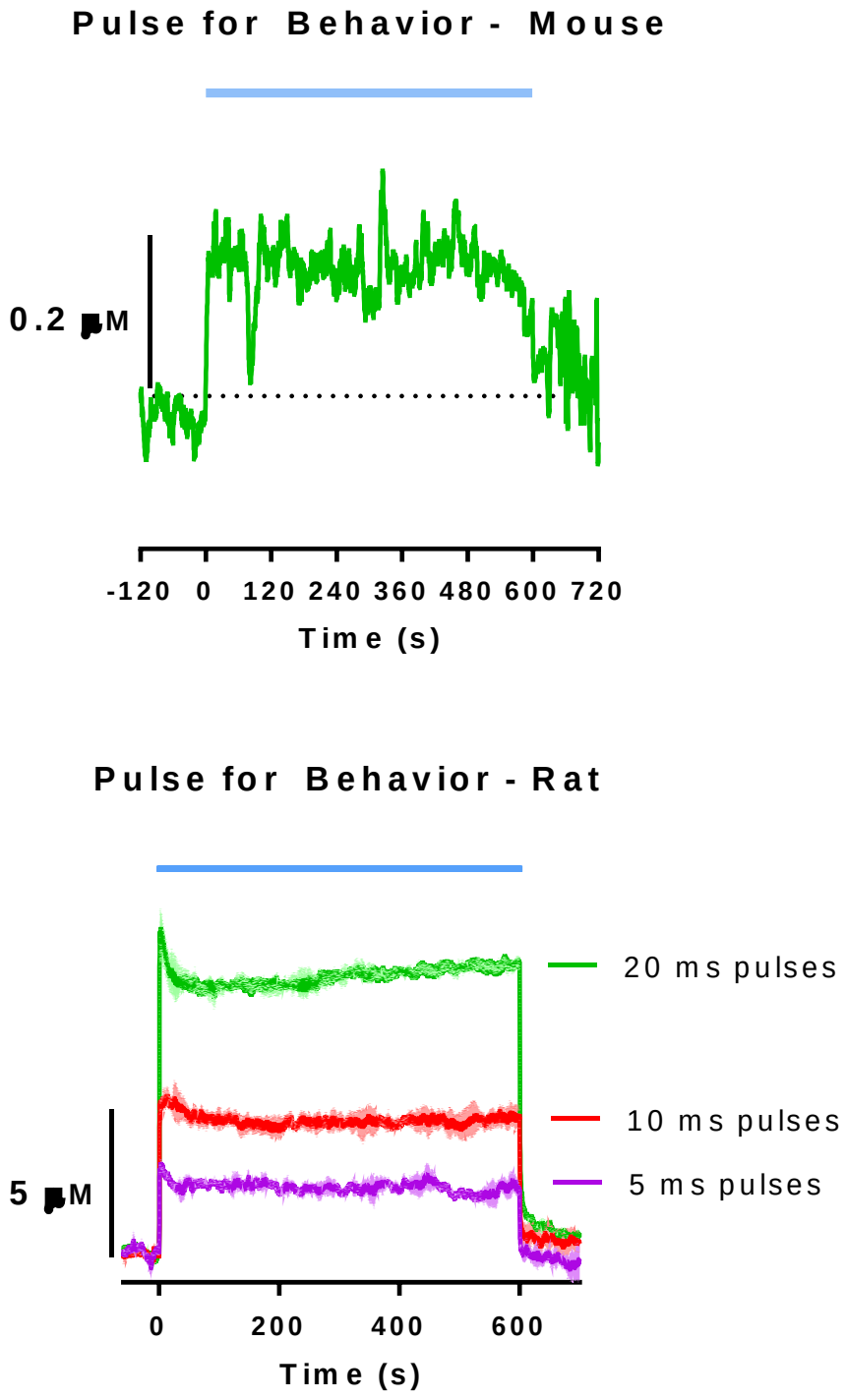


Figure 3.9: **Behavioral Parameters in anesthetized mice and rats**. A representative trace of the mouse response to the behavioral protocol (10 Hz, 5 mW, 5 ms pulse, 10 min) is shown above, with a modest increase in choline observed. In contrast, data from a single rat demonstrates significant increases in choline observed for 5 ms, 10 ms, and 20 ms pulse widths, keeping the rest of the protocol constant (2 repetitions of each stimulation train). Though the absolute magnitude varies between individuals, evoked acetylcholine rapidly reaches a peak during long stimulation protocols and maintains that level until the conclusion of the stimulation, whereupon it rapidly returns to baseline.

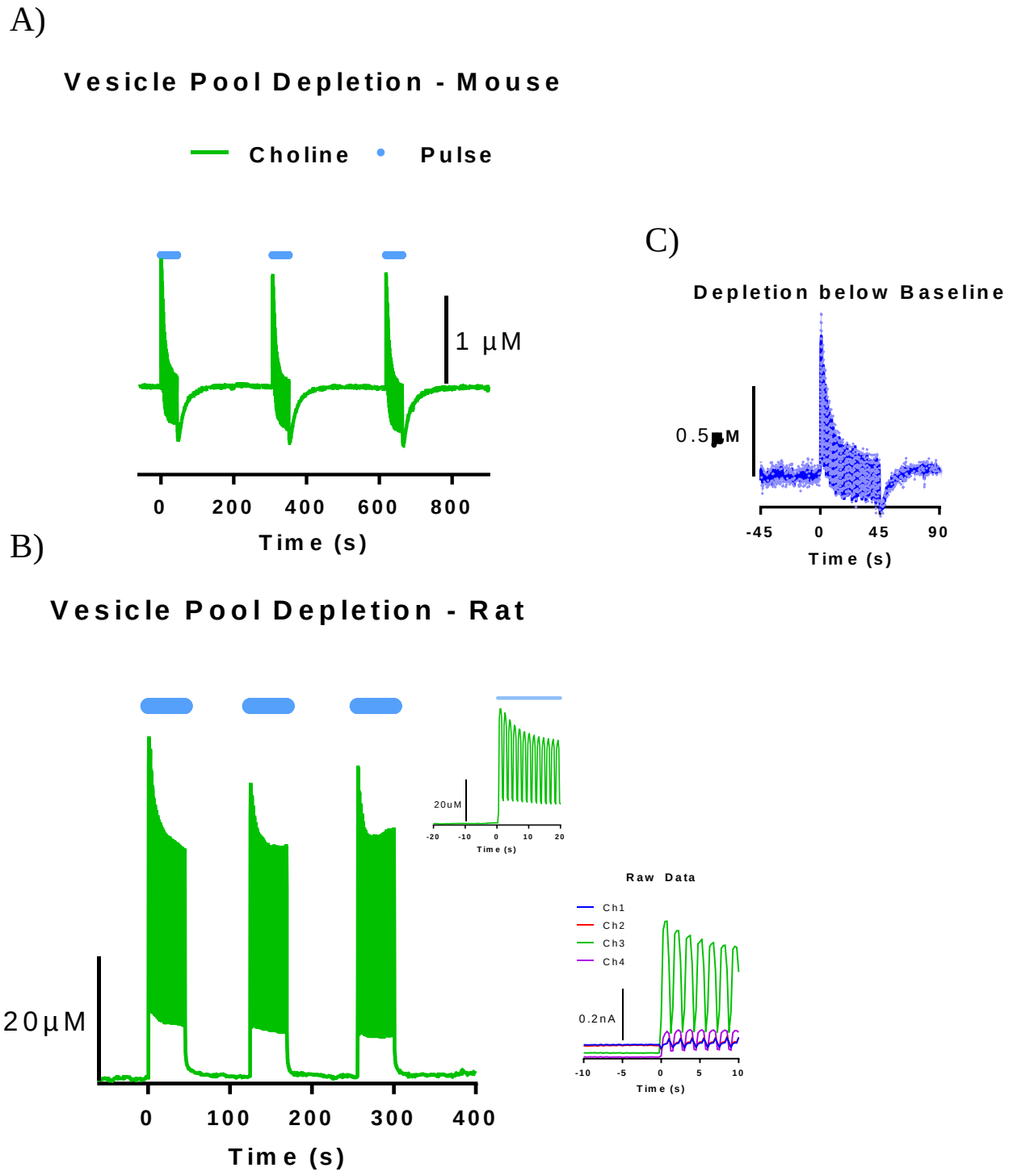
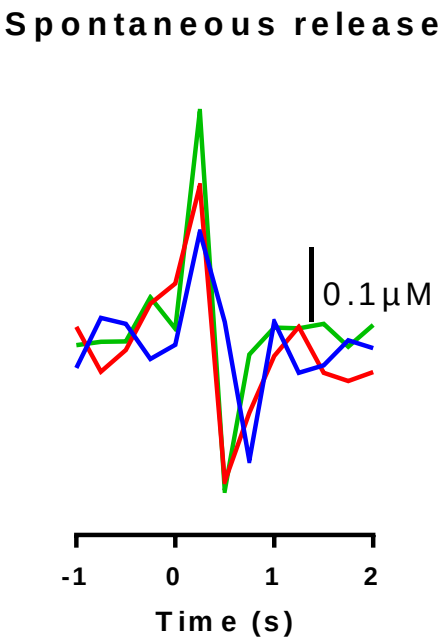


Figure 3.10: **Vesicle Pool Depletion Evidence in Mice and Rats**. (A,B) Similar evidence of vesicle pool depletion can be found in mice and rats with sufficiently high stimulation protocols. Inset on lower graph shows result of individual stimulation pulses, while lower right graph shows raw data (current) for the first trace. The stimulation protocol shown is one or more repetitions of 1 s on 0.5 s off for 45 s at 5 mW. In all examples, a reduction in release is evident both within stimulation trains (highest peak at beginning of stimulation) and between stimulation trains separated by at least a minute (highest peak at beginning of first train). This suggests that by driving release sufficiently hard, less ACh is available to release, in one case even dropping below baseline levels before recovering (C, Rat 3).

A)



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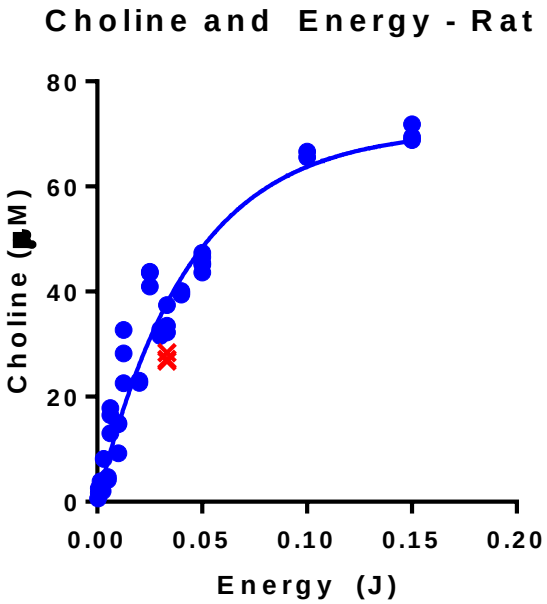

Figure 3.11: **Spontaneous Release and Energy**. (A) In one rat, at a relatively low level of isoflurane anesthesia (0.75-1%), spontaneous release events were observed. Three examples are superimposed above, though some were much larger (on the order of multiple µM). (B) For local stimulation of terminals, observed choline seems to scale with energy (J). For second and third maxima in the intense stimulation protocols in Figure 3.10, where vesicle depletion is hypothesized, observed choline begins to deviate from the expected energy relationship (red X's).

