ABSTRACT

THE AMYGDALA IS CRITICAL FOR TRACE, DELAY, AND CONTEXTUAL FEAR CONDITIONING

by Daniel Kochli

Numerous investigations have demonstrated amygdalar involvement in delay and contextual fear conditioning. However, much less is known about amygdala contributions to trace fear conditioning. The present experiments examine the contributions of amygdalar subnuclei to trace, delay, and contextual fear conditioning. Rats were trained using a 10-trial trace, delay, or unpaired fear conditioning procedure. Infusions of the protein synthesis inhibitor, cycloheximide, targeting the basal nucleus of the amygdala (BA) immediately following conditioning attenuated trace and contextual fear memory expression, but had no effect on delay fear conditioning. In a subsequent experiment, pre-training lesions targeting the entire basolateral amygdala (BLA) resulted in a deficit in trace, delay, and contextual fear conditioning. These data fully support a role for the BLA in all three types of fear memories and provide support for BA involvement in trace and contextual fear memory.
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Introduction

Pavlovian fear conditioning is one of the most extensively studied systems for investigating the neural mechanisms mediating learning and memory processes. It is a behavioral paradigm in which an organism learns to anticipate an aversive event by pairing that event (i.e., unconditioned stimulus; US) with a particular place or predictive stimulus (i.e., conditioned stimulus; CS). The amygdala serves a critical role in this fear learning; it receives both unimodal and multimodal sensory information and projects to a number of individual response circuits allowing for a coordinated fear response (e.g., Davis, 1997; 2006; Fanselow and LeDoux, 1999; Lee et al., 2001). More specifically, CS (e.g., tone, context) and US (e.g., footshock) sensory inputs converge in the basolateral amygdala (BLA) where the CS-US association is formed (Barot et al., 2009). Formation of this association requires protein synthesis in the amygdala (e.g., Bailey et al., 1999; Schafe and LeDoux, 2000; Maren et al., 2003; Kwapis et al., 2011). Once formed, this BLA-dependent association permanently supports the expression of fear memory (LeDoux, 1993; Fanselow and LeDoux, 1999; Davis, 2006; Gale et al., 2004; Amano et al., 2011). The BLA projects, both directly and indirectly, to the central nucleus of the amygdala (CeA), which in turn projects to brainstem and hypothalamic regions to trigger individual fear responses (LeDoux et al., 1988; Wilensky et al., 2006; Amano et al., 2011; Viviani et al., 2011).

Typically, fear conditioning to an auditory stimulus is performed using a delay procedure in which tone and footshock are temporally contiguous. There is a wealth of experiments that have demonstrated that this type of learning depends on the amygdala (e.g., Fanselow and LeDoux, 1999). Trace fear conditioning differs from delay conditioning in that a stimulus-free trace interval is inserted between the termination of the tone and the onset of footshock. Unlike delay conditioning (but see Quinn et al., 2008; 2009; Maren, 2008), acquiring of trace fear conditioning is critically dependent on several other structures, such as the medial prefrontal cortex and the hippocampus (e.g., McEchron et al., 1998; Quinn et al., 2002; Han et al., 2003; Chowdhury et al., 2005; Gilmartin and McEchron 2005a; Gilmartin and McEchron 2005b; Quinn et al., 2005; Quinn et al., 2008; Gilmartin and Helmstetter 2010). Surprisingly, little is known about amygdalar contributions to trace fear conditioning, and the few published studies are conflicting (Raybuck and Lattal, 2011; Kwapis et al., 2011; Gilmartin et al., 2012). More specifically, Raybuck and Lattal (2011) demonstrate that muscimol inactivation of the amygdala impairs delay but not trace fear conditioning, while Kwapis et al. (2012) show that inhibition of amygdalar protein synthesis disrupts both delay and trace fear conditioning. It is possible that this discrepancy arises from differential targeting of amygdalar subnuclei. As the coordinates used by Kwapis et al. fall in the lateral amygdala, the present study explores the role of protein synthesis in the basal nucleus of the amygdala. No studies to date have addressed possible differential contributions of amygdalar subnuclei to trace fear conditioning.
To further investigate the role of the amygdala in trace, delay, and contextual fear conditioning, we performed two experiments. In Experiment 1, rats received bilateral infusions of the protein synthesis inhibitor, cycloheximide, or vehicle into the basal nucleus of the amygdala (BA) immediately following trace or delay fear conditioning. This experiment allowed us to assess the role of de novo protein synthesis in BA in the consolidation of trace and delay fear conditioning, as well as simultaneously acquired contextual fear conditioning. Experiment 2 was similar except that rats received pre-training lesions of the basolateral amygdala or sham surgery prior to trace or delay fear conditioning. This allowed us to assess the collective contribution of the basolateral amygdalar nuclei to acquisition and/or expression of trace, delay, and simultaneously learned contextual fear conditioning.

Results

Experiment 1: Basal amygdalar protein synthesis is necessary for the consolidation of trace, but not delay, conditioned fear memory.

