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Separate basolateral amygdala projections to the hippocampal formation differentially modulate the consolidation of contextual and emotional learning

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SEPARATE BASOLATERAL AMYGDALA PROJECTIONS TO THE HIPPOCAMPAL FORMATION DIFFERENTIALLY MODULATE THE CONSOLIDATION OF CONTEXTUAL AND EMOTIONAL LEARNING

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CERTIFICATE OF APPROVAL

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To my husband, son, and our supportive families.

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ABSTRACT

Previous research investigating the neural circuitry underlying memory consolidation has primarily focused on single "nodes" in the circuit rather than the neural connections between brain regions, despite the likely importance of these connections in mediating different aspects or forms of memory. This focus has, in part, been due to technical limitations; however the advent of optogenetics has altered our capabilities in this regard, enabling optical control over neural pathways with temporal and spatial precision. The current set of experiments took advantage of optogenetics to control activity in specific pathways connecting brain regions in rats immediately after different kinds of learning.

Chapter 2 first established the use of optogenetics to manipulate activity in the basolateral amygdala (BLA), which has been shown to modulate memory consolidation for a variety of types of learning likely through its connections to various downstream regions. Using a one-trial inhibitory avoidance task, a simple and robust fear learning paradigm, we found that both post-training stimulation and inhibition of BLA activity could enhance or impair later retention of the task, respectively. Enhancement was specific to stimulation using trains of 40, but not 20, Hz light pulses.

Chapters 3 and 4 examined the projections from the BLA to the ventral hippocampus (VH) and medial entorhinal cortex (mEC) as the BLA's ability to influence the consolidation for many types of memory is believed to be mediated through discrete projections to distinct brain regions. Indeed, the BLA innervates both structures, and prior studies suggest that the mEC and VH have distinct roles in memory processing related to contextual and nociceptive (footshock) learning, such as those involved in contextual fear conditioning (CFC). Optogenetic stimulation or inhibition of the BLA-VH or BLA-mEC pathway after training on a modified CFC task, in

which the nociceptive or emotional stimulus (the footshock) and the context are separated, enabled experimental manipulations to selectively affect the consolidation for learning about one component and not the other. Optogenetic stimulation/inhibition was given to each candidate pathway *immediately after* the relevant training to determine its role in influencing consolidation for that component of the CFC learning. Chapter 3 results showed that stimulation of the BLA-VH pathway following footshock, but not context, training enhanced retention, an effect that was specific to trains of 40 Hz stimulation. Post-footshock photoinhibition of the same pathway impaired retention for the task. Similar investigations of the BLA-mEC pathway in Chapter 4 produced complementary findings. Post-context, but not footshock, stimulation of the pathway enhanced retention. In this particular case, only trains of 8 Hz stimulation were effective at enhancing retention.

These results are the first, to our knowledge, to find that BLA inputs to different structures selectively modulate consolidation for different aspects of learning, thus enhancing our understanding of the neural connections underlying the consolidation of contextual fear conditioning and providing a critical foundation for future research.

PUBLIC ABSTRACT

Multiple brains regions are known to be involved in emotionally influenced memory consolidation, an area of research of particular importance to brain-based disorders such as post-traumatic stress disorder (PTSD) and phobias, yet we know little about the specific and distinct neural pathways involved in different memory processes. We sought to better understand the neural circuitry underlying the consolidation of different aspects of memory involved in contextual fear conditioning, using a technique called optogenetics that allows activation or inactivation of certain brain regions or pathways via light-sensitive proteins expressed in the brain.

Our results show that posttraining manipulations of a region called the basolateral amygdala (BLA) can lead to both enhancement and impairment when memory is later tested. Importantly, the enhancement is only seen when a certain frequency of stimulation (here, 40 Hz) is used. Investigation of pathways between the BLA and ventral hippocampus suggest this pathway is important for footshock, but not context, learning in a task that requires both kinds of information. Conversely, the pathway between the BLA and medial entorhinal cortex is involved in context, but not footshock, learning.

This research is the first of its kind to examine different pathways from the BLA to determine their roles in the consolidation of different kinds of learning. This approach can be used to look at other pathways involved in learning and memory, but also pathways involved in other functions or disorders.

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LIST OF ABBREVIATIONS

AAV: Adeno-associated viral vector
ArchT: Archaerhodopsin
BLA: Basolateral amygdala
CeA: Central amygdala
CFC: Contextual Fear Conditioning
ChR2: Channelrhodopsin-2
DH: Dorsal hippocampus
IA: Inhibitory Avoidance
mEC: Medial entorhinal cortex
mPFC: Medial prefrontal cortex
VH: Ventral hippocampus

CHAPTER 1. INTRODUCTION

During the period immediately following an event, the memory for that event remains malleable, but over time, the strength of the memory increases, a process termed "memory consolidation." Due to the lack of immediate permanence of a memory, a window of opportunity exists during which the strength of memories for recent events can be modulated (either enhanced or impaired). Although considerable work has investigated individual structures underlying memory consolidation and their roles for different types of memory, little is known regarding how distinct pathways connecting diverse brain regions are involved in memory consolidation, particularly for different types of memories. In this introduction, I provide a brief background on the modulation of memory consolidation, with a particular focus on the roles of the basolateral amygdala (BLA) and the hippocampal formation. I then review interactions between the BLA and two regions of the hippocampal formation during memory consolidation with regard to different types of memories.

Memory Consolidation

The perseveration-consolidation hypothesis offered by Müller and Pilzecker in 1900 proposed that the memory for an event is initially unstable and, through ongoing processing, becomes more permanent, i.e., undergoes consolidation (Muller, 1900). Evidence supporting this idea indicates that disruption of normal brain processing has a detrimental effect on retention of recent events. A landmark study, conducted by Carl Duncan, supported the time-sensitive nature of memory consolidation when rats were given electroconvulsive shock following avoidance training. Manipulations given up to 15 minutes following training impaired retention as shown

by slower rates of learning across days (Duncan, 1949). However, electroconvulsive shock given after longer delays (> 1 h) had no effect on subsequent retention. Thus, Duncan concluded that memories are susceptible to disruption during this consolidation period and become more stable (and more difficult to disrupt) following this period, supporting a temporally limited period during which consolidation occurs.

In an effort to discover the ways in which memory is consolidated, manipulations of specific structures important for memory consolidation/modulation have been made using electrical stimulation (Gold et al., 1974), pharmacological intervention (Ellis and Kesner, 1983; McGaugh et al., 1990), and lesions (Parent et al., 1992; Parent et al., 1994; Parent et al., 1995). As consolidation is a time-dependent process, consideration of manipulation timing is necessary (McGaugh, 1966, 1989). Pre-training manipulations can affect the acquisition of a memory, leading to confounds such as effects on performance. In contrast, the use of posttraining manipulations ensures that any observed effects are due to a modulation of consolidation processes. Tests of long-term retention are made at a designated time after training (at least 24 h) or by measurements of learning across multiple days of training.

Evidence indicates that emotional arousal following an event also alters retention. Much work has investigated the mechanisms underlying this phenomenon, and studies suggest that emotional arousal activates the amygdala. The BLA in particular is critical for mediating the effects of emotional arousal on memory consolidation (Cahill and McGaugh, 1996, 1998). Prior work found emotionally arousing experiences increase circulating stress-related hormones including epinephrine and glucocorticoids. Systemic administration of these hormones immediately after

avoidance training, but not at longer delays, enhances retention (Gold and Van Buskirk, 1975; Gold and Van Buskirk, 1976), and amygdala lesions block these memory-enhancing effects (Cahill and McGaugh, 1990). Administration of glucocorticoid receptor agonists or β-adrenergic receptor agonists into the amygdala enhances retention (Liang et al., 1990; Introini-Collison et al., 1991). However, enhancement occurs only when injections are given immediately following an event but not at longer (30-120 min) delays. Thus, the involvement of the BLA in memory consolidation, especially following events inciting emotion, has led to a long and continued investigation into the BLA and memory modulation.

Basolateral Amygdala

The BLA is a heterogeneous structure composed primarily of pyramidal glutamatergic neurons (~90%), which serve as the principal projection cells for the BLA, with a much smaller proportion of inhibitory GABAergic interneurons (McDonald, 1984; Carlsen, 1988). The BLA modulates the consolidation of a wide variety of memory types including spatial and cued water maze tasks (Packard et al., 1994; Hatfield and McGaugh, 1999), contextual fear conditioning (LaLumiere et al., 2003; Huff and Rudy, 2004), conditioned taste aversion (Miranda et al., 2003), novel object recognition (Roozendaal et al., 2008b), and inhibitory avoidance (IA) (Lalumiere et al., 2004b; LaLumiere et al., 2005; Roozendaal et al., 2008a; Campolongo et al., 2009). The amygdala plays a modulatory role in different types of memory that are dependent on dissociable areas (McGaugh, 2002; Malin and McGaugh, 2006; Paz et al., 2006; Chavez et al., 2013), including those that do not involve fear responses (Liang and McGaugh, 1983; Introini-Collison and McGaugh, 1988; Salinas et al., 1993). Although manipulations of BLA activity and memory have occurred mostly in rodent models, human imaging studies support a BLA-memory

modulation link (Cahill et al., 1994; Nielson and Jensen, 1994; Cahill et al., 1995; Cahill et al., 1996). Whereas the BLA appears to modulate the consolidation for many different kinds of memories, other brain regions play more selective roles in the processing for specific kinds of memories and, in fact, the BLA is highly interconnected with many of these other structures that influence different kinds of memories (Pitkanen, 2000).

Contextual Fear Conditioning (CFC)

Considerable effort has been directed toward investigating the neurobiology of fear conditioning, including contextual fear conditioning (CFC). In a traditional CFC task, a particular context is paired with a footshock leading to a learned association between the two (Fanselow and Dong, 2010). Multiple footshocks can be presented in one training session and animals may undergo repeated days of combined context/footshock exposure. Re-exposing the animal to the context where conditioning previously took place and measuring the amount of the animals freezing can serve as an index of retention.

Substantial evidence from CFC studies indicates that a brief footshock with minimal time spent in a context produces poor retention of the training, an effect reversed by increased exposure to the context before or after footshock (Fanselow, 1986, 1990; Kiernan and Westbrook, 1993; Pugh et al., 1997; Liang, 1999; Stote and Fanselow, 2004; Landeira-Fernandez et al., 2006). Although the context and footshock are usually presented together, it is possible to present training for the context and footshock on separate days. This allows investigation into the function of a given structure or pathway in processing the separate mnemonic components of a task. Previous work has used separation of training to show dissociable learning about context vs. nociceptive stimulus (footshock) in a modified version of CFC (Liang, 1999; Stote and

Fanselow, 2004). Therefore, the current experiments used a CFC task that separates the context and footshock training (described in the Methods section below) to investigate whether efferent BLA projections to different brain regions modulate the consolidation of different CFC components.

Prior work indicates that BLA lesions alter freezing associated with fear memories as well as unconditioned freezing, suggesting a potential confound with using freezing as a measure of retention (Vazdarjanova et al., 2001). Utilizing multiple measures of retention for a memory allows a better understanding of this distinction. Vazdarjanova and McGaugh (1998) lesioned the BLA of rats prior to habituation in a Y-maze with contextually distinct arms. During training, rats were confined to only one arm while receiving footshocks. During retention testing, rats were permitted to explore the entire maze. Memory indices included freezing as well as approach, entries, and time spent in the shock-associated arm. Rats with BLA lesions showed decreases in freezing whether or not they received footshocks on the second day of training. However, BLA lesions increased the latency to enter the shock-arm and decreased number of arm entries and time spent in the shock arm compared to non-shocked controls. Importantly, this points to an inability to express the learning via freezing rather than not freezing due to inability to learn. Alternative measures of retention (like avoidance or time spent in a shock-associated chamber) allow this learning-performance distinction to be made and may provide better insights into the memory associated with contextual fear conditioning. The IA and modified CFC tasks used in the following experiments rely on an additional index of retention, latency to enter a shock chamber, in an effort to avoid potential freezing confounds.

Hippocampal Circuitry

The hippocampus is a prime candidate for differential modulation of context vs. footshock learning, as many parts of the hippocampal formation receive BLA input and are involved in memory modulation. The dorsal and ventral compartments of the hippocampus both play roles in memory but differ in the types of information processed during different tasks or types of learning (Fanselow and Dong, 2010). The dorsal portion of the hippocampus is important for spatial processing and navigation (Fanselow and Dong, 2010). The ventral portion of the hippocampus, however, is implicated in processing emotion and especially negative emotion. Projections from the BLA synapse onto ventral CA1 neurons terminating in multiple layers including the stratum pyramidale, stratum oriens, and stratum radiatum (Pikkarainen et al., 1999) and recent work supports the functionality of this connection (Felix-Ortiz and Tye, 2014). In contrast, there is no direct projection from the BLA to the dorsal hippocampus (DH); however, the entorhinal cortex (EC) receives projections from the BLA and then projects to the dorsal hippocampus providing an indirect connection between the BLA and DH (Pitkanen et al., 2000; Witter and Amaral, 2004). Recent work suggests that BLA inputs to the EC influence memory processing (Sparta et al., 2014) as will be discussed more below. This separation of anatomical connection supports functional separation of the dorsal and ventral regions of the hippocampus as well (Moser and Moser, 1998; Fanselow and Dong, 2010).

Dorsal hippocampus. The DH contains a large proportion of place cells, which are involved in processing spatial information (O'Keefe and Dostrovsky, 1971; Jung et al., 1994). For this reason, investigations of contextual information processing, including spatial components of many learning tasks, have highlighted the DH. CFC training (as previously described) has been

used to examine the DH during the contextual/spatial components of CFC learning. Pretraining blockade of NMDA receptors (Stote and Fanselow, 2004) or posttraining impairment of protein synthesis (Barrientos et al., 2002) in the DH relative to context pre-exposure training impair the facilitation of pre-exposure on the context-shock association. Indeed, spatial learning requires the integrity of the DH, but the information regarding spatial location does not appear to be processed in one specific DH location. Only a small percentage of DH tissue (~20-40%) in a nonspecific location was shown to be necessary for efficient learning in a Morris water maze task (Moser et al., 1995).

Other studies note a similar involvement of the DH following CFC conditioning, while ruling out involvement following other components of associative learning. For example, if DH lesions are made 1 day after a context-fear conditioning session, freezing is reduced when the rat is placed back in the context (Kim and Fanselow, 1992). Similar DH lesions following tone-fear conditioning did not affect freezing when the tone was re-presented, supporting a dissociation of memory components. Infusions of d-amphetamine into the DH following training on a water maze enhanced retention on a spatial, but not on a cued, version of the task (Packard et al., 1994). Important to the current investigation, this set of experiments showed BLA involvement in both the spatial and cued versions of the water maze.

Previous investigations of BLA-DH interactions in memory consolidation were limited by techniques available. Microinfusions of the muscarinic cholinergic agonist oxotremorine into the DH enhanced memory for the modified CFC task when given after context exposure, but not footshock, training (Malin and McGaugh, 2006). Oxotremorine injections in the BLA

immediately after both context and footshock training caused similar enhancements indicative of the BLA's involvement in both components of the task and more general memory modulation. Roozendaal and colleagues (1999) found that infusions of glucocorticoid agonists into the DH following IA training enhanced retention, an effect blocked by pretraining infusions of a β_1 adrenergic antagonist into the ipsilateral BLA. BLA lesions also block memory-enhancing effects of glucocorticoid administration into the DH following IA training (Roozendaal and McGaugh, 1997b).

Entorhinal cortex. As there is no direct projection from the BLA to the DH, the BLA projection to the EC provides an indirect pathway for BLA modulation of spatial memory information as information is passed from the EC to the DH (Fyhn et al., 2004). BLA projections reach the medial entorhinal subfield on the caudal end of the structure (Pikkarainen et al., 1999), and basal subdivisions of the amygdala provide heavier projections to the EC, and lateral amygdala regions provide lighter projections. In turn, there is a strong projection from the medial EC (mEC) to the hippocampus (Zhang et al., 2013). Much like the DH, the mEC contains cells in layers II and III called grid cells that fire in response to specific spatial locations (Hafting et al., 2005; Sargolini et al., 2006) and recent modeling work suggests that mEC grid cells are capable of providing the primary input to hippocampal place cells, though experimental work will need to investigate this possibility alongside other place cell inputs (Azizi et al., 2014). Grid cells are topographically organized with even spacing, similar size, and shared properties in local groups (Hafting et al., 2005). Collections of grid cells fire in similar patterns across trials in the same location, limiting the possibility that internal cues are providing these patterns; however, these grid-like patterns

persist even when the animal is in darkness suggesting internal cues are *supportive* of applying external contextual/spatial information to a given collection of grid cells.

Previous studies have examined EC involvement in memory; however, many performed pretraining manipulations (Maren and Fanselow, 1997; Bannerman et al., 2001) or examined extinction training (Ji and Maren, 2008; Baldi and Bucherelli, 2014) making connections to memory consolidation difficult. Many acquisition studies are in conflict with one another regarding the involvement of the EC in CFC memory as some negate EC involvement in context/spatial learning (Phillips and LeDoux, 1995; Good and Honey, 1997; Bannerman et al., 2001). In contrast, few studies examined *posttraining* manipulations and resulting effects on CFC memory consolidation. Baldi and colleagues (2013) used tetrodotoxin injections into the EC or fimbria-fornix (another hippocampus input) bilaterally following fear conditioning to both a tone and footshock. Temporary inactivation of the EC impaired CFC retention when the inactivation was made 15 min or 1.5 hours after training but not 24 hours later. Impairments in acoustic CS retention were only seen when TTX was given 15 min after initial training. Fimbria-fornix inactivation did not impair retention of either context or acoustic CS retention and freezing did not generalize to an alternative context alone pointing to EC involvement in CFC memory consolidation. Another investigation trained rats on a tone-shock conditioning task. Rats received extracellular signal-regulated kinase (ERK) cascade inhibitors into the EC 40 min after training (as ERK phosphorylation is increased following fear conditioning; peak 90 min; Hebert and Dash, 2004). Retention for contextual and cued memory was tested 48 hours later. Blocking the ERK cascade following training enhanced context, but not tone, fear retention. More recent work has used optogenetics to examine the BLA-mEC pathway during acquisition or expression

of CFC memory (Sparta et al., 2014). Inhibition of the pathway during acquisition significantly decreased freezing behavior when rats were later exposed to the original training context. Pathway inhibition during re-exposure did not affect freezing. Additionally, mice lacking the monosynaptic projection from the EC to the DH are impaired on a spatial working memory task (Suh et al., 2011). However, involvement of the BLA-mEC pathway in the consolidation of fear conditioning was unclear prior to the present studies.

