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Auditory and Contextual Contributions to Memory Lability and Synaptic Destabilization in the Amygdala

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AUDITORY AND CONTEXTUAL CONTRIBUTIONS TO MEMORY LABILITY AND SYNAPTIC DESTABILIZATION IN THE AMYGDALA

by

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ABSTRACT

AUDITORY AND CONTEXTUAL CONTRIBUTIONS TO MEMORY LABILITY AND SYNAPTIC DESTABILIZATION IN THE AMYGDALA

by

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Pavlovian fear conditioning provides a way to investigate memory formation and retrieval. During fear conditioning, a conditional stimulus (CS) is paired with an aversive outcome and the CS acquires aversive value over several pairings. The CS may then be presented during a retrieval session where fear responding is measured as an indicator of memory strength. Retrieval sessions may allow for the incorporation of new information into the original memory trace by destabilizing amygdala synapses. However, the specific circuits and neural inputs that contribute to memory lability and synaptic destabilization during a retrieval session are poorly understood. Previous work has shown that contextual novelty during an auditory retrieval session is necessary for memory lability, suggesting that brain regions encoding auditory and contextual information interact during memory retrieval. The dorsal hippocampus and auditory thalamus play selective roles in processing contextual and auditory information, respectively, during fear conditioning. In the current study, we manipulate functional inputs from each region to determine how each impacts memory lability at amygdala synapses. We found that 1) silencing auditory thalamic inputs in the amygdala during a brief retrieval session reduces fear to an auditory cue and leads to long lasting reductions in fear, and 2) inactivation of the dorsal hippocampus prior to training allows for memory impairment when anisomycin is infused into

the amygdala after a retrieval session in an anisomycin resistant memory. This work highlights an important role for brain regions processing sensory information during training and the impact on fear memory recall and modification.

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α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Adeno-associated viral vectors	AAV
Anisomycin	ANI
Archaerhodopsin-T	ArchT
Artificial cerebral spinal fluid	ACSF
Calcium-calmodulin dependent protein kinase II	CaMKII
Calcium impermeable AMPA receptors	CI-AMPAR
Calcium permeable AMPA receptors	CP-AMPAR
clasto-Lactacystin β-lactone	βlac
Conditioned response	CR
Conditional stimulus	CS
Dimethyl sulfoxide	DMSO
Dorsal hippocampus	DH
Intercalated cells	ITC
Lateral amygdala	LA
Long-term potentiation	LTP
Magnesium	Mg^{2+}
Medial geniculate nucleus of the auditory thalamus	MgN
N-methyl-D-aspartate	NMDA
Paired pulse facilitation	PPF
Post synaptic density	PSD
Post-Traumatic Stress Disorder	PTSD
Protein Kinase A	PKA

Ubiquitin-proteasome system	UPS
Unconditional stimulus	UCS
Vehicle	VEH

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Chapter 1: General introduction and background

Pavlovian Fear Conditioning

Understanding how memory for an aversive or traumatic event is formed can shed light on the dysregulation of fear responses that occurs in anxiety and stress related disorders such as PTSD. Expression of fear after trauma comes back in several different forms such as recall and avoidance of cues present during the event (Parsons & Ressler, 2013). Fear responses resulting from trauma can be generalized to safe stimuli or non-associated cues, resulting in maladaptive fear responding (Norrholm et al., 2011). Persistent changes in the neural circuitry and molecular mechanisms necessary for the formation of a fear memory and the expression of fear can provide a better understanding of treatments for fear related disorders.

Pavlovian fear conditioning can be used as a tool to measure and manipulate the molecular mechanisms underlying fear memory formation and modification in both rodents and humans (Fendt & Fanselow, 1999; Johansen et al., 2011; Parsons & Ressler, 2013). The memory formed from Pavlovian conditioning is quickly acquired, robust, and long lasting, making it appropriate for studying the neural mechanisms supporting learning and memory. During conditioning, a neutral stimulus, known as a CS, is paired with an aversive stimulus, known as a UCS. The subject learns that the CS predicts the UCS over several pairings, and the CS acquires aversive value. To test the long-term retention of the memory formed during conditioning, the CS can be presented independently of the UCS during a retrieval session where the CS will elicit a fear response (Fanselow, 1980). Specifically, the conditioning context, consisting of the collection of stimuli that make up the training environment (e.g. flooring, walls, lighting, odor), or a discrete auditory cue can often serve as a CS in fear conditioning. To measure the strength of the association between the CS and UCS in rodents, freezing behavior is often recorded as a

measure of fear responding. At retrieval, groups can be exposed to either the original training context or placed into a new context where the chamber, lighting, and odor are changed. The change in context allows us to separate tone specific fear from fear to the environment in which training occurred (Gilmartin & Helmstetter, 2010; Kwapis et al., 2011).

Early work studying memory storage showed that the associations formed from a learning experience can be disrupted with electroconvulsive shock immediately following training (Duncan, 1949; Misanin et al., 1968; Squire et al., 1984). A large number of rodent studies have shown that electroconvulsive shock or other disruptive treatments administered after training induce retrograde amnesia. Importantly, manipulations capable of disrupting memory need to take place within a time window close to when learning occurred, suggesting that memories transition to a less fragile state over time (Duncan, 1949). The "consolidation hypothesis" accounts for the temporally restricted fragility of a memory trace following learning and proposed that the stabilization of memory is characterized by the transition into a long-term state for permanent storage (McGaugh, 2000; Nadel & Land, 2000). Memory consolidation occurs at both a cellular and neural systems level. Cellular consolidation is generally referred to as the time period in which plasticity occurring in neurons storing the memory trace slows and becomes resistant to disruption. This normally occurs within a few hours from learning. Systems consolidation refers to the transition of memory storage to the cortex, which can take weeks to months. Pavlovian conditioning provides a way to study memory consolidation through the newly acquired CS-UCS associations. The memory formed from Pavlovian conditioning provides support for the consolidation hypothesis since these memories become increasingly resistant to disruption and modification as time after training increases in addition to changes in

the neural circuit important for memory storage (Anagnostaras et al., 1999; Bourtchouladze et al., 1998; Kida et al., 2002).

Neurobiological mechanisms of memory consolidation

Long-term changes in synaptic structure and cellular activity are thought to be a hallmark of memory storage. LTP is believed to be the cellular correlate for learning and memory and is characterized by long lasting increases in synaptic strength. It is believed that input-specific LTP is induced during fear conditioning and results in increased synaptic strength during memory consolidation (Kim & Cho, 2017). The plastic events that occur at potentiated synapses during the induction of LTP are thought to correspond to early time points of cellular consolidation, while plastic events during later stages of LTP have been linked to later phases of memory consolidation. For example, weak input (e.g. one train of 100 pulses at 100Hz) leads to increases in protein trafficking in synapses but the synaptic potentiation is not dependent on *de novo* protein synthesis or NMDA receptor activity and is relatively short-lived (Huang, et al., 1994; Lu et al., 2008). The potentiation of synapses independent of protein synthesis is referred to as early phase LTP (E-LTP) and might correspond to short-term memory (Bliss & Collingridge, 1993; Hernandez & Abel, 2009). On the other hand, strong inputs (e.g. multiple trains of 100 pulses at 100Hz) can increase synaptic efficacy lasting for several weeks and requires *de novo* protein synthesis as well as NMDA receptor activity. This is referred to as late phase LTP (L-LTP) (Abraham, 2003; Bliss & Collingridge, 1993; Krug et al., 1984; Lu et al., 2008). The ability to disrupt L-LTP is also temporally restricted, with a necessity for transcription occurring following the first tetanus but not 2-hours after, making the potentiated synapses stable 2-hours after induction (Nguyen et al., 1994). The extended duration of synaptic efficacy and the temporally

restricted fragility of L-LTP mimics memory stability and the persistence of long term memories over time.

The molecular events occurring during the early and late phases of LTP are key to understanding memory storage at a synaptic level. During associative L-LTP, NMDA receptors allow for calcium entry to activate a host of proteins associated with receptor trafficking and elevated protein expression in the PSD which in turn trigger synaptic plasticity. Activation of kinases, such as CaMKII or PKA, via NMDA receptor-dependent calcium influx plays a substantial role in the trafficking and function of AMPA receptors in the PSD during L-LTP (Bredt & Nicoll, 2003; Plant et al., 2006). AMPA receptors are trafficked into the PSD and are remain present for an extended duration following stimulation and have been used as markers for potentiated synapses because of their ability to regulate excitability during L-LTP. Generally, AMPA receptors can be functionally classified based on calcium permeability – GluR1 containing AMPA receptors are calcium permeable (CP-AMPAR), while GluR2 containing AMPA receptors are calcium impermeable (CI-AMPAR) (Bredt & Nicoll, 2003). AMPA receptors are organized into tetramers, namely the GluR1/1 homomeric receptors (CP-AMPAR) and GluR1/2 heteromeric receptors (CI-AMPAR). They are well studied and undergo activity-dependent trafficking. L-LTP induction initiates trafficking of CP-AMPARs into stimulated synapses via the second messenger, CaMKII, which is likely activated in response to NMDA receptor-regulated calcium influx (Barria et al., 1997; Barria & Malinow, 2005; Hayashi et al., 2000). At a later time point, CI-AMPARs replace the CP-AMPARs in the PSD during L-LTP expression (Kauer & Malenka, 2006; Plant et al., 2006). The presence of CI-AMPARs in the synapse is necessary for synaptic stabilization following the induction of L-LTP due to its lack of calcium permeability. Synaptic stabilization during L-LTP is also necessary during consolidation

and allows the memory to become resistant to disruption, therefore, the stabilization of potentiated synapses is critical for stabilization or long-term storage of a memory.

De novo protein synthesis is necessary for the stabilization of synapses. Previous work using a combination of techniques has shown a necessity for *de novo* protein synthesis during memory consolidation and LTP. For example, inhibition of gene transcription or RNA translation prior to or immediately following conditioning disrupts memory retention (Parsons et al., 2006b; Schafe et al., 2000). ANI is a protein synthesis inhibitor that blocks 60s ribosomal subunit activity and subsequent RNA translation and its use has provided much of the fundamental data on the role of protein synthesis-dependent plasticity in memory and LTP (Alberini, 2009; Davis & Squire, 1984). ANI will prevent the consolidation of several different types of memory, including but not limited to fear conditioning, inhibitory avoidance, contextual encoding, and object recognition, (Akirav, & Maroun, 2006; Bailey et al., 1999; Barrientos et al., 2002; Kwapis et al., 2011; Rossato et al., 2007; Stäubli et al., 1985), providing evidence that protein synthesis-dependence is a shared mechanism across a variety of memories. Infusions of protein synthesis inhibitors provides a way to study the timeline of memory consolidation and synaptic stabilization (Bailey et al., 1999; Bourtchouladze et al., 1998; Kwapis et al., 2011; Quevedo et al., 1999; Rossato et al., 2007; Schafe et al., 1999; Sharma et al., 2012). Delayed infusions of protein synthesis inhibitors have been useful when characterizing the temporal limitations of memory consolidation and have shaped our understanding of the role for synaptic stabilization following learning. As is the case with L-LTP, infusions of ANI can effectively block memory consolidation within 3 hours after a learning event (Bourtchouladze et al., 1998). The application of more modern techniques lend further support for the requirement of protein synthesis during memory consolidation. The incorporation of labeled puromycin molecules into

newly synthesized proteins have shown that protein synthesis occurs following learning and is necessary for successful consolidation (David et al., 2012; Hoeffer et al., 2011; Ma et al., 2013, Tudor et al., 2016).

Interruption of synaptic stability and long-term storage of information with broad-spectrum protein synthesis inhibitors has fueled research to find the specific proteins involved in memory and to determine their role in synaptic plasticity. The initial phase of memory consolidation is characterized by increases in intracellular calcium levels, which activate intracellular signaling cascades, such as CaMKII. As described previously, CaMKII can be activated in response to NMDA receptor-dependent Ca^{2+} influx and plays a critical role in synaptic potentiation and plasticity supporting memory formation (Jarome et al., 2016; Wang et al., 2003). Inhibiting the translation of CaMKII disrupts memory formation in addition to the maintenance of L-LTP which provides evidence that CaMKII translation is necessary for postsynaptic stabilization (Miller et al., 2000). It has been proposed that autophosphorylated CaMKII may bind to NMDA receptors to indirectly stabilize CI- and CP-AMPA receptors in the PSD (Barcomb et al., 2016; Barria & Malinow, 2005; Lisman & Zhabotinsky, 2001; Sanhueza et al., 2011; Sanhueza & Lisman, 2013). Collectively, this work shows a necessity for protein synthesis during synaptic stabilization, and specifically, a role for the synthesis of CaMKII during later phases of consolidation.

Memory consolidation and LTP share several characteristics. For example, potentiated glutamatergic synapses are thought to be critical for memory formation and are regulated by protein synthesis-dependent plasticity. Similar to during LTP, AMPA receptors undergo activity-dependent trafficking during memory consolidation and are persistently expressed in the PSD (Rumpel, et al., 2005; Yeh et al., 2006). PSD-95 is a synaptic scaffolding protein that also shows

persistently elevated expression and acts to stabilize CI- and CP-AMPA receptors at potentiated synaptic surfaces. Other scaffolding proteins, such as SHANK, are more dynamically regulated during and following memory phases but form complexes with a several other synaptic scaffolds to regulate AMPA receptor trafficking and spine morphology (Sala et al., 2001). CP-AMPARs are moved to the synaptic surface at the initial phases of consolidation and are associated with calcium-dependent phosphorylation of kinases supporting memory formation (Hayashi et al., 2000). At this time, PSD-95 and SHANK are also increased to accompany the increase in AMPA receptor synaptic expression (Jarome et al., 2011). CI-AMPAR replace CP-AMPAR in later stages of consolidation to stabilize the memory and the potentiated synaptic connections.

Recent evidence shows that a destabilization phase precedes the protein synthesis-dependent stabilization of synaptic change that accompanies learning. The destabilization phase of memory consolidation is dependent on the UPS and may regulate the necessity for protein synthesis discussed above. When active, the UPS targets proteins that are tagged by small protein modifiers, ubiquitin, for degradation. Specifically, the E1 ligase activates ubiquitin, allowing for the E2 ligase to bind and recruit E3 ligases. The E3 ligases bind to protein substrates, forming a polyubiquitin chain when linked at specific lysine residues (Jarome et al., 2013). Lysine-48 polyubiquitin tags are recognized by the UPS to target specific proteins for degradation and are thought to be the strongest UPS-recognized signal for protein degradation (Bingol & Sheng, 2011; Fioravente & Byrne, 2011; Hegde, 2010). The 26S proteasome is a multi-subunit structure containing a 20s catalytic core that is flanked by 19s regulatory particles. There are six Rpt subunits on the 19s caps, and phosphorylation of Rpt6 regulates 20s catalytic activity, suggesting phosphorylation of Rpt6 is critical for function of the UPS (Bedford et al., 2010). Previous work has shown that NMDA receptor-dependent CaMKII autophosphorylation activates the UPS, and

inhibition of NMDA receptor activity or CaMKII can prevent lysine-48 polyubiquitin tagging and phosphorylation of Rpt6 (Barria & Malinow, 2005; Jarome et al., 2016; Jarome et al., 2011). Proteins in the synapse that are tagged for UPS-dependent degradation are likely synaptic scaffolds to destabilize synapses to potentially allow for reorganization. SHANK is upregulated in response to learning and is also a proteasome target, providing a way for proteolytic activity to modulate AMPA receptor trafficking and stability in the synapse (Ehlers, 2003; Ferreira et al., 2015; Jarome et al., 2011; Lu et al., 2008). The activity dependent degradation of synaptic scaffolds that maintain stable markers of memory and synaptic potentiation emphasizes a critical role for the proteasome in memory.

