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# Neural Mechanisms Supporting Differential Auditory Fear Conditioning

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NEURAL MECHANISMS SUPPORTING DIFFERENTIAL AUDITORY FEAR  
CONDITIONING

by

Nicole C. Ferrara

A Thesis Submitted in  
Partial Fulfilment of the  
Requirements for the Degree of

Master of Science  
in Psychology

at

The University of Wisconsin Milwaukee

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## ABSTRACT

### NEURAL MECHANISMS SUPPORTING DIFFERENTIAL AUDITORY FEAR CONDITIONING

by

Nicole C. Ferrara

The University of Wisconsin—Milwaukee, 2015  
Under the Supervision of Professor Fred J. Helmstetter

Pavlovian fear conditioning provides an ideal way to study memory formation, retention, and updating. Plasticity in the auditory thalamus is required during the acquisition and consolidation of a fear memory when a tone signals a foot shock. The medial geniculate nucleus (MgN) of the auditory thalamus sends projections to the amygdala and auditory cortex and is functionally divided into two different regions, the medial division (MGm) and the ventral division (MGv). Traditionally, these divisions are thought to relay auditory information to the amygdala during fear-related associative learning. However, recent research has suggested a more complex role for the MgN when processing auditory stimuli during conditioning. In the current study, we identified synaptic activity necessary for memory formation in the MgN as well as how this plasticity impacts the amygdala. In order to target MgN plasticity, we used a discriminative fear conditioning procedure to recruit auditory thalamus plasticity during conditioning. We predicted that memory consolidation in the MgN requires mRNA translation, which will initiate amygdala receptor trafficking following auditory fear conditioning. We found that protein synthesis in the MgN is required for amygdala AMPA receptor surface expression and synaptic scaffolding necessary for auditory memory consolidation.

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## LIST OF ABBREVIATIONS

$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Anisomycin	ANI
Baseline	BL
Brachium of the inferior colliculus	BIC
Conditioned response	CR
Conditional stimulus	CS
Dorsal medial geniculate nucleus	MGd
Evoked excitatory postsynaptic potentials	EPSP
Evoked field potential	EFP
Extracellular regulated kinase	ERK
Gamma-aminobutyric acid	GABA
Long term potentiation	LTP
Medial geniculate nucleus	MgN
N-methyl-D-aspartate	NMDA
Posttraumatic stress disorder	PTSD
Unconditional stimulus	UCS
Vehicle	VEH
Ventral medial geniculate nucleus	MGv

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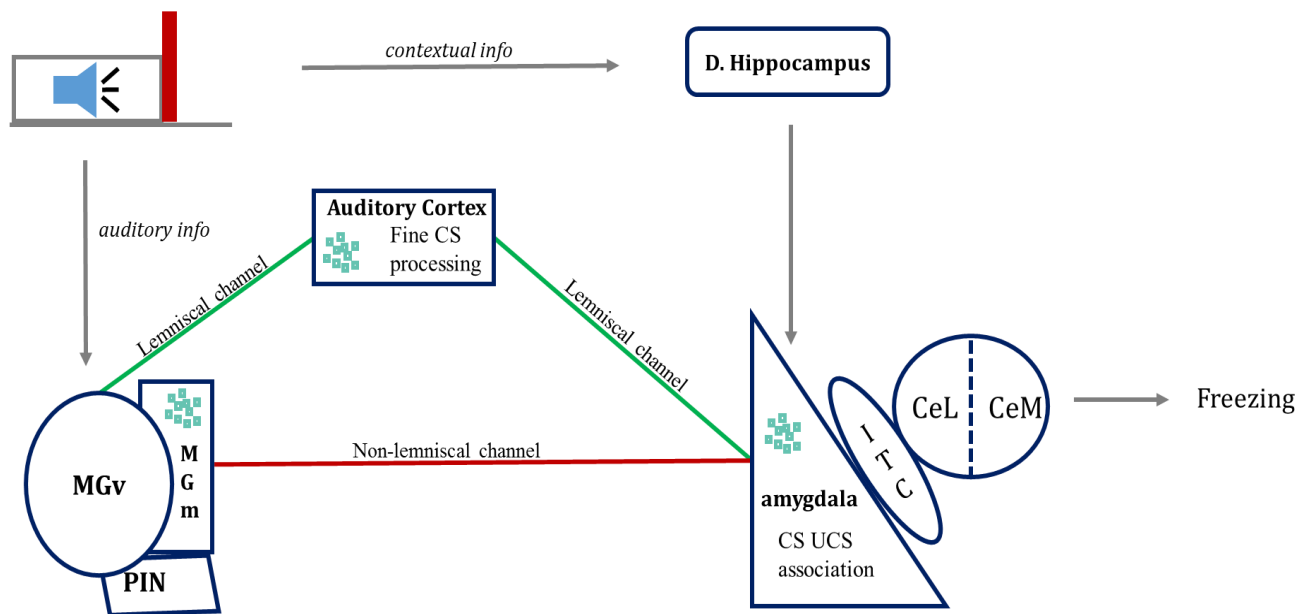
The study of fear memory formation and updating allows for the characterization of synaptic changes underlying maladaptive fear responses. Fear conditioning is a well-established Pavlovian conditioning procedure used to study the underlying neural mechanisms supporting memory formation that may help in the understanding of fear related psychiatric disorders (Fendt & Fanselow, 1999; Parsons & Ressler, 2013). During this procedure, a neutral CS is paired with an aversive, UCS. After a few pairings, the CS acquires aversive value and is able to elicit a fear response independently of the UCS when presented (Fanselow, 1980). During standard auditory fear conditioning, a tone (CS) and a foot shock (UCS) are paired. To measure the long term retention of this learning, a CS is typically presented alone in a novel environment at least 24-hours after training. The CS will elicit freezing behavior, indicating that the rodent remembers the fear learning. The fear memory formed with this procedure is rapidly acquired, strong, and long lasting which makes it ideal to study the molecular mechanisms occurring during memory consolidation.

Consolidation of an aversive memory requires activity in a network of brain structures, all of which are critical for conditioning and undergo protein synthesis-dependent plasticity (for review see Helmstetter et al., 2008). The amygdala is a critical site of synaptic plasticity during fear conditioning, and it has been generally accepted as a major site for sensory convergence of CS-UCS associations (Davis et al., 1997; Fendt & Fanselow, 1999; Maren, 1999; Blair et al., 2001; Kim & Jung, 2006). Inputs to the amygdala are necessary for memory consolidation, but the impact of presynaptic modulation in the amygdala has not been systematically characterized. Specifically, thalamic inputs to the amygdala have been shown to undergo learning-dependent plasticity during fear conditioning, but the plasticity in this structure and its impact on amygdala plasticity during fear memory acquisition and consolidation is limited. To have a comprehensive



understanding of fear memory formation, a better understanding of how inputs to the amygdala undergo synaptic plasticity is required. In the auditory thalamus, the MgN of the thalamus processes auditory and somatosensory input, making it a candidate structure for CS-UCS convergence during learning dependent-plasticity (Cruikshank et al., 1992; Edeline & Weinberger, 1992; Weinberger, 2011).

Several other brain areas encode temporal and contextual components during fear conditioning and require synaptic plasticity for fear memory formation. Specifically, inactivation of the dorsal hippocampus has been shown to prevent the encoding of contextual information during fear conditioning (Gafford et al., 2011), while inserting a stimulus free period (i.e., trace interval) between a tone and a shock recruits the medial prefrontal cortex, making the memory dependent on this structure as well (Gilmartin et al., 2013; Gilmartin & Helmstetter, 2010). This



**Figure 1. Auditory fear conditioning neural network.** Information about the auditory stimulus is transmitted to the MgN. From the MgN, information is sent to the amygdala through two different routes. The non-lemniscal channel contains broadly tuned neurons and begins in the MGm then projects directly to the amygdala. This channel typically shows the most learning-dependent plasticity and is the quickest route to the amygdala. The lemniscal channel originates in the MGv and indirectly projects to the amygdala via the auditory cortex. This channel contributes to the fine processing of auditory stimuli and is slower than the non-lemniscal channel.

work supports the idea that the amygdala requires inputs from a network of structures undergoing plasticity to form a fear memory (Figure 1).