Ventral hippocampus. There has been some controversy about the role of the VH in processing different types of memory (see Fanselow and Dong, 2010 for larger discussion). Some studies found VH involvement for tasks with a spatial memory component (Ferbinteanu and McDonald, 2001; Rudy and Matus-Amat, 2005), whereas others have discounted the VH as a site for spatial processing (Moser et al., 1995; Pothuizen et al., 2004).

However, the VH has consistent support in processing anxiety and emotional aspects of learning (Jung et al., 1994; Moser et al., 1995; Moser and Moser, 1998; Ferbinteanu and McDonald, 2001; Pothuizen et al., 2004; Zhang et al., 2004; Hunsaker and Kesner, 2008). For instance, lesions of the hippocampus confined to more ventral locations that do not connect or project to the dorsal/contextual-processing regions cause reduced anxiety responses on an elevated plus maze (Kjelstrup et al., 2002). Additionally, posttraining, but not pretraining, lesions of the VH impaired CFC and auditory conditioning and pretraining inactivation via muscimol impaired only auditory fear conditioning (Maren and Holt, 2004). Hippocampal damage in human populations is likewise associated with emotional dysregulation (as in depression and posttraumatic stress disorder; Bonne et al., 2008). This supports VH activity during CFC

learning, yet also suggests the VH *may* be playing a slightly more general role than the DH in processing separate components of a task. The current experiments, therefore, sought to provide evidence regarding whether BLA inputs to the VH are differentially involved in a task that involves both spatial and emotional components.

Optogenetics

Previous findings highlight the need for temporally precise control of neuronal activity, including within the BLA, in memory consolidation studies. During and following learning, BLA activity facilitates and couples with activity in downstream structures, including rhinal cortices, in the gamma frequency range (35-45 Hz; Bauer et al., 2007; Popescu et al., 2009). The strength of gamma frequency coupling between structures increases across learning trials, suggesting that concurrent activity in the BLA and downstream regions in this frequency range is a critical component of memory consolidation. However, these studies were primarily correlational and thus unable to determine the causal relationship between such activity and the resulting strength of memory. Thus, whether driving BLA inputs to other regions using gamma frequency stimulation will alter memory consolidation is unknown.

Optogenetic techniques allow control of activity in specific neurons via controlling light-sensitive proteins called opsins, and importantly, can also do so for distinct projections with temporal precision. Expression of an opsin can be safely delivered through an adeno-associated viral vector (AAV; Monahan and Samulski, 2000; Yizhar et al., 2011). The promoter region driving a given construct can limit the expression of opsins to a designated cell type. The CaMKIIα promoter limits expression to glutamatergic cells (Tye et al., 2011), the putative

projection neurons of the BLA (McDonald, 1982a; Millhouse and DeOlmos, 1983; McDonald, 1984). Importantly, spatial precision is also gained by delivering light to a specified target region. As virally transduced neurons will express light-sensitive opsins not only in their cell bodies but also down their axons (Gradinaru et al., 2010; Deisseroth, 2011; Mattis et al., 2012), illumination can be provided to a site of initial transduction or a downstream structure, either stimulating or inhibiting a given site or specific projection while leaving other axons unaffected (Yizhar et al., 2011). Perhaps most beneficial is the ability of optogenetics to be used in freely moving animals.

Two opsins were used in the current experiments based on their advances in temporal and spatial control. Channelrhodopsin-2 (ChR2) was developed from the algae *Chlamydomonas reinhardtii* and allows fast excitatory transmission via membrane-bound cation channels (Li et al., 2005). Although there are many mutants across a range of properties (e.g., power density), the engineered ChR2(E123A) mutant is advantageous due to faster channel closing resulting in reduction in double-spiking, the ability to follow light pulses up to 200 Hz, and elimination of plateau potentials (Gunaydin et al., 2010; Mattis et al., 2012). This makes ChR2(E123A) a good choice for ultrafast optogenetic control especially when a precise neural signal is necessary. This ChR2 mutant is maximally driven by blue light (peak activation ~470 nm; Gunaydin et al., 2010). ArchT (*archaerhodopsin-3* from *Halorubrum* strain TP009) is an outward proton pump capable of producing a hyperpolarized neuronal state (Chow et al., 2010; Mattis et al., 2012). Unmodified versions have problems with membrane trafficking, whereas the mutant version eArchT3.0 has reduced intracellular accumulation due to enhanced trafficking sequence modifications (Han et al., 2011; Mattis et al., 2012). This allows for increased photocurrent size

and increases in targetable tissue volumes as well as rapid activation (1.5-3 ms) of the pumps when driven by yellow-green light (peak activation ~566 nm; Chow et al., 2010; Han et al., 2011). Thus, eArchT3.0 allows better inhibitory control over the BLA and pathways to downstream targets.

Based on measurements from mammalian brains (Yizhar et al., 2011), light output of 10 mW at the fiber tip will produce ~1 mW/mm^2 of light (the minimum amount necessary to produce opsin activation), up to 1 mm directly away in a spherical shape centered 0.5 mm away from the fiber tip (Aravanis et al., 2007; Gradinaru et al., 2009; Yizhar et al., 2011). These parameters provide sufficient light for opsin activation in at least 0.4 mm^3 of tissue (Yizhar et al., 2011). Activation of opsins can be accomplished with very narrow/specific wavelength delivery matched to peak wavelength activation of the opsins as discussed above.

Summary

The BLA modulates memory consolidation for emotionally relevant events, yet does so in a very general manner, showing involvement across many types or components of memory. It has long been hypothesized that the BLA exerts this general modulation via projections to distinct regions throughout the forebrain. In contrast to the BLA, these downstream regions have more specific functions in memory modulation including the roles for the VH and EC in emotion and contextual/spatial information, respectively. However, investigating the specific roles of BLA inputs to these different regions, particularly in regard to different types of memory processing, during consolidation has been difficult. Selective control of the BLA using specific temporal parameters enables investigation into the causal nature of the relationship between neural activity

at specific frequencies and resulting changes in memory strength. Optogenetics was used to examine and target the BLA and specific pathways emanating from the BLA during the immediate posttraining period as part of an ongoing investigation into memory consolidation with increased temporal and spatial precision.

Therefore, the current set of experiments sought to address three major questions:

- How does stimulating vs. inhibiting the BLA influence the consolidation of learning following an IA task? **Chapter 2**
- How does stimulating vs. inhibiting the BLA→VH pathway influence the consolidation of learning for the context vs. footshock during a CFC task? **Chapter 3**
- How does stimulating the BLA→mEC pathway influence the consolidation of learning for the context vs. footshock during a CFC task? **Chapter 4**

General Methods

The following section provides the detailed methods for all experiments. Chapters 2-4 provide the experimental design and any individual variations.

Subjects. All rats were single housed in a temperature-controlled environment under a 12-hour light/dark cycle (lights on at 06:00) and allowed to acclimate to the vivarium at least three days before surgery. Food and water were available ad libitum throughout all training and testing. All procedures were in compliance with NIH guidelines for care of laboratory animals and approved by the University of Iowa Institutional Animal Care and Use Committee.

Surgery. Three to seven days after arrival, rats were anesthetized using ketamine HCL (10 mg/kg, i.m.) and xylazine HCl (3 mg/kg, i.m.) and placed in a stereotax (Kopf Instruments, Tujunga, CA). Rats received the appropriate virus microinjections [0.35 µl in all cases; AAV5-CaMKIIα-hChR2(E123A)-eYFP, the ChR2(E123A) control AAV5-CaMKIIα-eYFP; AAV5-CamKIIα-eArchT3.0-GFP, or its control AAV5-CamKIIα-GFP] delivered bilaterally into the BLA. In order to allow sufficient time for opsin expression in cell bodies or along the axons to the efferent structure prior to the start of behavioral training, rats underwent a second surgery two to four weeks after the first in which guide cannulae or fiber optic probes were bilaterally implanted, aimed at the BLA, VH, or mEC. The rats were given 1 week to recover from surgical procedures before behavioral training began.

Optical inhibition or stimulation. Stimulation experiments used CaMKIIα-hChR2(E123A)-eYFP and its respective control CaMKIIα-eYFP. The constructs for the inhibition experiments were either CAG-ArchT-GFP or CaMKIIα-eArchT3.0-eYFP and their respective controls CAG-GFP or CaMKIIα-eYFP. The CaMKIIα promoter limits gene expression to BLA glutamatergic cells (Tye et al., 2011), and the CAG promoter is a general cellular promoter. Illumination of BLA cell bodies was conducted at least 3 weeks after viral injection and illumination of BLA terminals in the VH or mEC was conducted at least 5 weeks after viral injection.

For the main experiments, two different sets of optics were used. For experiments in Chapter 2, optical probes were constructed by gluing an optical fiber (200 µm core, multimode, 0.37 NA) into a 24 ga internal cannula (Plastics One). The fiber extended 0.5 mm beyond the end of the cannula. When inserted into a chronically implanted 20 ga guide cannula, the fiber tip terminated

0.5 mm prior to the intended target site. For experiments in Chapters 3 and 4, optical probes were constructed in which the fiber optic (200 μm core, multimode, 0.37 NA) was glued into a stainless steel ferrule assembly with one side extending out of the end of the ferrule to be chronically implanted into tissue (modified from Sparta et al., 2012). The optical probe terminated 0.5 mm prior to the site intended to receive light (Stuber et al., 2011). The opposing side of the optical probe was polished and connected to a fiber optic leash via a ceramic split sleeve.

In all experiments, the fiber leash's other end (FC/PC connection) was threaded through a metal leash to protect the fiber from being damaged by a rat and attached to a 2:1 splitter allowing bilateral illumination. The splitter's single end was attached to an optical commutator allowing free rotation of the optic leashes connected to the rat. An insulated optical fiber connected the commutator to the appropriate laser source (DPSS, 300 mW, 473nm for ChR2(E123A) or 561 nm for eArchT3.0, with a multimode fiber coupler for an FC/PC connection). Light output was adjusted to 10 mW at the fiber tip based on previous work (Gradinaru et al., 2009; Yizhar et al., 2011; Deisseroth, 2012; Stefanik et al., 2013), as measured by an optical power meter. In most cases, the comparison control was be a "sham-control" in which no illumination was provided. Illumination was provided continuously (eArchT3.0 experiments) or controlled by a Master-8 stimulator (ChR2 experiments; as noted below). Rats received optical stimulation or inhibition in a separate holding chamber. All stimulation and inhibition was given bilaterally.

Behavioral training. Rats were handled individually 1 minute per day for 3 days prior to the start of training. All experiments used a standard IA chamber consisting of a trough-shaped box

divided into two sections: one-third made of white plastic and illuminated (30 cm) and second two-thirds (60 cm) made of stainless steel and darkened (Figure 1A). The dark chamber was connected to a shock generator and timer, controlled by the experimenter. The two sides were capable of being divided by a stainless steel door retractable through the floor.

Experiments in Chapter 2 utilized a standard IA task as outlined in Figure 1B. During training, rats were placed in the lit side, with the door fully retracted to allow free exploration. After a rat fully crossed into the dark compartment, the door was raised to prevent return to the lit compartment. When the rat reached the end of the dark compartment, it received a single inescapable footshock (0.5 mA, 1 s duration for ChR2(E123A) experiments; 1.0 mA, 2 s duration for ArchT experiments; different footshock intensities were used to prevent ceiling and floor effects, respectively) and was removed from the apparatus. For retention testing 48 h later, rats were placed in the lit side, with the door retracted. Rats' latencies to cross into the dark compartment were used as the index of retention with a maximum latency of 600 s.

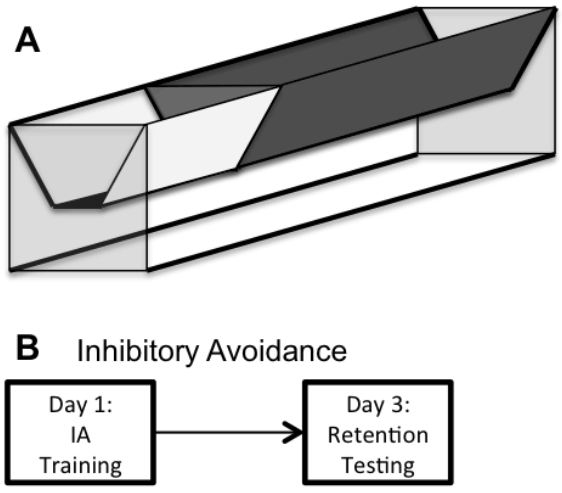

Figure 1. IA chamber and behavioral timelines.

A, Diagram of apparatus used in both IA and modified CFC training. **B,** Schematic diagram of the timeline for IA behavioral training and testing. **C,** Schematic diagram of the timeline for CFC behavioral training and testing.

Because retention latencies were expected to be low in inhibition experiments (preventing the observation of differences between groups), CMIA training was conducted

immediately after retention latencies were recorded, as detailed elsewhere (LaLumiere et al., 2003), to provide an additional index of retention of original CFC training. In brief, rats received a continuous mild footshock (0.35 mA) after crossing into the dark compartment until they returned to the lit compartment. The number of footshocks administered before a rat remained in the lit chamber for at least 200 s was recorded.

Experiments in Chapters 3 and 4 utilized a modified CFC task, which has been described previously and used in the past to provide this kind of dissociable learning (Liang, 1999; Malin and McGaugh, 2006). Figure 1C shows a general timeline of the behavioral training and testing. Briefly, rats first underwent context pre-exposure to the entire apparatus for 3 min (day 1). The following day, rats were confined to the darkened side of the chamber and receive an immediate inescapable footshock (day 2). Footshock parameters varied between stimulation (single 1 mA, 1s footshock) and inhibition (single 1mA, 2s or double 1 mA, 1s footshocks) experiments to prevent ceiling and floor effects, respectively. Immediately after *either* context or footshock training, rats received optical stimulation or inhibition of BLA terminals in the downstream target. Specific parameters are noted in experiments below. Retention was tested two days later (day 4) when rats were again placed in the illuminated compartment of the apparatus and allowed free access to the entire apparatus. Latency to cross over into the darkened compartment (maximum 600 s) was used as the primary index of retention.

Data analysis. Behavioral data was analyzed with one-way analyses of variance (ANOVAs) with post-hoc t-tests between groups with Tukey corrections to the p value ($p < 0.05$ for significance) or t-tests.

Verification of opsin expression and histology. Rats were killed with an overdose of sodium pentobarbital (100 mg/ml; i.p.) and then perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by PBS containing 4% paraformaldehyde. Brains were removed and stored at room temperature in 4% paraformaldehyde PBS for 24-48 hours until sectioning. The brains were coronally sectioned (50-75 μm) on a vibratome and mounted onto either gelatin-subbed slides for staining or stored in anti-freeze solution at -20º C until immunohistochemical procedures began. Verification of the optical probes' and cannula placement was performed with a standard Nissl stain preparation (Cresyl violet) and light microscopy according to the Paxinos and Watson atlas (2005). Any rat that's optical probe tip terminated outside of the target region was excluded from final analysis. Expression of opsins in the cell bodies and axon terminals were verified using immunohistochemistry procedures, as described previously (Stefanik et al., 2013). In brief, tissue sections were incubated in anti-GFP primary antibody solution for 48-72 h (PBS, 2% goat serum, 0.4% Triton-X, rabbit 1:20,000 primary antibody (Ab**cam**). Sections then were incubated for 1 h in a biotinylated anti-rabbit secondary antibody solution (K-PBS; 0.3% Triton-X; goat, 1:200, Vector Labs) and incubated in an ABC kit (Vector Labs) for 1 h. Sections were developed in DAB for ~5-10 minutes before being mounted onto gelatin-subbed slides. Slides were allowed to dry before being dehydrated with reverse alcohol washes for 1 minute each, soaked in citrosolv for a minimum of 5 minutes, and coverslipped with DePeX (Electron Microscopy Sciences). GFP/eYFP expression was assessed using a light microscope.

CHAPTER 2. POSTTRAINING OPTOGENETIC MANIPULATIONS OF BASOLATERAL AMYGDALA ACTIVITY MODULATE THE CONSOLIDATION OF INHIBITORY AVOIDANCE MEMORY IN RATS.

Considerable evidence indicates that the basolateral amygdala (BLA) modulates memory consolidation for a variety of different types of learning including spatial and cued water maze tasks (Packard et al., 1994; Hatfield and McGaugh, 1999), contextual fear conditioning (LaLumiere et al., 2003; Huff and Rudy, 2004), conditioned taste aversion (Miranda et al., 2003), novel object recognition (Roozendaal et al., 2008b), and inhibitory avoidance (IA) (Lalumiere et al., 2004b; LaLumiere et al., 2005; Roozendaal et al., 2008a; Campolongo et al., 2009). Such studies' conclusions have necessarily been limited by the caveats associated with the techniques. Microinjections do not permit temporally precise control of neuronal activity, either in terms of controlling neuronal spiking or in limiting the drugs' effects on BLA activity to a controllable time window, whereas electrical stimulation affects all neurons and axons within the vicinity of the electrode, including fibers of passage, and cannot be used for providing inhibition.