Network regulation of amygdala plasticity during memory formation

Human and rodent work highlight a role for the amygdala during fear conditioning. The amygdala is an integration site for CS-UCS neural activity through its connectivity with several different sensory processing regions. When applied immediately following fear conditioning, protein synthesis inhibition in the amygdala is known to disrupt both auditory and contextual fear memories and reduces synaptic plasticity (Bailey et al., 1999; Parsons et al., 2006a-b). For example, intracellular signaling pathways in the amygdala regulate AMPA receptor trafficking at select synapses following learning (Hayashi et al., 2000). Specifically, fear conditioning drives CP-AMPAR into synapses, which are persistently expressed in the amygdala (Yeh et al., 2006). Importantly, blockade of AMPA receptor surface expression at as little as 10% of synapses in the amygdala reduces fear learning (Rumpel et al., 2005), suggesting an important role for potentiated synapses in the amygdala for fear memory consolidation.

Brain structures such as the MgN and DH play distinct roles in the encoding of an auditory or contextual CS, respectively, and require local protein synthesis to successfully

encode information about auditory cues or the training context (Helmstetter et al., 2008). The learning-induced changes in synaptic plasticity in the amygdala are due to input from afferents throughout the fear circuit. The amygdala receives input from both the MgN and DH, and protein synthesis-dependent plasticity throughout the distributed circuit plays an important role in AMPA receptor presence in amygdala synapses (Davis & Squire, 1984; Ferrara et al., 2017; Fischer et al., 2004; Parsons et al., 2006c). CP-AMPAR expression at thalamo-amygdala synapses is critical for induction of LTP, showing a necessity for CP-AMPAR trafficking for thalamic modulation of amygdala synaptic plasticity (Clem & Huganir, 2010; Rumpel et al., 2005). Stimulation of MgN and auditory cortex terminals in the amygdala when paired with a UCS resembles the process of auditory fear conditioning and is dependent on glutamatergic synaptic transmission, suggesting MgN terminals can provide a representation of auditory information in the amygdala dependent on CP-AMPA receptor activity (Kwon et al., 2014).

The amygdala receives information from and is able to modulate synaptic plasticity in the DH (McGaugh, 2004; Richter-Levin & Akirav, 2001). For example, pharmacology and electrophysiology experiments have demonstrated that stimulation of the amygdala increases excitability in the hippocampus (Akirav & Richter-Levin, 1999; Ikegaya et al., 1995), and amygdala silencing also reduces hippocampal activity (Huff et al., 2006). Furthermore, there is increased theta synchrony between the DH and amygdala following fear learning, suggesting a critical role for amygdalo-hippocampal pathways during memory consolidation (Narayanan et al., 2007; Pape et al., 2005). While it is unclear how contextual information is relayed to the amygdala during Pavlovian conditioning, several studies highlight a role for the hippocampus in the encoding of contextual cues during learning, making the interactions between the amygdala

and hippocampus critical for a comprehensive understanding of memory consolidation (Lee et al., 2017; Maren et al., 1997, Redondo et al., 2014; Rei et al., 2015; Sanders et al., 2003).

Molecular mechanisms contributing to destabilization and restablization during reconsolidation

The retrieval of an established memory initiates a process known as reconsolidation, which may allow for the incorporation of new information into the original memory trace by destabilizing synapses supporting the memory (Lee et al., 2008; Lee, Nader, & Schiller, 2017). Reconsolidation is potentially useful because it could allow a stable, long-term memory to update with synaptic reorganization, but it is not universally accepted as a mechanism for memory updating (Biedenkapp & Rudy, 2004; Taubenfeld et al., 2001). It shares several features with the consolidation process and is characterized by temporally constrained destabilization and restabilization phases (Jarome et al., 2011; Milekic & Alberini, 2002). Destabilization during reconsolidation serves to make the memory and synapses labile through the degradation of proteins at synapses storing the memory trace. The restabilization serves to strengthen synaptic connections and potentially include new information into the memory trace.

During retrieval, NMDA-dependent calcium influx is believed to regulate UPS activity underlying synaptic destabilization, suggesting significant depolarization of neurons storing the memory are also required (Ben Mamou et al., 2006; Jarome et al., 2011). Specifically, the presence and activity of the NR2B containing NMDA receptors is necessary for synaptic destabilization via the UPS. The UPS targets several synaptic scaffolding proteins that maintain AMPA receptors in the post-synaptic density and degrades them, resulting in AMPA receptor internalization. SHANK is upregulated following consolidation to stabilize AMPA receptors and becomes polyubiquitinated following retrieval, providing evidence that the UPS degrades

synaptic scaffolds maintaining AMPA receptors during memory reconsolidation to regulate destabilization (Wang et al., 2011; Jarome et al., 2011). The internalization of AMPA receptors is thought to modulate memory lability and synaptic depotentiation following retrieval (Hong et al., 2013). Specifically, it is believed that the endocytosis of CI-AMPAR and insertion of CP-AMPAR allows for increased calcium into the synapse and phosphorylation of several signaling cascades regulating protein synthesis. Maintenance of CI-AMPAR in the synapse or inhibition of NMDA receptors or UPS activity during retrieval prevents the incorporation of new information into the memory trace, leaving the memory in a stable state that is unable to be strengthened or weakened (Dalton et al., 2008; Ferrara et al., *submitted*; Hong et al., 2013).

Restabilization is characterized by a requirement for protein synthesis. Infusions of the protein synthesis inhibitor, ANI, immediately following a retrieval session disrupts the original fear memory and prevents the return of CI-AMPA receptors to the PSD (Jarome et al., 2012; Lopez et al., 2015; Nader et al., 2000). Similar to consolidation, the return of AMPA receptors following fear memory retrieval is thought to be a good indicator of memory strength and changes in the pattern of CP- and CI-AMPAR expression in the PSD are sensitive to new information incorporated into the memory trace (Jarome et al., 2015). Specifically, AMPAR trafficking during retrieval can be disrupted with pre-exposure to the retrieval conditions, making AMPAR trafficking patterns during reconsolidation sensitive to encoding of sensory cues prior to learning (Jarome et al., 2015). The necessity for protein synthesis may be dependent on proteolytic activity by the UPS during destabilization. For example, blocking activity of the UPS or signaling events upstream from the UPS leaves the memory in a stable state and unable to be disrupted with protein synthesis inhibition at retrieval (Ben Mamou et al., 2006; Ferrara et al., *submitted*; Jarome et al., 2011). Examples of memory strengthening lend further support for UPS

activity and protein synthesis during reconsolidation. Specifically, inhibition of UPS activity leaves fear responding at the same level prior to retrieval-dependent strengthening, while inhibition of protein synthesis will reduce fear responding to baseline levels that were seen prior to conditioning (Fukushima et al., 2014; Lee, 2008). This work suggests protein synthesis inhibition can disrupt the original fear memory while inhibition of UPS activity leaves the memory in a stable state. Furthermore, inhibition of both UPS and protein synthesis keeps the memory stable and impairs the ability to strengthen the fear memory, suggesting protein degradation mediates protein synthesis-dependent plasticity underlying reconsolidation-mediated memory strengthening. The plasticity supporting reconsolidation-dependent memory strengthening is required throughout the fear circuit and is thought to be regulated by AMPA receptor activity (Fukushima et al., 2014).

Circuitry modulating retrieval-dependent destabilization in the amygdala

The presence of specific cues during memory retrieval is known to contribute to subsequent reconsolidation. It is believed that the incorporation of new information into the retrieval session is what drives memory lability and synaptic destabilization (Lee et al., 2008; Lee, Nader, &Schiller, 2017; but see Taubenfeld et al., 2001; Albereini, 2011). However, how the information presented at the retrieval initiates destabilization is unclear. During a retrieval session, information about the cue and the context are different from the training session. For example, the auditory cue presented during retrieval is typically no longer followed by shock and the context in which the cue is presented is normally “shifted”. The prediction error theory of reconsolidation emphasizes the importance of CS-UCS contingency as a variable for memory updating and synaptic destabilization and takes into account the unreinforced auditory cue (Díaz-Mataix et al., 2013). This theory highlights the temporal uncertainty of the shock presentation as

the critical factor triggering synaptic plasticity necessary for memory updating and focuses on the presentation of the discrete cue as the primary factor underlying the initiation of memory modification. The unreinforced cue presented at retrieval initiates enough temporal uncertainty concerning when the shock will be presented during the retrieval session to engage destabilization. Extinction is a supporting example of the importance of prediction error during memory modification. During extinction, cues are presented several times in the absence of the UCS. typically in a shifted/new context. The absence of the shock during fear extinction is sufficient to induce temporary synaptic depotentiation and destabilization, rendering the memory susceptible to ANI disruption (Dalton et al., 2008). If CS-UCS contingency is critical for memory modification, then presentation of the discrete CS in the absence of the UCS prior to conditioning should disrupt the ability for the CS to reliably predict the UCS. The disruption of the CS-UCS contingency would therefore make the memory resistant to modification and unlikely to see anisomcyin-related impairments in memory retention during retrieval. However, pre-exposure to tones prior to conditioning does not necessarily lead to resistance to memory disruption at retrieval, suggesting that the uncertainty of the cue-shock association is not the only factor triggering destabilization (Kim & Cho, 2017). As previously mentioned, a retrieval session not only typically involves an unreinforced CS but also occurs in a new/shifted context, suggesting that the shifted context in which the cue is presented may be an important factor in the initiation of reconsolidation.

Contextual novelty during memory retrieval provides another possible factor controlling synaptic destabilization and memory lability during reconsolidation. In this case, the novelty of the context in which the CS is presented would provide new information and engage reconsolidation-dependent memory modification. Specific examples manipulating context in the

absence of a discrete cue show that exposure to the context before non-aversive memory formation removes the necessity for protein synthesis (Biedenkapp & Rudy, 2004). On the one hand, this work raises concerns for the reconsolidation hypothesis and suggests reconsolidation may not be a process necessary for all types of memory. On the other, it may suggest that removal of retrieval novelty and lack of new information protects the memory from reconsolidation (Biedenkapp & Rudy, 2004). However, it is unclear if an altered context during retrieval plays an important role in the reconsolidation of an auditory fear memory. The reduction in fear responding following extinction is specific to the context in which extinction occurred (fear renewal), suggesting contextual cues play a critical role in the behavioral outcome of an auditory cue (Bouton & Bolles, 1979). Recent evidence shows novelty of the retrieval conditions (context and auditory cue) during a retrieval session is necessary for the initiation of reconsolidation (Jarome et al., 2015). Furthermore, exposure to the retrieval context and auditory cue prior to training is sufficient to prevent ANI induced disruption during retrieval (Jarome et al., 2015). Additionally, memories are resistant to ANI disruption with auditory fear overtraining, which has been linked to context generalization, suggesting that an excitatory context that may not be perceived as novel during retrieval may modulate the ability to disrupt an auditory fear memory (Wang et al., 2009). This work collectively shows that contextual cues guide destabilization and subsequent restabilization, and thus the brain structures that encode contextual cues and send information to the amygdala may be critical for fear memory reconsolidation.

The DH is known to be necessary for encoding of contextual information and may therefore play an important role in the initiation of reconsolidation. Protein synthesis inhibition in the DH prior to, or immediately following, fear retrieval disrupts long-term memory retention,

suggesting a critical role for protein-synthesis dependent plasticity during reconsolidation of contextual fear (Debiec et al., 2001; Suzuki et al., 2008). Specifically, retrieval of an auditory cue initiates AMPAR trafficking in the DH, suggesting contextual information during an auditory retrieval may be important for auditory reconsolidation (Sanders et al., 2003). This is further supported by increased theta synchronization between the DH and amygdala after auditory fear retrieval (Seidenbecher et al., 2003). More recent work shows strong auditory memories that are resistant to amygdala ANI disruption following a brief retrieval session are modulated by DH activity (Wang et al., 2009). Specifically, ANI resistant memories can be made labile and susceptible to ANI disruption when the DH is lesioned. As previously discussed, the NR2B subunit is important for initiating synaptic destabilization, and NR2B, but not NR1, expression in the amygdala is decreased when memories are resistant to disruption (Wang et al., 2009). The behavioral recovery of ANI-dependent disruption and NR2B subunit expression in the amygdala is mediated by the DH. This work highlights an important role for contextual information, regulated by the DH, in the long-term storage and maintenance of both auditory and contextual fear memories in the amygdala.

Activity in the MgN is critical for fear memory formation and additional learning, such as extinction (Orisini & Maren, 2009). Pharmacological interventions investigating a role for the MgN show that when information about CS-UCS associations between retrieval days is unchanged, plasticity in the MgN is not required for fear memory retention. However, electrophysiology work demonstrates maintained potentiation of the thalamo-amygdala pathway even following memory consolidation, suggesting activity in this pathway is critical after memory formation (Kim & Cho, 2017). The maintained potentiation and synaptic strength in this pathway may indicate an important role for plasticity at MgN-amygdala synapses for memory

maintenance. This would suggest that plasticity in the MgN, and specifically the thalamo-amygdala pathway, is critical when auditory CS-UCS associations are changed. This idea supports previous work showing a role for MgN plasticity during extinction learning and retrieval-dependent modification of CR during additional training (Ferrara et al., 2017; Orsini & Maren, 2009).

Extinction: inhibitory learning and depotentiation

Extinction refers to the repeated presentations of a previously trained CS in the absence of the UCS, resulting in a decrease in fear responding to the CS at a long-term test. Previous work has suggested extinction shares similarities with exposure therapies, making it therapeutically relevant. The understanding of the behavioral and neural mechanisms underlying extinction may help to minimize the return of fear expression seen in debilitating anxiety-related disorders. Currently, there are two major competing theories explaining how extinction results in decreased fear expression: inhibitory learning and depotentiation. Both views account for the reduction in fear at a long-term test, but the mechanisms through which the reduction in fear responding occurs is different.

Inhibitory learning

Fear extinction can be viewed as new inhibitory learning, resulting in a context-specific decrease in fear responding (Konorski, 1948; Rescorla, 1979). The associative inhibition view of extinction suggests contextual and temporal stimuli modulate excitatory CS-UCS association (Rescorla, 2004). This modulation of fear inhibits the excitatory CS-UCS association during extinction, resulting in a reduction in fear responding and a context specific CS-no UCS memory (Bouton & Bolles, 1979). The recovery of fear is, therefore, dependent on the specific context in which extinction occurred. It has been suggested that the extinction context encompasses the

physical context as well as the passage of time (Bouton, 2004). Spontaneous recovery refers to the return of fear at remote time points, while renewal demonstrates the context specificity of extinction. Fear renewal occurs when the CS is presented in the training or novel context after extinction and fear responding returns (Rescorla, 2004). The return of fear through spontaneous recovery and renewal strongly support that the original learning experience is retained following extinction.

As discussed throughout this document, the presence of AMPAR in amygdala synapses has been directly associated with memory strength and can serve as an index of synaptic potentiation. Following extinction, there is evidence of maintained potentiation of cortico- and thalamo-amygdala synapses (Kim & Cho, 2017) and increased inhibition from the mPFC in amygdala (Bloodgood et al., 2018). The inhibition from the mPFC is a result of long-range GABAergic neurons in the amygdala in addition to increased mPFC-interneuron synaptic strength (Bravo-Rivera et al., 2015; Rosenkranz et al., 2003). Recent work also shows a role for heterosynaptic inhibition of primary auditory input in the amygdala by mPFC terminals, resulting in decreased glutamate release (Cho et al., 2013). In this case, synaptic connections storing the CS-UCS memory, such as thalamo- and cortico-amygdala pathways, remain potentiated but are directly inhibited by mPFC projecting neurons. The result of multilevel inhibition from the mPFC in the amygdala is an imbalance between excitation and inhibition. In addition to increased activity from the mPFC, ITC neurons in the amygdala show increased activity following extinction and their activity is directly modulated by MgN input (Asede et al., 2015; Likhtik et al., 2008). For example, ITC cells can inhibit fear responding through projections to the central amygdala as well as by project back to the BLA to provide a negative feedback loop (Asede et al., 2015). During extinction, it is likely that MgN-driven activity in the

amygdala is reduced, which allows for increased activity from ITCs. The increase in ITC activity is inhibitory and feeds back to the amygdala to likely release GABA to presynaptically inhibit MgN input in the LA as well. The imbalance of extinction and inhibition is gated by context, encompassing the physical chamber as well as the passage of time, so when re-exposed to the CS in the training or novel context, excitation resumes resulting in a rapid return of fear (Cruz et al., 2014). This work strongly suggests extinction is not a result of the weakening of synaptic connections storing information about the CS-UCS association at cortico- and thalamo-amygdala synapses, but may be a result of increased inhibition in the amygdala (Figure 1).