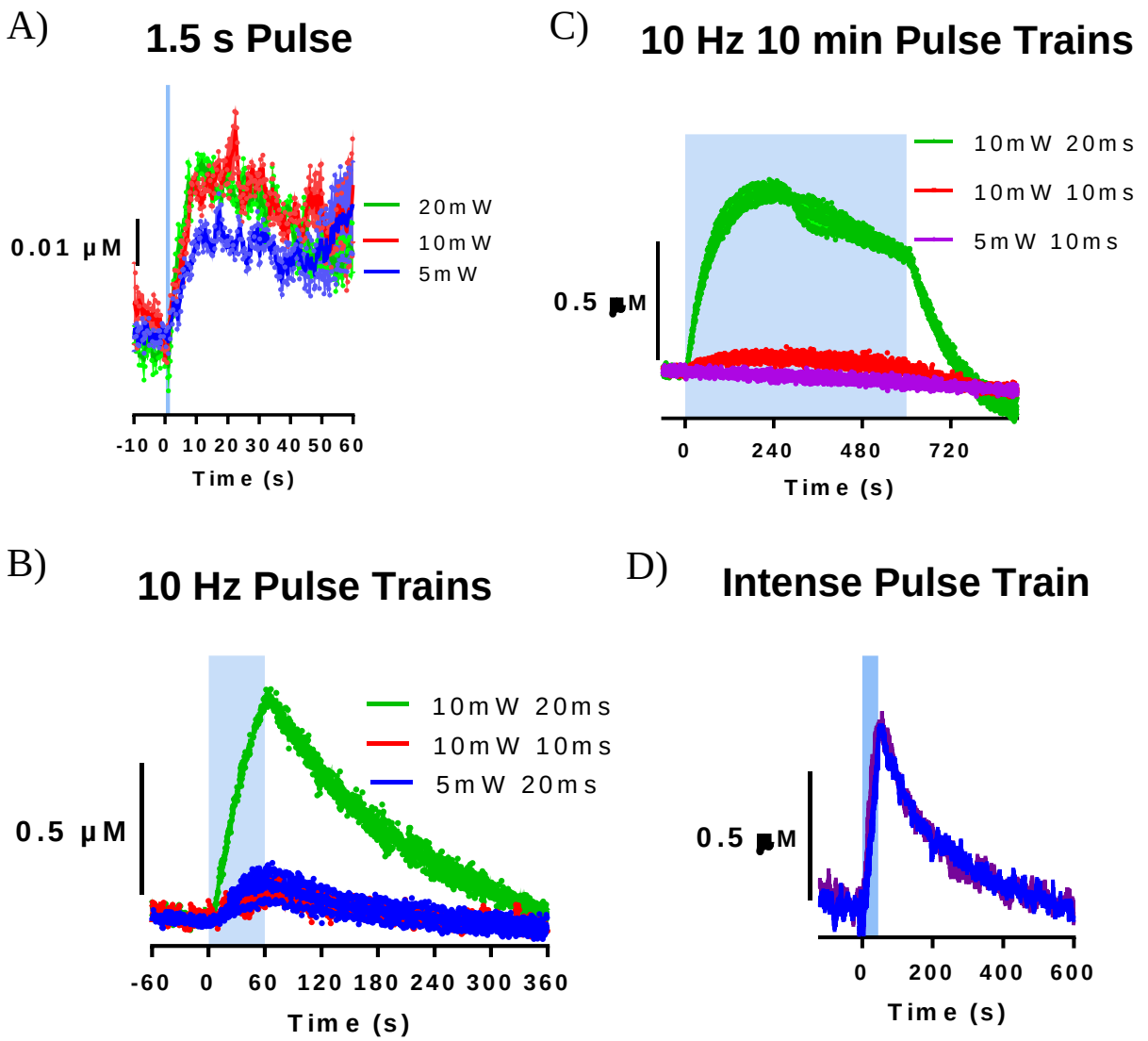

Figure 3.12: **Medial septal stimulation evokes ACh release in DH with distinct properties**. For one mouse, light stimulation in medial septum led to cholinergic release in the DH. (A) 1.5 s stimulation pulses led to mild, but sustained, release. (B,C) 10Hz pulse trains also led to sustained release that persisted for minutes after the conclusion of the light stimulation. (D) Intense pulse trains, which at terminals led to vesicle pool depletion, did not do so with cell body stimulation (repeated stimulation superimposed), but instead led to sustained elevation for many minutes. A characteristic that all cell body stimulation events had in common was sustained elevation of detected choline from seconds to minutes, suggesting distinct mechanisms from stimulating release from terminals in hippocampus.

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Chapter Four: Dissociation of cholinergic modulation in dorsal hippocampus and basolateral amygdala on stress-enhanced fear learning

Abstract

An exaggerated fear response can manifest as an anxiety disorder like post-traumatic stress disorder (PTSD). A fear memory consists of the contextual memory in which the event took place, stored in the dorsal hippocampus (DH), as well as the emotional component, stored in the basolateral amygdala (BLA). In both regions, acetylcholine (ACh) provides an important signal for this fear learning. Open questions are how these neural substrates change during maladaptive fear learning, and whether inhibiting ACh disrupts maladaptive fear. Using Stress-Enhanced Fear Learning (SEFL), which models many aspects of PTSD in rats, we tested whether ACh in the DH and BLA is required for maladaptive fear. To dissociate the effect of ACh in the DH and BLA, we infused scopolamine or artificial cerebrospinal fluid vehicle (aCSF) into these brain regions immediately before SEFL, and tested fear in both the trauma context and a novel context after a mild stressor. The results show that during learning, ACh acting within both the DH and BLA is required for sensitization of future fear learning. In the BLA, scopolamine blocks this sensitization to both contextual and discrete cues, but in the DH, scopolamine only blocks sensitization to contextual cues, leaving discrete cue sensitization intact. Rather than simply sensitizing the BLA, SEFL requires functional signaling in both the DH and BLA; this larger circuit, and the requirement for ACh, suggests future research and therapeutic targets for PTSD.

Introduction

For fear to be adaptive, it must rapidly and completely control behavior in situations that are predictive of threat (Fanselow and Lester, 1988). However, it must also be both titrated to the level of threat and relatively specific for threat-related stimuli. Both aspects of this fear responding are dysregulated in anxiety disorders such as post-traumatic stress disorder (PTSD), in which fear responses are enhanced and disrupt an individual's normal functioning (Bonne et al., 2004; Rosen and Schulkin, 1998).

Though the neural mechanisms for human PTSD acquisition are currently unknown and likely to be heterogeneous (Bennett et al., 2015; Roozendaal et al., 2009), circuit models of PTSD implicate the amygdala as an important structure for storage of traumatic memories and the influence of stress on emotional memory acquisition (Rosen and Schulkin, 1998; Waddell et al., 2008). In these models, acute or chronic stress leads to a 'hyperactive' amygdala, manifested by increased excitability of glutamatergic principle cells or reduced inhibitory drive from GABAergic inhibitory interneurons (Roozendaal et al., 2009). Previous work from our lab indicates that an upregulation of GluA1 in principal cells of the basolateral amygdala (BLA) after the traumatic event may be responsible (Perusini et al., 2015).

Though the amygdala is likely to be the core structure in the stress circuit leading to PTSD, the hippocampus has also been shown to play a role. MRI studies of PTSD patients frequently report substantial loss of gray matter in the hippocampus, though there is debate as to whether this is caused by the trauma or is a predisposition for diagnosis. In rodent studies, both circumstances have been observed (Bennett et al., 2015). Furthermore, in both human and rodent studies, the hippocampus is critical for the formation of contextual memories (Fanselow,

2010), and for the association of that memory with emotional valence, as occurs during both normal contextual fear learning and after trauma (Bennett et al., 2015; Orsini et al., 2011)

Acetylcholine (ACh) is a neuromodulator that affects learning processes in both the hippocampus and the amygdala; the majority of these projections originate from non-overlapping cell populations in the basal forebrain cholinergic system. Cholinergic inputs are critical for normal valence learning. Blockade of muscarinic receptors by scopolamine in the hippocampus prevents contextual fear learning but not tone fear learning (Gale et al., 2001) and in the amygdala impairs performance on a conditioned place preference but not a spatial radial maze task (McIntyre et al., 1998). Furthermore, stress may induce hyperexcitation of cholinergic circuits (Zimmerman and Soreq, 2006) particularly in the hippocampus (Finkelstein et al., 1988; Mitsushima et al., 2008; Pavlovsky et al., 2012; Stillman et al., 1997). This increased cholinergic tone after stress may be causally related to the effects of stress, as acetylcholinesterase inhibitors in some cases induce psychopathologies very similar to PTSD (Kaufer et al., 1998). These converging lines of evidence point to cholinergic signaling as a critical aspect of plasticity during learning under stress, and perhaps as an important target for disruption of this enhanced plasticity in order to reduce the deleterious effects of acute stress.

Animal models of PTSD such as stress-enhanced fear learning (SEFL) have recapitulated many aspects of human PTSD symptomology. The SEFL model recapitulates resistance to extinction therapy (Long and Fanselow, 2012) and sensitization to future mild stressors (Rau et al., 2005), and has also suggested molecular and cellular targets for future studies on traumatic stress (Ponomarev et al., 2010). The SEFL model exposes rats to an acute stress of 15 inescapable and unpredictable foot shocks in one environment. This exposure not only leads to high levels of fear to that environment, but to heightened levels of fear after a single shock in a

novel environment (Rau et al., 2005), compared with animals that did not receive the 15 shocks. This sensitization to future mild stressors was not disrupted by extinction of the original traumatic context, nor by blockade of N-methyl-D-aspartate receptors (NMDARs) during learning by intracerebroventricular (icv) infusion of APV before the trauma. These manipulations that change the valence of the traumatic contextual representation or disrupt storage of the traumatic contextual representation, respectively, do not disrupt sensitization to novel contexts. This suggests that the effects that SEFL has on exaggerated fear are mediated by circuit changes related to non-associative sensitization, in addition to formation of an associative memory. This is also supported by the lack of requirement for memory of the trauma in juveniles for expression of the phenotype as adults (Poulos et al., 2014).

Scopolamine is known to disrupt contextual processing, and more generally is often used as a model of cognitive impairment, but its effects in both the DH and BLA are intricate (Klinkenberg and Blokland, 2010). It has previously been shown to disrupt fear consolidation in the BLA (Baldi et al., 2007), but has mild or no effects on spatial learning consolidation in the DH (Popović et al., 2014), suggesting that it primarily mediates acquisition rather than consolidation of contextual learning. Furthermore, muscarinic receptor expression in the DH is heterogeneous and spatially complex, with four subtypes of receptors and both pre- and post-synaptic expression patterns (Levey et al., 1995), and projections to and feedback from other neurotransmitter systems further complicates the picture (Bergado et al., 2007). One study even suggests that scopolamine's effects on memory in the hippocampus may be mediated by increased ACh release and hyper-activation of nicotinic receptors. They demonstrated that co-infusion of a nicotinic antagonist ameliorated the scopolamine-induced memory impairment

(Newman and Gold, 2015); however, as this study was investigating working memory, conclusions may not be generally applicable to DH-dependent contextual learning.

The relationship between cholinergic signaling during the trauma and manifestation of the SEFL phenotype is currently unknown. In the first experiment, we infused scopolamine or artificial cerebrospinal fluid (aCSF) vehicle into the DH or BLA 1 hour prior to SEFL. We demonstrated that scopolamine in either brain region disrupted both trauma context memory formation and future contextual sensitization. In the second experiment, we administered a tone-shock pairing instead of a context-shock pairing as the novel mild stressor, and demonstrated that scopolamine in the DH did not disrupt sensitization to cues. Cholinergic signaling in the DH is involved in the development of sensitization after trauma to novel contexts, while cholinergic signaling in the BLA is likely critical for development of sensitization to a variety of novel stressors.