Recent findings highlight the need to have temporally precise control of neuronal activity, including within the BLA, in memory consolidation studies. Physiological recordings suggest BLA activity facilitates and couples with activity in downstream structures in the gamma frequency (35-45 Hz) range across learning trials (Bauer et al., 2007; Popescu et al., 2009). For example, if BLA activity is disrupted, gamma activity is reduced downstream in the striatum further supporting the notion that the BLA is promoting synchronous oscillations of activity in both structures (Popescu et al., 2009). The strength of gamma frequency coupling between structures appears to increase across learning trials and this synchronized activity likely induces

synaptic plasticity in target neurons (Bauer et al., 2007; Popescu et al., 2009). Whether stimulating the BLA and/or downstream structures at similar frequencies enhances retention has been impossible to determine with pharmacological manipulations. Similarly, whether BLA activity within precise temporal parameters is necessary for normal consolidation has been difficult to examine, despite previous studies suggesting that emotionally arousing stimuli produce changes in amygdala norepinephrine levels within a temporally limited window (Galvez et al., 1996; Hatfield et al., 1999).

The development of optogenetics has enabled selective control of neuronal activity with millisecond temporal precision and has been used in a number of contexts to examine the influence of neuronal activity on behavior (Gradinaru et al., 2009; Stuber et al., 2011; Tye et al., 2011). Thus, experimental manipulations using optogenetics have significant potential to inform investigations into memory consolidation through more precise control of neuronal activity as well as to address questions regarding the temporal aspects of BLA activity after learning that influence consolidation. However, no study has utilized optogenetics to modulate neuronal activity during the posttraining period, leaving unaddressed whether such manipulations will influence consolidation and under what conditions they will do so. Therefore, the present experiments examined whether posttraining stimulation and inhibition of BLA neurons modulates the consolidation of IA learning, using optical activation of either a "ChETA" version of the cation channel channelrhodopsin-2 (ChR2(E123A)) that permits high-frequency stimulation of neurons (Gunaydin et al., 2010; Yizhar et al., 2011) or the inhibitory outward proton pump ArchT (Chow et al., 2010), respectively. In particular, these experiments investigated whether stimulating BLA glutamatergic neurons at specific frequencies after IA

training enhances consolidation and whether inhibiting BLA activity for different lengths of time impairs consolidation.

Materials and Methods

Subjects. Male Sprague-Daley rats (200-250g at time of first surgery; Charles River, Wilmington, MA; n = 152) were used for this study.

Surgery. One week after arrival, rats received virus microinjections into the BLA (coordinates: 2.8mm posterior and 5.0mm lateral to Bregma, and 8.6mm ventral to skull surface). Injectors were left in place for 5 min to permit diffusion. Two weeks later, rats underwent the second surgery in which guide cannulae aimed bilaterally at the BLA (2.8 mm posterior and 5.0 mm lateral to bregma and 6.5 mm ventral to the skull surface) were implanted and secured by surgical screws and dental acrylic. Obdurators cut to the length of the guide cannulae with no projection were used to maintain openness and removed only during optical stimulation/inhibition. Rats were given 1 week to recover before behavioral training.

Behavioral procedures. One week after cannula implantation, rats were trained on an inhibitory avoidance (IA) task in a standard IA chamber. During training, rats were placed in the lit side, with the door fully retracted to allow free exploration. After the rat crossed into the dark compartment, it received a single inescapable footshock (0.5 mA, 1 s duration for ChR2(E123A) experiments; 1.0 mA, 2 s duration for ArchT experiments; different footshock intensities were used to prevent ceiling and floor effects, respectively) and was removed from the apparatus. During optical stimulation/inhibition, the probe was inserted into the previously implanted guide cannula, terminating ~0.5 mm dorsal to the virus injection site. For retention testing 48 h later,

rats were placed in the lit side, with the door retracted. Rats' latencies to cross into the dark compartment were used as the index of retention with a maximum latency of 600 s.

ChR2(E123A) experiments. Immediately after IA training, rats that had been injected with the ChR2(E123A) virus received 15 minutes of optical BLA stimulation using the following parameters: 2-s trains of either 20 or 40 Hz light pulses (pulse duration = 10 ms), given every 10 s. In a control experiment, rats that had been injected with the eYFP-control virus also received trains of 20 or 40 Hz light pulses. Other rats that had received the ChR2(E123A) virus underwent IA training but received no footshock during training and received 15 min of trains of 40 Hz pulses, identical to those of the main experiment.

ArchT experiments. Immediately following IA training, rats that had been injected with the ArchT virus received continuous optical BLA inhibition for either 1 or 15 min. For those rats that received 15 min of illumination, immediately after retention latencies were recorded, the rats underwent continuous multiple-trial inhibitory avoidance (CMIA) training as an additional retention test, as detailed elsewhere (Coleman-Mesches and McGaugh, 1995; LaLumiere et al., 2003). In brief, the rats received a continuous mild footshock when they crossed into the dark compartment until they returned to the light compartment. The number of footshocks administered before a rat remained in the light chamber for at least 200 s was recorded and used as an additional index of retention of their original IA training. Two control experiments were also conducted. Rats that had been injected with the GFP virus underwent IA training with 15 min of posttraining illumination. Another group of rats that had been injected with the ArchT virus underwent IA training but received the 15 min of optical inhibition 3 h after training.

Anesthetized optrode recordings. For recording experiments, rats underwent surgeries as described above except the anesthesia was 1-2% isoflurane. After 3-4 weeks of incubation time, rats were anesthetized with 1-2% isoflurane and single neurons were recorded using an optrode (200mm optical fiber glued to a ~8 MΩ recording electrode, tips separated by 300-500 mm). Signals were amplified and filtered (0.3-3 kHz bandpass) using a NeuroLog system (Digitimer, Hertfordshire, England). Single neuron spikes were discriminated and digital pulses were led to a computer for on-line data collection with the use of a laboratory interface and software (CED 1401 Plus, Spike2; Cambridge Electronic Design, Cambridge, England). Isolated single neurons were tested for light responsiveness (excitation or inhibition) at a range of stimulation frequencies (1 Hz – 40 Hz for ChR2(E123A) and prolonged tonic stimulation for ArchT) using the appropriate laser light source with ~10 mW light output. Multiple neurons per rat were tested to confirm robustness of expression (>3-4 responsive minimum).

Results

Optical activation of ChR2(E123A) in the BLA enhances retention of IA learning. Rats' latencies to enter the dark compartment on testing day were recorded and are shown in Figure 2. Panel A shows the latencies of rats that were given trains of either 20 or 40 Hz light pulses for 15 min immediately after IA training. A one-way ANOVA revealed a significant effect of stimulation ($F_{(2,33)} = 8.695$, $p < 0.001$). Those rats that received trains of 40 Hz light pulses had significantly higher latencies to enter the shock compartment than those of the sham-control rats ($p < 0.05$), whereas the latencies of those rats receiving trains of 20 Hz stimulation were not significantly different from either of the other two groups. Neither eYFP-expressing control rats

(Figure 2B) nor no-shock controls (Figure 2C) exhibited significant latency differences compared to their respective 40 Hz groups ($p > 0.05$ in both cases).

Optical activation of ArchT in the BLA impairs retention of IA learning. The latencies of rats that received 15 min of immediate posttraining BLA inhibition via activation of ArchT are shown in Figure 3A and are significantly different from those of sham-control rats ($t_{(20)} = 1.884$, $p < 0.05$). Thus, BLA inhibition for 15 min immediately after IA training impaired retention. Figure 3B shows that those rats that had previously received optical inhibition of the BLA required more footshocks to learn to stay out of the shock chamber compared to sham-control rats during CMIA training, as a t test revealed a trend toward a significant difference in the number of footshocks between the two groups ($t_{(20)} = 1.971$, $p < 0.07$). As with the ChR2(E123A) experiments, to ensure that illumination alone was not producing the impaired retention, the BLA of another group of rats was transduced with GFP alone. Figure 3C shows the latencies of rats expressing GFP alone in the BLA that were given 15 min of illumination immediately after training, and no significant difference between the sham-control group and the illumination group was found ($p > 0.05$). To determine whether a shorter period of inhibition would also impair retention, a group of rats received 1 min of posttraining BLA inhibition. Figure 3D shows the latencies of those rats. No significant difference between the latencies of the sham-control rats and those receiving 1 min of illumination was found ($p > 0.05$). To determine whether such inhibition produced long-lasting changes in the BLA that could have been responsible for the impaired retention, another group of rats received 15 min of posttraining inhibition of the BLA 3 h after training. Figure 3E shows the latencies of those rats. No

significant difference between the latencies of the sham-control group and the illumination group was found ($p > 0.05$).

Histology. Electrophysiology results verified robust influence of ChETA and ArchT on BLA neuronal activity. Figure 4A shows the activity of a BLA neuron in a ChR2(E123A)-transduced rat in response to trains of 20 and 40 Hz light pulses, indicating that the light pulses, at the behaviorally effective parameters, were able to drive BLA neuronal spiking. Figure 4B shows the inhibition of spontaneous activity of a BLA neuron in an ArchT-transduced animal in response to 1 min and 15 min of illumination. Figure 4C shows a diagram of the BLA with 20 randomly selected representative cannula placement sites. Optical fiber ends were intentionally placed dorsal to the BLA to allow for illumination of the structure ventral to the inserted optical fiber (i.e., the BLA), as evidence indicates that the parameters used in this study (see Materials and Methods) produce a spherical shape of light approximately 1 mm in diameter centered 0.5 mm away from the fiber tip (Yizhar et al., 2011). Figures 4D and E show representative images of the BLA expressing ChR2(E123A) and ArchT, respectively, as visualized using immunocytochemical staining procedures. In both images, the BLA is densely labeled, as a result of opsin expression in the cells and surrounding neuropil. In addition, moderate staining is visible outside the BLA as a result of opsin expression in BLA axons that either terminate in those regions or pass through them (Tye et al., 2011). Any rats that did not have at least one third of the BLA expressing GFP/eYFP were excluded (n = 6). More than 90% of rats showed staining throughout > 90% of the BLA.

Discussion

These results demonstrate that there is a critical frequency of BLA activity that modulates memory consolidation as well as limited period and provide the first evidence that posttraining optogenetic manipulations of neuronal activity modulate memory consolidation. In particular, the ChR2(E123A) findings are the first in which activity in the glutamatergic cells, the putative projection neurons of the BLA, was selectively controlled immediately after training to modulate memory consolidation, an exciting advance that will enable additional investigations into the causal nature of the relationship between neural activity at specific frequencies and the resulting changes in memory strength and plasticity.

Posttraining optical stimulation of BLA glutamatergic neurons, using trains of light pulses in the gamma frequency range (40 Hz in the present experiments), enhanced retention of IA learning. Stimulation with trains of 20 Hz pulses did not significantly enhance retention. These findings are consistent with previous recording work examining BLA activity across learning trials in which BLA-generated oscillation activity in the gamma frequency range becomes increasingly coupled with activity in downstream structures, including the striatum and rhinal cortices (Bauer et al., 2007; Popescu et al., 2009). Increased oscillation power between the BLA and these learning-associated structures correlates with increased behavioral measures of late-stage learning, suggesting that synchrony in these structures is a key driver of memory facilitation (Bauer et al., 2007; Popescu et al., 2009). Moreover, across auditory associative learning trials, increases in gamma oscillation activity in the auditory cortex are positively correlated with behavioral outputs of learning, similar to those mentioned above, as well as significant changes in receptive field frequencies in A1 (Headley and Weinberger, 2011). Gamma activity then may

predict not only the strength of memory for a particular stimulus association but also the plasticity associated with that learning. However, these studies, by virtue of being recording studies, were correlative in nature, and, causal validation was not possible, making the current study a critical novel expansion in both in our understanding of memory consolidation processes and in our ability to perform experiments addressing the causal relationships between activity and plasticity/memory. With the use of the ChR2(E123A) mutant, the present study was able to drive BLA glutamatergic neurons at the relevant frequency and determine that similar activity patterns provided after training enhances retention of learning. Whether the enhanced retention due to driving BLA activity at this frequency occurs through coordinated activity in downstream structures is an intriguing issue that can now be addressed in future experiments using the present study's techniques.

The ChR2 mutant utilized here has been previously characterized physiologically (Gunaydin et al., 2010); however, the present study is the first to use it in a functional manner to selectively control neuronal output. Posttraining electrical BLA stimulation has been used to influence memory consolidation (Gold et al., 1975; Bass et al., 2012), but those studies did not utilize specific frequencies of stimulation and therefore could not relate their findings to recording studies demonstrating precise temporal BLA activation during learning. Moreover, electrical stimulation studies are necessarily limited by simultaneous activation of all neurons in the region of interest (irrespective of phenotype) as well as fibers of passage. In the present findings ChR2(E123A) expression, and thus stimulation, was limited to BLA glutamatergic cells through the use of a glutamatergic neuron-specific promoter (CaMKIIα; Tye et al., 2011) allowing selective modulation of BLA glutamate neurons. Selective control of the projection neurons of

the BLA is a crucial ability in order to investigate how BLA activity during the consolidation period alters plasticity and/or activity in downstream structures. The nearby central amygdala (CeA) is primarily GABAergic (McDonald, 1982b), precluding ChR2(E123A) transduction in the CeA as well as in the GABAergic intercalated cells of the amygdala (Tye et al., 2011). Though ArchT expression may have occurred in such cells, previous findings indicate that posttraining CeA microinjections do not modulate memory consolidation (Quirarte et al., 1997; Roozendaal and McGaugh, 1997a; Lalumiere et al., 2004b). Therefore, regardless of any CeA expression, it is unlikely that the current results in either set of experiments can be explained by direct optical manipulation of CeA activity, and significant transduction of nearby structures was not observed. Selective modulation of BLA was further ensured due to the fact that the center of illumination was targeted in the BLA. Based on analyses of the spread of light within brain tissue, as described in the Materials and Methods, very little light affected tissue outside the BLA. Thus, with both illumination and viral transduction targeted at and mostly restricted to the BLA, it is unlikely that the observed effects in either set of experiments were due to opsin activation in nearby structures.

The ArchT results indicate that inhibiting BLA activity immediately after IA training for 15, but not 1, min impaired retention. The findings establish an important advance in the ability to discover the temporal relationships between emotional arousal and the modulation of memory consolidation as well as set the stage for future experiments to use optogenetic inhibition in combination with other techniques. The data from the ArchT experiments are consistent with previous studies showing that BLA inactivation impairs retention for learning (Parent and McGaugh, 1994; Zanatta et al., 1997), though it should be noted that the CAG promoter is a

general cellular promoter, limiting interpretation of the findings based on specific cell type. However, these previous studies were unable to determine the temporal window in which emotional arousal led to BLA activation and the subsequent modulation of memory consolidation, despite the need to determine the initiation time and duration of BLA activity for normal memory consolidation. The present results, therefore, are not only the first to control BLA activity with the "on/off" precision of optical inhibition during memory consolidation but also to demonstrate the critical nature of BLA activity for an immediate and extended (greater than 1 minute) posttraining period. Previous work indicates that a footshock, akin to those used in IA experiments, increases norepinephrine levels in the amygdala during the first 15 min after the shock, which then return to baseline by 30 min after the shock (Galvez et al., 1996; Hatfield et al., 1999). Together with such findings, the present work suggests that emotional arousal, as found with a footshock, activates the BLA for more than 1 min and at least 15 min after the learning event and that such activation throughout this extended time period is crucial for normal memory consolidation. As emotional arousal-driven increases in amygdala norepinephrine levels are believed to be critical in driving the strength of the memory consolidation (Ferry and McGaugh, 2000; McIntyre et al., 2002), it may be that norepinephrine-driven activation of the BLA occurs within this more specific temporal window, at least in terms of modulating the resulting memory consolidation. However, other work has shown increases in amygdala norepinephrine levels lasting up to 3 h after IA learning (McIntyre et al., 2002), despite the current work showing that 15 minutes of inhibition is sufficient to produce memory impairment. Future studies combining the present techniques with analysis of the pathways governing norepinephrine release may help to resolve this issue.

Of particular importance, the present studies include several crucial control experiments. Optical stimulation of rats that underwent "no-shock" training had no effect on retention, indicating that driving BLA glutamatergic neuronal activity alone did not induce aversion to the dark compartment or "create" a memory, consistent with the hypothesis that posttraining BLA manipulations modulate the consolidation of memories for events that have been acquired immediately prior to this time point (McGaugh, 2004). As illumination, especially continuous illumination as with the ArchT experiments, may produce undesirable effects such as heating (Yizhar et al., 2011), no-opsin illumination-alone control experiments were conducted. In the present experiments, illumination alone, matching the parameters used in the main experiments, had no effect on subsequent retention, decreasing concerns about tissue damage or alterations from prolonged illumination. Moreover, the results from the delayed posttraining optical inhibition of the BLA indicate that inhibiting ArchT-expressing neurons 3 h after training (1) no longer has an effect on retention as it falls outside of the limited consolidation time window and (2) does not produce prolonged alterations in BLA neuronal activity when illumination is suspended that could alter retention performance on day of testing.