Depotentiation

An alternative view of the neural substrates of extinction is the depotentiation of excitatory amygdala synapses. Depotentiation has been closely associated with memory erasure or "unlearning" which, unlike extinction, would not result in return of fear over time or and would not be dependent on the context in which depotentiation occurred (Hong et al., 2009; Kim & Cho et al., 2017; Kim et al., 2007). However, fear rarely returns to originally conditioned levels during spontaneous recovery, which may suggest some degree of unlearning (Delamater & Westbrook, 2014). Depotentiation has been largely characterized by decreased AMPAR presence in amygdala synapses and a more persistent reduction in fear responding over time as a result of extinction (Clem & Huganir, 2010; Hong et al., 2009; Kim & Cho et al., 2017; Kim et al., 2007). Support for this idea comes from manipulating CI-AMPAR internalization during extinction. Specifically, the inhibition of CI-AMPAR internalization plays an important role in memory modification, but not necessarily new learning (Kim et al., 2007). Peptides infused into the amygdala that block CI-AMPAR internalization during extinction can prevent the retention of extinction, suggesting modification of the original fear memory trace and possibly synaptic

depotentiation. However, these findings are not always consistent with the current extinction literature. For example, when AMPA: NMDA ratios were compared between *in vivo* depotentiation and extinction groups, extinction groups showed maintained potentiation at cortico- and thalamo-amygdala synapses while depotentiated synapses showed reductions in AMPAR presence (Kim & Cho, 2017). These findings lend support for distinct patterns of postsynaptic AMPAR expression in the amygdala following depotentiation and extinction, and further suggest that depotentiation is not a result of extinction (Figure 1).

Integrating inhibitory learning and depotentiation

Although depotentiation and inhibitory learning account for extinction differently, it is possible that some degree of both of these processes occur simultaneously during extinction. The formation of a fear memory requires associations between several different sensory, temporal, and emotional aspects of the training experience (Dunsmoor et al., 2015). It may be possible for some synapses storing select sensory information to undergo depotentiation, while others may

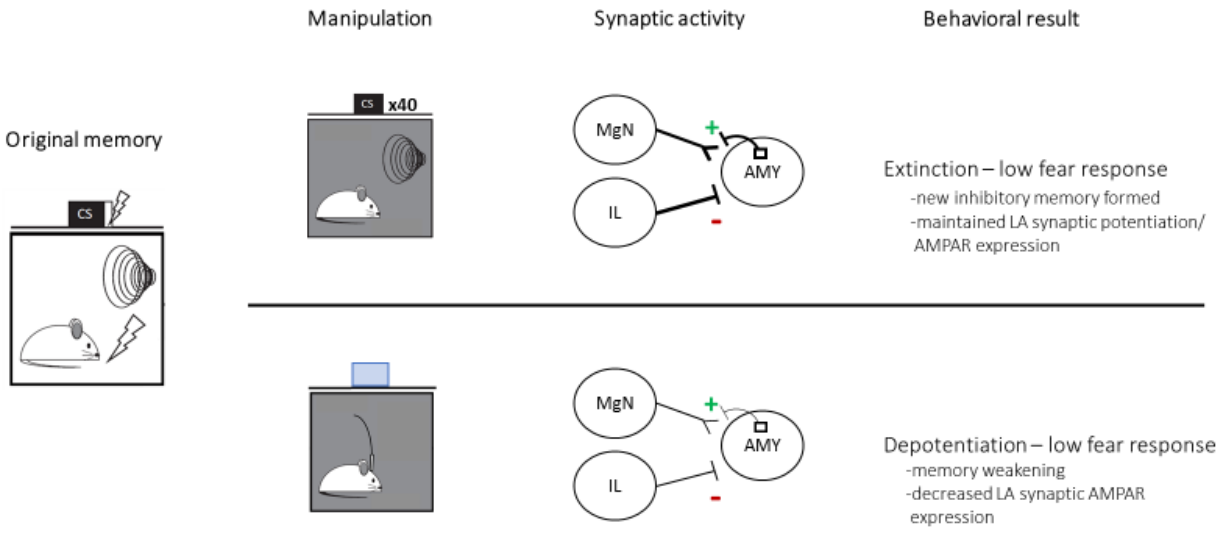

Figure 1. Extinction learning results in a new inhibitory memory formed. Increased inhibition in the amygdala from local inhibitory interneurons contributes to heterosynpatic inhibition of primary auditory inputs, and increased activity from the IL region of the mPFC also show increased activity of inhibitory projections to the basolateral amygdala. Extinction is characterized by context-dependent decrease in fear responding and maintained synaptic potentiation in the amygdala. Depotentiation using optogenetic techniques weakens inputs and results in a more permanent, persistent decrease in fear responding closely associated with memory erasure and characterized by decreased synaptic expression of AMPAR in the LA.

become potentiated. For example, while cortico-amygdala afferents may undergo depotentiation following extinction, mPFC-amygdala pathway may become potentiated, resulting in no difference in the overall degree of amygdala synaptic strength. With that being said, the evidence of cortico- and thalamo-amygdala changes in synaptic strength as a result of extinction remains inconsistent (Cho et al., 2013; Herry et al., 2008; Hong et al., 2009; Kim & Cho, 2017). This could be due to differences in fear conditioning training, extinction protocols, or how/when depotentiation is measured. For example, studies in support of depotentiation have used *ex vivo* paired-pulse low-frequency stimulation at cortico- and/or thalamo-amygdala synapses to show fear conditioned groups show synaptic depression while extinction and naïve groups do not (Hong et al., 2009; Kim et al., 2007). *In vivo* accounts of depotentiation show differences in AMPA:NMDA ratio at cortico- and thalamo-amygdala synapses during depotentiation but not following extinction (Clem & Huganir, 2010; Hong et al., 2009; Kim & Cho, 2017). Interestingly, retrieval-extinction designs result in CP-AMPAR mediated amygdala depotentiation and memory erasure, while this effect was absent in "traditional" single-day extinction groups, suggesting differences in multi- vs single-day extinction protocols may also explain the conflicting evidence of depotentiation in the extinction literature (An et al., 2017; Cain et al., 2003; Clem & Huganir, 2010).

Despite conflicting evidence for postsynaptic AMPAR modifications, both depotentiation and inhibitory learning views of extinction highlight a role for decreased presynaptic activity from the thalamus and cortex in the amygdala during extinction (Clem & Huganir, 2010; Cho et al., 2013; Hong et al., 2009; Kim & Cho, 2017). PPF provides a way to measure presynaptic activity. The depotentiation literature shows significant impairments in the ability to induce PPF using low-frequency stimulation, suggesting weak presynaptic activity as a result of extinction

(Clem & Huganir, 2010; Hong et al., 2009; Kim et al., 2007). Additionally, heterosynaptic inhibition of presynaptic activity in the amygdala from major auditory centers occurs following extinction (Bauer & LeDoux, 2004; Cho et al., 2013). Reductions in fear responding as a result of extinction may not necessarily be associated with postsynaptic loss of potentiation, but may be a result of increased inhibition of presynaptic activity from primary auditory centers. The loss of activity from presynaptic sites would account for changes in PPF following extinction as well as maintained potentiation at postsynaptic sites. Furthermore, this heterosynpatic inhibition of presynaptic activity occurs in response to priming of the mPFC-amygdala pathway, suggesting mPFC plays a role in presynaptic inhibition following extinction (Cho et al., 2013). Because the mPFC plays a large role in contextual fear, mPFC-dependent heterosynaptic inhibition of cortico- and thalamo-amygdala terminals after extinction is also likely context-dependent and may account for renewal of fear (Bruchey et al., 2007; Gilmartin et al., 2013; Herry et al., 2008).

Targeting and manipulating terminals using optogenetics

Optogenetics is a tool commonly used to study the precise time points at which specific brain circuits are necessary for memory formation and retention. Light exposure in the brain region expressing a particular opsin can activate or inhibit affected neurons within milliseconds (Boyden et al., 2005; Han et al., 2007). The AAV vector containing the ArchT insert has been used to silence neurons (Han et al., 2011). ArchT is a green-sensitive opsin proton pump and

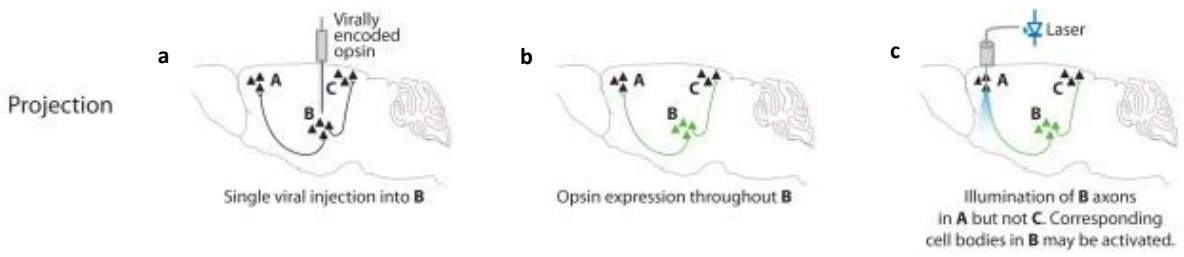

Figure 2. Viral infusions into a localized region (a) can undergo microtubule-dependent trafficking and expression can be visualized and manipulated to projecting regions (b). Light exposure to axons containing virus in distal regions can be manipulated (c). Adapted from Yizhar et al., 2011.

upon light exposure facilitates H+ exit, resulting in membrane hyperpolarization (Chow et al., 2010). After infusion into brain tissue, AAV vectors enter the neuron via endocytosis and are trafficked into endosomes and lysosomes (Castle et al., 2013). Once in endosomes and lysosomes, AAV can be moved to the Golgi and nucleus, or undergo dynein-dependent retrograde trafficking or kinesin-dependent anterograde trafficking, suggesting a microtubule mediated mechanism for axon terminal expression (Castle et al., 2013; Castle et al., 2014). Once infused locally (Figure 2a), the virus can be expressed throughout the neuron in a matter of weeks (Figure 2b). To silence or activate cells containing the opsin, light can be delivered locally through optical fibers that are implanted during a stereotaxic surgery (Sparta et al., 2011). Optical fibers can be implanted into regions where axons terminate to allow for manipulation of the connections between brain structures in a temporally precise manner (Johansen et al., 2012; Figure 2c). The present studies use an AAV9-CAG-ArchT-GFP virus to selectively silence input from the MgN in the amygdala (Bukalo, et al., 2015; Chow et al., 2010; Han et al., 2011; Kwon et al., 2014).

The goal of the following experiments was to determine the contribution of auditory and contextual modulation of synaptic destabilization during auditory fear memory recall. These experiments manipulated the MgN or DH during brief retrieval sessions and measured the effects of this manipulation on synaptic plasticity within the amygdala. Chapter 2 will focus on the contribution of auditory thalamic terminals in the amygdala during auditory fear memory retrieval and retention using optogenetics. Chapter 3 focuses on the role of DH activity during auditory fear conditioning and how this activity regulates the ability to disrupt a memory in the amygdala.

Chapter 2: Inhibition of thalamic terminals in the amygdala facilitates extinction-like learning

During auditory fear conditioning, synaptic connections between major auditory centers (e.g. the MgN) and the amygdala are strengthened. This is supported by work that has stimulated axons from the MgN in the amygdala as a CS to simulate auditory fear conditioning, suggesting an essential role for presynaptic plasticity from the MgN in the amygdala during conditioning (Kwon et al., 2014). The increase in activity in the thalamo-amygdala pathway initiates a series of postsynaptic events that contribute to protein synthesis, which is necessary for memory formation. Namely, increases in phosphorylation of several kinases, notably ERK and CREB, are necessary for the initiation of transcription and translation (Josselyn et al., 2001; Rashid et al., 2016; Schafe et al., 2000; Zhou et al., 2009). Additionally, potentiation of thalamo-amygdala synapses, measured by AMPAR presence in the synapse, is critical for auditory fear memory retention (Clem & Huganir, 2010; Rumpel et al., 2005). It has been suggested that the retention of auditory fear depends on the maintenance of potentiated synaptic connections from major auditory centers to the amygdala (Rumpel et al., 2005; Takemoto et al., 2017).

Extinction learning is largely characterized by a temporally-limited, context-dependent reduction in fear following repeated presentations of an unreinforced CS (Bouton, 2002). Renewal and spontaneous recovery are commonly used to test the contextual and temporal limitations of extinction and are associated with return of fear following extinction learning (Bouton & Bolles, 1979). The associative inhibition theory of extinction states that new inhibitory learning modulates the excitatory CS-UCS representation during extinction, and contextual changes reduce the inhibitory gate over the excitatory fear memory (Rescorla, 2004). Local and network changes result in increased inhibition in the amygdala during extinction,

resulting in decreased fear to the CS. Specifically, inhibitory regulation of the amygdala from the mPFC suppresses excitation and fear responding and are a result of long-range mPFC GABAergic neurons and strengthened mPFC-interneuron connections in the amygdala (Cho et al., 2013; Quirk et al., 2003; Senn et al., 2014). The increases in inhibition following extinction are associated with several plastic events in the amygdala (like the phosphorylation of ERK) that are necessary for the retention of extinction (Herry et al., 2006; Kwapis et al., 2014). However, phosphorylation of CREB in the amygdala is not critical for extinction, and levels of phosphorylated CREB have been associated with the strength of the CR, which potentially provides a dissociable molecular mechanism to measure changes between excitatory and inhibitory learning (Han et al., 2009; Lin et al., 2003; Porte et al., 2011; Tronson et al., 2012).

While behavior differs between brief retrieval sessions and extinction, they are both associated with maintained potentiation of amygdala synapses. This suggests synaptic strength is unchanged following extinction, which may explain rapid return of fear in cases such as renewal (Clem & Huganir, 2010; Herry et al., 2008; Kim & Cho, 2017). Modulation of excitatory thalamo-amygdala synapses during extinction may then be a presynaptically mediated process. There is evidence of heterosynaptic inhibition from the mPFC at thalamo-amygdala synapses that results in reduced glutamate release (Cho et al., 2013). Additionally, MgN terminals also modulate ITC activity, which provide a negative feedback loop to inhibit BLA neurons and show increased activity following extinction (Asede et al., 2015; Likhtik et al., 2008) Based on this, changes in activity in the thalamo-amygdala pathway likely contribute to an imbalance between excitation and inhibition that occurs during extinction learning, which has been suggested with a combination of physiology and pharmacology data (Clem & Huganir, 2010; Kim et al., 2007; Orsini & Maren, 2009), and mimicking activity in brain regions critical for extinction may be

sufficient to facilitate extinction learning (Bukalo et al., 2015; Do-Monte et al., 2015). Therefore, reducing activity from the MgN in the amygdala during a "shortened" extinction session may be able to facilitate extinction learning. The reduction in activity in the thalamo-amygdala pathway during retrieval should also lead to decreases in phosphorylated CREB but maintained CI-AMPAR expression in the amygdala. These results would support extinction studies demonstrating maintained potentiation of thalamo-amygdala synapses and changes in phosphorylated CREB corresponding to expression of fear. Because the reduction in fear from extinction is dependent on the context in which it occurred, the reduction in activity in the thalamo-amygdala pathway may also lead to the contextually- dependent return of fear if extinction is facilitated.