Inputs to the amygdala from the MgN transmit auditory information through lemniscal or non-lemniscal channels. These channels are the two primary pathways in the MgN. They both originate in the inferior colliculus and are thought to be involved in the discrimination of auditory cues (Hu, Senatorov, & Mooney, 1994; Ledoux et al., 1983). Both are unique and process distinct auditory information, giving rise to different responses as a result of associative learning (Hu et al., 1994; Edeline et al., 1992). Specifically, the lemniscal pathway includes neurons that are responsive to specific frequency tones and complex sounds, referred to as sharply tuned neurons residing in the auditory cortex that receive input from the MGv; this pathway does not directly project to the amygdala (Bordi & Ledoux, 1994). In contrast, the non-lemniscal pathway includes neurons that respond to a variety of types of tones as well as somatic information, known as broadly tuned multi-sensory neurons, residing in the MGm, which directly project to the amygdala (Bordi & Ledoux, 1994; Cruikshank et al., 1992). Broadly tuned non-lemniscal neurons throughout the MgN have been the primary focus during learning and memory research because they exhibit frequency selective increases in firing immediately and one hour after auditory fear conditioning, suggesting they encode auditory information following fear conditioning (Edeline & Weinberger, 1992; Poremba & Gabriel, 2001). While lemniscal neurons show increased spike activity immediately after auditory conditioning, this CS specific plasticity dissipates after one hour (Edeline & Weinberger, 1992), making it unlikely that these neurons are involved in the long-term consolidation the auditory memory. Collectively, these results suggest that neurons in the MGm, making up the non-lemniscal channel, are more closely

associated with the long-term synaptic changes necessary for memory formation due to neuronal spike activity increases that persist into a post-training consolidation window.

Based on the different projections of the lemniscal and non-lemniscal channels, several studies have characterized these as cortical and subcortical routes, respectively, during auditory fear conditioning (Apergis-Schoute et al., 2005; Hu et al., 1994; Li et al., 2013). For example, projections directly from the MgN to the amygdala through the non-lemniscal pathway are classified as subcortical; whereas, indirect projections from the MgN to the amygdala that rely on the auditory cortex are through a lemniscal channel and are classified as cortical routes. Preliminary studies attempting to characterize these projections began by focusing on different subregions of the MgN. Using anterograde tracer beads injected into the inferior colliculus to label the MgN, Hu and colleagues (1994) examined the distribution of glutamate receptors in the MGd and MGv regions of the MgN. In the MGd, NMDA and AMPA receptors were able to mediate synaptic transmission of EPSPs, while GABA receptor blockade was not needed to reveal these effects. Conversely in the MGv, extracellular recordings in the MgN showed that NMDA receptor blockade did not affect evoked monosynaptic stimulation of the BIC responses. However, AMPA receptor blockade with CNQX prevented evoked EPSPs. Furthermore, NMDA receptors were primarily found in non-lemniscal neurons, suggesting that lemniscal and non-lemniscal channels in the MgN are anatomically and behaviorally distinct.

#### *The role of the medial geniculate nucleus during auditory fear conditioning*

The investigation of lemniscal and non-lemniscal channels at a cellular level has provided a foundation for behavioral work studying the role of the MgN during auditory fear conditioning. Functional classifications of the lemniscal and non-lemniscal channels often focus on the role of

cortical or subcortical routes, respectively, for tone processing during auditory fear conditioning (Antunes & Moita, 2010; Kwon & Choi, 2009; Ledoux et al., 1983). Work characterizing these routes have used a wide variety of techniques to elucidate the role of the MgN locally and at a network level to aid in fear memory acquisition and formation (Antunes & Moita, 2010; Han et al., 2008; Kwon & Choi, 2009; Kwon et al., 2014; Maren et al., 2003; Parsons et al., 2006).

The functional role of routes from the auditory cortex and MgN to the amygdala have produced contradictory results for CS-UCS processing during the acquisition and consolidation of a fear memory. Initial studies investigating the role of the MgN used electrolytic lesions to study the role of the auditory system during fear conditioning and found that MgN but not the auditory cortex lesions reduced conditional responses, such as heart rate, lick suppression, and freezing (Ledoux et al., 1983). This provided preliminary evidence that subcortical rather than cortical pathways are a primary source for the classification of simple auditory stimuli; however, other work has shown that both pathways are necessary for fear conditioning, highlighting the controversy around these results (Boatman & Kim, 2006; Romanski & LeDoux, 1992). A number of studies have inactivated cortical or subcortical routes to the amygdala to pinpoint which routes are essential for memory consolidation, but often have opposing results, which may be related to the extent of the lesion or the pharmacological manipulation. The early work characterizing these routes show lesions to a wide range of nuclei, and therefore, may not have manipulated a specific region to determine the role in auditory fear conditioning, which may contribute to the discrepancies between results. While there is strong support for subcortical routes during auditory fear conditioning, recent research has suggested a larger role for cortical routes (Boatman & Kim, 2006) or both cortical and subcortical routes (Romanski & LeDoux, 1992) during acquisition of auditory fear conditioning.

Following the initial characterization of MgN involvement in auditory fear conditioning, several studies focused on the role of protein synthesis during the consolidation of an auditory fear memory. These studies suggest that protein synthesis is needed during, but not after, fear conditioning to disrupt fear memory consolidation (Apergis-Schoute et al., 2005; Maren et al., 2003; Parsons et al., 2006). Together, these results suggest that regions in the auditory thalamus are required for the initial association of auditory and somatosensory stimuli during training before projecting information to the amygdala, which may serve as the primary source of plasticity during fear conditioning.

Other views on the role of the MgN during fear conditioning suggest that the MGm is responsible for recoding auditory CSs according to their behavioral relevance (Cruikshank et al., 1992; Kwon & Choi, 2009; Kwon et al., 2014). During recoding, neurons that were not selectively responsive to a tone become disproportionately more responsive to the auditory CS after fear conditioning. Specifically, MgN neurons are not selectively responsive to a particular tone but initially encode tone-related information. After auditory fear conditioning, these neurons selectively fire above baseline to tones that were paired with a shock, while tones that were not paired with a shock during the same training session exhibit below baseline firing responses (Poremba & Gabriel, 2001). When this occurs, information about the initial encoding of the tone is updated or recoded according to its behavioral relevance or aversive nature, which can be measured by the level of differential freezing to a tone paired with a shock in comparison to auditory stimuli that were not paired with a shock. The recoding of auditory stimuli is likely dependent on the synthesis of new proteins in the MgN because local anisomycin infusions into the MgN reduce conditioned responses to tones, as well as modulate amygdala activity. In this case, synthesis of new proteins in the MgN would aid in the transport of newly synthesized RNA

from the MgN to the amygdala presynaptic terminals based on the consequence of the auditory cue during recoding, highlighting a requirement for direct input from the MgN to the amygdala for auditory fear conditioning.

Stimulation of MGm neurons, but not MGv neurons, have served as a UCS to elicit a conditional response when paired with an auditory CS (Cruikshank et al., 1992; Kwon & Choi, 2009), providing evidence that direct input from the MgN to the amygdala via non-lemniscal channels is necessary to support associative learning and is directly involved in coding CS-UCS information. Additionally, MGm stimulation paired with several auditory CSs is able to induce EFP changes in MGm-lateral amygdala projections, providing the first piece of evidence that plastic changes induced by MGm neurons can be measured in the amygdala as a direct consequence of associative fear learning (Kwon & Choi, 2009). The pairing of foot shocks and stimulation of projections from the auditory cortex and MgN in the lateral amygdala is sufficient to elicit conditioned responses. The expression of these conditioning responses, and the consolidation of auditory memories in general, is dependent on glutamatergic synaptic transmission in the amygdala (Kwon et al., 2014). Therefore, MgN neurons that are critical for auditory fear memory formation and are likely undergoing recoding, directly manipulate transmission of information to the amygdala. Because these synaptic connections are glutamatergic, MgN input to the amygdala underlying auditory fear conditioning is likely exerting its influence through the modulation of amygdala AMPA receptor trafficking. These findings provide strong evidence that monosynaptic transmission of information from the MgN during recoding is required to elicit freezing responses and to form amygdala-dependent CS-UCS associations.