The current results open new avenues of investigation into how the modulation of memory consolidation occurs (Johansen et al., 2012). That BLA activity in the first 15 minutes immediately following an event is necessary for normal consolidation suggests that the actions of stress hormones and activation of brainstem systems, such as the noradrenergic system, must influence the BLA throughout this time window. Future studies, therefore, can use optogenetic control of the BLA in combination with manipulations of these emotional arousal systems to determine the temporal and causal relationships among these systems during memory

consolidation. Moreover, as previous work suggests that the BLA influences consolidation through projections to downstream structures (McIntyre et al., 2012), future use of optogenetics to control neural pathways connecting structures, via illumination of opsin-transduced axon terminals in the downstream structure (LaLumiere, 2011; Yizhar et al., 2011), should prove particularly informative. In particular, the causal relationship between specific frequencies of BLA activity (i.e., 35-45 Hz) and its projections to different downstream targets remains unexplored but should provide a wealth of knowledge regarding the interactions and coherence of these systems during consolidation.

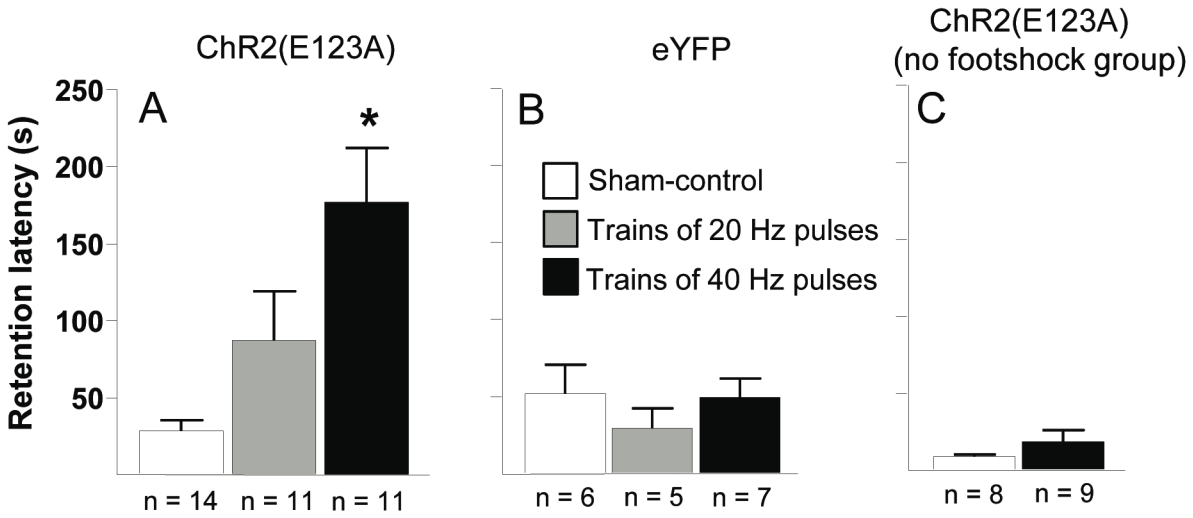

Figure 2. Retention effects of optical stimulation of ChR2(E123A)-transduced BLA neurons immediately after IA training.

A, Enhanced retention of rats given posttraining optical stimulation of the BLA, via activation of ChR2(E123A). The figure shows the mean latencies, in s (+/- SEM), to enter the shock compartment during the retention test as well as the n of each group underneath each bar. *$p <$ 0.05 compared to sham-control. **B**, No effect on retention of posttraining light pulses given to eYFP-expressing control rats. **C**, No effect of posttraining optical stimulation of the BLA on retention in ChR2(E123A)-expressing rats that did not receive a footshock during training.

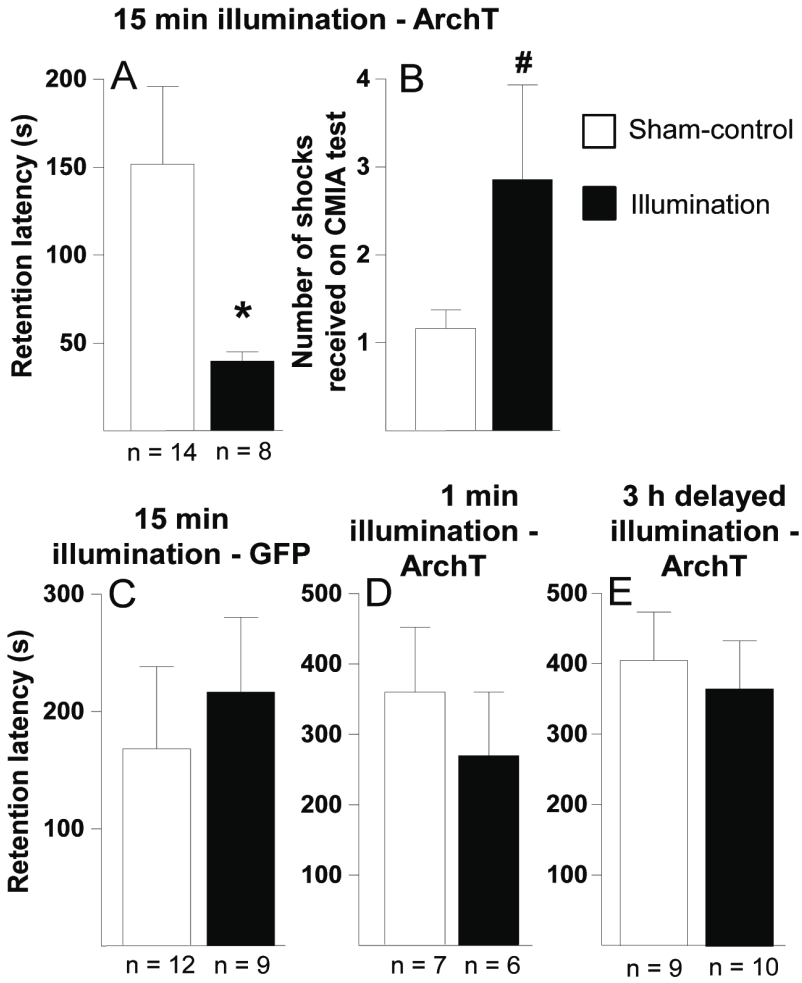

Figure 3. Retention effects of optical inhibition of ArchT-transduced BLA neurons immediately after IA training.

A, Impaired retention of rats given immediate posttraining optical inhibition of the BLA, via activation of ArchT. The figure shows the mean latencies, in s (+/- SEM), to enter the shock compartment during the retention test as well as the n of each group underneath each bar. *$p <$ 0.05 compared to sham-control. **B**, Impaired retention of the same rats from panel A, as assessed by CMIA training that immediately followed recording of retention latencies from panel A. #$p <$ 0.07 compared to sham-control. **C**, No effect of 15 min of illumination on retention in GFP-expressing control rats, following the same parameters as those used for the experiments in panel A. **D**, No effect of 1 min of posttraining optical inhibition of the BLA on retention. **E**, No effect of 15 min of optical inhibition of the BLA, given 3 h after training, on subsequent retention.

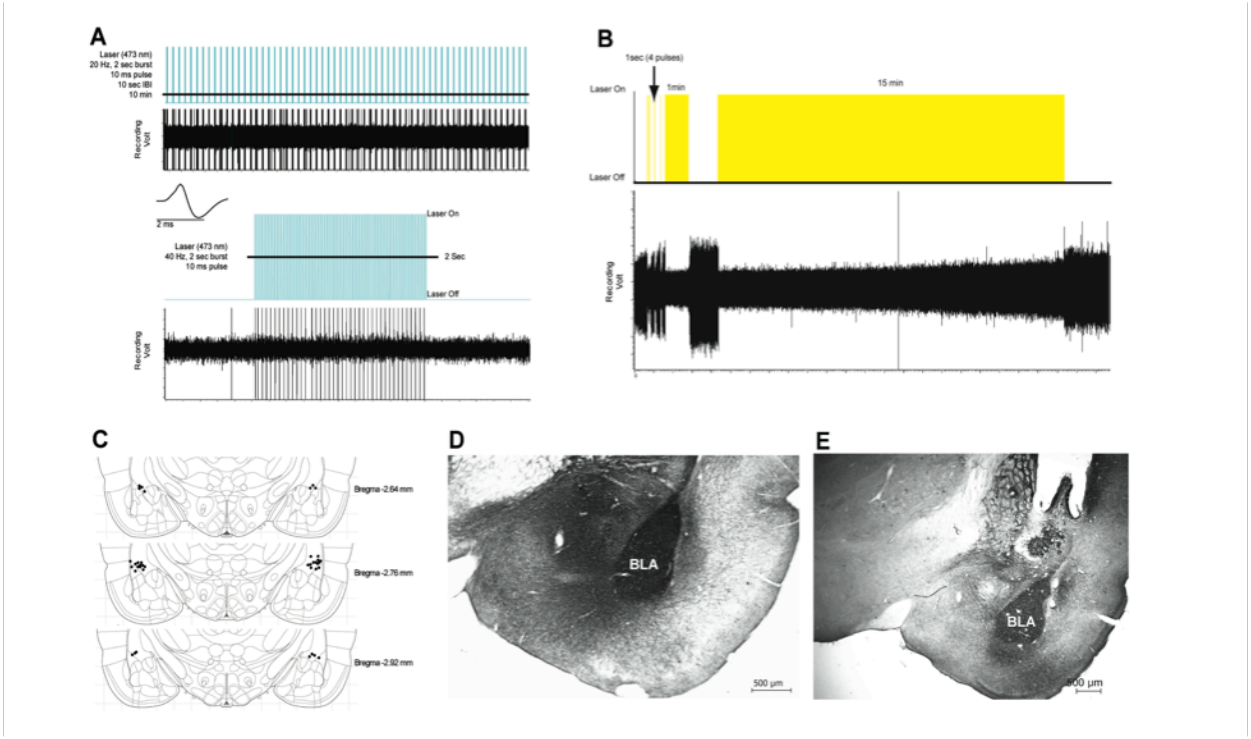

Figure 4. Electrophysiology and histology results.

A, Representative trace showing spike responses of a ChR2(E123A)-transduced BLA neuron to light pulses (10 ms duration, 473 nm wavelength) at either 20 Hz (top) or 40 Hz (bottom), given over 2 s. Middle inset shows representative action potential wave form. **B,** Representative trace showing spontaneous activity of an ArchT-transduced BLA neuron with or without light pulses of varying duration (561 nm wavelength). **C,** Diagram of estimated fiber optic track termination points in 20 randomly selected rats. **D,** Anti-eYFP immunocytochemical staining from a ChR2(E123A)-transduced rat. The BLA is densely labeled, whereas adjacent areas, including the central amygdala, show moderate staining from labeled axons. **E,** Anti-GFP immunocytochemical staining from an ArchT-transduced rat (image taken at lower magnification than panel D). The BLA is densely labeled, though stained fibers can be seen in surrounding regions, especially the central amygdala, representing ArchT-expressing axons

originating in the BLA. In addition, panel E shows the damage from the cannula immediately dorsal to the BLA.

CHAPTER 3. BASOLATERAL AMYGDALA PROJECTIONS TO VENTRAL HIPPOCAMPUS MODULATE THE CONSOLIDATION OF FOOTSHOCK, BUT NOT CONTEXTUAL, LEARNING IN RATS.

Manipulations of different brain regions can influence the consolidation of one type of learning without affecting others (Liang, 1999; Huff and Rudy, 2004; Malin and McGaugh, 2006). BLA connections with forebrain regions such as the hippocampus and striatum that are selectively involved in the consolidation of certain kinds of memories (McDonald, 1991b; Pitkanen, 2000; McGaugh et al., 2002; Malin and McGaugh, 2006; Paz et al., 2006; Chavez et al., 2013) suggest that efferent pathways from the BLA may be selectively involved in modulating the consolidation of specific kinds of information.

Indeed, much previous research investigated BLA interactions with the hippocampus during memory consolidation (Packard et al., 1994; Roozendaal et al., 1999; Malin and McGaugh, 2006) and that the BLA directly or indirectly influences activity and plasticity in different parts of the hippocampal formation (Ikegaya et al., 1995; Frey et al., 2001; McIntyre et al., 2005; McReynolds et al., 2014; Lovitz and Thompson, 2015). These findings suggest pathway mechanisms by which the BLA influences the consolidation of memories regulated by the hippocampal formation. However, the hippocampus is comprised of different subregions, including dorsal and ventral divisions, which are suggested to process different kinds of information (Moser and Moser, 1998; Bannerman et al., 2004; Fanselow and Dong, 2010). In rodents, the dorsal hippocampus (DH) is strongly implicated in spatial memories (Kim and Fanselow, 1992; Moser et al., 1995; Barrientos et al., 2002), whereas investigations into the role of the ventral hippocampus (VH) in spatial processing remain less clear. It is well established

that the BLA has projections to the VH and both of these structure have been linked with footshock-based learning tasks. Whereas the BLA appears to play a more general role in modulating both the contextual and emotional components of memory, the VH appears to be more specifically involved in the emotional component (Moser and Moser, 1998; Ferbinteanu and McDonald, 2001; Pothuizen et al., 2004; Zhang et al., 2004; Hunsaker and Kesner, 2008; Fanselow and Dong, 2010).

Some studies propose VH necessity for tasks involving spatial memory (Ferbinteanu and McDonald, 2001; Rudy and Matus-Amat, 2005), whereas others have discounted the VH as a site for spatial processing (Moser et al., 1995; Pothuizen et al., 2004). In contrast, the VH has been more clearly implicated in processing emotion and anxiety (Henke, 1990; Kjelstrup et al., 2002; Bannerman et al., 2004; Maren and Holt, 2004). Although much prior work has focused on the interactions between the BLA and DH during memory consolidation, the BLA sends a direct projection to the VH, but not the DH (Pikkarainen et al., 1999), and recent work supports the functionality of this projection (Felix-Ortiz and Tye, 2014). Yet, to our knowledge, no prior study has examined the interaction between the BLA and VH during memory consolidation, and, moreover, it is unknown whether BLA projections to the VH are selectively involved in the consolidation of *specific types* of information, but not others.

Contextual fear conditioning (CFC) involves learning regarding both contextual and emotional (footshock) information, suggesting the existence of distinct regions in the brain involved in dissociable components of this learning. Substantial evidence from CFC studies indicates that a brief footshock with minimal time spent in a context produces poor retention of the training, an

effect reversed by exposure to the context before the footshock (Fanselow, 1986, 1990; Kiernan and Westbrook, 1993; Landeira-Fernandez et al., 2006). Thus, the separate mnemonic components of the task can be investigated through presentation of the context and footshock on separate days. Prior work using this modified CFC paradigm has dissociated roles for the DH and anterior cingulate cortex. The DH, but not anterior cingulate, is involved in the consolidation of the context learning, whereas the anterior cingulate, but not the DH is involved in the consolidation of the footshock learning (Malin and McGaugh, 2006), establishing the utility of this paradigm in investigating the neurobiology underlying the consolidation of two different kinds of information. Although previous findings indicate a role for the VH in contextual fear conditioning (Bast et al., 2001; Gilmartin et al., 2012; Zhu et al., 2014), it is unknown whether BLA inputs to the VH influence memory consolidation for CFC and whether they do so in a manner related to the type of information being consolidated. Therefore, the present study used the modified CFC task to separate context and footshock learning (Liang, 1999; Malin and McGaugh, 2006) and provided optogenetic stimulation or inhibition of BLA axons in the VH immediately after training for either the context or footshock components of the task. It was hypothesized that the BLA projections to the VH would be involved in the consolidation for the footshock learning, but not the context learning.

Materials and Methods

Subjects. Male Sprague-Dawley rats (225-250 g at time of first surgery; Charles River; n = 265) were used for this study.

Surgery. One week after arrival, rats received virus microinjections [0.35 μL; rAAV5-CaMKIIα-hChR2(E123A)-eYFP, the ChR2(E123A) control rAAV5-CaMKIIα-eYFP, rAAV5-CaMKIIα-eArchT3.0-GFP, or the eArchT3.0 control rAAV5-CaMKIIα-GFP; University of North Carolina Vector Core] delivered bilaterally through a 33-gauge needle into the BLA (coordinates: 2.6 mm posterior and 4.9 mm lateral to Bregma and 8.3 mm ventral to skull surface). Following the virus injection, injectors were left in place for ≥ 5 min to permit diffusion.

Rats underwent a second surgery four weeks after the first in which fiber optic probes were bilaterally implanted, aimed at the VH (coordinates: 5.2 mm posterior and 5.5 mm lateral to Bregma and 7.5 mm ventral to skull surface). Coordinates were based on those areas of VH innervated by the BLA (based on Pitkanen, 2000 and modified empirically).

Optical stimulation or inhibition. Stimulation experiments used CaMKIIα-hChR2(E123A)-eYFP and its respective control CaMKIIα-eYFP. The constructs used in inhibition experiments were CaMKIIα-eArchT3.0-GFP and its respective control CaMKIIα-GFP. To enable sufficient time for robust opsin expression, illumination of BLA axons in the VH was conducted at least 5 weeks after viral injection. Figure 8A shows a schematic of viral injection placement, a 5-week incubation period, and fiber optic probe placement. In all cases, the comparison control was a

"sham-control" that had opsin expression but for which no illumination was provided except for one case (see below).

Behavioral training. All experiments used a standard inhibitory avoidance chamber, as depicted in Figure 4A and described in the general methods. Substantial evidence from CFC studies indicates that a brief footshock with minimal time spent in a context produces poor retention of the training, an effect reversed by exposure to the context before the footshock (Fanselow, 1986, 1990; Kiernan and Westbrook, 1993; Landeira-Fernandez et al., 2006). Although the context and footshock are usually presented together, it is possible to present context and footshock training on separate days, allowing investigation of the separate mnemonic components of the task. Therefore, the current experiments utilized a modified CFC task, which has been described previously and successfully used to produce dissociation of context and footshock learning (Liang, 1999; Malin and McGaugh, 2006). Briefly, rats first underwent context training in which they were placed in the illuminated side and allowed to freely explore the entire apparatus for 3 min (day 1). The following day, rats were confined to the darkened side of the chamber and received an immediate inescapable footshock (day 2; time in chamber < 15 s). Footshock parameters varied between stimulation (a single 1 mA, 1 s footshock) and inhibition (single 1mA, 2 s or two 1 mA, 1 s footshocks) experiments to prevent ceiling and floor effects, respectively. After *either* context (day 1) or footshock (day 2) training, rats received optical stimulation or inhibition of BLA axons in the VH in a separate holding chamber. Specific parameters are noted in individual experiments. Retention was tested two days later (day 4) when rats were again placed in the illuminated compartment and allowed free access to the entire

apparatus. Latency to cross into the darkened compartment (maximum 600 s) was used as the index of retention.