Here, we explore a role for the thalamo-amygdala pathway during fear recall. We found that when MgN terminals in the amygdala are silenced during retrieval there was a reduction in fear responding that was persistent at a 24-hour test. This reduction was dependent on the simultaneous pairing of optogenetic inhibition of MgN-BLA terminals and CS presentation. Further, fear renews when animals are re-exposed to the CS in the training context, and MgN-BLA silencing did not result in changes in AMPAR expression in amygdala synapses. We also measured changes in phosphorylation of CREB after the test and renewal because of CREB's dissociable expression following extinction and retrieval. We found that silencing MgN terminals in the amygdala at retrieval reduced levels of pCREB after the test and pCREB returned to control levels following renewal test. Collectively, these results highlight an important role for activity from the MgN in the amygdala during fear retrieval, and suggest decreased activity in the thalamo-amygdala pathway results in facilitated extinction learning.

Methods

Subjects

Subjects were male Long Evans rats from Envigo (n = 151; Indianapolis, IN) weighing approximately 350g at the time of arrival. Rats were individually housed with free access to water and rat chow. The animal colony was maintained at a 14:10-hr light/dark cycle with all experiments occurring under the light portion of the cycle. All experiments were approved by the Institutional Animal Care and Use Committee.

Optogenetics: Infusion of virus and implantation of optic fibers

Archaerhodopsin-T (CAG-ArchT-GFP) and control virus (CAG-GFP) recombinant adeno-associated virus (AAV) were produced and serotyped (AAV9) by Dr. Ed Boyden and packaged by the UNC vector core (ArchT titer: 3×10^{12}; control titer: 2×10^{12}). The control virus condition was identical to ArchT animals in every respect other than expression of ArchT. The promoter selected would be expected to activate expression in all local cell types. Immediately before surgery rats were anesthetized with 4% isoflurane and oxygen and after induction, isoflurane levels were maintained at 2 - 2.5% throughout the surgery. Virus was loaded into a 10µl Hamilton syringe with a 34-gauge needle (World Precision Instruments) and mounted onto a stereotaxic automated injector (World Precision Instruments). Groups received bilateral infusions of AAV_9-CAG-GFP or AAV_9-CAG-ArchT-GFP (0.5µL/side; 50 nanoliters/min) targeting the MgN (-5.3 mm posterior, +/-2.8 mm lateral, -5.6 mm ventral) relative to bregma (Paxinos & Watson, 2007). The needle was left in place for 10-min to allow for diffusion. Groups received a second surgery, approximately 8 weeks following virus surgery, to implant optic fibers (200µm diameter) into the LA (-3.0 mm posterior, +/-5.0 mm lateral, -7.0 mm ventral). Fibers were secured to the skull with four skull screws and were surrounded by

acrylic cement. Rats were given a minimum of 7 days after surgery to recover before behavioral training and testing.

Apparatus

Auditory fear conditioning was conducted in a set of four Plexiglas and stainless-steel chambers within sound-attenuating boxes (Context A) for Experiments 1-6. The floor contained 18 stainless steel bars connected to a shock generator (Coulbourn Instruments, Allentown, PA). Each chamber had speaker to allow delivery of white noise, overhead illumination with a 7.5 W bulb, and ventilation fans to provide a constant background noise (55 dB). The chambers were cleaned with 5% ammonium hydroxide solution between sets of rats. A set of similar chambers designated Context B served as a shifted context for auditory CS testing. Context B has several distinct features including dark Plexiglas flooring and ethanol cleaning solution.

Behavioral procedures and light delivery

Rats were transported, handled, and gently restrained for 3 days prior to behavioral training and testing. Rats were placed in Context A for delay fear conditioning. During training, rats received four white noise presentations (72dB, 10s) that were always paired with a footshock (1s, 1.0mA). The average inter-trial interval between each tone presentation was 110s. All auditory CS retrieval and testing sessions took place in Context B where rats received four discrete tone presentations of the CS (30s; 60s ITI) after a 60s baseline. A multimode patch cord was used to split the light for bilateral laser (λ = 532 nm, 20 mW, continuous) delivery and was controlled with TTL pluses (as described in Gilmartin et al, 2013). The laser was activated 1s prior to CS onset, remained on for the entire duration of the CS, and was turned off 1s after offset of the CS during retrieval sessions, with the exception of the ITI group where the laser was presented during the ITI and the laser only condition where the auditory cue was not presented

but the laser was during retrieval. All groups received a total of 128s of laser exposure. Freezing was defined as the cessation of all movement excluding respiration and was automatically scored in real-time with FreezeScan 1.0 detection software (Clever Sys, Inc., Reston, VA) calibrated to a trained human observer.

Synaptosomal membrane preparation

Animals were deeply anesthetized with isoflurane 90-minutes or 7-hours following test. Brains were immediately removed, flash frozen with dry ice, and stored at -80ºC until dissected. Crude synaptosomal fractions were obtained as previously described (Ferrara et al., 2017; Jarome et al., 2011). Amygdalae were dissected out and homogenized in TEVP buffer with 320mM sucrose and then centrifuged at 1000x g for 10 minutes. The supernatant was removed and centrifuged at 10,000 x g for 10-minutes, and the remaining pellet was denatured in lysis buffer (all in 100 ml DDH20; 0.605 g Tris-HCl, 0.25 g sodium deoxycholate, 0.876 g NaCl, 1 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 10 ml 10% SDS). Protein levels were measured with a protein assay kit (Bio-Rad laboratories, Hercules, CA, USA).

Immunofluorescence

Animals were deeply anesthetized with isoflurane 90-mins following retrieval. Brains were immediately removed and stored at -80ºC until sliced. Brains were sliced in 20-micron sections and were mounted onto charged slides. Slides were rehydrated in wash buffer (PBS + 0.05% Tween-20) and permeabilized (PBS + 0.3% Triton X) for 15-min, and incubated in blocking solution (PBS + 0.7% NGS). Slides were then incubated in phosphorylated CREB ser133 antibody (Cell Signaling, 1:100, #9198) solution (PBS + 0.3% Triton X + 5% NGS) overnight at 4 ºC. The next day, slides were incubated in secondary antibody solution for 2 hours and rinsed with wash buffer, a DAPI counterstain was applied, and slides were cover slipped.

Immunofluorescence microscopy and quantification

Specific anatomical locations were chosen based on a rat brain atlas (Paxinos & Watson, 2007). Amygdala images were captured on the Olympus Fluoview FV1200 confocal microscope using a 20x objective lens. Serial *z*-stack images covered a depth of 4.55μm through five consecutive sections (0.91μm per section) and were acquired using Fluoview software (Olympus). Three amygdala sections were analyzed bilaterally and were averaged for each rat (6 sections matched along the anterior-posterior axis for each rat).

Images were exported as 12-bit TIFF files and particles were quantified using ImageJ software (NIH, Bethesda, MD, USA). Images were quantified with ImageJ software by converting them to 32-bit, difference of Gaussian filtering (sigmas of 2 and 1.5), thresholding with the triangle method, and then counting particles greater than 4 pixels in diameter within the ROI. This results in a binary image with minimal background. All particle counts were averaged, bilaterally, across animals in each condition and normalized to the slices of animals infused with control virus using the "Analyze Particles" plugin in ImageJ.

Western blotting

Groups were trained and received a retrieval and test session as described above and sacrificed at 90-minutes or 7-hours following the test session. Following synaptosomal preparation, protein levels were normalized and loaded onto an SDS/PAGE gel and then to a membrane using a transfer apparatus (Bio-Rad). Membranes were incubated in blocking buffer for 1 hour before being incubated in GluR1 (Cell Signaling, 1:1000), GluR2 (Santa Cruz, 1:500), PSD95 (1:1000, Santa Cruz), or βactin (Cell Signaling, 1:1000) primary solutions overnight at 4 ºC. Membranes were then incubated in the appropriate secondary (Santa Cruz, 1:20,000)

antibody for one hour and prepped in a chemiluminescence solution for 3 minutes. Images were captured and densitometry performed using NIH Genesys.

Statistical analyses

All statistical analyses and graphing were conducted in Prism 7 software (Graphpad, San Diego, CA). Western blot samples normalized to actin levels are expressed as a percentage of control groups (no test). Behavioral and western blot statistical outliers were defined as being two standard deviations above or below group mean and were excluded from all subsequent analyses. The data is presented as group averages with standard error of the mean (SEM). Western blot and behavioral experiments were analyzed using a t-test or one-way Analysis of Variance (ANOVA).

Results

Auditory thalamic terminal activity in the amygdala is critical for fear memory retrieval and retention

To test whether activity from the MgN in the amygdala is required for fear retrieval, we used optogenetics to target terminals in the amygdala during CS presentation at retrieval. We infused virus (GFP or ArchT) into the MgN and implanted fibers targeting the lateral portion of the amygdala (example terminal image seen in Figure 3b). Groups were trained with auditory fear conditioning 8 weeks following viral infusion and received a retrieval session the next day. During retrieval, thalamo-amygdala terminals were silenced for the entire duration of auditory cue presentation. Groups were then tested for the retention of auditory fear (Figure 3a). There were no differences during the training session ($F_{(2, 51)} = 1.355$, $p = 0.27$; Figure 3c). The following day, groups received a CS retrieval session in a shifted context where thalamic terminals were silenced for the entire duration of the CS presentation. Silencing MgN terminals

in the amygdala at retrieval significantly reduced fear responding during CS presentation ($t_{(16)}$ = 2.45, $p < 0.05$; Figure 3d). Groups were presented with the CS the next day to test the long-term retention of fear following thalamo-amygdala terminal silencing. At the long-term laser free test, there was a persistent reduction in fear to the CS ($t_{(16)} = 4.74$, $p < 0.0005$; Figure 3e). This finding suggests that activity from the MgN in the amygdala is critical during fear memory retrieval and this activity is necessary for long-term retention.

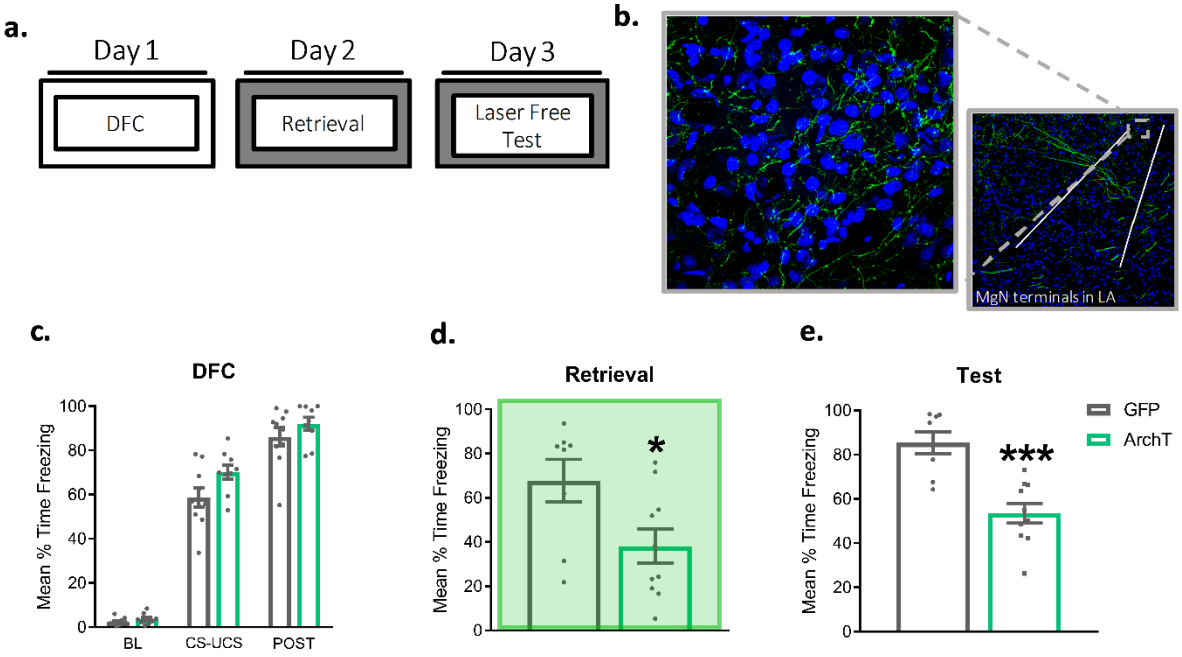

Figure 3. Auditory thalamic terminal activity in the amygdala is critical for fear memory retrieval and retention. Experimental design (a) and example MgN-LA terminal image (b). There were no differences in freezing during training (c). When MgN-LA terminals are silenced, there is a significant reduction in freezing during CS presentation during retrieval (d) and at a long-term test (e). *$p < 0.05$, ***$p < 0.0005$.

Recent work suggests that depotentiation of cortico- and thalamo-amygdala synapses does not require explicit pairing of stimulation-CS or memory reactivation during stimulation to see a long-term reduction in fear responding (Kim & Cho, 2017). We wanted to determine whether memory reactivation and/or explicit pairing of the CS with silencing of MgN terminals in the amygdala was required for reductions in fear at test. We tested explicit pairing of

stimulation with the CS in an ITI group, and the necessity of memory reactivation with stimulation in a laser-only group. The ITI groups received the same retrieval parameters as described above with the exception that the laser and CS were unpaired (Figure 4a). The laser-only group did not receive auditory cue presentation at retrieval but did receive laser presentation (Figure 4a). During retrieval, the laser only condition that did not receive any auditory cue presentations froze significantly less than the GFP group that did receive auditory cue presentations throughout the retrieval session ($p < 0.0001$) and there were no differences between the GFP and ArchT conditions (Figure 4b). At the long-term laser free test, there was no difference in freezing between any of the groups ($F_{(2, 21)} = 0.92$, $p = 0.41$; Figure 4c). These results suggest the auditory fear memory requires reactivation to see persistent reductions in fear due to thalamo-amygdala silencing. Furthermore, terminal silencing needs to be paired with CS

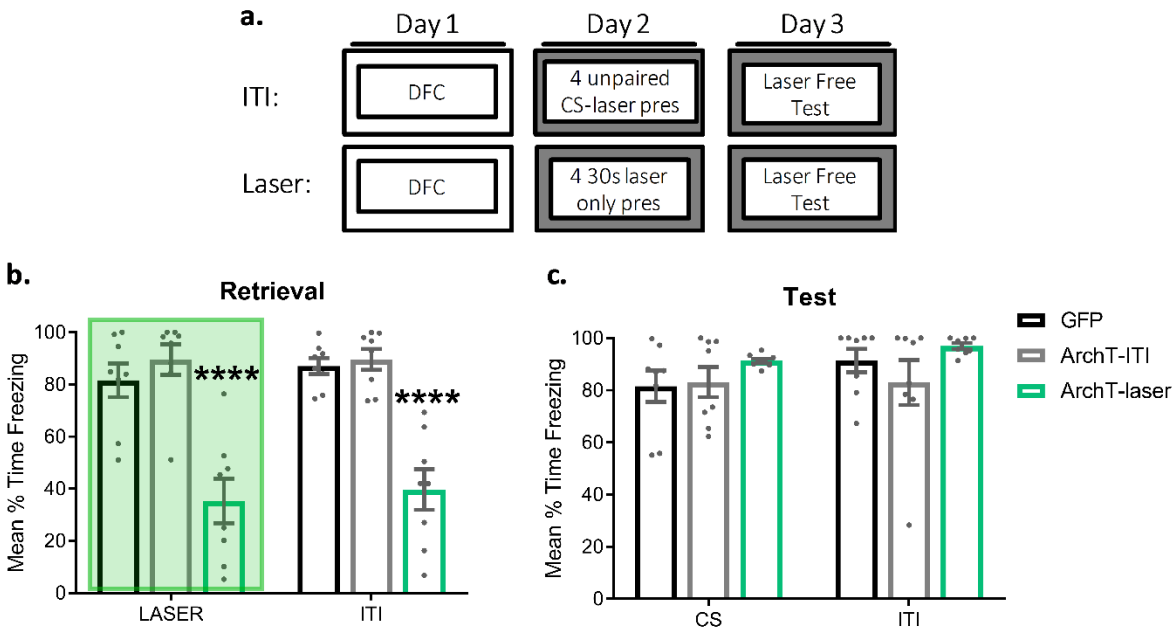

Figure 4. Inhibition of thalamo-amygdala terminals needs to be paired with auditory cue presentation for reduced fear responding. Experimental design (a). Laser only controls that did not receive a CS presentation during retrieval froze significantly less than the GFP group received CS presentations, and there were no differences in freezing between ArchT and GFP groups that received both CS and laser presentation (b). There were no significant differences between groups at a long-term test (c). ****$p < 0.0001$.

presentation during retrieval to see reductions in fear during retrieval and long-term test and thus is not a simple consequence of inhibition per se.