In line with evidence supporting auditory recoding of MGm neurons in response to fear conditioning, the number of phosphorylated CREB expressing neurons in the MGm/PIN, but not other regions of the auditory thalamus, is correlated with learning (Han et al., 2008), suggesting a molecular mechanism underlying recoding. When overexpressed, p-CREB in the MGm/PIN enhances the retention of an auditory fear memory (Han et al., 2008). Interestingly, this overexpression in the MGm/PIN was also associated with generalization of a conditional response to unconditioned tones after fear conditioning, indicating that the original efficacy of the memory was affected; however, the mechanisms dependent on tone generalization are not well understood (Han et al., 2008). It is possible that the overexpression of CREB, resulting in an abundance of mRNA, incorporated cells into the auditory code that would not have originally participated. This would indicate that the number of cells and the degree of their activity is directly correlated with conditioned response to a tone. For example, the inhibition of protein or mRNA synthesis would result in a reduction of activity and inhibition of recoding, therefore, a low freezing response to auditory cues. However, the overexpression of protein or mRNA would indicate hyperactivity, and a high freezing response that is generalized across multiple tones. This suggests that MgN neurons undergoing recoding are dependent on precise level of neuronal transcriptional and translational activity. The mechanism of auditory memory generalization through overexpression of proteins in the MgN is not well characterized; however, it is likely dependent on MgN modulation of amygdala activity. The abundance of mRNA produced in the MgN due to overexpression of CREB could be used to strengthen presynaptic terminals in the amygdala, the critical site for long term memory storage. However, the mechanism of synaptic modulation of MgN activity on the amygdala remains unknown.

Despite exhibiting learning-dependent plasticity, several studies have argued that the MgN may not be a critical site for memory formation or retrieval, making it an unlikely source for memory storage (Apergis-Schoute et al., 2005; Maren et al., 2006). Furthermore, it has been suggested that the amygdala is the primary site for memory storage during fear conditioning, potentially holding the memory ‘engram’ (Blair et al., 2001; Davis et al., 1997; Fanselow & LeDoux, 1999; Fendt & Fanselow, 1999; Maren, 1999; Maren, 2001; Kim & Jung, 2006). Specifically, infusions of muscimol into the amygdala prevent conditioned CS elicited spike firing in the MGm, suggesting that reciprocal projections originating from the amygdala sent to the MGm are required for learning-dependent MGm plasticity to support an auditory memory trace (Maren et al., 2001). The extracellular regulated kinase (ERK) is phosphorylated in response to associative learning, occasionally via glutamate receptor activity, in a series of brain structures involved in the regulation of memory formation (Adams & Sweatt, 2002; Apergis-Schoute et al., 2005; Herry et al., 2006). ERK phosphorylation in the MgN is required for induction of long term potentiation (LTP), a neural correlate of memory, in the amygdala (Apergis-Schoute et al., 2005). However, phosphorylated ERK in the lateral amygdala is not necessary for conditioned-induced synaptic plasticity in the MgN (Schafe et al., 2005), highlighting a molecular mechanism in which presynaptic influence from the MgN to the amygdala, but not amygdala to MgN, is required for auditory memory processing.

A majority of auditory fear conditioning studies have focused on auditory conditioning with a single tone. Because the MgN may use a precise neuronal code to process auditory cues, a larger neuronal network may be recruited for a procedure using multiple tones in comparison to a single tone study. Specifically, delay fear conditioning may engage a neuronal code in the MgN that is much smaller than a code that would be engaged for training using multiple tones during



memory formation. It is possible that using a more complex auditory conditioning paradigm (e.g. a tone discrimination paradigm in which one tone is paired with shock (CS+) and one is not (CS-)) may be sufficient to recruit maximal MgN plasticity, allowing for the investigation of CS recoding and generalization in the MgN during a single training session with multiple auditory cues. Differential auditory fear conditioning procedures, using multiple tones, may be able to engage non-lemniscal and lemniscal auditory CS processing within the same training session. This allows for the manipulation of crude or precise processing of auditory cues and the recoding of these cues during conditioning in relation to cortical versus subcortical channels.

The generalization of fear responses to safety cues from aversive cues is commonly seen in individuals diagnosed with PTSD (Jovanovic et al., 2012), and differential fear conditioning provides a mechanism to study the generalization of fear between learned safety and aversive signals. The heightened fear to a safety cue, or a reduction in discrimination between safety and aversive signals, provides a model to study the behavioral characteristics underlying fear related disorders. Specifically, differential fear conditioning provides a way to behaviorally measure learned fear responses to auditory cues following pharmacological manipulation, as well as the factors contributing to the generalization of fear between auditory cues (Antunes & Moita, 2010; Ghosh & Chattarji, 2014). This generalization of fear is commonly classified as a heightened fear to a safety signal (CS-) as well as maintained fear to an aversive signal (CS+), which mirrors the maladaptive fear expression seen in individuals with PTSD (Jovanovic et al., 2012). Furthermore, differential fear conditioning provides a way to elucidate the neural mechanisms underlying generalization of fear and highlight the switch from discrimination to generalized fear (Ghosh & Chattarji, 2014).

Recent work has used electrolytic lesions to investigate the potentially unique roles of the MGm and MGv during differential auditory conditioning, finding that the MGm, but not the projections from the MGv to the auditory cortex, is necessary to support the consolidation and extinction of discriminative fear (Antunes & Moita, 2010). This study (Antunes & Moita, 2010) taken together with previous work showing that the auditory cortex is not necessary for the generalization of differential fear (Ghosh & Chattarji, 2014), this work suggests that direct projections from the MGm, but not indirect projections from the MGv, to the amygdala are required to support auditory discriminative fear memory. This also provides evidence that subcortical projections to the amygdala from the MGm are required for further processing of the CS-UCS associations during auditory fear conditioning. Furthermore, these projections have been classified as predominantly non-lemniscal, indicating that they are dependent on NMDA and AMPA receptors (Farb & LeDoux, 1998; Hu et al., 1994; Li et al., 1996). This suggests a critical role for the MGm to elicit appropriate behaviors to a CS+ or CS-, while also providing a more precise model and mechanism in which recoding of auditory stimuli may occur.

The molecular mechanisms supporting differential conditioning are not well characterized, and little work has been done on how auditory discrimination learning influences MgN plasticity. Initial work investigating amygdala influence on the MGm used discriminative avoidance conditioning paired with electrolytic lesions or muscimol in the amygdala to abolish training induced neuronal plasticity in the MGm, these studies concluded that amygdala activity is necessary for MGm training induced neuronal activity (Poremba & Gabriel, 1997; Poremba & Gabriel, 2001). Furthermore, MGm training induced neuronal activity is dependent on the number of trials given during conditioning (Talk et al., 2000). During discriminative avoidance conditioning, amygdala activity only affects MGm plasticity selectively during later training

trials (60-120 trials) but not initial trials (Talk et al., 2000). Additionally, training induced neuronal activity in the amygdala selectively increases to a CS+ with little to no change in response to a CS- after discriminative avoidance conditioning, whereas training induced neuronal activity in the MGm increases to a CS+ and decreases to a CS- (Poremba & Gabriel, 2001). This highlights that the synaptic plasticity occurring in these structures are fundamentally different but dependent on one another. To reconcile these results with other literature, Porema et al. (2001) suggests that amygdala-dependent MGm plasticity may be influenced through the auditory cortex, or lemniscal pathway, to sustain MGm plasticity during conditioning. Specifically, the auditory input from the MGm at the beginning of training contributes to plasticity in the amygdala, the amygdala activates frequency-specific neurons in the auditory cortex, and the auditory cortex then sends this information back to the MGm to elicit discriminative training induced plasticity.