ChR2(E123A) experiments. Immediately after either context or footshock training, rats that had been injected with the ChR2(E123A) virus received 15 min of optical stimulation of the BLA→VH pathway using the following parameters: 2-s trains of either 20, 40, or 80 Hz light pulses (pulse duration = 5 ms), given every 10 s as used previously (Huff et al., 2013) or, in one experiment, 4-s trains of 20 Hz light pulses (pulse duration = 5 ms). For most experiments, the control group was a "sham-control" that received no illumination. However, in the main experiment, rats that had been injected with the eYFP control virus also received trains of 40 Hz light pulses.

Based on positive results, several control experiments were conducted, most of which used only the effective stimulation parameters from the main experiment. We investigated whether the results with the 40 Hz light pulses were due to the number of pulses, rather than the frequency of the pulses. Therefore, a group of rats received 20 Hz light pulses extended to 4 s trains (twice the original 20 Hz train length), every 10 s over 15 min immediately following footshock training. In a different control experiment, a group of rats received exposure to an alternate context (an operant chamber) on day 1 and 15 min of trains of 40 Hz pulses following footshock in the darkened side of the inhibitory avoidance chamber on day 2. Other rats that had received the ChR2(E123A) virus underwent CFC training but received no footshock during training followed by 15 min of trains of 40-Hz pulses, identical to those of the main experiment.

eArchT3.0 experiments. Complementary loss-of-function experiments were conducted to investigate the *necessity* of the BLA→VH pathway for consolidation. Rats expressing eArchT3.0 received continuous optical inhibition of the BLA→VH pathway for 15 min following context or footshock training, with different delays following the training. Because it was possible that the optical manipulations for the inhibition experiments were producing long-lasting dysfunction in the BLA→VH circuit that was responsible for the reduced latencies at the retention test, we conducted a control experiment in which the manipulation was given 3 h after training, when the consolidation window is typically observed to be closed (McGaugh and Roozendaal, 2009). We did not conduct a similar control experiment with the stimulation study, as it was considered unlikely that dysfunction in the circuit created by stimulation was responsible for the observed increase in latencies at the retention test. Prior studies using pharmacological manipulations to produce memory enhancement have typically not observed effects with 3 h posttraining manipulations (Lalumiere et al., 2004b; McGaugh and Roozendaal, 2009).

Electrophysiological verification**.** Electrophysiological activity was gathered to confirm that hippocampal neurons could be influenced by optogenetic manipulation of projections from the BLA to the VH (n = 4). Rats were initially anesthetized with 4% isoflurane followed by i.p. injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). The scalp was retracted and the skull leveled between Bregma and Lambda. Craniotomies were made at target sites in the right hemisphere. For a given rat, two sites could be optogenetically targeted to manipulate either BLA cell bodies or axons in the VH.

For verification of axon stimulation, one fiber-optic cannula was aimed at the terminal fields of BLA neurons in the VH (5.1 mm posterior, 3.7 mm lateral, and 7.4 mm ventral to Bregma, at a 10° angle in the lateral plane). For verification of inhibition, one fiber-optic cannula was lowered and fixed in place with cyanoacrylate (SloZap; Pacer Technologies) accelerated by ZipKicker (Pacer Technologies) and with methyl methacrylate (i.e., dental cement; A-M Systems). This cannula was aimed at cell bodies of BLA neurons (2.6 mm posterior, 3.1 mm lateral, and 8.8 mm ventral to Bregma, at 12° angle in the anterior plane) and was attached by patch cable (Doric) to a 473-nm laser (OptoEngine) driven by a pulse generator (custom-made in the Narayanan lab). The second site inhibited the terminal fields of BLA neurons in the VH (5.1 mm posterior, 3.7 mm lateral, and 7.4 mm ventral to Bregma, at a 10° angle in the lateral plane). This cannula was attached to a patch cable (Doric) and a 561-nm laser (OptoEngine) and was held in place during the recording session by one of the two stereotactic arms. A third craniotomy was for a microwire electrode targeting layer CA1 of the VH (AP -5.1, ML±7.1, DV -7.9@10° in the lateral plane). Recording was done with 2x8 multi-electrode arrays of 75 μm tungsten wires (250 μm between wires and rows; impedance measured *in vitro* at 1000 kΩ; MicroProbes for Life Science). A final craniotomy was a small hole drilled for insertion of the ground wire.

Once the electrode was lowered into location in the VH, potential neuronal units were identified on-line using an oscilloscope and audio monitor. Neuronal recordings were made using a multi-electrode recording system (Plexon). After a pause to ensure that the recording was stable, the following optogenetic protocol was initiated in order to determine if VH cells responded to BLA stimulation and inhibition. Stimulation was provided 5 min at a time with no light, 20, or 40 Hz pulses of 473 nm light either alone or in combination with continuous 561 nm light.

Data analysis. Retention latencies for all behavioral experiments were analyzed using either a t-test or a one-way ANOVA with a Tukey post hoc test. $P < 0.05$ was considered significant. All measures are expressed as mean ± SEM, and each group's n is indicated in the figure below its respective bar.

Neurophysiological Recordings: Neuronal ensemble recordings in the VH were made using a multi-electrode recording system (Plexon, Dallas, TX). Putative single neuronal units were identified on-line using an oscilloscope and audio monitor. The Plexon off-line sorter was used to analyze waveforms off-line and to remove artifacts. Principal component analysis (PCA) and waveform shape were used for spike sorting. Single units were identified as having 1) consistent waveform shape, 2) separable clusters in PCA space, 3) average amplitude estimated at least three times larger than background activity, 4) a consistent refractory period of at least 2 ms in interspike interval histograms, and 5) consistent firing rates around optical stimulation (as measured by a runs test of firing rates across trials around optical stimulation; neurons with $|z|$ scores > 4 were considered 'nonstationary' and were excluded). Spike activity was analyzed for all cells that fired at rates above 0.1 Hz. Analysis of neuronal activity and quantitative analysis of basic firing properties were carried out using NeuroExplorer (Nex Technologies, Littleton, MA), and with custom routines for MATLAB.

Results

To confirm prior work indicating the necessity of both context training (pre-exposure to the entire apparatus) and footshock training (immediate shocks paired with one portion of the apparatus) for retention of the CFC task, naïve rats (i.e., did not undergo surgeries of any kind) either received context training on day 1 with no footshock on day 2, alternate-context training on day 1 with a footshock on day 2, or both the context training on day 1 and footshock training on day 2 (i.e., the complete CFC training). Figure 5C shows the day 4 retention latencies for all three groups. A one-way ANOVA revealed a significant effect ($F_{(2,18)} = 5.304$, $p < 0.05$) with post hoc analyses indicating significantly higher latencies for those rats receiving the complete CFC training (both context and footshock training) compared to those of rats receiving either alternate-context training on day 1 or no footshock on day 2. The retention latencies for the alternate context and no-footshock groups did not differ from each other.

Optical activation of BLA axons in the VH enhances retention of footshock learning for a modified CFC task. To determine whether post-footshock stimulation of the BLA→VH pathway enhances consolidation for CFC, rats underwent the modified CFC training and received posttraining optical stimulation of ChR2(E123A)-transduced BLA terminals in the VH on day 2, as outlined in Figure 6A. The BLA-VH pathway was stimulated using trains of light pulses (2-s trains, given every 10 s) at either 20, 40, or 80 Hz (5-ms pulse width, 473 nm light) over 15 min. Figure 6B shows the retention latencies for rats that underwent the full modified CFC training and received optical stimulation of the BLA axons in the VH immediately following footshock training on day 2. For this experiment, the sham-control group expressed either ChR2(E123A) or eYFP-alone and received no illumination, and these groups were combined for analysis as their

latencies did not significantly differ from one another ($t_{(12)} = 0.057$, $p > 0.05$; mean ± SEM for each: ChR2(E123A) = 37.50 ± 10.30; eYFP = 36.5 ± 9.78). A one-way ANOVA revealed a significant difference between groups ($F_{(4,38)} = 7.057$, $p < 0.001$). Rats that received trains of 40 Hz pulses had significantly increased retention latencies compared to sham controls ($p < 0.01$), the 20 Hz group ($p < 0.01$), and eYFP controls ($p < 0.01$). No other groups were significantly different from each other. To determine whether the effect observed with the trains of 40 Hz stimulation was due to the number of light pulses received (rather than the frequency), a separate experiment (matched pulses) was conducted in which ChR2(E123A)-transduced rats received 20 Hz stimulation in 4 s trains following footshock training and thus received an equivalent number of light pulses. The latencies of these rats were not significantly different from the latencies of their sham-control animals, as shown in Figure 6C ($t_{(19)} = 0.34$, $p > 0.05$).

To further demonstrate the necessity of both components of the task, a separate group of rats underwent context training in an alternate context (an operant chamber) on day 1 and then received optical stimulation using trains of 40 Hz light pulses following standard footshock training on day 2. Retention latencies for the stimulation group, as shown in Figure 6D, were not significantly different from those of the sham-control group ($t_{(6)} = 0.082$, $p > 0.05$). In addition, to ensure that the optical stimulation of the BLA-VH pathway alone did not induce aversion for the shock chamber, a control experiment was conducted in which rats received normal context training, but on day 2, were simply placed into the shock compartment with no footshock administered. They were then given optical stimulation identical to that found to be effective at enhancing retention in Figure 6B. Retention latencies for those rats, shown in Figure 6E, again did not differ between the sham-control and stimulation groups ($t_{(13)} = 0.64$, $p > 0.05$).

Optical inhibition of BLA axons in the VH impairs retention of footshock learning for a modified CFC task when given at a 25 min delay. To determine whether post footshock inhibition of the BLA→VH pathway impairs consolidation of a CFC task, rats underwent CFC training and received posttraining optical inhibition of eArchT3.0-transduced BLA terminals in the VH following footshock training on day 2 as outlined in Figure 7A. Optical inhibition was obtained using continuous 561-nm light for 15 min. The latencies of rats that received 15 min of immediate post-footshock training inhibition are shown in Figure 7B, but no significant difference was observed (single footshock: $t_{(18)} = 0.34$, $p > 0.05$). Due to the low control group latencies and the potential for a "floor" effect, we repeated the experiment using two footshocks. Latencies for those rats are shown in Figure 7C, and again no significant difference was observed ($t_{(20)} = 0.076$; $p > 0.05$).

Regardless of the footshock parameters delivered on day 2 (a single 2 s, 1 mA footshock or two 1 s, 1mA footshocks), the control group's latencies were very low, potentially creating floor effects that prevented observation of any memory impairment. Therefore, we considered that having a small delay between the footshock and the optical manipulations might lead to higher control latencies, as we have previously observed that immediate posttraining optical manipulations produce depressed control baselines (Huff et al. 2013). Moreover, prior work suggests that BLA activity approximately 30 min following a learning event may be especially important (McIntyre et al. 2002; Pelletier et al. 2005). Therefore, we conducted an additional experiment in which optical inhibition was given beginning 25 min following the footshock training (rats were returned to their home cage during the delay), which was successful in

producing higher control group latencies. Figure 7D shows the retention latencies of those rats, and a t-test revealed a significant difference ($t_{(21)} = 2.781$, $p < 0.05$), indicating that inhibiting BLA axons in the VH 25 min after footshock training impaired retention. In another experiment, optical inhibition was given 3 h after footshock training to determine whether such inhibition produced long-lasting changes in the BLA→VH pathway, which could have been responsible for the impairments in retention latencies. As shown in Figure 7E, no significant difference in latencies was observed between sham-control and illumination groups ($t_{(31)} = 1.599$, $p > 0.05$). Finally, to determine whether the effects were due to illumination alone, another group of rats expressing GFP alone (i.e., no opsin controls) received 15 min of post-footshock training illumination at a 25 min delay. Latencies for these rats were no different from those of the sham-control group (panel F; $t_{(13)} = 1.252$, $p > 0.05$).

Optical stimulation and inhibition of BLA axons in the VH did not affect retention of the context learning for the modified CFC training. Following the completion of the experiments in which optical stimulation/inhibition was given after footshock training, we conducted additional experiments to determine whether similar manipulations given after the context training (day 1) would alter retention. Rats underwent CFC training and received either posttraining optical stimulation of ChR2(E123A)-transduced BLA terminals in the VH or posttraining optical inhibition of eArchT3.0-transduced BLA terminals in the VH following context training on day 1 (see Figure 8A and C for outline of training and testing). Figure 8B shows retention latencies for rats that received 15 min of stimulation immediately following context training on day 1. A one-way ANOVA revealed no significant difference between groups regardless of stimulation frequency ($F_{(3,16)} = 0.19$, $p > 0.05$). Photoinhibition of BLA terminals in the BLA provided for 15

min at a 25 min delay after context training also had no significant effect on retention (Figure 8D; $t_{(18)} = 0.70$, $p > 0.05$).

Figure 9 shows histological verification of opsin expression in BLA axons in the VH as well as electrophysiological confirmation of robust influence of stimulation and inhibition of BLA axons (via illumination of ChR2(E123A)- and eArchT3.0-expressing BLA axons) over VH neuronal activity. Figure 9B show representative images of the BLA (left) or BLA axons in the VH (right) expressing eArchT3.0 or ChR2(E123A), respectively. The right panel also shows a representative fiber optic track. Figure 9C shows a representative raster plot of neural excitation in the VH in response to light delivery to BLA axons in the VH in a ChR2(E123A)-transduced rat. Figure 9D shows a raster plot of the inhibition of BLA axons in the VH in a rat transduced with both ChR2(E123A) and eArchT3.0. Illumination (473 nm) was provided to the BLA cell bodies to stimulate BLA neurons while illumination (561 nm) was simultaneously provided to the VH to inhibit BLA axons. Inhibition was found to reduce VH neuronal activity driven by the cell body stimulation.

Discussion

The current study extends previous work examining BLA modulation of memory consolidation and provides evidence that the projection from the BLA to the VH plays a selective role in the consolidation of specific components of contextual fear conditioning. The findings indicate that immediate posttraining optogenetic stimulation of this pathway following footshock, but not context, training increased retention latencies for CFC. This modulation was specific to stimulation using trains of 40, but not 20 or 80, Hz light pulses. Optical inhibition of the same pathway following footshock, but not context, training impaired retention when inhibition was

given 25 min after training. Together, these results indicate that the BLA modulates the consolidation for the footshock component of CFC through efferent projections to the VH and that this pathway does not play a similar role in the consolidation of the contextual learning for this task.

Prior studies indicate that the BLA modulates the consolidation for many different types of learning including inhibitory avoidance (Lalumiere et al., 2004b; LaLumiere et al., 2005), CFC (LaLumiere et al., 2003; Huff and Rudy, 2004), conditioned taste aversion (Miranda et al., 2003; Guzman-Ramos and Bermudez-Rattoni, 2012), spatial and cued-response learning (Packard et al., 1994), and novel object recognition (Roozendaal et al., 2008b; Bass et al., 2012). However, evidence also indicates that different types or kinds of learning involve separate and distinct brain regions, as, for example, the hippocampus and caudate are involved in the consolidation of spatial and cued water maze learning, respectively (Packard et al., 1994). The BLA interacts with a variety of structures both cortical (e.g. anterior cingulate cortex, insular cortex) and subcortical (e.g. caudate, nucleus accumbens) during memory consolidation, potentially accounting for its diffuse involvement in consolidation across tasks and types of memory (for recent review see McIntyre et al., 2012). However, prior work was unable to directly manipulate activity in distinct efferent BLA pathways to determine whether such pathways were selectively involved in specific kinds of memory consolidation.

The present work used a modified CFC paradigm, in which the consolidation of context and footshock learning are separate (Liang, 1999). Prior work using this paradigm has found that, whereas the BLA itself modulates the consolidation for context and footshock components, the

dorsal hippocampus and anterior cingulate are selectively involved in the consolidation for the context and footshock learning, respectively (Malin and McGaugh, 2006). Thus, the present findings, indicating a role for the BLA→VH pathway in the consolidation of the footshock, but not contextual, component for the task, provide evidence for the selective roles of different efferent pathways from the BLA, depending on the type of learning. Although the present work found a role for BLA projections to the VH only in footshock learning, as Malin and McGaugh observed for the anterior cingulate, there is no direct projection from the VH to the anterior cingulate or vice versa. Of note, however, is the consistent co-activation of the anterior cingulate and VH in fear memories including expression/retrieval (Cullen et al., 2015), a relationship likely mediated through the nucleus reuniens of thalamus (Vertes, 2006; Nieuwenhuis and Takashima, 2011). Nonetheless, a wealth of findings provides evidence supporting a promiscuous influence for the BLA on memory consolidation and more discrete roles for other brain regions depending on the component or type of learning involved. Moreover, they also suggest that the BLA, through its different projections, interacts with specific brain regions in a manner dependent on the component of the CFC investigated, a hypothesis supported by the current research.