Persistent reductions in fear due to thalamo-amygdala terminal silencing is not a result of depotentiation

Reductions in fear at a 24-hour time point can be a result of depotentiation *or* extinction. Persistent reductions in synaptic expression of AMPAR in the amygdala provide evidence of depotentiation, which is associated with persistent reductions in fear. In amygdala synaptosomal fractions, expression of AMPAR subunits GluR1 and GluR2 are reduced 90-min after a brief retrieval session and return to previous levels at the end of the reconsolidation window (at least 6-hr following retrieval), suggesting maintained synaptic and memory strength. (Jarome et al., 2015). During depotentiation, synaptosomal expression of GluR1 and GluR2 are persistently reduced during reconsolidation and outside of the reconsolidation window, contributing to long-term reductions in fear. To determine whether we are engaging a depotentiation mechanism in response to thalamo-amygdala terminal silencing, in crude synaptosomal fractions, we conducted western blots to look at the synaptic expression of AMPA receptors at 90-min and 7-hr following the laser free test (Figure 5a). At the 90-min timepoint following test, there were no differences in GluR1 ($t_{(14)} = 0.45$, $p = 0.66$), GluR2 ($p = 0.58$), or PSD95 ($p = 0.88$), indicating there were no differences in AMPAR synaptic expression during reconsolidation (Figure 5b). At the 7-hr time point, there were also no differences in GluR1 ($t_{(15)} = 1.05$, $p = 0.31$), GluR2 ($p = 0.15$), or

PSD95 (p = 0.25) (Figure 5c). Collectively, these results suggest the reductions in fear seen during test may not be the result of depotentiation or a reduction in the number of synapses.

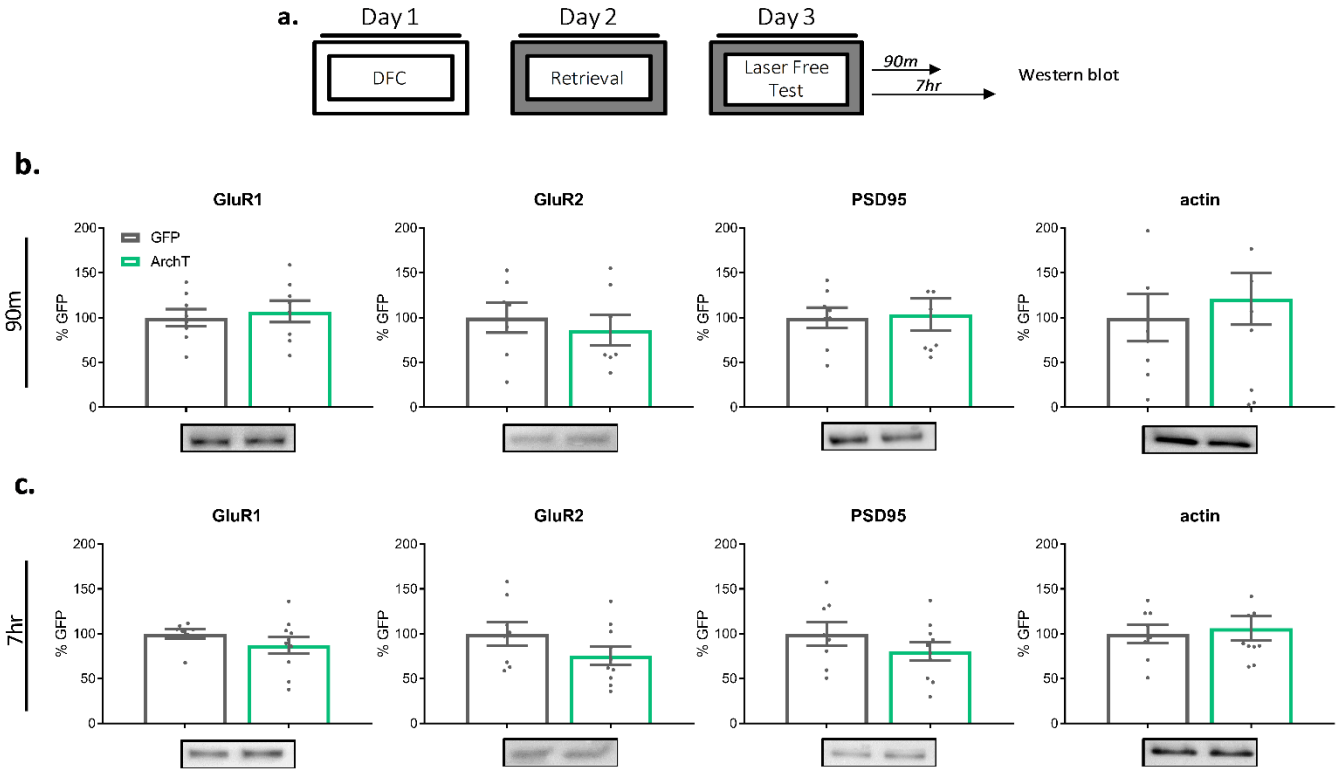

Figure 5. Persistent reductions in fear due to thalamo-amygdala terminal silencing is not a result of depotentiation. Experimental design (a). There were no differences in LA synaptosomal expression of AMPAR or PSD95 between GFP and ArchT groups (b). Long-term reductions in fear responding were not associated with differences in LA synaptosomal AMPAR expression or PSD95 outside of the reconsolidation window (c).

Thalamo-amygdala silencing during retrieval facilitates extinction learning

During extinction learning, there is an imbalance between excitation and inhibition in the amygdala. Several studies show that extinction is not a result of depotentiation (Kim & Cho, 2017, but see Hong et al., 2009; Kim et al., 2007), but instead could result from reduced activity from primary excitatory inputs onto the amygdala. Extinction can be characterized by the return of fear through a shift in context or passage of time. To test if silencing excitatory input from the MgN in the amygdala facilitates extinction processes in comparison to groups without thalamo-amygdala manipulations during retrieval, we tested for the renewal of fear when rodents were

placed back into the original training context and presented with the auditory CS (Figure 6a). Similar to previous results, silencing thalamo-amygdala terminals at retrieval leads to a reduction in fear behavior during retrieval ($t_{(26)} = 3.05$, $p < 0.05$) and this reduction persists during a subsequent laser-free test ($p < 0.05$; Figure 6b-c). Interestingly, fear renews when groups are placed back into the training context and presented with the auditory CS ($p = 0.17$; Figure 6d), suggesting silencing of thalamic terminals in the amygdala may result in facilitated extinction learning. We cannot rule out the possibility that silencing the thalamo-amygdala pathway during fear retrieval may be a transient effect where fear responding would recover to control levels over a sufficient amount of time. However, we think it is unlikely that the return of fear seen during renewal is dependent on the passage of time because pharmacological manipulations of the MgN or amygdala after initial learning are associated with persistent reductions in fear responding, and return of fear as seen in spontaneous recovery designs would not be expected until at least 7 days following retrieval.

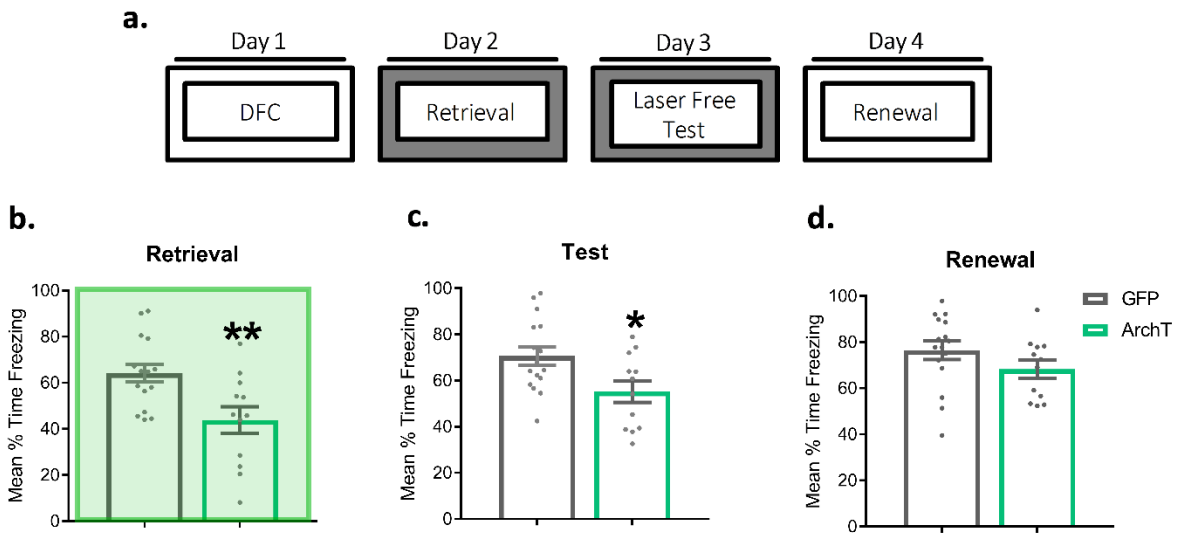

Figure 6. The reduction in fear responding as a result of thalamo-amygdala silencing is context-dependent. Experimental design (a). Silencing MgN-LA terminals decreases fear responding to the CS during retrieval (b) and at a long-term test (C). There were no significant differences between ArchT and GFP groups when the CS was tested in the training context (d). *$p < 0.05$, **$p < 0.005$.

Next, we measured phosphorylated CREB to further assess a role for thalamo-amygdala terminal silencing as a method for facilitating extinction learning. Prior work suggested CREB undergoes dephosphorylation following extinction learning, and levels of phosphorylated CREB in the amygdala are directly correlated with the degree of fear responding, unlike other plasticity markers such as phosphorylated ERK (Baumgärtel et al., 2008; Hagiwara et al., 1992; Han et al., 2009; Kwapis et al., 2014; Lin et al., 2003; Porte et al., 2011; Tronson et al., 2012). We used immunofluorescence to measure the amount of phosphorylated CREB in the amygdala at 90-min following the retrieval, laser free, and renewal tests (Figure 7a). If silencing thalamo-amygdala terminals at retrieval results in facilitated extinction, we would expect to see reduced in phosphorylated CREB in the amygdala at the retrieval and laser free test but not renewal test. After retrieval, there was a significant reduction in phosphorylated CREB when terminals from the MgN in the LA were silenced ($t_{(20)} = 2.12$, $p < 0.05$; Figure 7c). There was also a significant

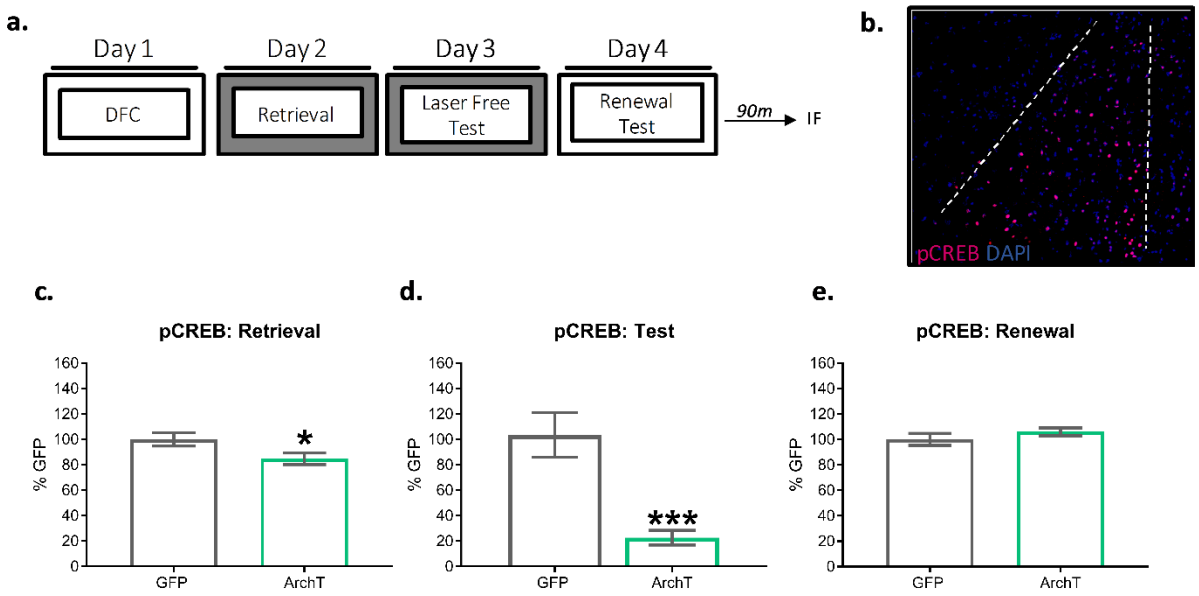

Figure 7. Silencing MGN-LA terminals during retrieval reduces LA phosphorylated CREB expression after retrieval and a laser-free test but not after a renewal test. Experimental design (a). Representative image of phosphorylated CREB expression in the LA in a GFP animal following laser-free test. Phosphorylated CREB is reduced after the retrieval session where MgN-LA terminals are silenced (c) and after the laser-free test (d). There are no differences in phosphorylated CREB expression in the LA after a renewal test (e). *$p < 0.05$, ***$p < 0.001$.

reduction in phosphorylated CREB after the laser-free test when thalamic terminals in the LA were silenced ($t_{(22)} = 5.39$, $p < 0.001$; Figure 7d). After renewal, there were no differences in phosphorylated CREB between groups ($t_{(16)} = 1.05$, $p = 0.31$; Figure 7e). These results show silencing thalamo-amygdala terminals during retrieval decreases levels of phosphorylated CREB in the amygdala at a long-term test but not after a renewal test, similar to our behavioral results.

Discussion

In the current experiments, we assessed a role for MgN terminal activity in the amygdala during fear retrieval and the impact on long-term retention of fear. We show thalamo-amygdala activity is necessary for fear retrieval and retention, and this persistent reduction in fear is likely not due to depotentiation in the amygdala. However, this effect may be a result of facilitated extinction learning. We show a renewal effect when the CS is presented in the training context and additionally show changes in pCREB that are similar to changes in the behavioral expression of fear at a laser-free test and during renewal of fear. This work highlights an important role for excitatory input, specifically from the MgN, in the amygdala for long-term fear responding to auditory cues.