Materials and Methods

Subjects. A total of 128 naïve male Long-Evans rats (64 DH, 64 BLA), weighing 270-300 g (Harlan, Indianapolis, IN) were individually housed and maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*. Animals were handled daily (one-two min per rat) for at least one week prior to the start of behavioral training and and/or surgery. The procedures used in this study were in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Surgery. One week after housing, rats received surgical implantation of two guide cannulae aimed at the dorsal hippocampus or basolateral amygdala. DH and BLA cannulae placements are shown in Figure 4.1 and 4.2; rats with one or both cannula tracts that missed the DH or BLA

were not included in the behavior analysis. Rats were first anesthetized with sodium isoflurane (1-5%) and mounted in a stereotaxic frame. Guide cannulae (26-gauge, 7 mm or 9mm; Plastics One) were then lowered to the dorsal hippocampus (3.8 mm posterior to bregma, 2.5 mm lateral to bregma, and 1.8 mm ventral to dura) or basolateral amygdala (0.2 mm posterior to bregma, +4.6 mm lateral, and 7.4 mm ventral from dura). For infusions, the internal cannulae would extend 1.0 mm beyond the tip of the guide cannulae. Dental acrylic was used to fix cannulae to the skull, and dummy cannulae (33-gauge, 7 mm or 9mm) were inserted into the guide cannulae.

Apparatus. Behavioral training used a set of four identical fear conditioning chambers (30 x 25 x 25 cm, Med-Associates, Inc St. Albans, VT), equipped with a Med-Associates VideoFreeze system. Individual boxes were enclosed in sound-attenuating chambers in an individual, dedicated experimental room. The SEFL context was comprised of chambers with aluminum sidewalls and a clear Plexiglas rear wall. The grid floor consisted of 16 stainless steel rods (4.8 mm thick) spaced 1.6 cm apart (center to center). Pans underlying each box were sprayed with a thin film of 50% Windex® to provide the context with a scent. Chambers were individually lit from above by white lights and cleaned with 50% Windex in between squads. Fans mounted above each chamber provided background noise (60 dB). The experimental room was brightly lit with overhead white light. Animals were transported to the context in squads of eight in their home cages, which were slid onto hanging racks mounted to a portable cart and covered with a white sheet or black sheet. All aspects of the context were altered to create a distinctive single shock context. This context was comprised of an alternating large small or height-staggered grid floor and a black plexiglass A-frame. The context light was off, the experimental room light was red, and chambers were cleaned and scented with a 7%

acetic acid solution. Rats were transported to the context in groups of 4 in a black tub with individual dividers and bedding on the floor. Many aspects of the context were altered again for the cohort of rats that received tone conditioning and tone test in a separate, third context. This context consisted of a white plexiglass floor, white curving plexiglass rear wall in order to make the chamber shaped like a semicircle, context light off, red and white experimental chamber lights concurrently on, and cleaned with Pine Sol. Groups of four rats were transported to this context together in a large transparent plastic tub with blue pads on the floor. All chambers were cleaned with a 10% bleach solution following each day of behavioral testing.

Procedure. SEFL Context procedure is detailed in Figure 4.3, while SEFL Tone procedure is detailed in Figure 4.6. On Day 1, prior to the fear conditioning procedure, rats received bilateral infusions of either scopolamine hydrobromide (50 mg/ml in aCSF) or the same volume of aCSF. Rats were held by experimenters while injection cannulae (33-gauge; 8 mm or 10mm), connected to 10-ml Hamilton syringes with PE-20 polyethylene tubing (Plastics One) and mounted on a microinfusion pump (Harvard Apparatus, South Natick, MA), were inserted into the guide cannulae. Scopolamine or aCSF (DH total volume was 1 µl, BLA total volume was 0.25 µl) was infused (DH rate of 0.25 µl/min, BLA rate of 0.1 µl/min). Injection cannulae were left in place for an additional minute to facilitate diffusion. Dummy cannulae were then reinserted. Rats were returned to home cage for one hour prior to being placed in conditioning chambers. Rats receiving the SEFL protocol received 15 unpredictable foot shocks (1 sec, 1.0 mA) pseudorandomly spaced across a 90 min conditioning session. The first shock occurred after 3 min in the chamber. Rats receiving the No Shock protocol were placed in the context for 90 min but received no foot shocks. All rats were returned to their home cages after each

experimental session. To assess the level of fear to the trauma context, on Day 2 rats were returned to the same context for a 5 min context test. To assess the response to a novel stressor, on Day 3 rats were placed in the single shock context. Three min after entering the context, they received a single foot shock (1sec, 1.0mA), and were removed from the context 1 min later. On Day 4, rats were returned to the single shock context and freezing was assessed over the 8 min context test. A subset of rats received the tone training protocol, which differed on Days 3 and 4. On Day 3, rats received a single tone presentation (80 dB, 20 sec) co-terminating with a foot shock (1mA, 1 sec) after three min in the context. They were removed from the context 1 min after the shock. On Day 4, these rats were exposed to the novel tone test context, and experienced the same protocol as on Day 3 but without the foot shock, in order to test fear of the tone. The use of a novel conditioning chamber to asses tone freezing precludes any contribution of contextual freezing to this measure. For assessment of fear, statistical analyses on freezing behavior were performed using an automated near infrared (NIR) video tracking equipment and computer software (VideoFreeze, Med-Associates Inc., St. Albans, VT),

Histology. To assess cannulae placements, rats were anesthetized with isoflurane and decapitated. Brains were removed from the skull and placed in a 10% formalin/30% sucrose solution for 3 days prior to sectioning. Coronal sections (40 mm thick) were taken throughout the extent of the cannula track and mounted on slides. Injection sites were reconstructed using bright field microscopy. Rats that had one or both cannulae or injector tracks outside the target structure were excluded from analysis (Figure 4.1, 4.2).

Results

Scopolamine in the BLA or DH blocks fear acquisition to SEFL context

Rats received an infusion of either scopolamine (SCOP) or vehicle (aCSF) into the DH or BLA, and 1 hour later experienced the 15-shock procedure (SEFL) or an equivalent duration context exposure with no shock (NS) (Procedure, Figure 4.3). As rats receiving no shock showed no freezing during the session and often fell asleep, their within-session freezing behavior during the contextual exposure is not reported. Of rats who received the SEFL procedure, scopolamine retarded fear acquisition. This led to different levels of freezing between SEFL-SCOP and SEFL-aCSF rats during the beginning of the session, but similar levels of freezing by the end of the session (Figure 4.4). This effect was similar in the DH ((main effect of Drug ($F(1, 420) = 76.08$, $p < 0.0001$) and Shock ($F(14, 420) = 5.295$, $p < 0.0001$), and a significant two-way interaction ($F(14, 420) = 2.482$, $p < 0.01$); follow up t-test for first post-shock epoch ($p<0.05$) and last epoch ($p>0.05$)) and the BLA ((main effect of Drug ($F(1, 420) = 34.63$, $p < 0.0001$) and Shock ($F(14, 405) = 4.192$, $p < 0.0001$), but no interaction ($p > 0.05$)). BLA cannulated aCSF rats have slightly lower levels of fear (~ 20%) overall than DH cannulated aCSF rats. Even small amounts of damage to the BLA have been shown to disrupt context fear (Flavell and Lee, 2012), which indicates that this is likely due to mild bilateral damage from cannulation implants, which did not prevent fear learning. Overall, these results demonstrate that while scopolamine has an amnestic effect, it does not prevent within-session responding to a dangerous environment.

Though the effect of scopolamine infusion during trauma was mild, the effects on trauma memory recall were profound (Figure 4.5). While DH SEFL-aCSF rats demonstrated high fear to the trauma context (~85% freezing), scopolamine led to a significant reduction in freezing in

SEFL rats, but not in non-shocked controls (main effect of SEFL ($F(1, 32) = 22.91$, $p < 0.0001$), main effect of Drug ($F(1, 32) = 12.55$, $p < 0.01$), significant interaction between SEFL and Drug ($F(1, 32) = 5.643$, $p < 0.05$); SCOP reduced fear in SEFL rats ($p < 0.0001$) but not non-shocked rats ($p > 0.05$)). A similar result was seen with scopolamine infusion into the BLA. Scopolamine led to a significant reduction in freezing in the SEFL rats, but not non-shocked controls (main effect of SEFL ($F(1,43) = 22.30$, $p < 0.0001$), no main effect of Drug ($F(1, 43) = 1.636$, $p > 0.05$), and a significant interaction between SEFL and Drug ($F(1, 43) = 5.393$, $p < 0.05$); scopolamine reduces fear in SEFL rats ($p < 0.05$) but not non-shocked rats ($p > 0.05$)). These findings suggest that cholinergic signaling in the BLA and DH, specifically at muscarinic receptors, is essential for the formation of a strong traumatic memory.

Scopolamine in the BLA or DH blocks sensitization to a novel context CS

While scopolamine is sufficient to disrupt the memory of a traumatic experience, it was unknown whether this temporary disruption would affect future fear learning. Off drug, these rats were exposed to a novel context and given a single shock, and contextual freezing was measured the following day in the same context.

In aCSF rats, the SEFL procedure led to a disproportionate amount of fear to the new conditioning context, demonstrating the sensitizing nature of trauma to future fear learning (Figure 4.6). However, scopolamine infusion two days prior prevented this sensitization, both in DH rats (main effect of SEFL ($F(1, 25) = 26.29$, $p < 0.0001$) and Drug ($F(1, 25) = 7.442$, $p < 0.05$), significant interaction between SEFL and Drug ($F(1, 25) = 9.337$, $p < 0.01$); SEFL-SCOP rats froze significantly less than SELF-aCSF rats ($p<0.01$)) and in BLA rats (main effect of SEFL ($F(1, 42) = 8.085$, $p < 0.01$) and Drug ($F(1, 42) = 5.078$, $p < 0.01$), significant interaction

between SEFL and Drug ($F(1, 42) = 9.349$, $p < 0.01$); SEFL-SCOP rats froze significantly less than SEFL-aCSF rats ($p<0.001$)).

`There is some evidence to suggest that sensitization of the BLA underlies SEFL, and that this sensitization requires CORT signaling in the BLA and an upregulation of GluA1 (J. Perusini, in prep; Perusini et al., 2015). These data demonstrate that cholinergic signaling in the BLA during trauma is also required for SEFL to develop.

Cholinergic signaling in both the DH and BLA is required for strong fear memory to the trauma context. Yet the effects of DH scopolamine infusion suggest that cholinergic signaling in the DH is also required for sensitized responding to new context-shock pairings after trauma. In order to dissociate the influence of the DH and BLA in the development and expression of SEFL, we tested the effects of these manipulations on sensitization to new discrete cues.

Scopolamine in the DH fails to prevent sensitization to a novel tone CS

Instead of a context-shock pairing, a cohort of rats received a single tone-shock pairing after the SEFL procedure. The following day, they were exposed to a novel context and given a tone test (Procedure, Figure 4.7).

In DH rats, scopolamine during trauma did not prevent future sensitization to discrete cues (Figure 4.8). During the tone, SEFL-SCOP and SEFL-aCSF rats had a disproportionate freezing response, and did not differ in their freezing levels ($p>0.05$). Though some rats in the NS-SCOP group had higher freezing to the tone, which drove an interaction, the majority of non-shocked rats had very low responding to the tone (main effect of SEFL, $F(1,24) = 27.54$, $p<0.0001$, main effect of DRUG, $F(1,24) = 4.965$, $p<0.05$, interaction, $F(1,24) = 6.279$, $p<0.05$). The disproportionate response of the SEFL groups continued in the period of time after the tone ended, while the non-shocked group maintained normal levels of fear (main effect of SEFL

($F(1,24) = 19.21$), $p < 0.001$), no main effect of DRUG ($p > 0.05$), no interaction ($p > 0.05$)). These data demonstrate that while blocking muscarinic receptors in the DH during trauma disrupts contextual sensitization, it does not affect sensitization to tones.

The selective blockade of contextual sensitization after SEFL was also evident when testing contextual generalization. A subset of rats were administered the first three days of the Tone SEFL protocol, and on the fourth day were tested for contextual generalization for ten minutes in a novel, third context. While SEFL-ACSF rats generalized to this third context, SEFL-SCOP rats did not (ttest, $p < 0.05$), demonstrating that contextual generalization after trauma was also blocked by scopolamine administration before trauma (Figure 4.9, A).