Taken together, the plasticity occurring between the MgN and amygdala have produced contradictory results for CS-UCS processing during training (Edeline & Weinberger, 1992; Maren et al., 2001; Poremba & Gabriel, 2001; Schafe et al., 2005; Weinberger, 2011). While a majority of MgN fear conditioning literature has attempted to determine where the recoding of auditory stimuli occurs during fear conditioning, few have considered the interaction between the amygdala and MgN. The aim of the current proposal is to delineate the role of MgN and amygdala interactions supporting CS-UCS associations. To investigate auditory CS recoding after conditioning and amygdala influence on the MGm, the current study characterized the neural mechanisms occurring during auditory fear conditioning in comparison to contextual fear conditioning. We tested the hypotheses that 1) protein synthesis in the MgN will be necessary to support differential auditory fear conditioning 2) protein synthesis blockade in the MgN will

prevent AMPA receptor trafficking and stability in the amygdala following auditory but not contextual fear memory consolidation.

## Method

### *Subjects and Surgery*

The current experiments were performed on 96 adult Long-Evans male rats (300-375g). Rats were individually housed and had access food and water at all times. All experiments were approved by the Institutional Animal Care and Use Committee.

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. Using stereotaxic coordinates, animals were implanted with bilateral cannulae targeting the MGm (-5.3 mm posterior, +/-2.8 mm lateral, -5.6 mm ventral) according to bregma. These coordinates were determined based on the rat brain atlas (Paxinos & Watson, 2007). Each cannulae was secured to the skull with a screw and surrounded by acrylic cement. A dummy cannula was screwed into the cannula guide to prevent occlusion.

### *Microinjection of Drugs*

After a full recovery (4-7 days), animals were adapted to transport handling procedures for 3 days before conditioning. The transport handling procedures included a gentle restraint during the sound of the infusion pump.

Thirty-minutes before or immediately after conditioning, rats received bilateral microinjections of anisomycin (125 µg/µl, Tocris), or vehicle (ACSF). The injection was delivered at a rate of 0.5 µl/min and at a volume of 0.5 µl/hemisphere. Rats were returned to their home cages after injections.

### *Fear Conditioning*

Conditioning occurred in a set of four Plexiglas and stainless steel chambers within sound-attenuating boxes (Context A). The floor included 18 stainless steel bars lining the floor, which are connected to a shock generator (Grason-Stadler, West Concord, MA). Each chamber has a speaker to allow for the pure tone (1kHz, 7kHz) and ventilation fans to provide a constant background noise (55 dB). Between rats, the chambers were cleaned with 5% ammonium hydroxide.

Rats were placed in Context A chambers for a CS+/CS- training paradigm. During this training paradigm, rats received ten counterbalanced CS+ presentations, which included a pure tone paired with a shock (0.5mA), and ten CS- presentations, which included a pure tone not paired with a shock. The average inter-trial interval between each tone presentation was 70-s. Context B served as a shifted context to test the strength of the auditory memory. This chamber consists of Plexiglas flooring and was cleaned with an acetic acid solution. The remaining materials were identical to Context A.

Rats were tested for the strength of the auditory and contextual memory 24-hours after conditioning. To measure auditory learning, rats were placed in Context B where they received 5 discrete tone presentations of the CS+ and CS- (30-s each) separated by a 60 second ITI while freezing rates were recorded.

### *Behavioral Analyses*

Freezing is a response that indicated learning and memory of a fearful event during training and testing sessions; this was measured as the cessation of all movement, excluding respiration. Freezing was automatically scored in real-time with FreezeScan 1.0 detection software (Cleverly Sys, Inc., Reston, VA).

### *Statistical Analyses*

All statistical analyses and graphing were conducted in GraphPad. For experiment 1, a mixed model 2 (CS freezing) x 2 (drug) ANOVA was used to compare within subject CS freezing and between subject drug effects. Fisher's LSD post hoc was used to compare vehicle and drug infused animals within CS+ or CS- presentations. For experiment 2, a 2 (drug) x 3 (training type) ANOVA was used to compare drug by type of fear conditioning. To compare drug groups during training, the averages for the 6-minute baseline period, CS-UCS pairings period, and the 4-minute post CS-UCS pairings period were analyzed between drug groups using a 2 (drug) x 3 (freezing during training period) ANOVA. The Western blot groups were grouped by drug and were compared based on training (context, delay, or differential fear conditioning). Tukey's multiple comparisons were used when appropriate to compare protein expression within a drug group between context, delay, and differential fear conditioned groups.

### *Histology*

Upon completion of testing, rats were deeply anesthetized with isoflurane, and transcardially perfused with 0.9% saline immediately followed by 10% buffered formalin. Brains were kept in 10% buffered formalin. After 2 days, brains were transferred to 20% sucrose formalin before being frozen for coronal sectioning. Slices were then be mounted onto glass slides and stained with Cresyl violet in order to verify cannula placement.

### *Western Blot Methods*

Animals were deeply anesthetized with isoflurane 30-minutes following training. Brains were immediately removed and stored at -80°C until dissected. Amygdala and MgN sections were homogenized in TEVP buffer with 320mM sucrose, and were 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 DDH2O; 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). 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), SHANK (StressMarq, 1:1000), or actin (Cell Signaling, 1:1000) primary solutions overnight at 4 °C. Membranes were then incubated in the appropriate secondary antibody for one hour and prepped in a chemiluminescence solution for 3 minutes. Images were captured and densitometry performed using NIH Genesys.

## Results

### Experiment 1

Anisomycin infusions into the MgN disrupt the consolidation of delay fear conditioning when infusions are made prior to the training session, but not immediately following training (Parsons et al., 2006, Maren et al., 2003, Apergis-Schoute et al., 2005). Recent evidence suggests

that the MgN is required for differential fear conditioning

(Antuntes & Moita, 2010;

Ghosh & Chattarji, 2014).