Inhibitory activity from the mPFC in the amygdala contributes to the imbalance of excitation and inhibition during extinction learning and retention (Bloodgood et al., 2018; Cho et al., 2013). Plasticity in the MgN is also critical for fear extinction learning, and recent work shows heterosynaptic inhibition of primary auditory inputs in the amygdala may contribute to fear extinction learning (Cho et al., 2013; Orsini & Maren, 2009). In the current experiment, inhibition of thalamo-amygdala connections during retrieval results in extinction-like behaviors and molecular profiles. We found a persistent reduction in fear at a long-term test following thalamo-amygdala terminal silencing, which is renewed when retested in the training context. Previous work shows terminals from the mPFC in the lateral amygdala suppress activity from

sensory inputs (Rosenkranz & Grace, 2001). There are also increases in synaptic strength between the mPFC and amygdala interneurons following extinction that inhibit auditory input (Cho et al., 2013). Extinction, then, can result in suppression of auditory input by the mPFC in the amygdala. Previous work also shows increases in ITC activity following extinction, which can feedback and inhibit BLA neurons (Asede et al., 2015). The synaptic connections between the MgN and ITCs may contribute to changes in fear responding seen following extinction because of the ability of ITCs to modulate EPSPs in the BLA, suggesting a feedback loop of ITC cells onto BLA neurons. Based on this, it is possible that inhibition of the thalamo-amygdala pathway during retrieval may mimic inhibition via modulation of ITCs or by mimicking silencing that would occur in the presence of increased mPFC activity that occurs during extinction to result in extinction-like behaviors.

We also found MgN terminal silencing in the amygdala needs to be paired with the CS during retrieval to observe reductions in auditory fear, suggesting the ability to reduce fear responding in the thalamo-amygdala pathway during retrieval is temporally restricted to the period of CS presentation. Further, the reduction in fear responding seen at long-term test is likely dependent on glutamate receptor activity in the MgN (Kwon et al., 2014; Orsini & Maren, 2009). Although much of the pharmacology work minimizes a role for the MgN during fear retention, the current data shows a strong role for the maintenance of thalamo-amygdala synaptic connections at long-term time points for persistent expression of fear (Apergis-Schout et al., 2005; Kwon et al., 2012). These results are consistent with the previous work discussed suggesting an important role for the silencing excitatory input in the amygdala during extinction learning and further suggest thalamo-amygdala activity is critical during CS presentations after fear learning.

Our behavioral and biochemical results lend support for thalamo-amygdala silencing as a critical mechanism for maintained fear responding. Following thalamo-amygdala silencing, we did not see changes in AMPAR expression during or outside of the reconsolidation window. These results suggest that the persistent reductions in fear are not due to depotentiation, suggesting synaptic strength is maintained despite the reduction in fear, which is similar to previous work showing maintained potentiation of cortico- and thalamo-amygdala synapses after extinction (Kim & Cho, 2017). Levels of pCREB have been correlated with the strength of conditioned fear expression, and here, we report a significant reduction in pCREB following thalamo-amygdala silencing (Baumgärtel et al., 2008; Hagiwara et al., 1992; Han et al., 2009; Lin et al., 2003; Porte et al., 2011; Tronson et al., 2012). Collectively, our reduction in pCREB following the retrieval and test as well as maintained expression of AMPAR in amygdala synapses suggest thalamo-amygdala silencing during a brief retrieval session leads to persistent reductions in auditory fear but maintained post synaptic potentiation.

In summary, we provide several pieces of evidence suggesting a critical role for MgN activity in the amygdala during fear recall. We found thalamo-amygdala silencing during retrieval results in a context-specific decrease in fear, reduction in pCREB, and maintained potentiation of amygdala synapses. These results support a strong role for excitatory input from primary auditory centers in the amygdala during long-term fear responding.

Chapter 3: Contextual novelty, dorsal hippocampus, and amygdala-dependent synaptic destabilization and memory lability

Introduction

Memory retrieval provides an opportunity to include new information into the original memory trace, providing a unique therapeutically relevant opportunity to modulate debilitating fear-related disorders (Lee et al., 2008; Lee, Nader, &Schiller, 2017). Retrieval is characterized by the degree of fear expression to the CS, AMPAR trafficking at amygdala synapses, and sensitivity to protein synthesis inhibition. Specifically, protein synthesis inhibition in the amygdala following a brief retrieval session is associated with long-term memory impairment and is often used to characterize memory lability during reconsolidation (Jarome et al., 2012; Lopez et al., 2015; Nader et al., 2000). The internalization of CI-AMPAR during reconsolidation is thought to regulate memory lability, as measured by anisomycin-dependent impairments in the long-term retention of fear. For example, inhibition of CI-AMPAR internalization prevents the retrieval related anisomycin reduction in fear memory retention (Hong et al., 2013). Interestingly, the internalization of AMPAR is influenced by sensory cues present during retrieval. Specifically, pre-exposure to the retrieval conditions prior to training prevents internalization of AMPAR during reconsolidation and anisomycin-dependent memory impairment at a long-term test, suggesting novelty of the retrieval conditions influences memory lability and synaptic destabilization during retrieval (Jarome et al., 2015).

The DH and amygdala interact during fear memory formation and retrieval, and specifically activity in the amygdala impacts long-term plastic events in the DH (Ikegaya et al., 1995; McIntyre et al., 2005; McReynolds et al., 2009). While the amygdala is critical for auditory and contextual fear memory formation and retention, the necessity for the DH in contextual and auditory fear memory retention is dependent on the type of fear conditioning. For example, inactivation or protein synthesis inhibition in the DH during training in auditory delay fear conditioning shows selective impairment in context fear retention without impacting

auditory CS fear (Debiec et al., 2001; Helmstetter et al., 2008; Suzuki et al., 2008), while a trace fear training requires the DH for both auditory and contextual fear retention (Chowdhury et al., 2005; Quinn et al., 2008). Even though DH activity is not necessary for a delay auditory fear memory, plastic changes in the DH have been reported in response to cued fear retrieval, suggesting DH plasticity occurs during auditory fear retrieval and may be important for memory lability during retrieval in the amygdala (Sanders et al., 2003; Seidenbecher et al., 2003). Interestingly, auditory memories resistant to impairment with amygdala anisomycin infusions during retrieval are regulated by DH activity (Wang et al., 2009). While evidence suggests an interaction between the dorsal hippocampus and amygdala during auditory delay fear memory retrieval, how the dorsal hippocampus may influence later recall of an auditory fear memory is unclear. It is possible that contextual information processed by the DH during delay fear conditioning regulates the ability of an auditory fear memory to become labile during retrieval (Jarome et al., 2015).

The goal of the following experiments was to directly test whether contextual novelty during auditory fear memory retrieval is necessary for memory lability, as indicated by the requirement for protein synthesis in the amygdala. To test this, groups received delay fear conditioning, retrieval, and test in the same context. Infusions of anisomycin were delivered to the amygdala immediately following retrieval. Consistent with previous work, we show contextual novelty is critical for memory susceptibility to anisomycin impairment following a retrieval session. We next tested whether the contextual restraint on memory lability was dependent on DH activity. We found that DH inactivation during training allowed for amygdala anisomycin impairments during retrieval. Because internalization of CI-AMPAR are necessary for memory lability during retrieval, we wanted to determine if contextual novelty regulates

AMPAR trafficking during retrieval. We found inactivation of the DH during training allows for internalization of AMPAR in the amygdala when the context is not shifted. These results suggest contextual novelty during retrieval is critical for memory lability and synaptic destabilization in the amygdala.

Methods

Subjects

Subjects were male Long Evans rats from Envigo (n = 95; Indianapolis, IN) weighing approximately 350g at the time of arrival. Rats were individually housed with free access to water and rat chow. The animal colony was maintained at a 14:10-hr light/dark cycle with all experiments occurring under the light portion of the cycle. All experiments were approved by the Institutional Animal Care and Use Committee.

Surgery

Immediately before surgery, rats were anesthetized with 4% isoflurane and oxygen, and after induction, isoflurane levels were maintained at 2 - 2.5% throughout the surgery. LA cannula were implanted at a 10° lateral angle (-3.0 mm posterior, +/-6.5 mm lateral, -7.6 mm ventral) and DH (-3.6 mm posterior, +/-2.6 mm lateral, -2.0 mm ventral) according to bregma (Paxinos & Watson, 2007). Cannula were secured to the skull with four screws and surrounded by acrylic cement. Rats were given a minimum of 7 days after surgery to recover before behavioral training and testing.

Apparatus

Auditory fear conditioning was conducted in a set of four Plexiglas and stainless steel chambers within sound-attenuating boxes (Context A). The floor contained 18 stainless steel bars connected to a shock generator (Coulbourn Instruments, Allentown, PA). Each chamber had a

speaker to allow delivery of white noise, overhead illumination with a 7.5 W bulb, and ventilation fans to provide a constant background noise (55 dB). The chambers were cleaned with 5% ammonium hydroxide solution between sets of rats. A set of similar chambers designated Context B served as a shifted context for auditory CS testing in some conditions. Context B has several distinct features including dark Plexiglas flooring and 5% acetic acid cleaning solution.

Drug preparation and infusion

Animals were adapted to transport handling procedures for 3 days before conditioning, which included gentle restraint during the sound of the infusion pump. Drugs were prepared on the day of infusion. Groups received bilateral microinjections of lidocaine (40 μg/μl, Sigma), or vehicle (sterile saline) at a rate of 0.5 μl/min and at a volume of 0.5 μl/hemisphere into the DH 10-minutes prior to training. Amygdala injections occurred immediately following a retrieval session (Anisomycin: 125 μg/μl, or ACSF vehicle). Drugs were infused through 33-ga injection cannulae extending 0.5-0.7 mm beyond the guide cannulae. Injectors remained in place for 90s following infusion to ensure drug diffusion.

Behavioral procedures

Rats were placed in Context A for delay fear conditioning. During training, rats received four white noise presentations (72dB, 10s) that were paired with a footshock (1s, 1.0mA). The average inter-trial interval between each tone presentation was 110s. Auditory CS retrieval and testing sessions took place in Context A or B where rats received four discrete tone presentations of the CS (30s; 60s ITI) after a 4-min baseline. Freezing was defined as the cessation of all movement excluding respiration and was automatically scored in real-time with FreezeScan 1.0 detection software (Clever Sys, Inc., Reston, VA), which was calibrated to a trained human observer.

Synaptosomal membrane preparation

As described in Chapter 2.

Western blot method

As described in Chapter 2.

Statistical analyses

All statistical analyses and graphing were conducted in Prism 7 software (Graphpad, San Diego, CA) software. Western blot samples were normalized to actin levels expressed as a percentage of control groups (no reactivation). Behavioral and western blot statistical outliers were defined as being two standard deviations above or below group mean and were excluded from all subsequent analyses. The data are presented as group averages with standard error of the mean (SEM). Western blot and behavioral experiments were analyzed using a one-way Analysis of Variance (ANOVA).

Results

Memory lability in the amygdala is regulated by contextual novelty and DH activity

To directly test the necessity of contextual novelty on auditory fear memory lability, training, retrieval, and test conditions were the same across days (Figure 8a). The dorsal hippocampus is critical for processing contextual information during training, so we inactivated the dorsal hippocampus with lidocaine during training to determine if we could remove the constraint of contextual novelty on retrieval-dependent memory lability. Because lidocaine was "on board" during training, we compared freezing responses, or performance, between vehicle and lidocaine groups during conditioning. There were no significant performance effects during training while the DH was inactivated ($F_{(6, 58)} = 0.39$, $p = 0.88$; Figure 8b) or during the retrieval

session ($F_{(3, 29)} = 2.047$, $p = 0.13$; Figure 8c). To more closely look at an effect of DH inactivation on purely contextual fear during the retrieval session, lidocaine and vehicle groups were directly compared during the baseline period. There was a modest reduction in freezing in groups that received lidocaine infusions into the dorsal hippocampus ($t_{(31)} = 1.56$, $p = 0.06$).

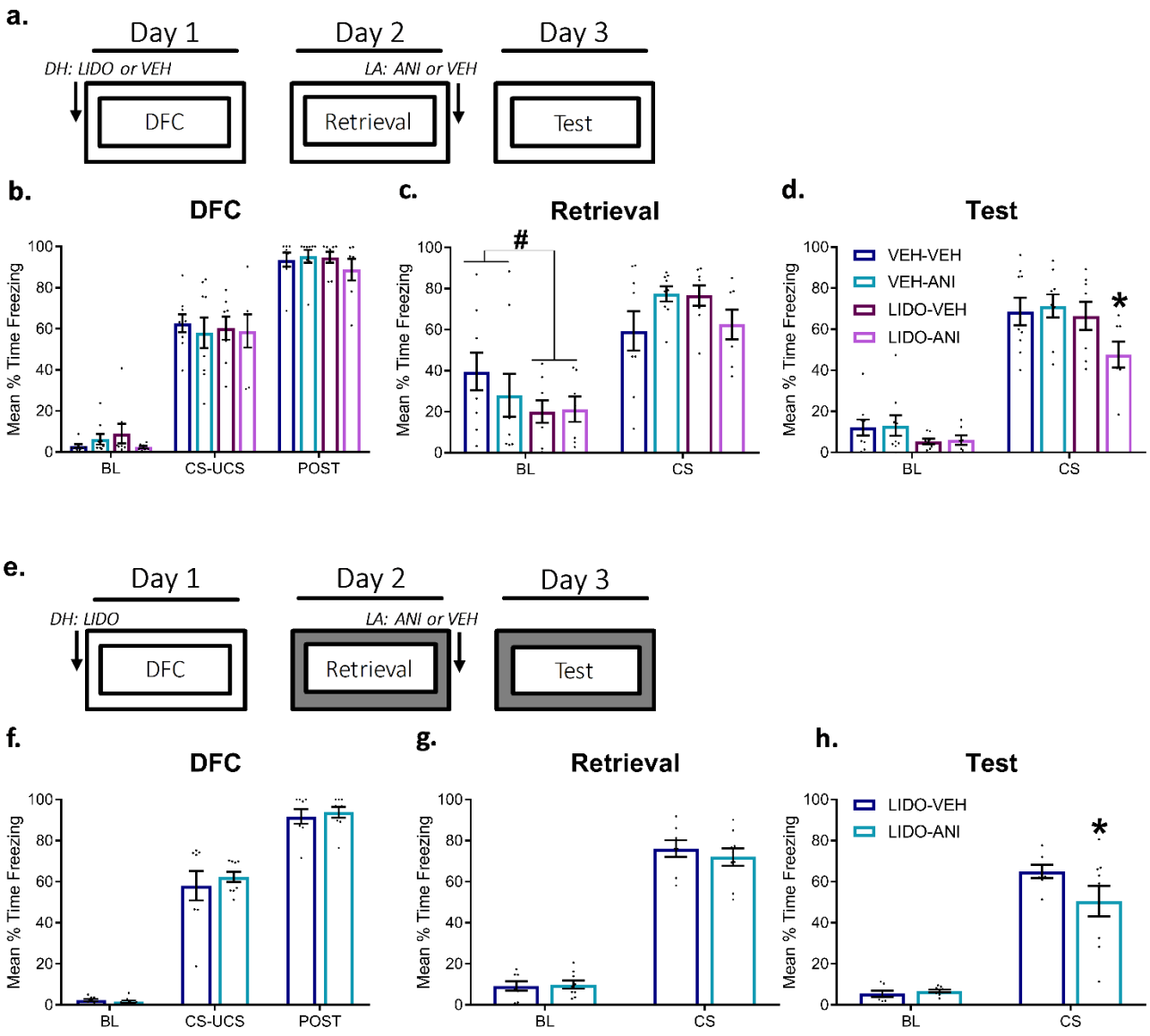

Figure 8. Memory lability in the amygdala is regulated by contextual novelty and DH activity. Experimental design (a&e). Groups were infused with Lidocaine (LIDO) or Vehicle (VEH) into the DH prior to training and there were no significant performance effects during training (b&f). When LIDO and VEH groups are compared, there is a trend for a significant reduction in context but not auditory CS fear when groups received LIDO into the DH (c). Groups that received LIDO into the DH and anisomycin (ANI) into the amygdala froze significant less to the CS in comparison to the VEH-VEH condition (d). There were no significant differences in baseline or auditory CS fear during retrieval in a shifted context when groups received LIDO infusions into the DH prior to training (g). The group that received an amygdala ANI infusion immediately after retrieval froze significantly less to the CS than the VEH group during the test (h). #$p = 0.06$, *$p < 0.05$.