Although the BLA is well known for its role in modulating emotionally influenced memory consolidation, the BLA has also been shown to modulate consolidation for learning that occurs in the absence of any observable affective component, including object-in-context learning and contextual learning (Malin and McGaugh, 2006; Barsegyan et al., 2014). However, it is impossible to rule out the possibility that any learning involves at least minimal levels of emotional arousal. Nonetheless, using the same behavioral paradigm used here, Malin and

McGaugh (2006) found that post-context intra-BLA microinjections of a muscarinic cholinergic agonist enhanced retention for CFC. Thus, the present work, finding no role for the BLA→VH pathway in the consolidation of the contextual learning, provides important information about the role of the BLA in memory modulation and suggests that other efferent pathways from the BLA are involved in the consolidation of the contextual learning for this CFC task.

The VH receives a significant innervation from the BLA and, in light of the well-established roles for the hippocampus in memory, appeared to be a likely candidate for at least some BLA influences on memory consolidation for CFC. Indeed, prior work suggests that posttraining inactivation of the VH impairs CFC and auditory fear conditioning (Maren and Holt, 2004). However, previous investigations have produced conflicting evidence regarding the role of the VH in specific aspects or types of memory (see Fanselow and Dong, 2010 for larger discussion). The role of the VH in spatial information/memory processing has been unclear (Ferbinteanu and McDonald, 2001; Pothuizen et al., 2004; Rudy and Matus-Amat, 2005), likely due to differences in the specific regions of the hippocampus targeted or identified as "VH" as well as differences in techniques and behavioral paradigms. Yet it is generally agreed that the VH plays a significant role in emotional processing including fear and anxiety (Moser and Moser, 1998; Ferbinteanu and McDonald, 2001; Bannerman et al., 2003; Pothuizen et al., 2004). For example, hippocampal lesions that are confined to more ventral locations that do not connect or project to the dorsal/contextual-processing regions show reduced anxiety responses as evidenced by more time spent in the open arms of an elevated plus maze (Kjelstrup et al., 2002). Moreover, optogenetic manipulations of BLA inputs to the VH indicate a role for this pathway in altering anxiety-related behavior (Felix-Ortiz and Tye, 2014). In agreement with some of these previous

results, the present findings provide evidence that BLA inputs to the VH modulate the consolidation for the emotion-related aspect of contextual fear conditioning (i.e., the footshock learning) but are not involved in the contextual component. However, it is critical to note that our findings do not address the role of the VH itself but rather provide evidence for the role of the *pathway* from the BLA to the VH during memory consolidation.

The present findings also provide further evidence for the importance of gamma frequency activity in memory consolidation. Prior work from our laboratory indicates that optical stimulation of the BLA, using bursts of 40 Hz light pulses, enhances retention, whereas similar stimulation using bursts of 20 Hz light pulses has no effect (Huff et al., 2013). The present work extends those findings to the BLA→VH pathway and indicates that bursts of 80 Hz light pulses also have no effect. Gamma oscillations (35-45 Hz) are thought to be predictive of memory and underlying plasticity (Popescu et al., 2009; Headley and Weinberger, 2011) and coordinated neuronal activity is suggested to play a role in regulating synaptic plasticity (Bauer et al., 2007). However, whether stimulation and inhibition of BLA axons in the VH is modulating underlying plasticity within the VH or elsewhere remains unknown. It is possible that the current modulation of BLA→VH activity produces a spread of activity beyond the VH and that critical plasticity occurs in these downstream regions.

Methodological considerations. The target stimulation parameters chosen for these experiments were based on our previous findings indicating that the parameters for stimulation and inhibition are effective in modulating consolidation following a one-trial inhibitory avoidance task (Huff et al., 2013). Here, we were able to extend these stimulation parameters to the BLA→VH pathway

during memory consolidation of footshock learning. Importantly, a range of stimulation frequencies, including the most effective frequency (40 Hz) for the enhancement of consolidation for footshock learning and inhibitory avoidance learning in general (Huff et al., 2013), was used to investigate the BLA→VH pathway during context learning, yet no frequency was effective for enhancing the consolidation of the context learning in this pathway. However, it remains possible that an effective frequency exists, as different frequencies of activity may be important for different types of learning. Using 4 s trains of 20 Hz light pulses (to match number of light pulses in 2 s trains of 40 Hz stimulation) had no effect and implies that it is the frequency, and not the number of light pulses, that is important for the enhanced retention observed with bursts of 40 Hz light pulses. Nonetheless, effective parameters for other downstream targets may differ. Although a 3-h posttraining control experiment was conducted for the inhibition work in order to exclude the possibility that the inhibition was producing long-lasting dysfunction in the circuit, we did not perform a similar control with the stimulation experiments, as studies using pharmacological manipulations to produce memory enhancement do not observe effects on retention when given 3 h after training (McGaugh and Roozendaal, 2009). Nonetheless, although unlikely, it is possible that the stimulation-induced memory enhancement occurred as a result of effects of the stimulation on performance during the retention test 2 d later, rather than on the memory consolidation.

In contrast to our previous work indicating that immediate posttraining BLA inhibition impairs retention of inhibitory avoidance learning, the present study found that 15 min of inhibition given 25 min after training, but not immediately after training, impaired retention of the footshock learning. As we have observed, both in the present study and our past work (Huff et

al., 2013), that connecting a rat to the optical tether immediately after training reduces latencies in sham-control rats, relative to connections following a delay, this findings may reflect a "floor effect," in which latencies were too low in the control group to observe an impairment. Alternatively (and perhaps in concert with the floor effect), the present results may reflect the importance of activity in the BLA or this pathway specifically following a delay after training. Indeed, previous work suggests that emotional arousal during training leads to sustained enhancement in BLA activity for at least 30 min following stimulus presentation (Pelletier et al., 2005) and that inhibitory avoidance training induces peak norepinephrine release in the amygdala approximately 30 min after training (McIntyre et al., 2002).

In contrast to many CFC studies that use freezing as the index of retention, retention in the current study was assessed via latency to cross from the illuminated compartment to the dark compartment. Because any increase in freezing would be reflected by increases in retention latencies, the use of latencies as the index of retention provides a broad measure to capture differences in memory. Nonetheless, it remains possible that our measure of retention may have failed to detect a group difference in behavior during the retention test. It is also possible that other retention measures may reflect different components of the memory that were separately modulated and yet were not observable in our particular paradigm.

Conclusions

The present study strongly indicates that the BLA projections to the VH play a dissociable role in memory consolidation, as our results suggest that posttraining optical stimulation and inhibition of this pathway enhanced and impaired, respectively, the retention of the footshock learning.

Such manipulations, however, had no effect on the retention of the context learning. These findings are, to our knowledge, the first to provide direct support for the long-held hypothesis that the modulation of memory consolidation by the BLA for so many different kinds of learning occurs through distinct efferent pathways from the BLA to downstream regions.

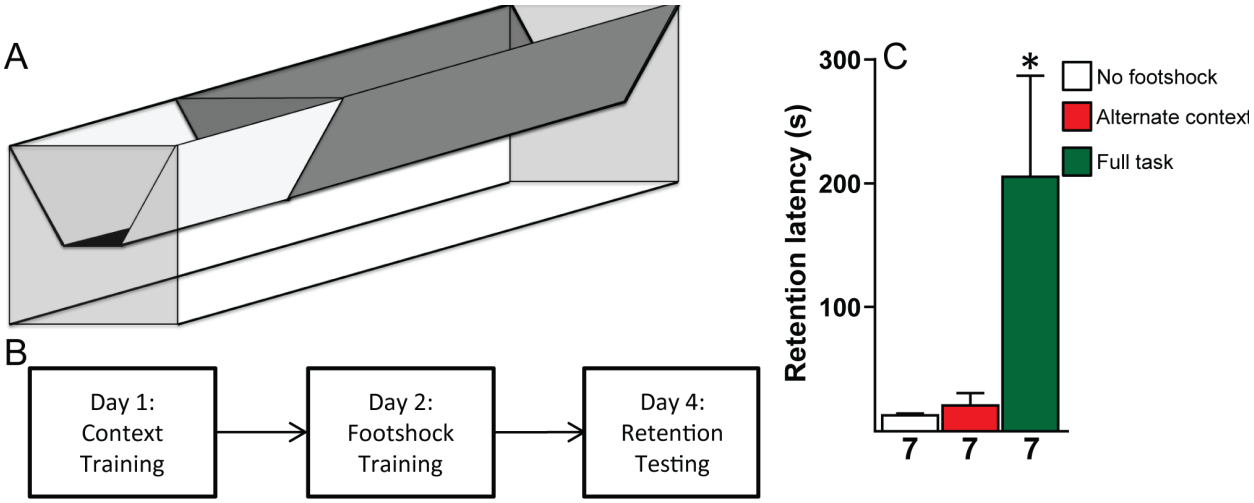

Figure 5. Experimental design.

A, Diagram of inhibitory avoidance apparatus used in the modified CFC training. **B,** Schematic diagram of the timeline for behavioral training and testing. **C**, Naïve rats (i.e., rats that did not undergo surgeries) underwent the modified CFC task. Rats received either 1) Context training on day 1 but no footshock on day 2; 2) Alternate-context training on day 1 and footshock training on day 2; or 3) Context training on day 1 and footshock training on day 2 (i.e., the full CFC training). The figure shows the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. Those rats that received context and footshock training in the same apparatus had significantly higher retention latencies than the other two groups (*$p < 0.05$), which did not differ from each other.

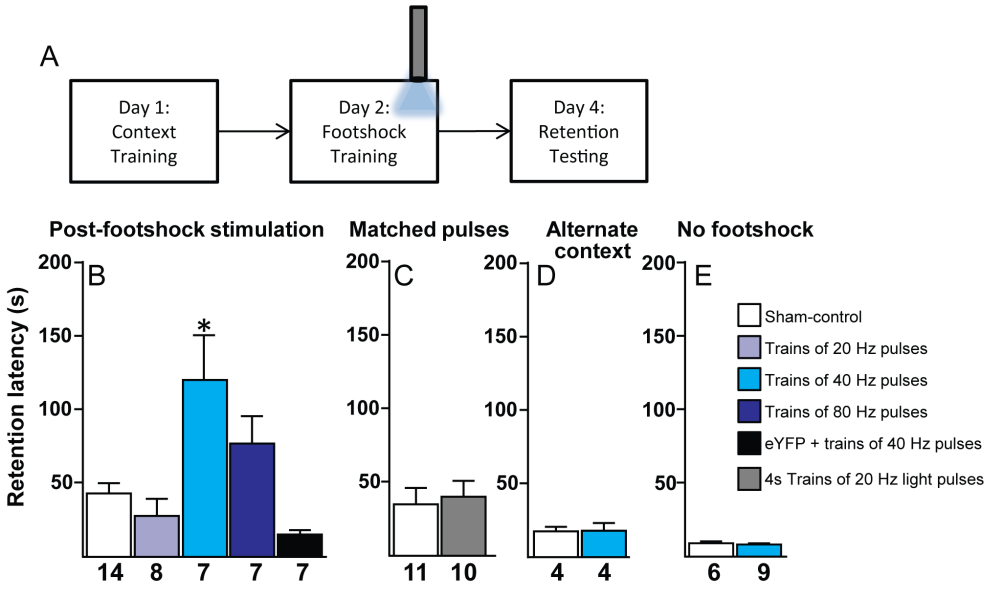

Figure 6. Retention effects of optical stimulation of ChR2(E123A)-transduced BLA axons in the VH immediately after footshock (day 2) training.

Panels B-E show the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. **A,** Schematic diagram of the timeline for experimental training as well as optical stimulation following footshock training. **B,** Increased retention latencies of rats given post-footshock training optical stimulation of BLA axons in the VH via activation of ChR2(E123A). Retention was significantly increased in rats that received trains of 40 Hz light pulses following training. *$p < 0.05$ compared to sham-control, 20 Hz, and eYFP-control groups. **C,** No effect on retention when identical total numbers of light pulses were given to rats after footshock training. Rats received 4 s trains of 20 Hz light pulses, thus matching the total number of light pulses given to rats in panel B that received 2 s trains of 40 Hz pulses. **D-E,** No effect of optical stimulation of the BLA→VH pathway following day 2 training on retention if either component of the task (context pre-exposure or footshock delivery, respectively) was altered/omitted.

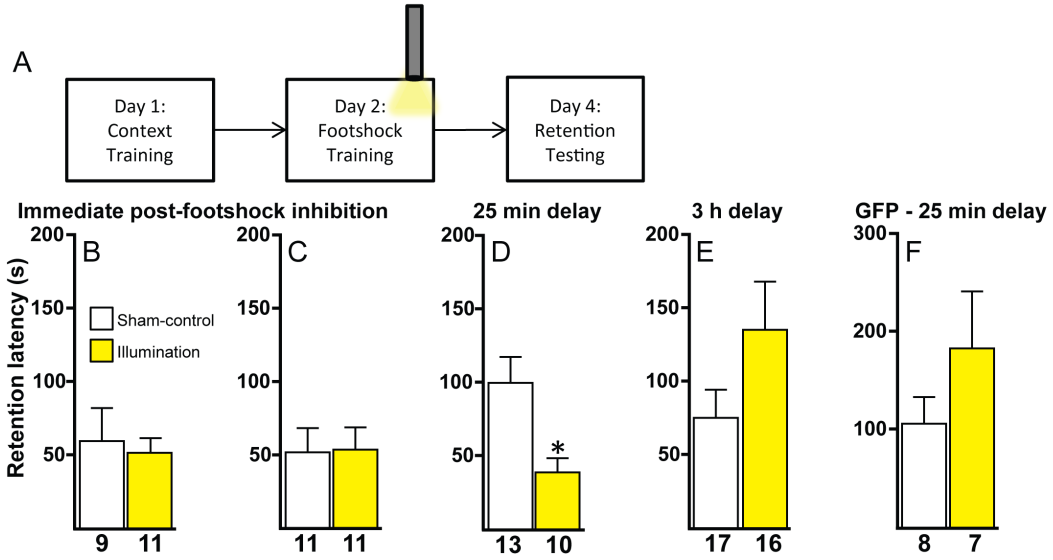

Figure 7. Retention effects of optical inhibition of eArchT3.0-transduced BLA axons in the VH after footshock (day 2) training.

Panels B-F show the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. **A,** Schematic diagram of the timeline for experimental training as well as optical inhibition following footshock training. **B-C,** No effect on retention was observed when optical inhibition was provided immediately after footshock training regardless of footshock strength and duration (**B**: A single 1 mA, 2 s footshock; **C**: Two 1 mA, 1 s footshocks). **D,** Impaired retention of rats given post-footshock optical inhibition of BLA axons in the VH at a 25 min delay. * $p < 0.05$, compared to sham control. **E,** No effect on retention of 15 min of continuous optical inhibition of the BLA→VH pathway given 3 h after footshock training. **F,** No effect of illumination on retention in GFP-alone control rats following the experimental parameters in D.

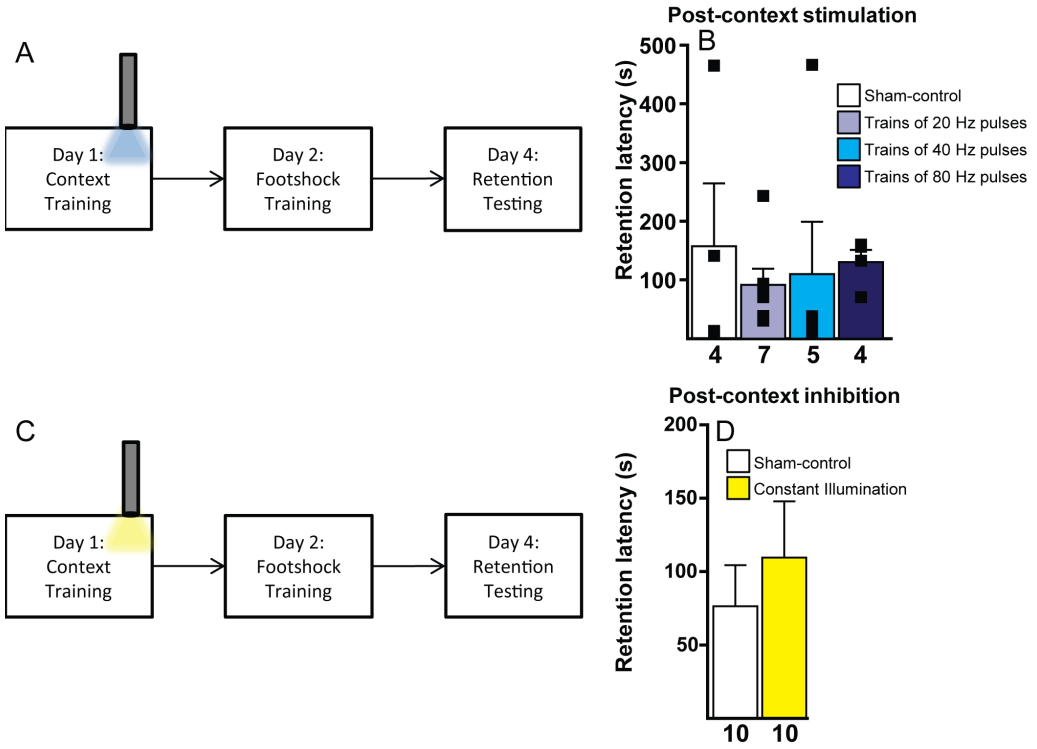

Figure 8. Retention effects of optical stimulation or inhibition given after context training on day 1.

All graphs show the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. **A,** Schematic diagram of the timeline for experimental training as well as optical stimulation following context training. **B,** Retention latencies of rats given post-context training optical stimulation of BLA axons in the VH via activation of ChR2(E123A). Due to the low n in this experiment, the individual data points are also plotted. Such stimulation had no effect on retention regardless of stimulation frequency used. **C,** Schematic diagram of the timeline for experimental training as well as optical inhibition following context training. **D,** Retention latencies of rats given optical inhibition in the BLA→VH pathway via activation of eArchT3.0 at a 25 min delay following context training. No effect of optical inhibition using the same parameters as Figure 3D was observed for retention.