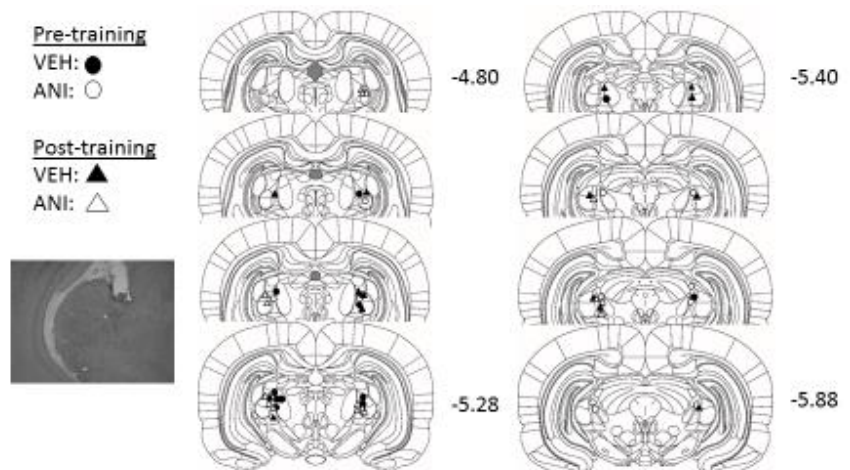
Furthermore, a higher degree of

auditory processing may be

required to support a memory

trace for multiple tones, which

would may suggest a shift in

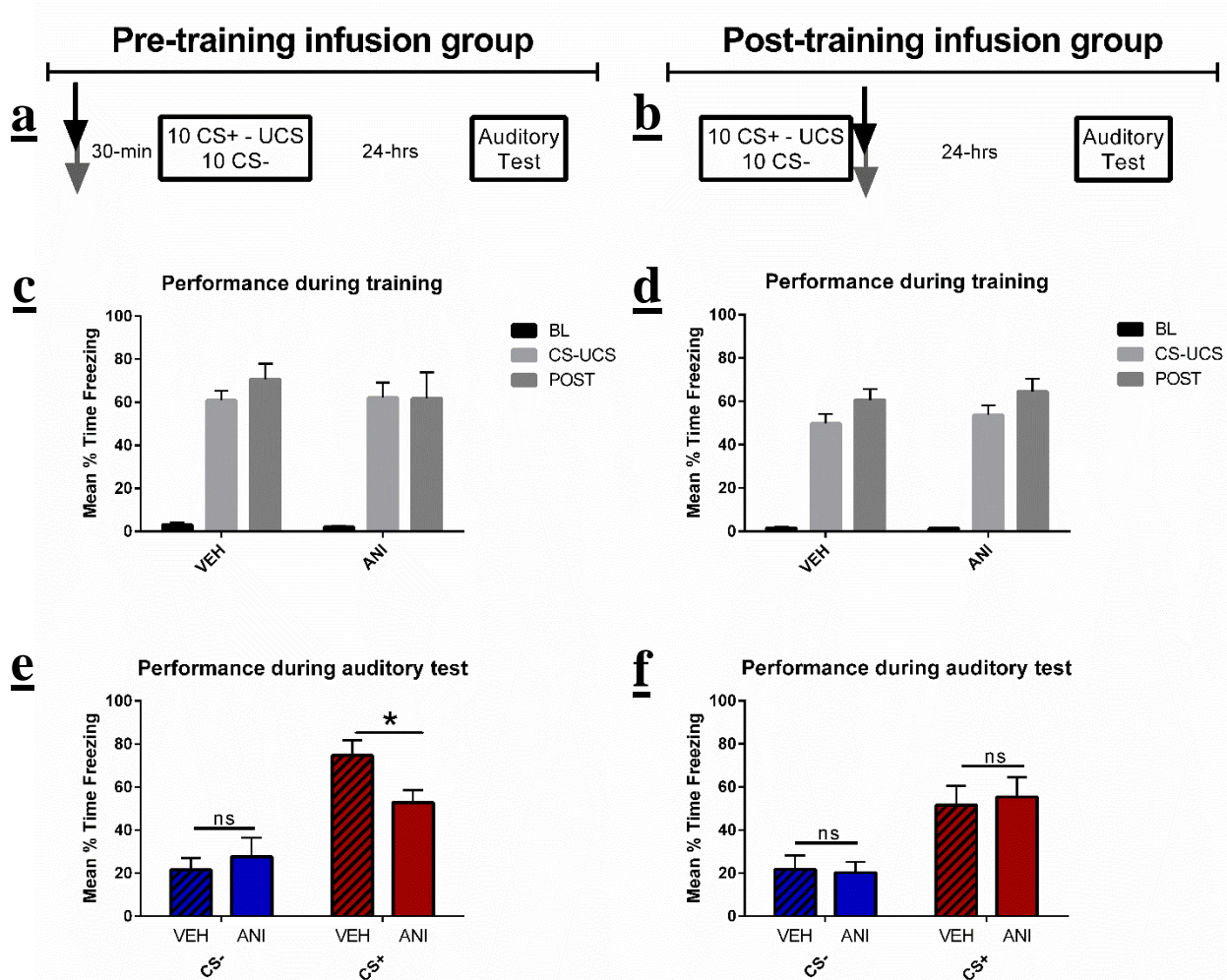


**Figure 2. Infusion sites for animals in Experiment 1.** Sample Nissl stained coronal slice to show infusion site. Animals were accepted for further analysis if placements were within the MgN. Circles = infusions before differential fear conditioning. Triangles = infusions immediately following training. White symbols = ACSF vehicle. Black symbols = Anisomycin (ANI).

the network of brain structures required for memory formation. The requirement for protein synthesis is often used as a marker for brain structures involved in or storing the memory trace. The cannulae guiding the injectors were targeted towards the MGm (Figure 2); however, based on the infusion volume, it is likely that regions of the MGv received anisomycin and were susceptible to protein synthesis disruption. The current study tested whether the MgN was required for an extended period of time for differential fear conditioning, in comparison to delay fear conditioning, by testing the requirement for protein synthesis before and after training. To test this, animals were infused with anisomycin prior to or immediately following differential fear conditioning (Figure 3a-b). There were no significant differences between vehicle and drug infused groups for animals infused prior to ( $F_{(2, 36)} = 0.5607, p = 0.5757$ ) or immediately following ( $F_{(2, 40)} = 0.1681, p = 0.8459$  for the baseline, CS-UCS period, and post shock period during training (Figure 3c-d). Recent evidence suggests that the MgN is required for differential fear conditioning for the retention to the tones the following day. There was a significant main effect for freezing responses to CS ( $F_{(1, 15)} = 35.74, p < 0.0001$ ) as well as a near statistically significant interaction between CS and drug ( $F_{(1, 15)} = 4.521, p = 0.0505$ ). Specifically, Fisher's LSD post hoc revealed a significant difference within CS+ freezing between vehicle and anisomycin drug groups ( $p = 0.0351$ ) but not CS- freezing behavior ( $p = 0.5445$ ) (Figure 2e). Animals that received infusions of anisomycin or vehicle following training showed a significant main effect for freezing responses to the CS ( $F_{(1, 17)} = 22.68, p = 0.0002$ ) but not an interaction between CS and drug ( $F_{(1, 17)} = 0.1607, p = 0.6935$ ). However, Fisher's LSD post hoc showed that animals infused with anisomycin or vehicle immediately following training showed equal levels of freezing to the CS+ ( $p = 0.7245$ ) and CS- ( $p = 0.8800$ ) during the auditory retention test (Figure 2f). This result demonstrates that protein synthesis blockade in the MgN prior to, but not



immediately following, training disrupts the discrimination between CS+ and CS-, suggesting that protein synthesis in the MgN is only required during the training period for auditory discrimination.