During the long-term retention test, there was a significant main effect of drug ($F_{(3, 58)} = 3.17$, $p < 0.05$; Figure 8d). This main effect revealed a significant reduction in fear between groups that received lidocaine-anisomycin infusions in comparison to vehicle-vehicle infusions ($p < 0.05$). These results support the idea that context shifts are required for a memory to be susceptible to protein synthesis inhibition (Jarome, et al 2015). Furthermore, neural activity in the dorsal hippocampus gates or modifies anisomycin-dependent memory lability in the amygdala.

To rule out potential confounding lidocaine-anisomycin interactions and ensure DH inactivation does not impair auditory fear memory retention, we included a condition where all groups receive lidocaine DH infusions and the context between training and retrieval/test is shifted (Figure 8e). There were no differences between groups during training ($F_{(2, 30)} = 0.33$, $p = 0.72$; Figure 8f) or retrieval ($F_{(1, 15)} = 0.53$, $p = 0.48$; Figure 8g). At test, there was a near statistically significant interaction ($F_{(1, 14)} = 3.29$, $p = 0.09$), and a main effect for time ($F_{(1, 14)} = 140.80$, $p < 0.0001$). Post hoc analysis revealed a significant reduction in freezing in groups that received anisomycin infusions immediately following retrieval ($p < 0.05$; Figure 8h).

Activity in the dorsal hippocampus during training regulates amygdala AMPA receptor trafficking during reconsolidation

The trafficking of AMPA receptors at synapses in the amygdala following retrieval has been linked to memory lability and modification. Previous work shows reduced expression of GluR1 and GluR2 in the amygdala at 90-min following retrieval (Jarome et al., 2015). To determine if inactivation of the dorsal hippocampus restores AMPA receptor trafficking patterns in the absence of contextual novelty, we inactivated the dorsal hippocampus with lidocaine prior to training and sacrificed groups 90-min following retrieval (Figure 9a). There was a significant reduction in GluR2 ($p < 0.05$) and nearly significant reduction in GluR1 ($p = 0.07$) expression

between groups that received a context shift (B) and groups that did not (A-VEH) (Figure 9b). There was no significant difference between the context shift (B) condition and groups that received lidocaine infusions but received a retrieval session in the training context (A-LIDO) when measuring GluR1 ($p = 0.81$) or GluR2 ($p = 0.52$) expression (Figure 9b).

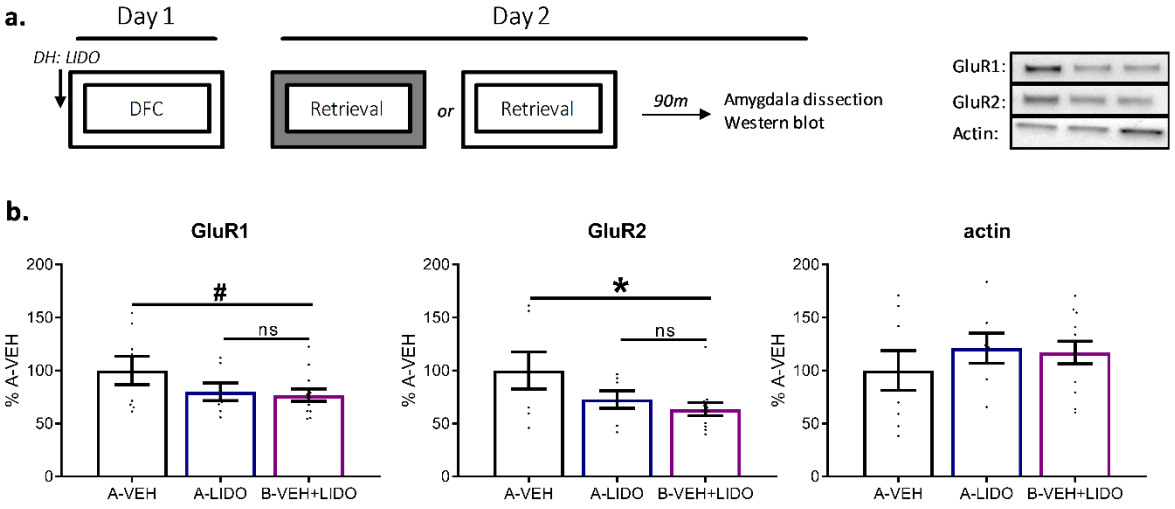

Figure 9. Activity in the DH during training regulates amygdala AMPA receptor trafficking during reconsolidation. Experimental design and representative western blot bands (a). Groups that received a CS presentation in a shifted context or received DH LIDO infusions prior to training without a training-retrieval context shift show reduced GluR1 and GluR2 expression in comparison to the DH VEH group without a context shift (b). #$p = 0.07$, *$p < 0.05$.

Discussion

In the current experiment, we manipulated contextual novelty by having groups undergo training, retrieval, and test in the same context. The DH is known to selectively encode contextual information during delay fear conditioning and may play an important role during the initiation of reconsolidation (Helmstetter et al., 2008). Memory susceptibility to disruption using protein synthesis inhibitors has also been linked to contextual novelty (Jarome et al., 2015). To target hippocampal activity, we infused lidocaine into the dorsal hippocampus prior to auditory fear conditioning. Groups received a retrieval session 24-hrs later and infusions of either vehicle or anisomycin in the amygdala immediately after retrieval. During the retrieval session, groups

that received lidocaine infusions into the dorsal hippocampus show modest reductions in context fear in comparison to groups infused with vehicle. This could be due to generally low fear responding during baseline in the vehicle conditions (i.e. less than 35% freezing), which may not be sensitive enough to detect decreases in fear. At a long-term test, groups that received lidocaine into the dorsal hippocampus and anisomycin into the amygdala showed a deficit in CS retention, suggesting activity in the dorsal hippocampus during training allows for amygdala-dependent memory lability following retrieval. We next measured AMPA receptor expression in the amygdala to determine if dorsal hippocampal inactivation is critical for AMPA receptor internalization during reconsolidation. The group that received lidocaine infusions into the DH without a shift in context showed a similar pattern of AMPA receptor expression in the amygdala in comparison to the shifted retrieval condition. Collectively, this work suggests activity in the DH during training can mediate memory lability and synaptic destabilization in the amygdala during retrieval when contextual novelty is removed.

Several studies demonstrate an important role for crosstalk between the dorsal hippocampus and amygdala during fear memory formation (McIntyre et al., 2005; McReynolds et al., 2009; Sanders et al., 2003; Seidenbecher et al., 2003; Wang et al., 2009). During delay fear conditioning, activity in the dorsal hippocampus is necessary for contextual processing, and the amygdala integrates a broad spectrum of sensory information for long-term storage (Helmstetter et al., 2008). Furthermore, the plastic events occurring in the DH and amygdala seem to impact one another, suggesting bidirectional modulation of plasticity between these regions during learning and memory (McIntyre, 2005; McReynolds et al., 2010 Richter-Levin & Akirav, 2001). For example, the amygdala and hippocampus show increased synchrony following fear learning, and inactivation of the amygdala prevents increases in hippocampal IEG expression (Huff et al.,

2006; Narayanan et al., 2007; Pape et al., 2005). While some work suggests the amygdala is a critical local site for memory storage (Gale et al., 2004; Kim et al., 1993), other work suggests that the amygdala plays a modulatory role for memory storage in the DH (Bevilaqua et al., 1997; McIntyre et al., 2005; McReynolds et al., 2009). Specifically, lesions or inactivation of the amygdala are known to impair memory formation, and LTP occurs at thalamo- and cortico-amygdala synapses following fear conditioning, providing evidence that the amygdala is a key site for memory storage and requires persistent potentiation of synapses (Apergis-Schoute et al., 2005; Gale et al., 2004; Kim & Cho, 2017). However, other work suggests the amygdala plays a modulatory role in memory storage by providing emotional valence or arousal. In this case, the amygdala would only be important for emotional or motivational aspects of the memory whereas the hippocampus would be the primary site for memory storage. For example, norepinephrine in the amygdala is thought to contribute to enhancements in hippocampal-dependent tasks, highlighting an important role for the amygdala in the modulation of memory elsewhere (McReynolds et al., 2010). Our results are consistent with work highlighting an important role for the amygdala during permanent memory storage but do not attempt to rule out the necessity of other brain regions in the consolidation and retention of a fear memory. Instead, our results emphasize that the DH and amygdala work together during fear memory formation and recall. Specifically, our results show that inactivation of the DH does not prevent DFC memory formation but impacts the ability to modulate this memory with amygdala manipulation, therefore the DH may gate the ability to modify a DFC fear memory in the amygdala during retrieval.

Amygdala AMPA receptor trafficking during retrieval has been linked to the initiation of memory lability (Jarome et al., 2012; Lopez et al., 2015). Rapid internalization of CI-AMPAR

during reconsolidation allows for synaptic plasticity underlying destabilization of synaptic connections to allow for the incorporation of new information into the original memory trace (Hong et al., 2013; Migues et al., 2016). The pattern of AMPAR trafficking and amount of AMPAR that return to the synapse after reconsolidation are sensitive to the cues present during retrieval (Jarome et al., 2015). Consistent with this, we demonstrate that contextual novelty during retrieval is an important factor for AMPAR internalization and this is regulated by DH activity during training. Specifically, when the retrieval context is novel, CI-AMPAR internalize and the memory is susceptible to protein synthesis inhibition, and when the context is not shifted, CI-AMPAR are maintained in amygdala synapses and do not allow for memory modification. Thus, the contextual information encoded and regulating lability is dependent on hippocampal activity, which then influences memory persistence in the amygdala.

In the current study, we used anisomycin to inhibit protein synthesis and track memory lability. Anisomycin has been referred to as a "messy" drug due to its ability to activate stress related kinases, apoptosis, and result in temporary behavioral reductions in fear and state-dependent effects (Bradley & Galal, 1988; Iordanov et al., 1997; Lattal & Abel, 2004; Rudy et al., 2006). However, many of these reports do not locally infuse anisomycin and none have reported such effects using cued fear conditioning or retrieval. Work from our lab supports the effectiveness of anisomycin used at the current dose in inhibition of protein synthesis in the amygdala for memory formation and retention (Parsons et al., 2006b). Furthermore, we found a specific reduction in auditory fear during a context shift or inactivation of the DH and not a global impairment in behavioral performance in other groups also infused with anisomycin, so it is unlikely changes in fear from anisomycin in this case was related to any potential adverse effects.

Collectively, these results provide insight for contextual novelty during retrieval-dependent memory modification and lend further support for dorsal hippocampal-amygdala interactions during learning and memory. Our work adds to existing work showing the amygdala is a critical site for memory storage and plasticity here is critical for retrieval-dependent memory updating which is gated by contextual information processed by the DH.

Chapter 4: General discussion

The goal of these experiments was to elucidate the contributions of auditory and contextual information to fear retention and post-retrieval modification. In two different aims we examined a role for contextual novelty and the maintenance of thalamo-amygdala pathway activity during auditory fear retrieval (Figure 9).

We found MgN activity in the amygdala is critical for fear retrieval and retention. This provides some of the first direct evidence for thalamo-amygdala pathway activity during auditory fear retrieval and retention. We originally predicted persistent reductions in fear would be associated with depotentiation of amygdala synapses as a result of MgN-LA terminal silencing during retrieval. However, the persistent reductions in fear do not seem to be a result of depotentiation, at least as inferred from synaptic expression of AMPAR (Figure 3) or of memory erasure (Figure 4). Previous work measuring depotentiation isolated the specific synaptic connections that were manipulated to measure the presence of amygdala AMPAR after manipulation, suggesting our method for measuring depotentiation may not be sensitive enough to detect significant changes in AMPAR expression (Kim & Cho, 2017). This explanation for our results is unlikely given that we do not see evidence of memory erasure (Figure 4). Our reductions in fear during retrieval and test as a result of MgN-LA terminal silencing are

consistent with extinction-like behavior that is limited to the retrieval context (Bouton, 2004). During extinction learning, heterosynaptic inhibition of primary auditory inputs in the amygdala contributes to the reduction in fear responding at long-term tests (Bauer & LeDoux, 2004; Cho et al., 2013; Rosenkranz & Grace, 2001). Heterosynaptic inhibition reduces presynaptic activity from regions such as the MgN and ACx while maintaining potentiation of cortico- and thalamo-amygdala synapses. Inhibition of thalamo- and cortico-amygdala pathways during extinction could occur from a variety of different sources. For example, mPFC input to the amygdala is increased following extinction and can excite local interneurons, which release GABA. The GABA release binds to pre- and post-synaptic sites and results in inhibited activity from primary auditory inputs in the amygdala. An alternate explanation for our extinction-like behaviors comes from manipulation of MgN-ITC synapses. Neurons from the MgN project to ITC neurons in the amygdala to directly manipulate ITC activity. ITC activity is increased following extinction and can feedback onto BLA projection neurons to inhibit them. The increase in ITC activity during extinction may then be based on decreased activity from the MgN. This increase in ITC activity would then allow for inhibition of BLA projecting neurons via GABA release, which would also presynaptically inhibit MgN inputs in the LA. Based on this, silencing MgN-LA terminals during a retrieval session may therefore facilitate extinction learning by mimicking the heterosynaptic inhibition that occurs during extinction. Our results are consistent with several behavioral and molecular profiles collectively showing activity from the MgN in the amygdala plays an essential role in the balance between excitation and inhibition that regulates auditory fear retention extinction, such as maintained postsynaptic potentiation of amygdala neurons, changes in amygdala pCREB expression correlated with fear responding, and fear renewal

(Baumgärtel et al., 2008; Hagiwara et al., 1992; Han et al., 2009; Lin et al., 2003; Kim & Cho, 2017; Porte et al., 2011; Rescorla, 2004; Tronson et al., 2012).

We found DH activity and contextual novelty regulate amygdala synaptic destabilization and memory lability during retrieval. We demonstrate a necessity for contextual novelty during retrieval for memory lability, and further show that DH activity during training contributes to amygdala-dependent memory lability in the absence of contextual novelty (Figure 6). CI-AMPAR internalization in the amygdala is necessary for synaptic destabilization during retrieval and is regulated by contextual novelty (Hong et al., 2013; Jarome et al., 2015). We provide evidence that DH activity during training can regulate amygdala AMPAR trafficking during reconsolidation when groups receive training and retrieval in the same context, suggesting contextual cues encoded by the DH during training can regulate amygdala synaptic destabilization during auditory fear retrieval (Figure 7). Collectively, these results support the idea that contextual novelty initiates synaptic destabilization and memory lability in the amygdala, and additionally show that the contextual information that regulates later amygdala destabilization may be encoded by the DH.

Final conclusions

In conclusion, memory retrieval provides a unique time for memory modification. Activity throughout a distributed circuit is critical for recall, expression, and reconsolidation of memory during a retrieval session. Sensory input to the amygdala has been implicated in the formation of a memory, but how these processes contribute to the stability of a memory after consolidation are unclear. Based on our findings, we show retrieval-dependent memory updating is dependent on the amygdala and 1) activity from the auditory thalamus in the amygdala during retrieval regulates fear responding and has lasting impacts on long-term fear retention and 2) the

lasting modifications of a fear memory via protein synthesis inhibition or through manipulation of amygdala inputs are gated by contextual information encoded during conditioning.