Minimal contribution of state-dependent and consolidation effects in the DH

One potential explanation for the freezing deficit in the trauma context for rats that received scopolamine is that there is a state-dependent effect of scopolamine. In effect, the state of experiencing scopolamine in DH may be salient, and therefore incorporated into the contextual representation. Therefore, a lack of freezing in the trauma test context could be due to recognition that the context is different from training, due to the lack of drug. In order to test this, we subjected a subset of DH rats who had received scopolamine during SEFL to a second trauma test; this test occurred after an infusion of scopolamine. No differences were seen between those that re-experienced the context under scopolamine or an aCSF infusion (Figure 4.9, B, $p > 0.05$), suggesting that state-dependent effects are not the main mediator of the low levels of context fear seen in the scopolamine DH rats after SEFL.

For the following experiment, we used a subset of DH rats who had previously received one shock in order to conserve research subjects. Though this mild fear-inducing experience

may subtly affect future fear learning, the use of only subjects who had received this experience minimized the between-group effects on variance.

Scopolamine could exert its effects by disrupting memory formation, by disrupting consolidation, or by a combination of the two. We tested this distinction by administering the SEFL protocol and infusing scopolamine or aCSF into the DH within 10 minutes after the protocol concluded. Though prior intra-DH scopolamine infusion is disruptive for both the trauma test as well as for contextual sensitization after a single shock, post-SEFL scopolamine did not disrupt freezing in either test condition (Figure 4.9, C, $p>0.05$). This test demonstrates that scopolamine in DH disrupts plasticity processes occurring during acquisition, but does not exert its effects on trauma by blockade of consolidation.

Discussion

Humans diagnosed with PTSD are a heterogeneous population, due to variety in intensity and duration of the trauma, as well as underlying genetic, epigenetic, and environmental differences. In this study, we used an intense acute trauma in rats that produces robust and repeatable fear to the original trauma context, as well as sensitization to novel stressful contexts, as a model for these particular aspects of human anxiety disorders. For the first time, we demonstrated the requirement of cholinergic signaling at muscarinic receptors in the BLA or DH for both trauma memory and future sensitization. We also showed that these effects are unlikely to be merely state-dependent effects of scopolamine, nor are they primarily effects on consolidation. In the hippocampus, we further demonstrated that scopolamine does not fully block the development of the SEFL phenotype. Rats in this group, despite showing markedly reduced fear to the SEFL context, still show sensitization to tone stimuli.

Blockade of muscarinic receptors in the BLA disrupted contextual fear learning as well as sensitization. Muscarinic receptor activation in the BLA is known to have both direct and indirect effects on principle cells (Egorov et al., 2006; Washburn and Moises, 1992), and activation increases signal to noise ratio for principle cells (Unal et al., 2015). This increased signal-to-noise ratio may underlie sensitized responding of BLA circuitry after SEFL, as these effects were mediated by muscarinic receptor activation. Furthermore, a recent study demonstrated that acetylcholine during learning enhanced fear memory durability; increasing cholinergic release during learning led to slower extinction due to impaired retention, and this effect was also mediated by both muscarinic and nicotinic receptors (Jiang et al., 2016). Overall, these data suggest a model for enhanced BLA function under stress, where elevated levels of ACh lead to heightened BLA circuitry response to the current stressor and an enhancement in responsiveness to future stressors.

It remains to be seen whether sensitization to discrete stimuli differs from sensitization to contextual stimuli after muscarinic blockade during stress. Our study demonstrated that scopolamine before SEFL disrupted sensitization to novel contexts after stress. Due to the importance of cholinergic signaling in the BLA for both tone and contextual fear conditioning, one hypothesis is that muscarinic blockade during stress will prevent sensitization to both classes of novel stimuli after stress. This remains to be tested.

Blockade of muscarinic receptors in the DH disrupted contextual fear learning and future contextual sensitization, but had no effect on sensitization to a discrete tone cue. Tone and context learning have differential requirements for hippocampal processing, as NMDAR blockade in the DH disrupts context but not tone learning (Bast et al., 2003). Furthermore, hippocampal ACh has a particular role in contextual processing. Higher ACh release in the DH

is observed in behavioral designs where context contingency with shock is higher than tone, and lower when tone is a better predictor of shock than context (Calandreau et al., 2006). In addition to this correlation, decreasing hippocampal ACh in the same study disrupted context conditioning and improved tone conditioning; increases had the opposite effect. Our study replicates these effects, demonstrating that scopolamine in the DH disrupts even very intense contextual conditioning. However, it also gives the DH a new role in sensitizing contextual learning circuitry during stress, leading to elevated responses to future contextual learning episodes. ACh signaling at muscarinic receptors during stress is critical for this sensitization.

In addition to considering effects of scopolamine infusions into the DH or BLA on local circuit function, muscarinic blockade in one of these brain regions can disrupt communication in a network of regions involved in fear learning under stress. Scopolamine disrupts resting state functional connectivity between mouse brain regions involved in memory (Shah et al., 2015), and this disruption of inter-region communication may be important for its ability to disrupt SEFL. One of the persistent effects of the trauma itself is increased inter-regional communication, which may play a role in enhanced responding to threatening cues after stress. Soldiers with PTSD (compared to those without) have inter-regional hypersynchrony at high frequencies (80-150 Hz), as well as a decrease in signal variability; this was most evident in the network containing hippocampus and amygdala (Mišić et al., 2016). There is some evidence that these connectivity changes after trauma may be related to cholinergic signaling. Individual differences in cholinergic gene expression mediate functional connectivity differences in humans between the basal forebrain, amygdala, and hippocampus during processing of emotional stimuli (Gorka et al., 2015). These differences in cholinergic gene expression may explain some of the variability in individuals' responses to traumatic events, as the hyperactive cholinergic signaling

during stress is given greater or lesser influence on learning and memory circuitry. Investigations of individual differences in these genes and the ability to be resilient in the face of stress is a promising future area of research.

Our study did not investigate sex differences in muscarinic blockade during SEFL, but much evidence exists that the stress response is divergent between males and females. One such example is that males have higher increases in ACh release after restraint stress than females, while females have higher corticosterone release; the ACh effect in particular is dependent upon gonadal hormones (Mitsushima et al., 2003, 2008). This suggests that scopolamine may have distinct effects in males and females on disruption of the SEFL phenotype. As anxiety disorders, including PTSD, are currently more prevalent in females (Breslau et al., 1997; McLean and Anderson, 2009), the likely distinct changes in female neural circuitry after stress are an important component of future research.

Anxiety disorders are equally common in young and old adults, yet there may be distinct mechanisms in their development. Some of the hallmarks of stress, such as increased CORT and ACh release, are only evident in younger rats, and absent from aged rats (Mizuno and Kimura, 1997). Future studies might also shed light on changes in circuit sensitization with age.

ACh, while critical for the SEFL phenotype, is by no means the only neuromodulator that is important for this effect. Corticosterone is required (Perusini et al., 2015) and likely interacts with ACh effects described in the current study (Gilad et al., 1985; Paul et al., 2015). Another neuromodulator that likely plays a role is norepinephrine (NE). Studies have demonstrated that there is also enhanced release of NE during mild stressors after trauma (Ronzoni et al., 2016), but did not look at release levels during trauma. Mimicking enhanced noradrenergic activity after trauma by giving an alpha2 adrenoceptor antagonist (yohimbine)

after standard fear conditioning, lead to an enhanced and generalized fear memory (Gazarini et al., 2014), suggesting that NE signaling during and after trauma may be important for traumatic memory formation. Furthermore, NE signaling might have a critical role in resilience to chronic stress (Isingrini et al., 2016). Interactions between ACh and NE during and after stress remain an important avenue of research.

As our effects were on traumatic memory formation, direct translation to human therapeutics remains a distant target. As demonstrated in the current study, scopolamine after trauma, at least in the DH, does not have the same disruptive effect on circuits underlying context fear and sensitization after SEFL. However, scopolamine and other cholinergic drugs are already under investigation for treatment of fear-related disorders. One promising method is to use low-doses of scopolamine to create context-independent extinction memories (Zelikowsky et al., 2013). Another potential use might be in memory erasure, either by blockade of re-consolidation or other methods (Maren, 2011).

Cholinergic inputs to memory circuitry exert powerful effects on memory processes. Under stress, these inputs become hyperactive, leading to long-term changes in multiple brain regions that lead to treating future mild events as traumatic. Though anxiety disorders such as PTSD are clearly maladaptive, they are the result of an evolutionary process where hyper-responding to non-threatening stimuli leads to survival, and failure to respond to a real threat leads to death (Nesse, 2005; Rosen and Schulkin, 1998). Taken in this context, cholinergic signaling offers a potential way to recalibrate circuitry that is simply functioning too well.

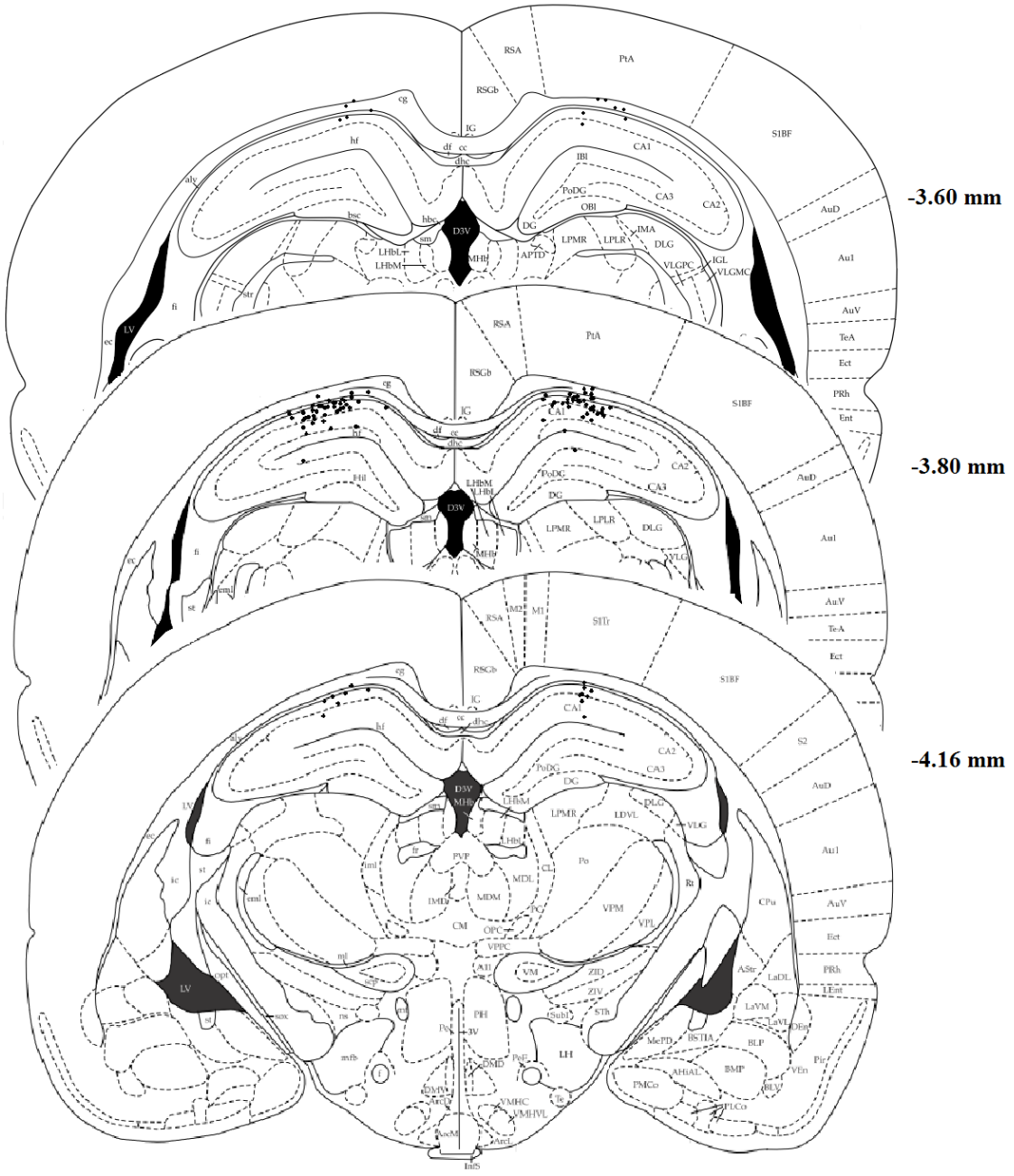


Figure 4.1: **Dorsal Hippocampus Cannula Placement.** All hippocampal cannula tip placements are shown above. Rats with one or both internal tips outside DH were excluded from analysis. (n=65 included).