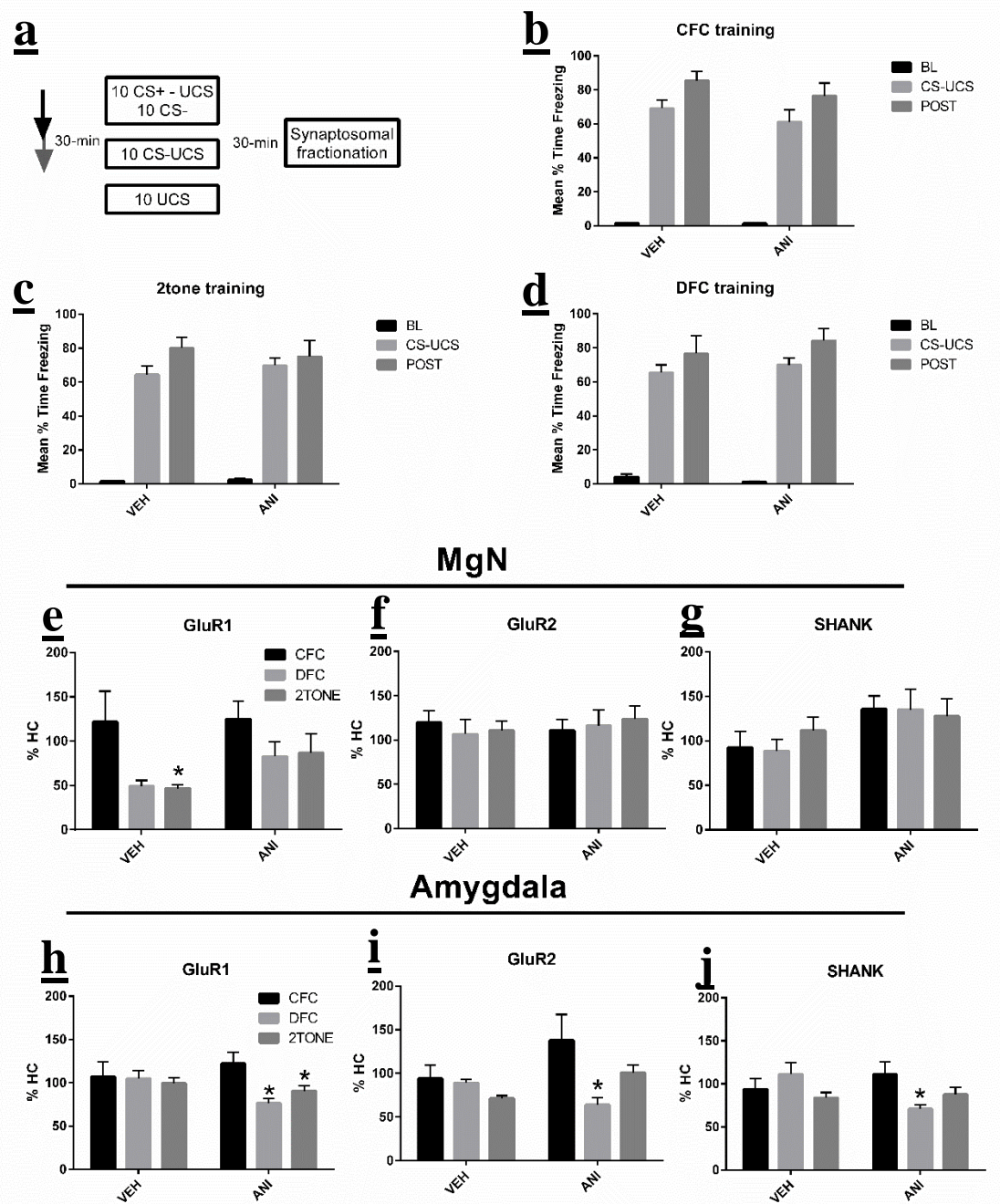
**Figure 3. Differential conditioning does not require extended periods of protein synthesis in the MgN for memory formation.** (a) Experimental design for c & e. Animals were infused with anisomycin (n = 8) or vehicle (n = 8) 30-minutes before differential fear conditioning. (b) Experimental design for d and f. Animals were infused with anisomycin (n = 9) or vehicle (n = 10) immediately after differential fear conditioning. (c & d) There were no differences between groups during acquisition of differential fear conditioning. (e) Animals infused with vehicle 30-minutes prior to differential fear conditioning show elevated freezing in response to the CS+ and significantly low freezing to the CS- during the retention test. Infusions of anisomycin reduce CS+ / CS- discrimination seen in vehicle groups. (f) Animals infused with vehicle or anisomycin immediately following differential fear conditioning discriminate between CS+ and CS- tones. All data are shown as mean percentage of time spent freezing ( $\pm$ SEM). BL = 6-min baseline period, CS-UCS = 10 CS+-UCS and CS- presentations; Post = 4-min post shock period. \* denotes  $p < 0.05$ .

## Experiment 2

Gene transcription and mRNA translation regulate auditory, but not contextual, memory formation in the MgN (Parsons et al., 2006, Maren et al., 2003, Apergis-Schoute et al., 2005). Little work has been done to elucidate synaptic changes in the MgN following auditory conditioning in comparison to contextual fear conditioning. Additionally, the mechanisms in which the MgN modulates synaptic activity in the amygdala necessary for auditory memory formation remain unclear. The goal of this experiment was to determine which protein synthesis-dependent mechanisms in the MgN are required for auditory memory formation and how this activity modulates amygdala activity. Animals were implanted with cannula targeting the MgN and were infused with anisomycin or vehicle 30-minutes before contextual, delay, or differential fear conditioning (Figure 4a). Infusions of drug or vehicle did not affect acquisition behavior during delay ( $F_{(2, 36)} = 0.5686$ ,  $p = 0.5713$ ), contextual ( $F_{(2, 32)} = 0.5772$ ,  $p = 0.5672$ ), or differential ( $F_{(2, 36)} = 0.5993$ ,  $p = 0.5546$ ) conditioning (Figure 4b-d). Animals were sacrificed 30-minutes after training, and synaptic fractions from MgN and amygdala tissue were obtained. We first looked at alterations between trained groups and naïve home cage animals. We used a one-way ANOVA followed by Tukey HSD *post hoc* multiple comparisons to detect differences between home cage and all trained groups regardless of infusion. GluR1 levels in the MgN ( $F_{(6, 57)} = 2.171$ ,  $p = 0.059$ ) and amygdala ( $F_{(1, 57)} = 1.624$ ,  $p = 0.157$ ) did not differ between groups. Even with a null ANOVA result, multiple comparisons did not show significant differences between home cage and any training group for both amygdala and MgN. GluR2 levels in the MgN ( $F_{(6, 58)} = 0.646$ ,  $p = 0.693$ ) were not different between groups. Amygdala GluR2 levels were different between groups ( $F_{(6, 56)} = 2.743$ ,  $p = 0.021$ ), but Tukey HSD *post hoc* multiple comparisons did not show a significant difference between home cage and context vehicle ( $p =$

1.000), context anisomycin ( $p = 0.498$ ), delay vehicle ( $p = 0.999$ ), delay anisomycin ( $p = 0.589$ ), differential vehicle ( $p = 0.785$ ), or differential anisomycin ( $p = 1.000$ ) groups. MgN SHANK levels ( $F_{(6, 58)} = 1.346$ ,  $p = 0.252$ ) and amygdala ( $F_{(6, 57)} = 1.963$ ,  $p = 0.086$ ) were not different between groups.

While there were no significant differences between home cage and trained groups, anisomycin infusions into the MgN caused amygdala alterations in AMPA receptor trafficking and synaptic scaffolding in auditory fear conditioned groups that are known to support memory formation. Therefore, we proceeded with a 2x3 factorial design. This design allowed us to compare context, delay, and differential training within anisomycin and vehicle drug infused groups. Context fear conditioned animals were used as the comparison group since it has been established that the MgN selectively effects auditory fear conditioning. Results revealed a main effect for MgN GluR1 ( $F_{(2,49)} = 4.977$ ,  $p = 0.0108$ ) but not for GluR2 ( $F_{(2, 50)} = 0.077$ ,  $p = 0.926$ ) or SHANK ( $F_{(2, 51)} = 0.105$ ,  $p = 0.901$ ). Tukey's multiple comparisons *post hoc* test showed a significant reduction in GluR1 surface expression for vehicle differential fear conditioned animals when compared to context vehicle infused animals (Figure 4e-g). Delay fear conditioned animals showed a comparable reduction in GluR1 surface expression (Figure 4e). However, the mean difference between context and delay fear conditioned animals was not as large, and therefore, was not significant with follow-up *post hoc* tests. For the amygdala, there was a modest main effect for GluR1 ( $F_{(2, 47)} = 3.175$ ,  $p = 0.051$ ), and GluR2 ( $F_{(2, 47)} = 3.137$ ,  $p = 0.053$ ), and a significant interaction for SHANK ( $F_{(2, 48)} = 3.583$ ,  $p = 0.036$ ). Tukey's multiple comparisons *post hoc* tests revealed a significant reduction in amygdala GluR1 levels for both differential and delay fear conditioned animals in comparison to context fear conditioned animals