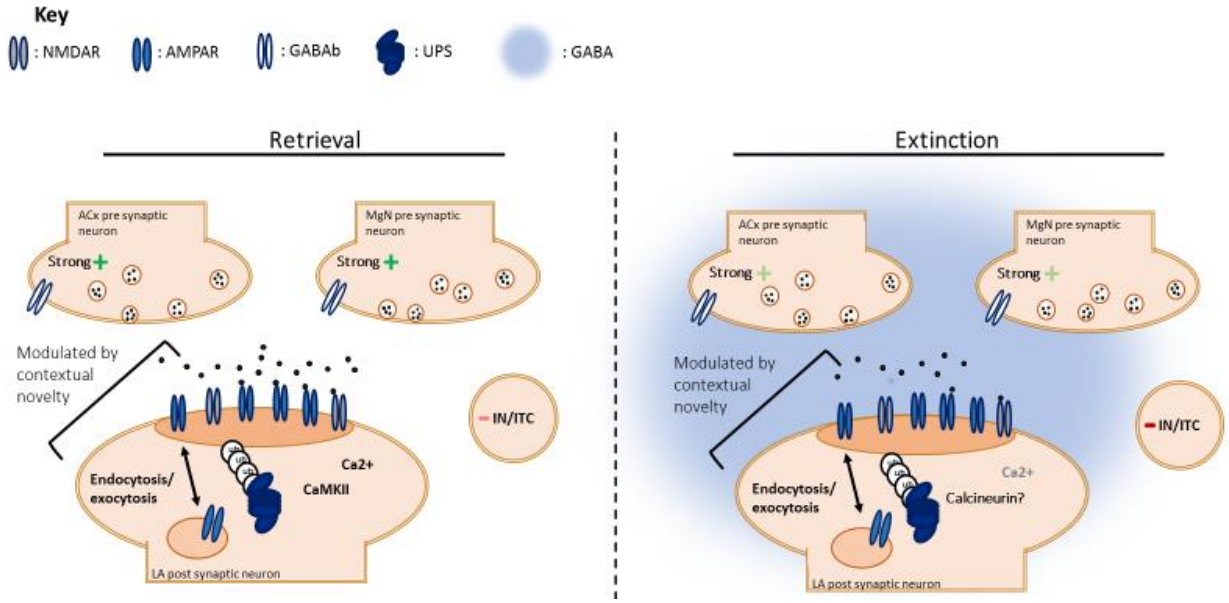

Figure 10. During retrieval, presynaptic activity from primary auditory centers release glutamate, which binds to AMPAR and NMDAR. When in a new context, the original memory can be updated and results in the phosphorylation of kinases, increases in UPS activity, and AMPAR trafficking. When contextual novelty is removed, AMPAR do not internalize and the memory is not able to be modified. During extinction, activity from inhibitory interneurons or ITCs can release GABA. GABA spillover binds to presynaptic GABAb receptors on primary auditory inputs in the amygdala to stop glutamate release. The reduction in neurotransmitter release from primary auditory centers contributes to the reduction in fear seen during extinction learning and retention. Inhibition of excitatory presynaptic inputs during retrieval may therefore mimic this process. This reduction in fear is limited to the context in which extinction was learned and can undergo renewal when the context is changed. Contextual novelty following extinction learning may release the inhibitory regulation of primary auditory centers, which would allow the rapid return of fear.

Future work needs to address *when* contextual information processed by the DH is important. Our work highlights an important role during learning and previous work shows contextual encoding prior to conditioning is important, but does DH processing of contextual cues need to occur during retrieval to allow for memory lability? If DH activity is necessary for auditory memory lability during retrieval, then this would provide an avenue to prevent the context-dependent return of fear and memory susceptibility to disruption. This approach would provide more information about how contextual cues influence memory modification and the

context-dependent return of fear, and when the DH interacts with the amygdala for memory disruption.

Traumatic memories can be associated with debilitating behaviors generalized to cues not predictive of an aversive event. Current treatment options, such as exposure therapies, are not always effective and require the repeated presentation of stimuli evoking substantial fear responses. Therefore, shorter more effective treatment sessions would reduce the amount of trauma re-exposure and may have fewer contextual constraints. Retrieval sessions allowing for the modification of the original fear memory provide a way to shorten exposure therapies and understand the limitations and neural mechanisms underlying long-term reductions in fear. Based on our results, shorter therapies could be more effective by reducing excitatory presynaptic activity in the amygdala during recall and ensuring novelty of the therapeutic environment.

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Taubenfeld, SM., Milekic, MH., Monti, B., & Alberini, CM. (2001). The consolidation of new but not reactivated memory requires hippocampal C/EBPβ. *Nature Neuroscience, 4*: 813-818.

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CURRICULUM VITAE

NICOLE FERRARA, M.S.

EDUCATION

University of Wisconsin – Milwaukee **2012 - present**
Doctoral Candidate
Major in Neuroscience
Minors in Neurobiology, Cognition and Perception
Advisor: Fred Helmstetter, Ph.D.
Dissertation: Auditory and contextual contributions to memory lability and synaptic destabilization in the amygdala

University of Wisconsin – Milwaukee **2015**
M.S. in Experimental Psychology—Neuroscience
Advisor: Fred Helmstetter, Ph.D.
MS Thesis: Neural mechanisms supporting auditory differential fear conditioning

Albion College, Albion, MI
B.A. in Psychology **2008-2012**
Neuroscience Concentration
Minor in the Gerstacker Institute of Business and Management
Honors Thesis: The Effects of Scopolamine on Escape and Avoidance Learning in Earthworms

AWARDS & HONORS

UWM | APF fellowship submission department nominee	**2017**
UWM | Neuroscience Mini-symposium Abstract Winner	**2017**
UWM | Psychology Summer Fellowship	**2016**
UWM | Student Travel Award	**2013 – 2014**
Albion | Dean's List	**2008, 2010 – 2012**
Albion | Webster Scholarship	**2008 – 2012**
Albion | Music Scholarship	**2008 – 2012**

RESEARCH EXPERIENCE

Helmstetter Neurobiology of Learning and Memory Laboratory **2012-present**
Graduate Student
University of Wisconsin—Milwaukee

Pankey Infectious Disease Laboratory **Summer**, **2012**
Volunteer
Ochsner Hospital

Wilson Invertebrate Behavior Laboratory **2011-2012**
Research Assistant
Albion College

TEACHING EXPERIENCE

UWM | **Physiological Psychology, Teaching Assistant** **Spring 2017**
(Ira Driscoll, Ph.D.)

UWM | **Physiological Psychology, Teaching Assistant** **Fall 2012, 2013, 2016**
(James Moyer, Jr., Ph.D.)

UWM | **Research Methods, Teaching Assistant** **Spring 2013**
(Marcellus Merritt, Ph.D.)

Albion College | **Research, Design, and Analysis II Lab Assistant** **Spring 2012**
(Jacque Carlson, Ph.D.)

PROFESSIONAL MEMBERSHIPS

President & Founder, AAUW UW-Milwaukee Chapter **2017-present**
Molecular Basis of Memory Association **2015-Present**
President 2015-2017
Vice President 2017-Present
Secretary, Graduate Students in Neuroscience **2014-present**
Secretary, Association for Graduate Students in Psychology **2014**
Student Affiliate, Society for Neuroscience **2011-present**
Psi Chi Member, National Honors Society of Psychology **2011-present**
Sigma Tau Delta, National English Honorary Society **2012-present**
Albion | Gerstacker Institute of Business and Management **2009-2012**

PUBLICATIONS

Ferrara, N.C.*, Jarome, T.J.*, Cullen, P.K., Kwapis, J.L., & Helmstetter, F.J. Activity-dependent protein degradation and GluR2 endocytosis in the amygdala regulate reconsolidation-dependent reevaluation of a contextual fear memory. *Submitted to Scireports*. **(*Authors contributed equally).**

Ferrara, N.C., Gilmartin, M.R., Reis, D.S., & Helmstetter, F.J. Region-specific amygdala increases in ERK and mTOR phosphorylation regulate memory formation. *In prep.*

Ferrara, N.C., Cullen, P.K., & Helmstetter, F.J. (2017). Medial geniculate nucleus input modulates amygdala encoding of fear memory discrimination. *Learning & Memory.*

Cullen, P.K., **Ferrara, N.C.**, Pullins, S.E., Helmstetter, F.J. (2017). Context memory formation requires activity-dependent protein degradation in the hippocampus. *Learning & Memory.*

Kwapis, J.L., Jarome, T.J., **Ferrara, N.C.**, & Helmstetter, F.J. (2017). Updating procedures can reorganize the neural circuit supporting a fear memory. *Neuropsychopharmacology*.

Jarome, T.J.*, **Ferrara, N.C.***, Kwapis, J.L., & Helmstetter, F.J. (2016). CaMKII regulates proteasome phosphorylation and activity and memory destabilization following retrieval. *Neurobiology of Learning and Memory*. **(* Authors contributed equally).**

Jarome, T.J.*, **Ferrara, N.C.***, Kwapis, J.L., & Helmstetter, F.J. (2015). Contextual information drives the reconsolidation-dependent updating of retrieved fear memories. *Neuropsychopharmacology*. **(* Authors contributed equally).**

Wilson, W.J., **Ferrara, N.C.**, Blaker, A.L., & Giddings, C.E. (2014). Escape and avoidance learning in the earthworm *Eisenia hortensis*. *Peer J*.

ABSTRACTS

Ferrara, N.C., Cullen, P.K., Pullins, S.E., & Helmstetter, F.J. (2017). Thalamic inputs to the amygdala regulate fear memory retrieval.
Poster presentation at the Society for Neuroscience annual meeting in Washington DC

Cullen, P.K., **Ferrara, N.C.,** Pullins, S.E., & Helmstetter, F.J. (2017). Neural activity in the ventrolateral periaqueductal gray provides a feedback mechanism to modulate fear network activity.
Poster presentation at the Society for Neuroscience annual meeting in Washington DC

Pullins, S.E., Cullen, P.K., **Ferrara, N.C.,** Cruz, W.J., & Helmstetter, F.J. (2017). Contributions of retrosplenial cortex to event-related and contextual fear learning.
Poster presentation at the Society for Neuroscience annual meeting in Washington DC

Ferrara, N.C.*, Jarome, T.J.*, Cullen, P.K., Kwapis, J.L., & Helmstetter, F.J. Activity-dependent protein degradation and GluR2 endocytosis in the amygdala regulate reconsolidation-dependent reevaluation of a contextual fear memory.
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA

Ferrara, N.C., Cullen, P.K., Rotondo, E.K., & Helmstetter, F.J. (2016). Medial geniculate nucleus input modulates amygdala encoding during fear memory discrimination.
Poster presentation at the RIKEN Institute Brain Science Institute Summer Program
in Tokyo, Japan.
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA

Ferrara, N.C.*, Jarome, T.J.*, Cullen, P.K., Kwapis, J.L., & Helmstetter, F.J. (2016). Activity-

dependent protein degradation and GluR2 endocytosis in the amygdala regulate reconsolidation-dependent reevaluation of a contextual fear memory. **(* Authors contributed equally)**
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA

Cullen, P.K., **Ferrara, N.C.**, Pullins, S.P., Hintz, J.M., & Helmstetter, F.J. (2016). Behavioral
expression of a fear memory is maintained by neural activity in a distributed brain network throughout CS presentation.
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA

Ferrara, N.C., Cullen, P.K., Rotondo, E.K., & Helmstetter, F.J. (2015). Neural mechanisms
supporting auditory differential fear conditioning.
Poster presentation at the Society for Neuroscience annual meeting in Chicago, IL.

Cullen, P.K., **Ferrara, N.C.**, Pullins, S.E., & Helmstetter, F.J. (2015). Neural activity in the
lateral amygdala driven by auditory CS onset critically determines memory retrieval
and behavioral expression of fear.
Poster presentation at the Society for Neuroscience annual meeting in Chicago, IL.

Cullen, P.K., **Ferrara, N.C.**, Callif, B.L., & Helmstetter, F.J. (2014). Hippocampal protein degradation in required for context memory formation.
Poster presentation at the Society for Neuroscience annual meeting in Washington DC.

Ferrara, N.C., Gilmartin, M.R., Reis, D.S., Lee, J.L., & Helmstetter, F.J. (2014). ERK-mTOR interactions in the lateral, basolateral, and central amygdala during fear memory consolidation.
Poster presentation at the Pavlovian Society annual meeting in Seattle, WA and Society for Neuroscience in Washington DC.

Cullen, P.K.*, **Ferrara, N.C.*,** & Helmstetter, F.J. (2014). Using optogenetics to alter fear and molecular signaling within the amygdala. **(* Authors contributed equally)**
Poster presentation at the Pavlovian Society annual meeting in Seattle, WA.

Kwapis, J.L., Jarome, T.J., **Ferrara, N.C.**, & Helmstetter, F.J. (2013). Updating a memory can change the way it is stored.
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA.

Ferrara, N.C., Gilmartin, M.R., Reis, D.S., Lee, J.L., & Helmstetter, F.J (2013). Interactions between the mammalian target of rapamycin (mTOR) signaling pathway and the extracellular signal-regulated kinase (ERK) in the basolateral amygdala during fear memory consolidation.

Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA.

Gilmartin, M.R., **Ferrara, N.C.**, Callif, B.L., Schraufnagel, E.E., & Helmstetter, F.J. (2013). Divergent intracellular signaling in the amygdala supporting trace and delay fear conditioning: role of protein kinase-A and extracellular-signal regulated protein kinase pathways.
Poster presentation at the Society for Neuroscience annual meeting in San Diego, CA

Ferrara, N.C. (2011). The Effects of Nicotine on Earthworms.
Poster presentation at the Society for Neuroscience annual meeting in Washington, D.C.

Ferrara, N.C., & Blaker, A. (2011). Escape and Avoidance Learning in the Earthworm.
Poster presentation at the Society for Neuroscience annual meeting in Washington, D.C. and the Pavlovian Society's annual meeting in Milwaukee, WI.

INVITED COLLOQUIA, SYMPOSIA, & PROGRAMS

Ferrara, N.C. (2018). Thalamic terminals in the amygdala regulate fear memory retrieval and retention
Presentation for the Association for Graduate Students in Psychology

Participated in the Engaging Girls in STEM workshop (2017 & 2018).

Organized and participated in Brain Awareness Week at St. Joseph Academy (2017).

Ferrara, N.C. (2017). Reconsolidation dependent mechanisms of memory updating.
UWM neuroscience mini-symposium
Presentation for the Association for Graduate Students in Psychology

Ferrara, N.C. (2016). Medial geniculate nucleus input modulates amygdala encoding during fear memory discrimination.
UWM neuroscience seminar presentation

Awarded invitation and financial sponsorship to the RIKEN Brain Science Institute Summer lecture series (2016).

Ferrara, N.C. (2016). Neural Mechanisms Supporting Differential Fear Conditioning
Presentation for the Association for Graduate Students in Psychology

Ferrara, N.C. (2015). Neural Mechanisms Supporting Differential Fear Conditioning.
UWM neuroscience seminar presentation

Ferrara, N.C. (2014). The Brain.
Presentation for the Science Club at Atwater Elementary School

Ferrara, N.C. (2014). Interactions between the ERK and mTOR pathways during memory consolidation in the amygdala.
Presentation for the Association for Graduate Students in Psychology

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LABORATORY SKILLS

Confocal microscopy
Designer Receptors Exclusively Activated by Designer Drugs
Fear conditioned training and testing
Fluorescent *in situ* hybridization
Immunohistochemistry
Immunofluorescence
Microinjections
Optogenetics
Stereotaxic surgery
Western blotting

RELEVANT COURSE WORK

Experimental Design
Neurobiology of Learning and Memory
Advanced Psychological Statistics
Proseminar in Biological Psychology
Behavioral Neuroscience
Conditioning and Learning
Eukaryotic Gene Regulation
Cognitive Neuroscience
Cellular and Molecular Toxicology
Cellular and Molecular Mechanisms of Neurodegenerative Disease