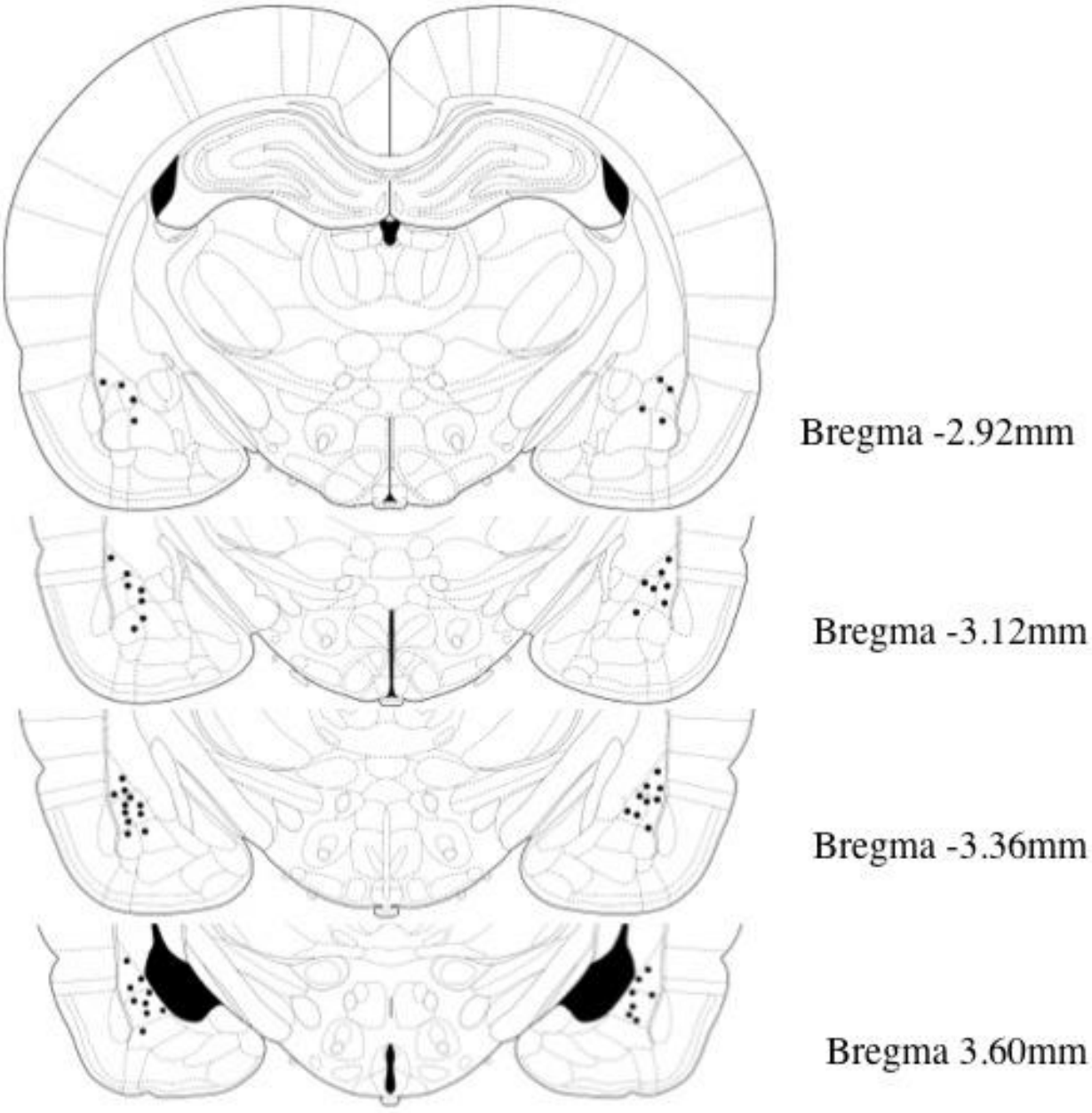


Figure 4.2: **Basolateral Amygdala Cannula Placement.** All amygdala cannula tip placements are shown above. Rats with one or both internal tips outside BLA were excluded from analysis. (n=42 included).

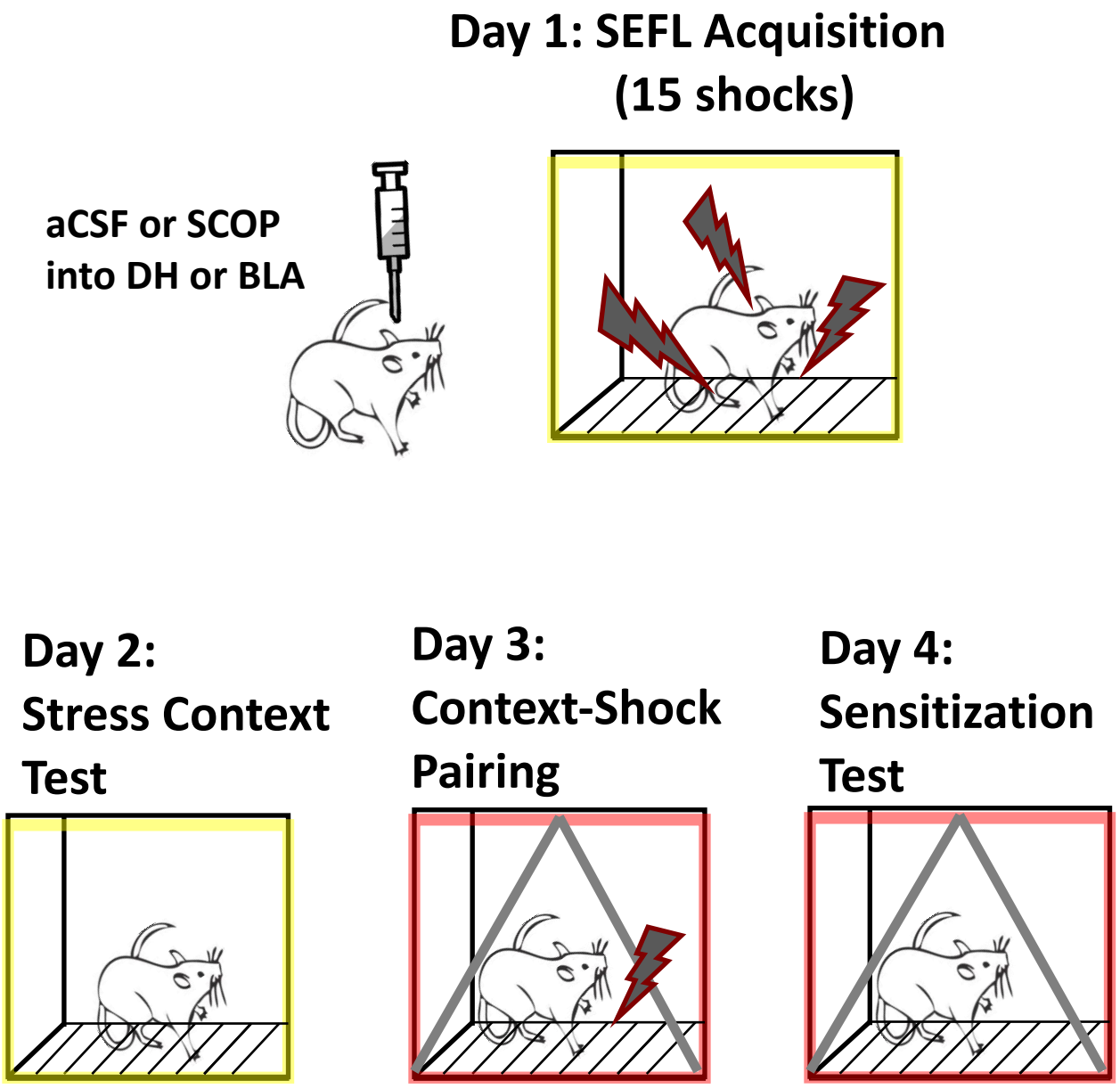
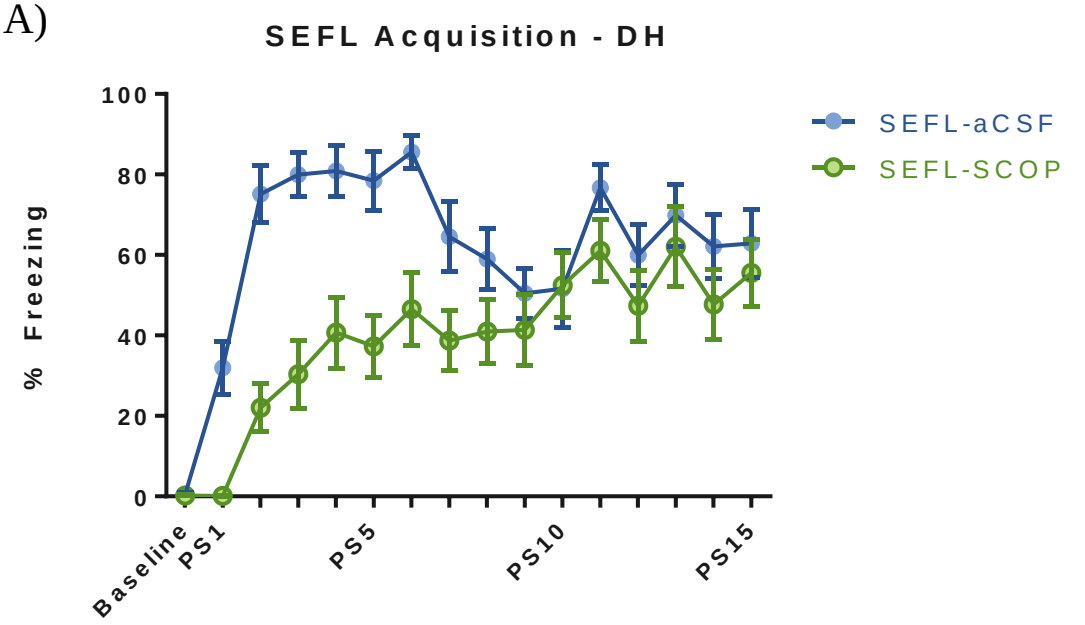


Figure 4.3: **SEFL Context Procedure.** Schematic representation of the stress-enhanced fear learning procedure, which in the SEFL group produces both high levels of fear in the trauma context as well as sensitization to a new context previously paired with shock.