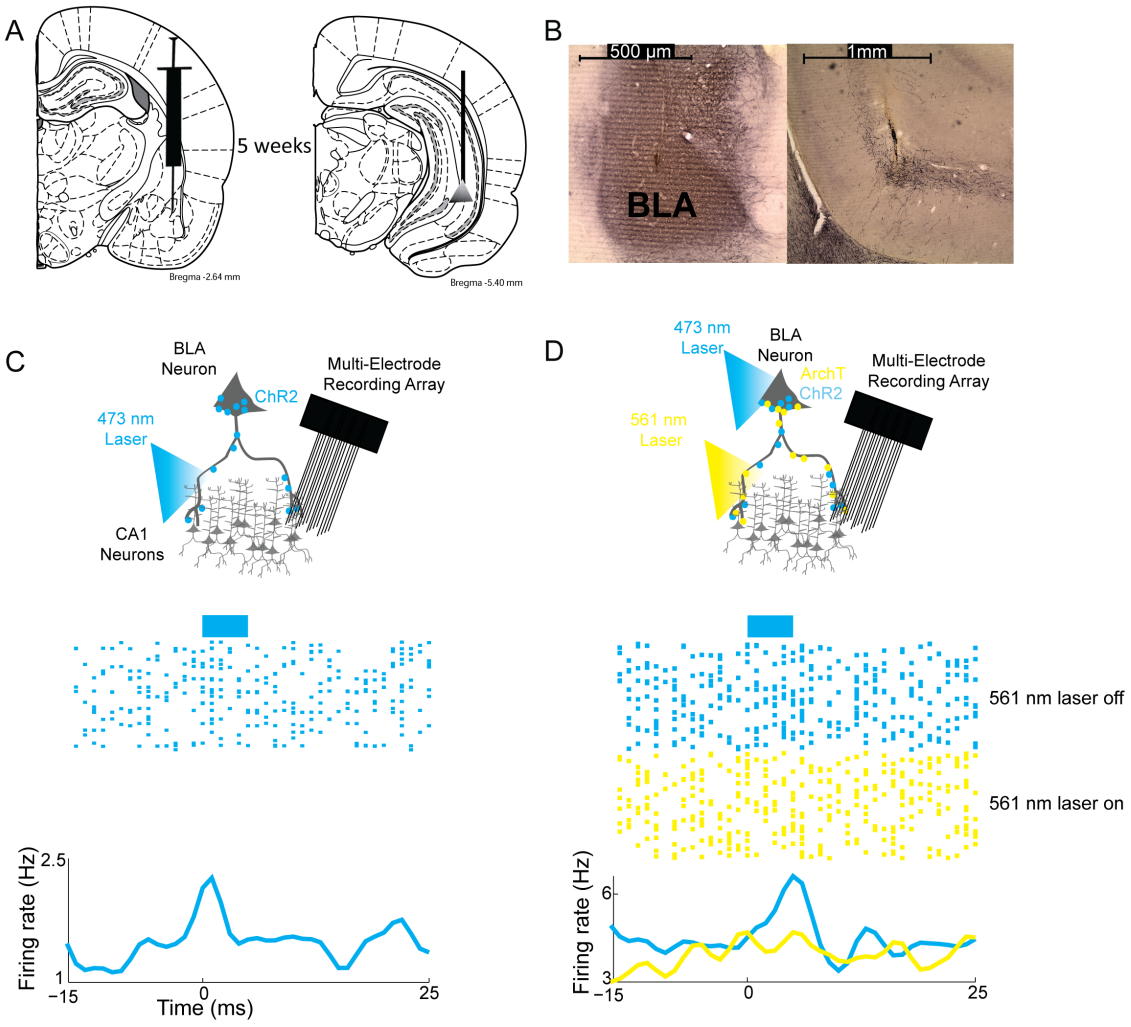

Figure 9. Histology and electrophysiological verification.

A, Schematic diagram of BLA injection site, incubation time, and optic probe placement in VH. **B,** Anti-GFP immunohistochemical staining from the injection site in a eArchT3.0-transduced rat (left) and anti-eYFP immunohistochemical staining of BLA axons in the VH in a ChR2(E123A)-transduced rat. Additionally, damage from the fiber optic probe implant can be seen terminating dorsal to the innervating fibers. **C,** Raster plot of a VH neuron modulated by ChR2-transduced BLA axon stimulation. Blue rasters (above) and histogram (below) are from 40-Hz stimulation with the 473-nm laser (ChR2-targeted). **D,** Raster plot of a VH neuron modulated by both ChR2 and ArchT activation. Blue rasters (above) and histogram (below) are from 40-Hz stimulation

with the 473-nm laser (ChR2-targeted). Yellow rasters (above) and histogram (below) are from simultaneous 40-Hz stimulation with the 473-nm laser and constant illumination with the 561-nm laser (ArchT targeted).

CHAPTER 4. BASOLATERAL AMYGDALA PROJECTIONS TO MEDIAL ENTORHINAL CORTEX MODULATE THE CONSOLIDATION OF CONTEXTUAL, BUT NOT EMOTIONAL, LEARNING IN RATS.

It has been hypothesized that the connections of the BLA with downstream regions permit its modulation of memory consolidation across many different kinds of learning (McGaugh, 2002; McIntyre et al., 2012). There has been long-standing interest in BLA-hippocampus interactions during memory consolidation (Packard et al., 1994; Roozendaal et al., 1999; Huff and Rudy, 2004), and the functional separation between the dorsal and ventral subdivisions of the hippocampus has been a critical component of these investigations (Moser and Moser, 1998; Fanselow and Dong, 2010), as prior evidence suggests that the dorsal hippocampus (DH) plays a large role in spatial processing whereas the ventral hippocampus (VH) processes negative emotion (Henke, 1990; Kjelstrup et al., 2002; Bannerman et al., 2004; Maren and Holt, 2004).

In support of BLA-hippocampus interactions and the selective role for the VH in particular types of processing, our own previous work indicates that the BLA-VH pathway is selectively involved in the footshock, but not context, learning during a modified CFC task (Huff et al., 2016). However, using this same task, other work suggests that the DH is important for the consolidation of the contextual, but not footshock, information (Malin and McGaugh, 2006), a finding that confirms prior studies indicating a role for the DH in other models of contextual fear conditioning (Barrientos et al., 2002; Stote and Fanselow, 2004). Previous investigations have examined the BLA-DH relationship. Activating β-adrenergic receptors in the BLA immediately following an IA task not only enhances retention, but also leads to an increase in Arc expression

in the DH (McIntyre et al., 2005). As the DH does not receive a direct innervation from the BLA, the nature of the BLA-DH interaction is difficult to investigate directly.

The mEC, however, receives a moderate projection from the BLA (Pitkanen et al., 2000) and then projects to the DH (Andersen et al., 1969; Steward and Scoville, 1976), providing a means by which the BLA influences DH activity. Moreover, previous results suggest a role for the mEC in memory consolidation. EC inactivation immediately following training for an avoidance response task (Baldi et al., 1999), CFC (Baldi et al., 2013), and an IA task (Ardenghi et al., 1997; Zanatta et al., 1997) impair long-term retention. Supporting interactions between the BLA and the mEC, past work indicates that BLA lesions attenuate the memory-enhancing effects of 8-Br-cAMP infusions into EC (Roesler et al., 2002) and that BLA neurons fire in phase with both EC delta and theta oscillations (Pare and Gaudreau, 1996). Recently, Sparta and colleagues (2014) found that BLA inputs to the EC influence memory acquisition, supporting the functional connection between these two brain regions. However, the role of the BLA-mEC pathway during consolidation of different components of a CFC task remains unknown. Previous work from our lab has shown the importance of temporal and spatial precision in investigation of memory consolidation including the BLA (Huff et al., 2013) and its projections to other regions (Huff et al., 2016). Therefore, the current set of experiments examined whether optogenetic stimulation of the BLA→mEC pathway affects the consolidation of context, but not footshock, memory during a modified CFC task.

Materials and Methods

Subjects. Male Sprague-Dawley rats (225-250 g at time of first surgery; Charles River; n = 161) were used for this study.

Surgery. One week after arrival, rats received virus microinjections [0.35 μL; rAAV5-CaMKIIα-hChR2(E123A)-eYFP or the ChR2(E123A) control rAAV5-CaMKIIα-eYFP] into the BLA. Rats underwent a second surgery four weeks after the first in which fiber optic probes were bilaterally implanted, aimed at BLA terminals in the mEC (coordinates: 7.2 mm posterior and 5.6 mm lateral to Bregma and 6.5 mm ventral to skull surface) or on one case, the VH (coordinates: 5.2 mm posterior and 5.5 mm lateral to Bregma and 7.5 mm ventral to skull surface). Coordinates were based on those areas innervated by the BLA (based on Pitkanen 2000 and modified empirically). The rats were given 1 week to recover from surgical procedures before behavioral training began.

Optical stimulation. Stimulation experiments used CaMKIIα-hChR2(E123A)-eYFP and its respective control CaMKIIα-eYFP. To enable sufficient time for robust opsin expression, illumination of BLA axons in the mEC was conducted at least 5 weeks after viral injection. Figure 10A shows a schematic of viral injection placement, a 5-week incubation period, and fiber optic probe placement. In all cases, the comparison control was a "sham-control" that had opsin expression.

Behavioral training. All experiments used a standard IA chamber, as described in the general methods and shown in Figure 1A. Briefly, rats first underwent context training in which they

were placed in the illuminated side and allowed to freely explore the entire apparatus for 3 min (day 1). The following day, rats were confined to the darkened side of the chamber and received an immediate inescapable footshock (day 2; time in chamber < 15 s). All experiments used a single 1 mA, 1 s footshock. After *either* context (day 1) or footshock (day 2) training, rats received optical stimulation of BLA axons in the mEC or VH in a separate holding chamber. Specific parameters are noted in individual experiments. Retention was tested two days later (day 4) when rats were again placed in the illuminated compartment and allowed free access to the entire apparatus. Latency to cross into the darkened compartment (maximum 600 s) was used as the index of retention.

Immediately after either context or footshock training, rats that had been injected with the ChR2(E123A) virus received 15 min of optical stimulation of the BLA→mEC pathway using the following parameters: 2-s trains of 8, 20, 40, or 80 Hz light pulses (pulse duration = 5 ms), given every 10 s as used previously (Huff et al., 2013; Huff et al., 2016). For all experiments, the control group was a "sham-control" that received no illumination.

Based on positive results, control experiments were conducted, which used only the effective stimulation parameters from the main experiment. A group of rats received exposure to an alternate context (an operant chamber) on day 1 and 15 min of trains of 8 Hz pulses following footshock in the darkened side of the inhibitory avoidance chamber on day 2. An additional group of rats had fiber optic probes implanted in the VH as previous investigations used more limited frequencies.

In all experiments, rats received optical manipulations immediately after either the context *or* footshock training. In brief, upon being removed from the training apparatus, bilaterally implanted fiber optic probes were connected to optical fibers through which 473 nm light was delivered. The rat was then placed into an open-top box with opaque sides and the appropriate illumination was provided.

Results

Figure 10B shows the retention latencies for rats that underwent the full modified CFC training and received optical stimulation of the BLA axons in the mEC immediately following context training on day 1. A one-way ANOVA revealed a significant difference between groups ($F_{(3,36)} = 2.963$, $p < 0.05$). Post hoc analyses indicated no difference between sham controls and any frequency; however, post hoc analyses indicated significantly higher latencies for those rats that received trains of 80 Hz light pulses compared to rats that received trains of 20 Hz light pulses. As the previous results showed no significant effects compared to the sham-control rats, a separate group of rats received post-context stimulation using trains of 8 Hz light pulses. Retention latencies for rats that received post-context stimulation with trains of 8 Hz pulses were significantly higher than those of sham controls ($t_{(23)} = 2.148$, $p < 0.05$, Figure 10C). To ensure that the light pulses alone were not responsible for the enhanced retention, a control experiment was conducted in which the BLA was transduced with eYFP alone and 8 Hz light pulses were applied to BLA terminals in the mEC. Figure 10D shows the retention latencies for this experiment. The latencies for the illumination group were not significantly different from those of the sham controls ($t_{(21)} = 1.768$ $p > 0.05$).

To determine whether stimulation of the BLA-mEC pathway alters the consolidation of the footshock learning, rats were trained on the full CFC task but received stimulation following footshock training (day 2) across the full range of stimulation frequencies previously used (8-80 Hz). A one-way ANOVA revealed no difference in retention latencies among groups regardless of the frequency stimulation provided, as shown in Figure 11A ($F_{(4,40)} = 0.6082$, $p > 0.05$). Additionally, as previous results indicating a role for the VH in footshock, but not context, learning did not examine theta-frequency stimulation (Huff et al., 2016), a separate experiment was conducted in which rats received theta stimulation in the BLA-VH pathway following context training. Retention latencies were not significantly different between sham controls and those rats that received theta stimulation in the BLA-VH pathway ($t_{(15)} = 0.6161$, $p > 0.05$; panel B). In a final control experiment, rats underwent exposure to an alternate context (an operant chamber) on day 1 before receiving trains of 8 Hz stimulation to BLA-mEC pathway. Retention latencies for rats that received stimulation following alternate context exposure were not significantly different from sham controls ($t_{(9)} = 0.4073$, $p > 0.05$; Figure 11C).

Discussion

The current experiments build on previous work from our lab investigating the role of the BLA in optogenetic manipulation of memory consolidation and provide further evidence that separate BLA projections play different roles during the footshock and context components of a CFC task. These findings indicate that stimulation of BLA projections to the mEC following context, but not footshock, training significantly enhanced retention for a modified CFC task. In particular, stimulation using trains of 8 Hz pulses, but not 20, 40, or 80 Hz pulses, was effective. Similar stimulation provided to BLA terminals in the VH following context training did not

enhance retention. Together, this points to a role for the BLA-mEC pathway in modulating the consolidation for the context, but not footshock learning, in the modified CFC task used here.

The mEC is involved in spatial processing and memory consolidation and the BLA is involved in consolidation of spatial memories, supporting investigation of the functional connection between the two brain regions following a spatial learning component of a modified CFC task. Although previous work has investigated the BLA-mEC pathway during the *acquisition* of fear learning (Sparta et al., 2014), the present experiments are, to our knowledge, the first to examine at the BLA-mEC pathway during the posttraining consolidation period. Additionally, use of the modified CFC task permitted examination of the BLA-mEC pathway following separate components of CFC task again, allowing us to better define the role of the BLA-mEC pathway during CFC learning. As the mEC is known to process information from the BLA and communicate with the dorsal hippocampus through either the indirect trisynaptic pathway (Andersen et al., 1969) or a direct pathway (Steward and Scoville, 1976), it is not surprising that the present findings are consistent with previous findings using the same modified CFC task in which BLA and DH were both involved in the consolidation of spatial memories (Malin and McGaugh, 2006).

Our findings in Chapters 2 and 3 indicated that gamma frequency stimulation was effective in enhancing retention following a one-trial IA task when applied to BLA cell bodies and for the modified CFC task when applied to BLA terminals in the VH after footshock training (Huff et al., 2013; Huff et al., 2016). As parameters for projections to downstream targets may differ, a wider range of stimulation parameters (8-80 Hz) was used to identify the target frequency for the

BLA-mEC pathway. Indeed, gamma frequency stimulation of BLA terminals in the mEC following context learning had no effect. However, stimulation in the theta frequency range (here, 8 Hz) increased retention latencies. This is consistent with research investigating the role of theta rhythms in memory consolidation, although many of these studies examined changes in theta during sleep (Popa et al., 2010; Ognjanovski et al., 2014; Boyce et al., 2016). Evidence suggests that theta rhythm activity in the EC and hippocampus is also important for spatial memory (Buzsaki, 2005; Cappaert et al., 2009; Buzsaki and Moser, 2013). Theta-gamma coupling occurs during spatial learning in the hippocampus proper and especially the dorsal CA1 region that receives input from the EC (for review see Colgin, 2015). For example, in one study, rats performed a spatial alternation task while investigators recorded from the CA1 subfield of the hippocampus (Nishida et al., 2014). Theta-gamma coupling increased across learning trials suggesting the importance of both frequencies in memory consolidation.

Combined with previous work supporting a specific role for the BLA-VH pathway in the modulation of footshock, but not context, learning in the same modified CFC task (Huff et al., 2016), the present data provide further evidence that the BLA modulates memory consolidation via distinct projections to different targets. Therefore, it is likely that a network of connections emanating from the BLA are involved in various aspects or kinds of learning (footshock vs. context vs. positively valenced stimuli, etc.) for different tasks. Additionally, the present findings suggest that both theta and gamma frequency activity will be of importance in other pathways and types of learning and memory, as they are found throughout the brain in connection to learning (for review see Buzsaki and Wang, 2012).