**Figure 4. MgN protein synthesis modulates amygdala synaptic scaffolding and AMPA receptor trafficking required for auditory memory formation.** (a) Experimental design for b-j. Animals were infused with anisomycin or vehicle 30-minutes before contextual, delayed, or differential (2 tone) fear conditioning. MgN and amygdala was collected 30-minutes following training and processed for crude synaptosomal membrane fractions. (b, c, d) Drug infusions prior to training did not affect freezing responses. (e, f, g) Infusions of anisomycin altered GluR1 receptor trafficking in the MgN but did not change GluR2 or SHANK expression. (h, i, j). GluR1, GluR2, and SHANK expression were reduced in the amygdala following auditory fear conditioning in response to MgN anisomycin infusions. \* denotes  $p < 0.05$  from Context Fear Conditioned groups.

within the anisomycin group (Figure 4h). Additionally, GluR2 and SHANK levels were reduced in the anisomycin group for delay fear conditioned animals in comparison to context fear conditioned animals (Figure 4i-j). Differential fear conditioned groups that were infused with conditioned animals (Figure 4i-j). Differential fear conditioned groups that were infused with anisomycin showed similar reductions as delay fear conditioned groups for both GluR2 and SHANK; however, this was not statistically significant. Anisomycin infusions have been known to activate stress related kinases and induce apoptosis (Lordanov et al., 1997). However, the current results were restricted to auditory fear conditioned groups, so we believe that the anisomycin infusions were not nonspecifically effecting molecular mechanisms underlying auditory fear conditioning. These results indicate that protein synthesis dependent activity is largely influenced by GluR1 trafficking in the MgN. Furthermore, these synaptic alterations in the MgN modulate GluR1, GluR2, and SHANK levels for auditory, but not contextually, fear conditioned animals in the amygdala.

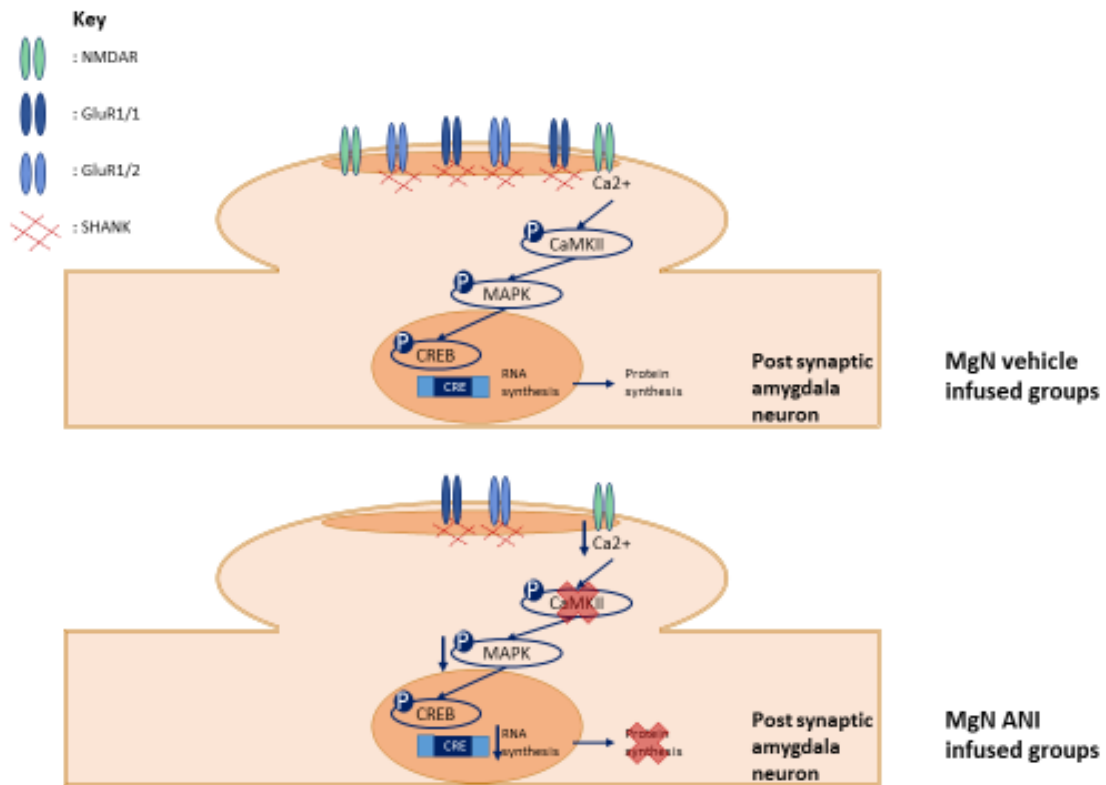
## **Discussion**

Here, we present evidence of MgN modulation of amygdala synaptic activity following auditory fear conditioning. We found that protein synthesis inhibition prior to differential fear conditioning reduces freezing to the CS+. This suggests that the information processed in the MgN regulates responses to auditory cues that signal shock. Interestingly, inactivation or PKA inhibition in the lateral amygdala following differential fear conditioning also reduces discrimination; however, the reduction in discrimination is characterized by an increase in freezing to the CS- (Ghosh & Chattarji, 2014). Collectively, these results suggest that MgN input to the amygdala is necessary for processing aversive signals, whereas signaling from other structures to the amygdala may be required for safety signal processing. Supporting these data,

overexpression of CREB in the MgN prior to conditioning results in an increase and generalization in freezing responses to multiple tones (Han et al., 2008). Therefore, overexpression of proteins or kinases results in enhanced generalization of fear responses characterized by an increase in freezing while inhibition of proteins or kinases results in a decreased freezing and generalized fear responses characterized by a reduction in fear responses to aversive auditory cues. Based on this information, MgN activity shares a direct correlation between the degree of fear response generalization to auditory cues and protein expression. These data show that the regulation of freezing to an auditory cue paired with a footshock is due to MgN input to the amygdala. We also characterized the effects of protein synthesis inhibition during training on amygdala activity. We show that protein synthesis inhibition in the MgN during auditory, but not contextual, fear conditioning is critical for synapse stabilization and AMPA receptor trafficking in the amygdala (Figure 5). A reduction in amygdala GluR1 and GluR2 surface expression as well as synaptic scaffolding levels have also been characterized by a decrease in conditioned responses (Gerlai et al., 1998; Jarome et al., 2011; Reisel et al., 2002; Yeh et al., 2006). Together, these data support a reduction in CR expression characterized by decreased synaptic plasticity in the amygdala is due to inhibition of MgN protein synthesis.

Much of the previous work on the MgN has focused on auditory delay fear conditioning recent attention has established a role for the MgN in differential fear conditioning as well (Anutnes & Moita et al., 2010; Ghosh & Chattarji, 2014). The recent attention has established a role for the MgN in differential fear conditioning as well (Anutnes & Moita et al., 2010; Ghosh & Chattarji, 2014). The present study attempted to distinguish a distinct role for the MgN during

delay or differential fear conditioning. Auditory fear conditioning procedures, such as trace fear conditioning and trace fear extinction, are known to engage additional structures, which also shifts the storage of these memories to other brain regions (Gilmartin et al., 2013; Kwapis et al., 2014). Therefore, differential fear conditioning may also engage and shift memory storage to additional structures that were not previously recruited for delay fear conditioning. Based on this information, we hypothesized that the requirement for protein synthesis may be extended for a more complicated auditory fear conditioning training procedure. However, we found that infusions of anisomycin only disrupted memory formation when made prior to, but not



**Figure 5. Projected effects of protein synthesis-dependent MgN activity on amygdala synaptic plasticity underlying auditory memory formation.** Glutamatergic synaptic transmission from MgN presynaptic terminals elicits activity-dependent trafficking of GluR1 and GluR2 subunits into the PSD, which are stabilized by SHANK. The calcium influx from NMDA receptors activates a series of signaling cascades, which initiates gene transcription and RNA translation. When anisomycin is infused into the MgN, there is a reduction in the expression of amygdalar GluR1, GluR2, and SHANK in synaptosomal fractions. This may also reduce the degree of calcium influx and degree of transcription and translation occurring, all of which are critical for memory formation.

immediately after, differential fear conditioning. Even though our data cannot conclusively support a distinct role for the MgN in various auditory fear conditioning paradigms, we show that protein synthesis inhibition modulates amygdala activity that is critical for differential memory formation, similar to delay fear conditioning. This highlights a more general role for MgN activity during auditory fear memory processing. Because the amygdala is a primary structure involved in the formation and updating of memories for both delay and differential fear conditioning, our data suggest that MgN activity during the training session modulates fear memory formation through the manipulation of amygdala activity.