A)



B)

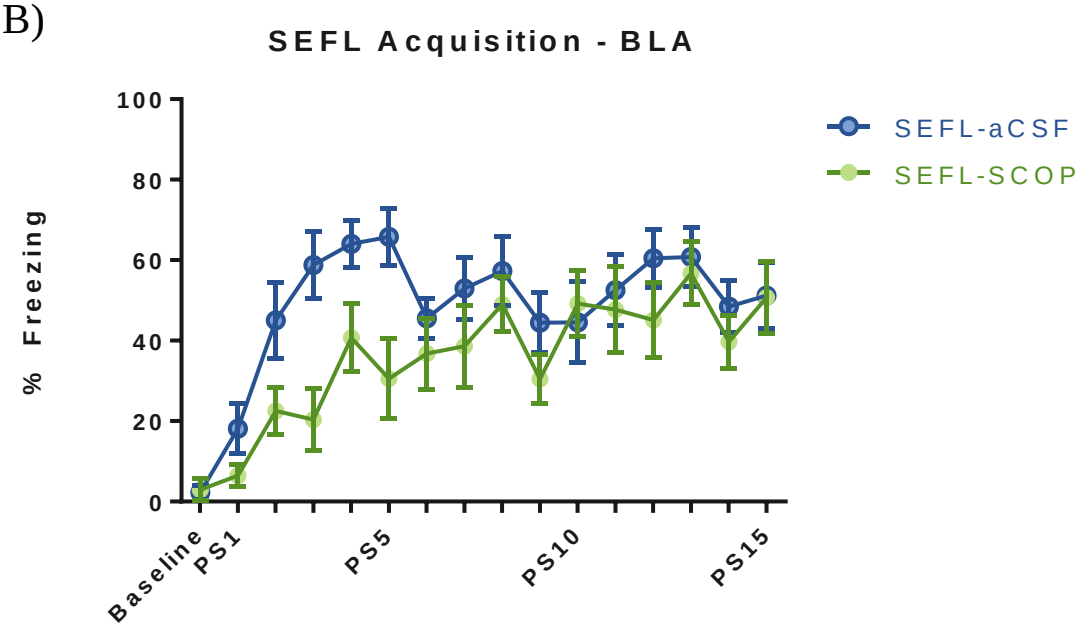


Figure 4.4: **SEFL Acquisition under scopolamine or aCSF in DH or BLA.** A,B) Baseline fear is recorded for the first three minutes of the session before the first shock, while "PS" designates post-shock freezing, as measured for 30 s beginning 30 s after the end of a shock. Non-shocked (NS) rats received a context exposure of the same length, but without any shocks (data not shown). Both DH and BLA cannulated rats acquire fear, as measured by post-shock freezing. Both scopolamine and aCSF groups demonstrate post-shock freezing, though scopolamine rats have a slower rate of learning, as measured by lower levels of fear toward the beginning of the session. Both scopolamine SEFL groups are not significantly different from aCSF SEFL groups by the end of the conditioning session ($p>0.05$). Error bars indicate S.E.M.

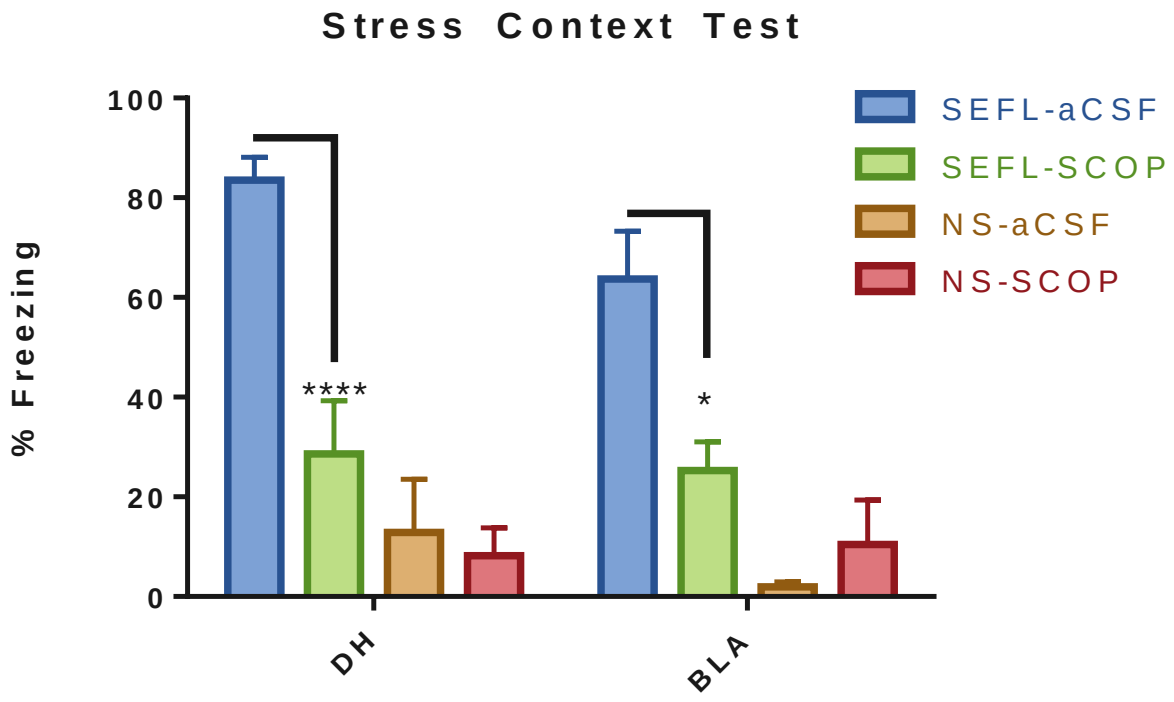


Figure 4.5: **Stress Context Test for DH and BLA rats after SEFL.** Data shown is averaged across an 8 min test. While SEFL-aCSF rats have very high levels of fear, scopolamine attenuated the context fear to the trauma test in both DH and BLA SEFL rats. Non-shocked animals have very low levels of fear. This demonstrates that scopolamine before fear learning markedly reduces contextual fear at test. Error bars indicate S.E.M.

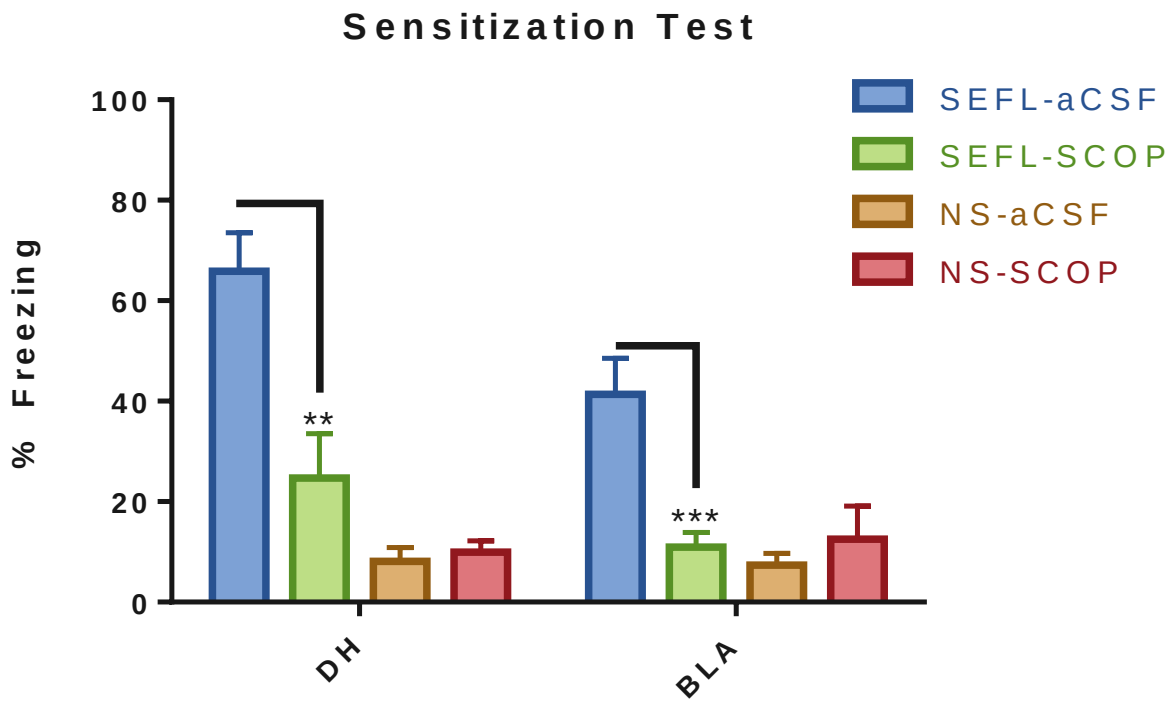


Figure 4.6: **Sensitization Test for DH and BLA rats after SEFL.** Data shown is averaged across an 8 min test. After all rats received a single shock in a novel context, contextual fear to that context was measured the following day in a sensitization test. SEFL-aCSF rats show profound sensitization to the context, as fear in these rats is elevated above rats that did not receive SEFL. However, this sensitization was attenuated in DH-scopolamine rats and eliminated in the BLA-scopolamine rats, indicating that disruption of cholinergic signaling with scopolamine in either DH or BLA before SEFL significantly reduces sensitization of future fear learning. Error bars indicate S.E.M.

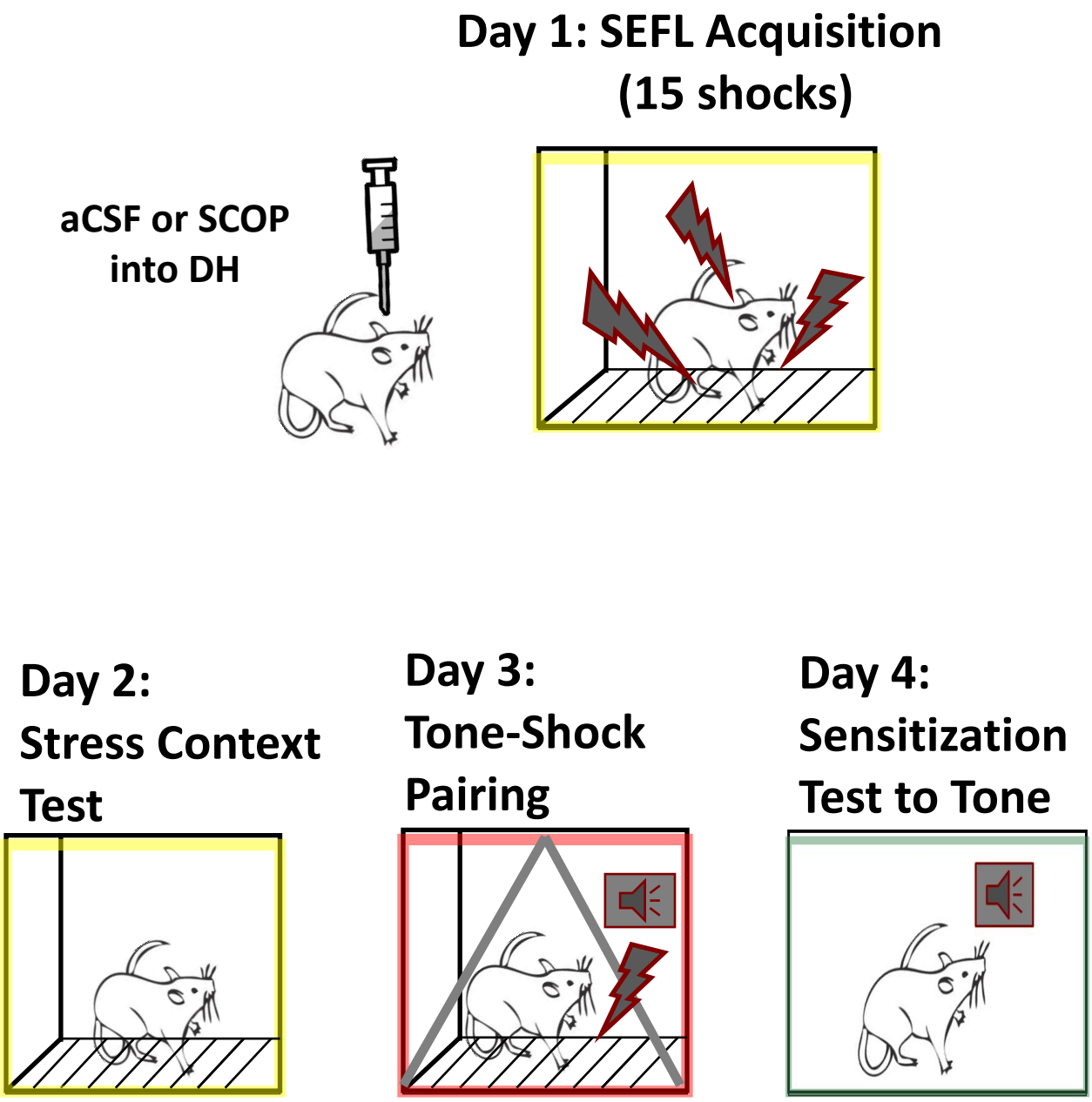


Figure 4.7: **SEFL Tone Procedure.** Schematic representation of the stress-enhanced fear learning procedure, which in the SEFL group produces both high levels of fear in the trauma context as well as sensitization to a new tone previously paired with shock. This procedure was used for DH-cannulated rats.