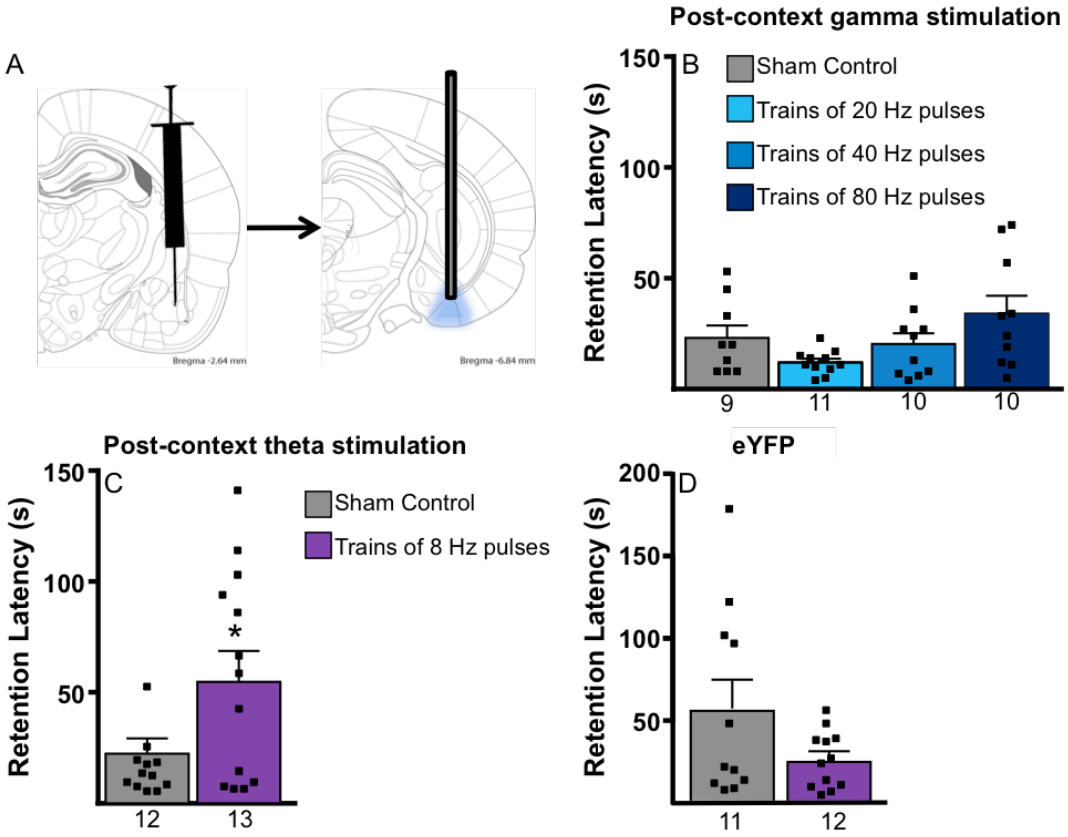

Figure 10. Retention effects of optical stimulation of ChR2(E123A)-transduced BLA axons in the mEC immediately after context (day 1) training.

Panels B-D show the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. **A,** Schematic diagram of BLA injection site and optic probe placement in mEC. **B,** Retention latencies of rats given post-context training gamma frequency optical stimulation of BLA axons in the mEC via activation of ChR2(E123A). There was no significant effect on retention regardless of gamma frequency stimulation used. **C,** Increased retention when theta frequency stimulation was given to rats after context training. *p < 0.05 compared to sham-control. **D,** No effect on retention of posttraining light pulses given to eYFP-expressing control rats.

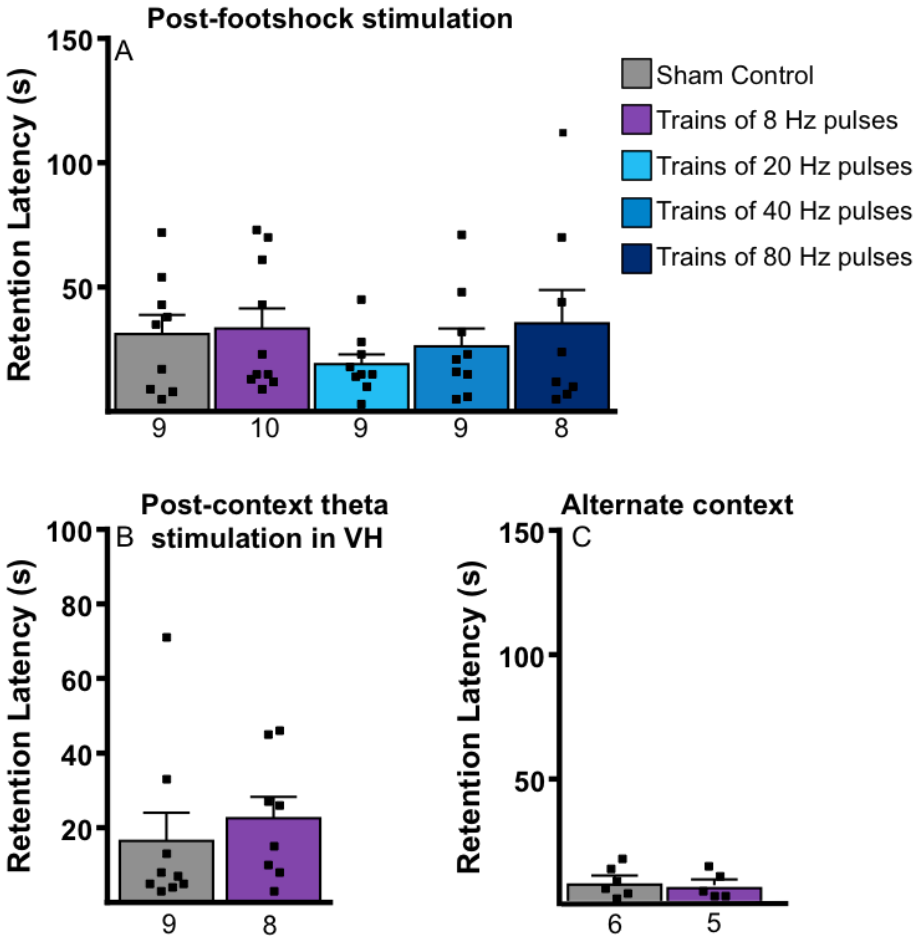

Figure 11. Retention effects of ChR2(E123A)-transduced BLA axons in the mEC after footshock training on day 2.

All graphs show the mean retention latencies, in seconds (± SEM), as well as the number of rats in each group under the respective bar. **A,** Retention latencies of rats given post-footshock training optical stimulation of BLA axons in the mEC via activation of ChR2(E123A). Such stimulation had no effect on retention regardless of stimulation frequency used. **B,** Retention latencies of rats given theta frequency stimulation following context (day 1) training in the BLA-VH pathway. **C,** No effect of optical stimulation of the BLA-mEC pathway following day 1 training on retention if context pre-exposure was altered.

CHAPTER 5. GENERAL DISCUSSION

Investigations of memory consolidation began over a century ago and BLA involvement in modulating consolidation has been heavily implicated in much of this research (Roozendaal and McGaugh, 2011). The ability to be involved in consolidation for a variety of learning tasks including fear conditioning (LaLumiere et al., 2004a) or less arousing tasks like object recognition (Roozendaal et al., 2008b) likely results from the many BLA efferent projections. Previous explorations of the BLA involved techniques that, by their very nature, limited interpretation. The development of optogenetics significantly increased the spatial and temporal precision for investigating single structures and the pathways between various structures. One-trial associative learning paradigms like IA and modified CFC allow robust learning, the consolidation of which can be enhanced or impaired immediately following training.

In chapter 2, we established the use of optogenetics in manipulating the BLA to influence memory consolidation. Rats that received stimulation of BLA cell bodies immediately after IA training showed enhanced retention for the task when tested 48 h later (Huff et al., 2013). Importantly, this enhancement was found using trains of 40, but not 20, Hz stimulation. Optical inhibition of the BLA for 15 min, but not 1 min, following training impaired retention. These results indicate that posttraining optogenetic manipulations can be used to both enhance and impair memory consolidation and help to define the temporal dynamics involved in the BLA during the consolidation period.

Chapter 3 examined the projection from the BLA to the VH, a known site of emotional processing. Optical stimulation of the BLA-VH pathway following footshock, but not context,

training enhanced retention for a modified CFC task. This enhancement occurred with trains of 40, but not 20 or 80, Hz light pulses, similar to the effective stimulation parameters found in Chapter 2 for the BLA cell bodies following an IA task. Optical inhibition of the same pathway *immediately* following footshock training was not effective. However, if inhibition began 25 min after training, retention latencies were significantly reduced. Again, this effect was specific to optical inhibition provided to the post-footshock, but not post-context, training period. This was the first investigation, to our knowledge, to directly manipulate this pathway during memory consolidation supporting the idea that different BLA pathways are involved in dissociable aspects of memory consolidation.

To extend these findings, experiments in Chapter 4 examined the involvement of the BLA-mEC pathway in the same modified CFC task, as the mEC is a known site of spatial processing and consolidation. Optical stimulation using trains of 8, but not 20, 40, or 80, Hz light pulses following context training enhanced retention. No stimulation frequency used for the BLA-mEC pathway enhanced retention following footshock training. This suggests the BLA-mEC pathway is selectively involved in consolidation of context learning and further indicates that different BLA pathways are involved in consolidation for different types of learning.

Stimulation patterns

Gamma oscillations (30-100 Hz) occur throughout the brain and have been linked to processing sensory stimuli (Adrian, 1942; Mather and Sutherland, 2011) and attention (Bouyer et al., 1981; Engel et al., 2001; Fries et al., 2001), and more recently it has been argued that gamma plays a large role in processing emotion (Jutras et al., 2009; Luo et al., 2009; Headley and Weinberger,

2011). Activity in the theta frequency range (4-10 Hz) is prominent throughout the hippocampal formation and has also been linked to ongoing behavior (Grastyan et al., 1959; Stumpf, 1965; Vanderwolf, 1969) and consolidation (Montgomery et al., 2008; Popa et al., 2010). Through investigation of BLA projections to the VH and mEC, our work indicates that the stimulation patterns, specifically the frequency of stimulation, effective at enhancing memory consolidation are not identical between different pathways, suggesting that the endogenous activity within the pathways important for memory consolidation is also different. Although gamma frequency stimulation was effective in modulating consolidation when applied to BLA cell bodies following an IA task and the BLA-VH pathway following footshock training, only theta frequency stimulation was effective in modulating consolidation of context learning when applied to the BLA-mEC pathway. This difference may be due to the type of information being processed in a particular area or pathway. Gamma frequency stimulation was effective in areas (BLA and BLA-VH pathway) modulating footshock whether in a combined or separated manner consistent with gamma frequency activity during emotional learning. Theta frequency stimulation in the BLA-mEC pathway was effective in increasing retention only following context training, suggesting theta may be more important in areas processing context/spatial learning.

Previous work concurrently recorded the BLA and some target structures. Gamma oscillations between the BLA and striatum synchronize and the power of oscillations increases in later stimulus-response learning trials (Popescu et al., 2009). Amygdalostriatal gamma coherence also correlates with the degree of learning. Similar recordings in the BLA and rhinal cortices during and following learning found an increase in gamma frequency power during late learning and a

weak correlation between theta and gamma power (Bauer et al., 2007). Although gamma and theta activity are sometimes detected alone, the two types of activity are often observed during the same tasks (e.g., exploratory behaviors, working memory, consolidation), especially in the hippocampus and entorhinal cortex (Buzsaki et al., 1983; Chrobak and Buzsaki, 1998; Buzsaki et al., 2003; Jensen and Colgin, 2007; Colgin et al., 2009). Theta-gamma cross-frequency coupling also exists within the prefrontal cortex (PFC) and hippocampus during learning. Increases in theta-gamma coupling in the prefrontal cortex appear to be related to better performance during working memory (Li et al., 2012) and enhanced retrieval of a spatial memory task (Shirvalkar et al., 2010). The strength of theta-gamma coupling also increases across training days both within the medial PFC (Shearkhani and Takehara-Nishiuchi, 2013) and dorsal hippocampus (Tort et al., 2009). The EC is known to entrain theta activity in the rest of the hippocampal formation (Holsheimer et al., 1982; Buzsaki et al., 1983; Leung, 1984; Brankack et al., 1993) and hippocampal theta is know to entrain of other portions of the cortex (Sirota et al., 2008). EC lesions reduce theta amplitudes and lead to decreases in hippocampal gamma power (Bragin et al., 1995).

It is hypothesized that gamma frequency activity in the hippocampus helps to route information through the hippocampus to areas of neocortex (Colgin et al., 2009) and theta oscillations modulate gamma frequency activity (Canolty et al., 2006). Additionally, the BLA, hippocampus, and mPFC all show levels of theta-gamma coupling during tasks involving fear and anxiety (Stujenske et al., 2014). As both gamma and theta activity have been detected during learning and memory in many species (Fell et al., 2001; Sederberg et al., 2007; Jutras et al., 2009; Tort et

al., 2009), they remain good candidate frequencies to obtain positive results when optogenetically applied during time points of interest.

Alternative pathways

Depending on the projection investigated, the BLA will likely have very different roles in various features of learning or types of learning (Janak and Tye, 2015). Although two candidate BLA targets in the hippocampal formation were chosen here, other structures with distinct BLA innervations could show similar dissociations for multifaceted learning tasks. One of these structures is the nucleus accumbens, which has two subdivisions, the core and shell, both receiving separate BLA projections. Indeed, previous work has investigated the well-defined anatomical connections between the BLA and the accumbens shell and core and examined the roles of these subdivisions during memory consolidation, including for footshock-based learning (Setlow et al., 2000; Roozendaal et al., 2001; LaLumiere et al., 2005). Evidence indicates that the BLA provides a relatively dense innervation of both the accumbens shell and core (Kelley et al., 1982; Christie et al., 1987; Robinson and Beart, 1988). Other findings suggest that stressful stimuli, including footshock stress, and footshock-associated tones increase dopamine levels in the accumbens and that BLA activity also increases dopamine levels (Kalivas and Duffy, 1995; Pezze et al., 2002). Indeed, blockade of dopamine receptors in the accumbens shell prevents the ability of the BLA to modulate memory consolidation for IA learning, another footshock-based learning task (LaLumiere et al., 2005). As the BLA projections to the accumbens modulate the local release of dopamine (Floresco et al., 1998; Howland et al., 2002; Stuber et al., 2011), changes in dopamine levels following stress may be due, in part, to BLA projections' activity.

Other findings suggest that the accumbens shell is also necessary in spatial and contextual learning tasks (Ito et al., 2008), which is not surprising given the inputs to the accumbens shell from the hippocampus (Kelley and Domesick, 1982; Roozendaal et al., 2003). In contrast to the findings with the accumbens shell, studies examining the accumbens core indicate that contexts previously associated with footshocks, but not footshocks themselves, increase dopamine in the accumbens core (Kalivas and Duffy, 1995; Pezze et al., 2001). Physiological recordings also suggest that BLA activity facilitates and couples with activity in downstream structures, including the striatum, in the gamma frequency range (35–45 Hz) across learning trials (Popescu et al., 2009).

Another BLA target, the mPFC, also comprises subdivisions that play more specific roles in different types of learning (ranging from more sensory to limbic to executive function). The BLA has well-known separate projections to the four subdivisions of the mPFC (McDonald, 1991a; Hoover and Vertes, 2007). IA training increases Arc and/or c-fos expression in many of these regions, and blocking protein synthesis in these areas impairs memory consolidation (Zhang et al., 2011). The dorsal mPFC has been implicated in processing emotional information like those involving fear (Baeg et al., 2001; Gilmartin and McEchron, 2005) but also driving drug seeking behavior (Peters et al., 2009). Indeed, interactions between the BLA and dorsal mPFC are well known. For example, intra-mPFC injections of the glucocorticoid receptor agonist RU 28362 enhanced IA retention, an effect blocked by BLA lesions (Roozendaal et al., 2009). The ventral mPFC appears to play an inhibitory role in fear learning, as activity in that region is necessary for fear suppression and extinction (Milad and Quirk, 2002; Milad et al., 2004).

Utilizing optogenetics, investigations of some of these BLA pathways have been completed for tasks associated with positive and negative consequences. Following reward conditioning, the BLA projection to the accumbens core showed increasing synaptic strength following reward conditioning and stimulation of the same pathway caused positive reinforcement (Namburi et al., 2015). Studies with mice found increased optical self-stimulation of the BLA-accumbens core pathway and reduced motivation for a sucrose reward when the pathway is inhibited (Stuber et al., 2011). Stimulation of the BLA-mPFC pathway also increased anxiety-like behavior as measured by a reduction in time spent in the open arms of an elevated-plus maze and less time in the center of an open field (Felix-Ortiz et al., 2016). Conversely, BLA-mPFC pathway inhibition increased time spent in the center zone during an open field test. Beyeler and colleagues (2016) identified BLA projections to three regions (the mPFC, VH, or CeA) with different response profiles to positive and negative stimuli. BLA projections to both the accumbens core and VH had more excitatory responses to positive cues and reduced inhibitory responses. BLA neurons projecting to the CeA were more excited (and less inhibited) by the negative-associated cue. The authors suggest this diversity in neuronal response may account for the different behavioral responses observed when different BLA projection ensembles are recruited (Beyeler et al., 2016).

However, to further complicate the interactions between the BLA and targets discussed herein (VH, mEC, nucleus accumbens, and mPFC), reciprocal projections from the VH, mEC and portions of the mPFC to the BLA exist (Pitkanen et al., 2000; Vertes, 2004). These descending pathways have also been shown to be functional in learning and memory (Rosenkranz et al.,

2003; Milad et al., 2004; Mouly and Di Scala, 2006; Orsini et al., 2011; Lubkemann et al., 2015) and could thus demonstrate a complex neural network for emotional learning. Additionally, there are connections between the hippocampus and mPFC (Jay et al., 1995; Takita et al., 1999; Paz et al., 2007) making the possible network for associative learning even more complex.

Conclusions

The current findings were the first to directly examine the pathways from the BLA to the VH and mEC during memory consolidation. Collectively, these data show different BLA efferents play distinct roles during consolidation of different components of the same CFC task. Importantly, photoinhibition of both the BLA itself and the BLA-VH pathway 3 h after training, and presumably outside the "consolidation window," had no significant effect, suggesting a temporal limit for the role of the BLA and its projections during consolidation. Together, this work helps to narrow the specific role for different pathways during various tasks or components of tasks but also expands our perspective of differential pathways working in tandem during consolidation.

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