The role of protein synthesis in the MgN during formation of auditory fear remains unclear (Apergis-Schoute et al., 2005, Maren et al., 2001; Maren et al., 2003; Parsons et al., 2006). Protein synthesis inhibition prior to fear conditioning disrupts auditory memory formation without showing behavioral deficits during acquisition (Parsons et al., 2006); however, when inhibited immediately following conditioning, protein synthesis blockade has no effect on memory formation (Maren et al., 2003). These previous data are consistent with the results in the present study, suggesting that MgN function does not alter with changes in auditory fear conditioning parameters. Alternatively, gene transcription inhibitors applied prior to or immediately following conditioning result in auditory memory disruption (Apergis-Schoute et al., 2005). The selective effect of protein synthesis inhibition suggests a temporally distinct role for the MgN during auditory memory formation. It is possible that newly synthesized proteins are involved in the trafficking of mRNA to amygdala terminals for translation during the training session, making transcription of genes critical before and after conditioning but protein synthesis selectively necessary during training. This view emphasizes the importance of microtubule transport of mRNA necessary for presynaptic strength and stability in the amygdala. Work using



U0126 to regulate MEK1/2 phosphorylation shows that inhibition prior to and following conditioning disrupt auditory memory formation in the MgN (Apergis-Schoute et al., 2005). ERK is a known regulator of gene transcription, but also plays a role in axonal differentiation and microtubule stability (Goold & Gordon-Weeks, 2005) as well as retrograde BDNF signaling that modulates MAP kinase phosphatase-1 induction and stabilization (Jeanneteau et al., 2010). Interestingly, axonal differentiation, microtubule stability, and MAP kinase phosphatase-1 are all factors critical for axonal remodeling. Collectively, these data suggest that protein synthesis in the MgN is critical for memory formation to support microtubule transport of mRNA to presynaptic terminals in the amygdala, which may be dependent on ERK activity.

Understanding the mechanism of information transmission in amygdala synapses is critical for the complete analysis of fear memory. Human and rodent literature suggest a major role for the amygdala in fear responsiveness and generalization (Parsons & Ressler, 2013). Activity in the MgN and the amygdala is critical for auditory memory formation. The stimulation of MGm cell bodies during conditioning is sufficient to serve as a UCS during auditory fear conditioning, suggesting that the MgN is a major convergence zone for auditory and somatosensory information (Cruikshank et al., 1992; Edeline & Weinberger, 1992; Kwon & Choi, 2009). Additionally, the modulation of terminal activity from the MgN and auditory cortex to the amygdala regulates auditory fear memory formation, and the inhibition of phosphorylated ERK in the MgN regulates amygdala activity, suggesting that ERK-dependent input from the MgN is critical for fear conditioning (Apergis-Schoute et al., 2005; Kwon et al., 2009; Kwon et al., 2014). However, the mechanism of MgN ERK modulation on amygdala activity is unclear. Whether ERK is involved in the trafficking of molecules from the MgN or gene transcription, MgN cell body and terminal activity are necessary components for memory formation. These

data demonstrate that auditory processing from the MgN is able to regulate amygdala activity during fear conditioning and is dependent on phosphorylated ERK and glutamatergic synaptic transmission.

Auditory thalamic input to the amygdala is known to be critical for memory formation and synaptic plasticity, but the degree to which the MgN modulates this activity is unclear. Because the MgN and amygdala are monosynaptically connected, alterations in receptor trafficking and synaptic scaffolding provide a way to measure the initial modulation of amygdala activity by the MgN. Several studies have shown that memory consolidation can be characterized by increased surface expression of GluR1, GluR2, and synaptic levels of SHANK in the amygdala following conditioning, suggesting that these are critical factors necessary to support memory consolidation (Jarome et al., 2011; Miguez et al., 2010; Yeh et al., 2006). Loss of GluR1 has been characterized by an inability to induce LTP as well as impaired spatial working memory task (Schmitt et al., 2003). In the amygdala, fear conditioning increases the surface expression of GluR1, and this expression is maintained for up to 24 hours following conditioning, suggesting a mechanism supporting long term memory formation (Yeh et al., 2006). The destabilization of synapses is thought to be dependent on calcium influx (Jarome et al., 2011). GluR2 subunits are calcium impermeable, thereby preventing destabilization while maintained at the synapse surface. Additionally, GluR2 surface expression is maintained by PKM $\zeta$ , a primary memory maintenance molecule involved in fear conditioning, following fear conditioning in the amygdala (Kwapis et al., 2012; Miguez et al., 2010). Therefore, the maintenance and stability of a long term fear memory is thought to be regulated by the degree of GluR2 expression found in the synapse following conditioning (Miguez et al., 2010). Several post synaptic scaffolding proteins form slots for AMPA receptors to traffic into following

learning. Inhibition of scaffolding proteins often results in aberrant spine morphology and receptor expression. SHANK provides the synaptic scaffolding necessary to stabilize AMPA receptor surface expression as well as the regulation of spine morphology, providing a link between synaptic activity and the postsynaptic cytoskeleton (Lu et al., 2007; Sala et al., 2001). Interestingly, electrophysiological work has shown that isoforms of SHANK colocalize with presynaptic proteins such as synaptophysin (Sala et al., 2001). Furthermore, the overexpression of postsynaptic SHANK resulted in the enhancement of vesicular release, indicating that SHANK modulates presynaptic function (Sala et al., 2001). Collectively, AMPA receptor trafficking SHANK expression and provide a direct measure of synaptic activity and long term memory formation.

The present results are consistent with previous work showing that protein synthesis in the MgN is critical for AMPA receptor trafficking and synapse stability in the amygdala following conditioning. Specifically, the inhibition of protein synthesis in the MgN prior to auditory fear conditioning, reduces the surface expression of GluR1, GluR2, and SHANK in the amygdala, indicating that MgN modulation of long term memory formation exerts influence over amygdala AMPA receptor trafficking and synapse stability. In the current study, vehicle trained groups did not show training-induced increases of AMPA receptor surface expression or SHANK levels, but this is consistent with previous data looking at identical time points (Migues et al., 2010; Yeh et al., 2006). A majority of time points chosen for Western blot experiments targeting synaptic changes in the amygdala occur at or beyond 1 hour post conditioning to see learning induced changes. The 30-minute time point used for the Western blot experiment in the current study was chosen in an attempt to capture MgN activity but ultimately was not optimal for conditioning induced increases in either the MgN or amygdala. Despite the lack of learning

induced increases in AMPA receptor surface expression in vehicle trained animals, it is reasonable to suggest that the reduction in anisomycin trained groups would continue to show significantly lower levels of synaptic protein expression from a trained vehicle group at later time points. These vehicle trained groups would likely show significant increases from a naïve group at later time points following training based on prior work characterizing the dynamic activity of AMPA receptor expression (Migues, 2010; Yeh et al., 2006).

Amygdala AMPA receptor trafficking and synapse stability are critical for auditory memory formation and are dependent on MgN protein synthesis. We show that MgN protein synthesis is required during differential fear conditioning, which is characterized by a reduction in freezing to an aversive auditory cue. This disruption of auditory fear memory formation is accompanied by reduced surface expression of GluR1, GluR2, and synaptic levels of SHANK in the amygdala. These results demonstrate that synaptic activity from the MgN is likely modulating fear responses during memory formation, and changes in glutamatergic surface expression and synapse stability in the amygdala are the underlying mechanisms regulating MgN-dependent long term memory formation.

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