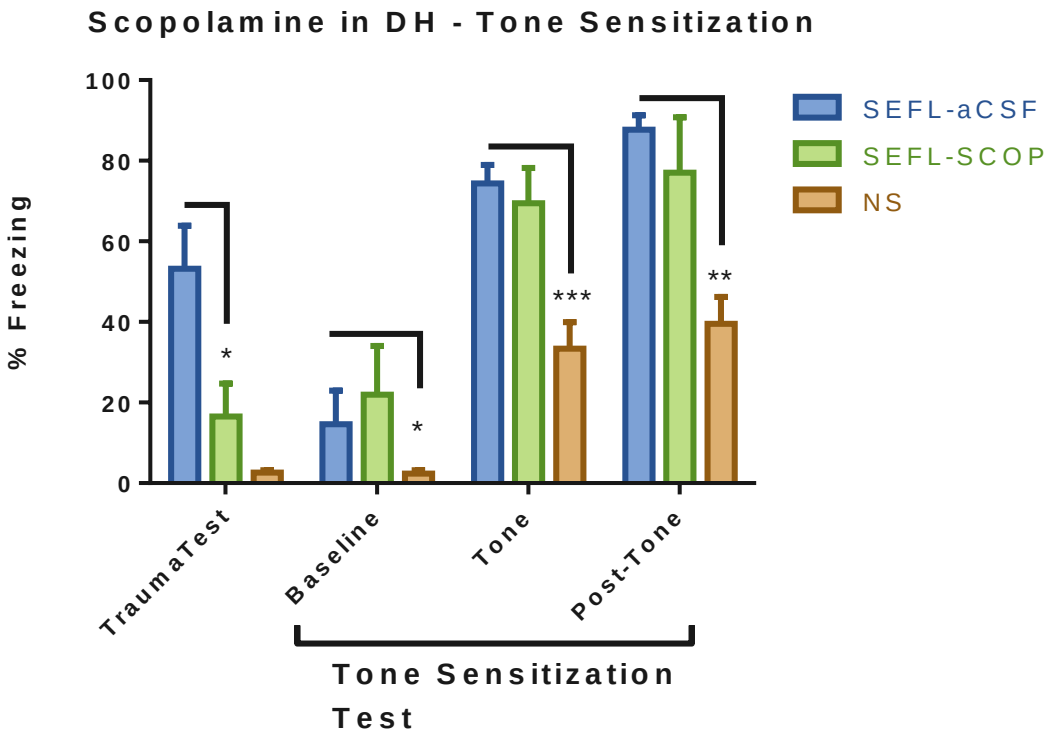


Figure 4.8: **Tone Sensitization after SEFL in DH.** Data is shown for the trauma test (first bar cluster) and for the tone test (remaining bar clusters). In this cohort, we replicated our initial finding in the Trauma Test, namely that scopolamine attenuated fear of the SEFL context. After a tone shock pairing, there was some generalization to the tone-test context in both SEFL groups, but fear levels were low before tone onset. During the tone and in the post-tone period, all SEFL rats show elevated fear compared to rats that did not receive SEFL. These data indicate that while scopolamine into DH protects against future sensitization to contexts, it does not protect against future sensitization to tones. Error bars indicate S.E.M.

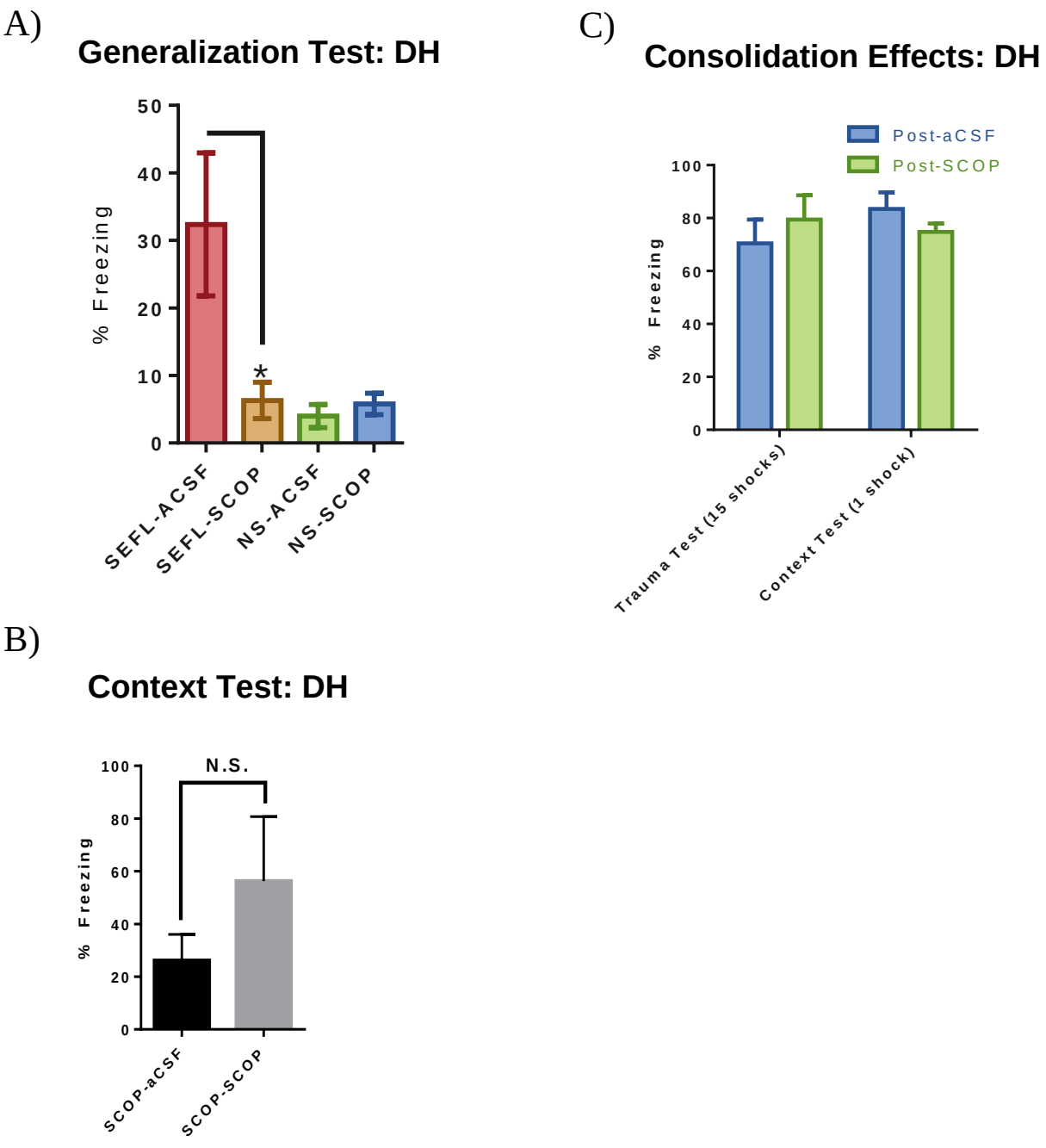


Figure 4.9: **Test of state-dependency and effects on consolidation in DH.** A) Rats that had received the first three days of the SEFL protocol with tones (n=6 or 7 per group) were tested in a novel, third context for generalization. While SEFL-ACSF rats generalized, SEFL-SCOP rats did not ($p<0.05$). B) DH cannulated rats that had received SEFL were given an infusion of scopolamine and re-tested in the trauma context (n=3 per group). Rats that had previously received scopolamine did not freeze significantly more than rats that had previously received aCSF (ttest, $p=0.31$), suggesting that scopolamine's disruptive effect on trauma test freezing after SEFL is not likely to be due to state-dependent effects. C) DH cannulated rats that had previously experienced one shock were administered the SEFL protocol, and received post-SEFL administration of scopolamine or aCSF. Both groups showed similar levels of fear to the trauma context one day later ($p>0.05$) and the one-shock context two days later ($p>0.05$). Error bars indicate S.E.M.

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Chapter 5: General Conclusions

Rapid, accurate, and lasting contextual fear learning and recall are essential for survival. These memories vary in strength, due to how effectively the stimuli that make up the context are processed into a configural representation, as well as the strength, frequency, and unpredictability of the aversive US(s) paired with the context. If the US is strong, frequent, and unpredictable, in addition to a strong contextual fear memory, circuitry participating in that memory formation will be sensitized, and future fear learning will result in disproportionately high fear responding.

In the DH and BLA, the current consensus for recent fear memories is that activity in a proportion of the neurons activated by the learning episode is important for the recall of that episode. Many neural populations will have similar activity during encoding and recall due to simple sensory processes, but a dissociation must be made between "sensory" reactivation, such as primary sensory cortical cells that respond to a repeated stimulus presentation with the same activity, and memory-related reactivation. For recent time points, I dissociated this sensory account with associative memory formation by comparing neural reactivation in an immediate shocked group with a delayed shocked group. Though both experienced the same sensory inputs (context exposure and shock), the temporal relationship determined whether fear memory was formed (Ch2). Though reactivation in these two groups was the same in the DH, this does not suggest that firing patterns of DH cells are not sensitive to fear conditioning information. Multiple lines of evidence suggests that place fields are sensitive to contextual fear (Moita et al., 2004; Wang et al., 2012, 2015); however, the remapping observed in these studies consists of changes in the firing fields of the same cells that were active before pairing. Use of catFISH

reactivation metrics does not provide information about the spatial properties of the cells sampled. Therefore, it can be assumed that though DH cells are likely remapping in the delayed shock group and not in the immediate shock group, reactivation of the same population of cells is not dependent upon associative learning per se, but upon returning to a previously-experienced context. In addition to recall of fear, these DH cells are reactivated, and many shift firing fields, during extinction in the conditioning context, but not extinction in another context (Wang et al., 2015). As extinction is known to be context-dependent, it is likely that the initial learning population in DH is important for forming new, neutral-valence representations of the initially fearful context (Redondo et al., 2014), though future studies are needed to explore these ideas in more detail.

Though the BLA has long been believed to be the location where context-shock associations are stored (Fanselow and LeDoux, 1999), and is required at all temporal intervals for recall of associative fear (Gale et al., 2004), my recent work provides evidence that BLA cells active during learning are important for recall at both recent, and preliminary evidence suggests also remote, time points (Ch2). This is also the first evidence that activity in this population is only required for initial activation of context-dependent fear behavior; after these cells are allowed to become active for a short time (between 30 s and 2 min), ongoing fear behavior is established and their activity is no longer required (Ch2).

Arc-Cre mice were considered for these experiments, in order to confine expression to principle cells rather than including interneurons (which express Fos but not Arc). However, current genetic lines have expression at baseline, before induction with 4-OHT, and so lack the specificity required (Guenthner et al., 2013; Hersman, unpublished observations). The use of inhibition, rather than excitation, with the Fos-Cre mice partially controls for this discrepancy.

BLA interneurons have high levels of activity during learning (Bienvenu et al., 2012), and many tagged cells in the current study have interneuron morphology (Figure 2.5), suggesting the tagged population was made up of both BLA principle cells and interneurons. However, based on the current understanding of memory coding in the BLA, inhibition of a subset of interneurons is likely to be less disruptive to fear behavior than the inhibition of the BLA principle cells activated during learning. This might not have been the case with driving the activity of this heterogeneous population, which could have had bidirectional effects, making interpretation difficult.

An important factor in this study (Ch2) is that fear behavior was not completely inhibited during inhibition of the initial learning cellular population. This is likely a combination of three factors. The first is a procedural one: connecting cables to the laser in the context took some seconds, during which time sampling of contextual stimuli was certainly possible. However, the activity of the experimenter was disruptive to normal mouse exploration behavior during this time, distracting the mouse from optimal contextual processing. Secondly, placement of the ferrules dorsal to the BLA complex meant that spread of light could potentially have been incomplete. Anterior or posterior aspects of the BLA, and particularly very ventral parts of the basal complex, may have not received light intensity sufficient to prevent all neural activity. Weak activity of this population could potentially drive the low levels of fear observed during light stimulation. This hypothesis could be tested with DREADD-receptor mediated inhibition. However, in this case the tight temporal aspect of the requirement of these cells during only the initial exposure to the context would not be observed, due to temporal dynamics of CNO spread and metabolism. Thirdly, even complete lesions of the BLA do not eradicate all contextual fear, but spare anywhere from 10-30% average freezing (for specifics, see Gale et al., 2004), which is

not different from average freezing values during the initial light-ON session in this study (~ 30%). Light-ON freezing in this case, therefore, may not depend upon BLA activation at all, but on weakly-compensatory cortical projections to other parts of the amygdalar complex (Krasne et al., 2011). Future modeling, electrophysiology, and projection-specific silencing studies may be able to disentangle these alternate pathways.

Population-level neural activation, in the BLA and DH, is similar for encoding and recall of contextual fear memories. This leaves open the question of what signals during learning lead to the ability of these populations to reactivate during recall. This dissertation has tested the contribution of one important neuromodulator, acetylcholine (ACh), to these encoding processes in the DH and BLA (Ch3, Ch4). ACh is known to be released in DH during novel environment exploration, due to both motor activity during exploration as well as attentional processes related to novel stimulus processing (Giovannini et al., 2001). This ACh release hugely increases in magnitude in the case of an acutely traumatic event, dependent upon glucocorticoid signaling during stress (Mitsushima et al., 2008). ACh is also released in the amygdala in response to stress; however, the temporal profile differs from hippocampal release. While DH release occurs during stress, amygdala release occurs upon the release from stress (Mark et al., 1996), suggesting a differing role for ACh processing in the two regions.

Enhancing cholinergic release in DH during novel environment exploration led to formation of a stronger contextual memory, as indexed by fear after later pairing with shock (Ch3). This manipulation was confirmed using choline biosensors, which recorded evoked ACh after multiple different stimulation parameters. Enhanced ACh in the DH did not simply lead to enhanced exploration or sampling of more details, as crossings and rearings during stimulation did not differ between transgenic mice and controls. Nor did enhanced ACh in the DH act like

an aversive US, as simple pairing did not lead to fear behavior (Ch3). This suggests that the enhanced ACh release in the DH observed during stress is not itself sufficient to produce a stressful state. Rather, it serves to enhance encoding of details that may be relevant to identifying the likelihood of future stressful events. It is important to keep in mind, however, that this stimulation protocol caused a relatively minor enhancement in ACh release in DH. Driving the system into more elevated levels of ACh release may indeed be sufficient to produce some of the effects of stress; future studies will have to address this possibility.

Due to the organization of the present set of experiments, it is difficult to dissociate whether enhanced ACh during encoding led to improved confidence in memory details or improved the ability to rapidly recall the contextual representation formed. Both interpretations would lead to a stronger context-shock association on Day 2, and elevated fear on Day 3 (Figure 3.2). Future studies should test the selectivity of the enhanced memory with a contextual generalization test after learning. In this case, it is essential to train controls with a stronger training protocol, such that the natural memory and "enhanced" memory are expressed equally in the conditioning context, in order to selectively test generalization of the memory. Consistent with the explanation that enhanced ACh leads to improved confidence in memory details, an equally strong "enhanced" memory should generalize less to a novel context, as the discrepancies between contexts will be easier to identify. However, in the case of traumatic contextual memories, the effect of the trauma may trump this deeper knowledge of contextual details, leading to generalization of fear in even distinct environments (Lopresto et al., 2015).

Contextual memories set the stage for the events that take place in them; this is particularly true for traumatic memories. Acute traumatic experience leads to widespread changes in neurotransmission, both during and after trauma, which are important for the

observed effects of trauma (Mark et al., 1996; Mora et al., 2012). Using a previously-developed model of acute stress (Rau et al., 2005), my work demonstrates a new requirement for ACh release at muscarinic receptors for both the formation of a traumatic contextual memory and for future sensitization to mild contextual stressors (Ch4). These effects are not due to state-dependent effects on learning, nor to disruption of consolidation. Furthermore, I demonstrate for the first time that DH cholinergic signaling during trauma is important for sensitization to new contexts, but not for sensitization to simple stimuli such as tones. Therefore, despite low levels of fear in the trauma context and normal responding to new contextual cues, rats that received scopolamine in DH before SEFL still have some circuit changes due to the trauma. These changes are manifested in sensitization to new tones after shock (Ch4).

These data situate the DH and BLA in a circuit activated by acute traumatic stress, a circuit important for changing future responding to contextual stressors. Scopolamine has been shown to disrupt resting state functional connectivity between mouse regions involved in memory (Shah et al., 2015); this disruption may be an important aspect of the effect of scopolamine on SEFL. The importance of cholinergic signaling on interregional communication has been demonstrated in humans. Individual differences in cholinergic gene expression was demonstrated to mediate differences in functional connectivity between the basal forebrain, amygdala, and hippocampus, during processing of emotional stimuli (Gorka et al., 2015). These differences may underlie sensitivity to traumatic events, as soldiers diagnosed with PTSD had enhanced interregional hyper-synchrony in the network containing the hippocampus and amygdala (Mišić et al., 2016).

These different lines of data suggest a model for cholinergic signaling under stress. Before stress, individual differences in cholinergic gene expression may exert control over how

correlated DH and BLA activity may be. In situations that do not reach some threshold of stress, enhanced correlation may adaptively improve identification and response to emotional stimuli. However, under cases of acute stress, massive release of ACh throughout the circuit (though with variable temporal profiles, see Mark et al., 1996) not only leads to intense activation of cells involved in formation of a traumatic memory, but synchronizes interregional firing in a manner that likely serves to greatly increase the impact of direct cellular activation. After the stressful episode concludes, this does not only lead to recall of a very strong contextual fear memory. It also leads to alteration of the plasticity of neural populations involved in memory, potentially reducing the threshold required for future memory formation. Yet it also leads to enhanced synchrony between these same regions, allowing this hypersensitivity to emotional stimuli to persist long after the conclusion of the trauma. Animal models of anxiety disorders, such as SEFL, will be important for testing these ideas about the relationship between cholinergic signaling, sensitization of future fear learning, and interregional synchrony after trauma.

Though understanding of contextual fear learning circuitry is important for fundamental understanding of normal human episodic memory, clinical applications of this research take two forms. The first is for therapeutic memory enhancement; both in storing stronger memories, and in preventing the loss of older memories. Degenerative disorders such as Alzheimer's disease (AD) and frontotemporal dementia (FTD), and to a lesser degree normal cognitive aging, are characterized by impairments in memory. This research may have applications for formation of stronger memories. The second application is for therapeutic erasure, or at least degradation of the emotional component, of acute traumatic memories leading to anxiety disorders such as PTSD. This research relates to this problem as well.

Many of the symptoms of AD, in particular, have long been attributed to disruptions of cholinergic function (Coyle et al., 1983). Parallels to Parkinson's disease have been drawn, where instead of selective loss of dopaminergic neurons, there is selective loss (at least in early stages) of cholinergic innervation in AD. My work suggests that enhancing cholinergic release may be able to rescue some of the deficits observed in early human AD, particularly for the recall of complex episodes that share characteristics with rodent contextual memory. Indeed, preliminary trials of deep brain stimulation of the cholinergic nucleus basalis of Meynert have shown improvements in some patients that persist almost a year after the conclusion of the stimulation. This improvement is seen over and above concurrent patient medications also intended to increase cholinergic activity (Kuhn et al., 2015). This promising trial suggests that DBS of forebrain cholinergic populations may stave off cognitive decline due to loss of cholinergic populations in the course of the disease. Open questions include whether there is a difference in benefit for previously-formed memories and novel memory encoding, as well as whether different cholinergic subregions should be targeted for memory of facts and memory of entire episodes. My work, as well as other work in rodents, suggest that cholinergic impact may differ in these different cases. Future studies using rodent models of AD (Oddo et al., 2003), as well as examining normal cognitive decline in rodents due to aging (Cansev et al., 2015), may be able to test some of these hypotheses.

The influence of cholinergic signaling on traumatic memory, anxiety disorders, and PTSD is a relatively recent topic of study (Zimmerman and Soreq, 2006). Traumatic memories can become debilitating to an individual's daily functioning, and methods to erase or reduce the impact of these traumatic memories has been a topic of some debate (Maren, 2011). Much of this debate has focused on the neural differences underlying extinction and reconsolidation, and

how to improve the effectiveness of the two for human therapeutic improvement. The influence of cholinergic modulation on extinction has already shown some positive results.

During extinction, patients are exposed to contextual stimuli relating to the traumatic event in a safe setting, and fear expression typically decreases with repeated exposures. However, the challenge is that this extinction learning is context-dependent; fear often renews in alternative contexts. Cholinergic influences in this learning have already shown therapeutic promise, as low doses of scopolamine during extinction training in rats led to reductions in fear renewal in a novel context (Zelikowsky et al., 2013). My data suggest that an alternative method of enhancing extinction may be, paradoxically, to increase ACh during extinction learning. Computationally, secondary learning (such as extinction) is shown to be weaker than initial learning, and does not replace the original fear representation. Rather, this conflicting memory that the context is safe can only weakly exert control over behavior (Krasne et al., 2011). With enhanced ACh, it is possible that extinction will be promoted, as the absence of the shock would be encoded more strongly. This has the potential to facilitate the weak extinction memory in competing with the original fear memory, in order to control future expression of fear behavior.

Beginning with a contextual fear learning circuit of the DH, BLA, and mPFC, I went on to show how preventing the reactivation of the fear learning cells in the BLA can reduce the magnitude of fear recall at both recent and remote time points. This signaling has only transient importance during a recall event; once BLA fear-context cells are reactivated, downstream regions (potentially CeA) maintain the expression of fear even if the BLA inputs are silenced. Signaling and activation during learning leads to the ability to recall the memory at a later date, and ACh amplifies this learning signal in the presence of both neutral (context exploration) and highly salient (stress-enhanced fear learning) contextual experiences. If ACh release is

enhanced, stronger contextual memories are formed. If this signaling is blocked, it reduces the ability to learn about context. In DH and BLA, this leads to a loss of recognition of the context where a stressful event occurred the previous day. Furthermore, blockade of muscarinic transmission not only disrupts ongoing memory formation, but blocks the mechanism of sensitization to future contextual learning, while leaving (in DH) stimulus sensitization intact. This work deepens our understanding of contextual fear learning circuitry at multiple time points, as well as the influence of ACh on this circuitry during contextual memory formation and the formation of traumatic memories. I hope this work will lead to future research on titrating the strength of contextual memory formed by altering cholinergic signaling, and that this will lead to human therapeutic intervention in both cases of impaired memory formation and formation of maladaptively strong traumatic fear memories.

Chapter Five: References

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