Brains were sliced and stained with cresyl violet to verify cannulae placements via bright field microscopy. Three rats were excluded from statistical analysis due to misplaced cannulae. The cannulae placements in the remaining 51 rats were deemed acceptable and included in all statistical analyses.

Immediately following trace, delay or unpaired fear conditioning, rats received bilateral infusions of either cycloheximide or vehicle targeting the basal nucleus of the amygdala. Over the next two days, rats were tested for freezing to both tone and context in separate sessions (see Figure 2A). During the 180sec baseline period of the tone test, no differences were observed among groups \( F(4,46) = 1.58, p = 0.195 \). Further, among trace and delay conditioned animals, there were no main effects of training \( F(1,38) = 1.01, p = 0.322 \) or infusion \( F(1,38) = 1.88, p = 0.178 \) and no interaction \( F(1,38) = 1.26, p = 0.268 \) (Figure 2B).

Average freezing during the test tones was significantly different in vehicle-infused rats as a function of training condition \( F(2.27) = 16.04, p < 0.001 \). Pairwise comparisons revealed that both trace and delay vehicle-infused rats froze significantly more than unpaired controls \( p < 0.05 \), demonstrating that the freezing in trace and delay animals results from associative processes. Among trace and delay conditioned rats, there was a significant main effect of training \( F(1,38) = 9.78, p < 0.01 \), and a significant training X infusion interaction \( F(1,38) = 6.37, p < 0.05 \), but no main effect of infusion \( F(1,38) = 0.28, p = 0.598 \). Pairwise comparisons revealed a significant deficit in tone freezing for cycloheximide infusions in trace, but not delay, conditioned animals (Figure 2B).

During the trace interval (or trace interval equivalent for unpaired and delay conditioned animals), freezing differed significantly in vehicle-infused rats as a function
of training condition \[ F(2,27) = 17.42, p < 0.001 \]. Pairwise comparisons revealed that both trace and delay vehicle-infused rats froze significantly more than unpaired controls \((p < 0.05)\), showing that the freezing during this period continues to be a result of associative learning. Among trace and delay conditioned rats, there was a significant main effect of training \([F(1,38) = 12.61, p = 0.001]\), and a significant training X infusion interaction \([F(1,38) = 4.77, p < 0.05]\), but no main effect of infusion \([F(1,38) = 1.75, p = 0.194]\). Pairwise comparisons revealed a significant deficit in trace interval freezing for cycloheximide infusions in trace, but not delay, conditioned animals (Figure 2B).

**Experiment 1: Basal amygdalar protein synthesis is necessary for the consolidation of simultaneously learned contextual fear memory.** The average percentage of time spent freezing over the entire 8 minutes of the context test was calculated (Figure 2C). Among vehicle-infused rats, there were no significant differences in context freezing as a function of training \([F(2,27) = 0.76, p = 0.478]\). In trace and delay conditioned animals, there was a significant main effect of infusion \([F(1,38) = 8.65, p < 0.05]\), but no main effect of training \([F(1,38) = 0.26, p = 0.613]\) and no training X infusion interaction \([F(1,38) = 1.31, p = 0.259]\). *A priori* planned comparisons revealed that cycloheximide infusions following trace conditioning attenuated freezing compared with vehicle infusions \((p < 0.05)\). However, following delay conditioning, cycloheximide had no significant effect on context freezing \((P > 0.05)\).

**Experiment 2: Basolateral amygdalar lesions disrupt tone fear memory in trace and delay conditioned rats.**

Lesion extent was quantified in a manner similar to that described previously (Quinn et al., 2013). Briefly, three brain slices throughout the extent of the BLA were stained using immunofluorescence for the neuronal nuclei marker NeuN and Glial Fibrillary Acidic Protein (GFAP). Lesion extent was visualized via fluorescent microscopy, and was quantified using ImageJ (NIH) software. Five rats were excluded from statistical analyses; four cases were excluded due to unilateral lesions, and one case was excluded due to lesion misplacement. The lesion extents of the remaining 31 rats were deemed acceptable and included in all statistical analyses. Overall, lesion extent covered 53% of the BLA, with trace animals averaging 59% and delay animals averaging 47%. Lesion extents were primarily confined to the BLA, but seven cases extended laterally into adjacent temporal cortices (five trace, two delay) and six cases extended medially into the lateral portion of the CeA (four trace, two delay). Additionally, eight cases had at least unilateral sparing of the most anterior portion of the BLA (four trace, four delay).

Prior to trace or delay fear conditioning, rats received bilateral neurotoxic lesions of the basolateral amygdala or sham surgery. Tests for freezing to both tone and context occurred across two consecutive days following training (see Figure 4A). Despite very low levels of freezing during the 180sec baseline period of the tone test,
there was a significant main effect of surgery \([F(1,27) = 6.17, p < 0.05]\), but no main effect of training \([F(1,27) > 0.01, p = 0.973]\) and no training X surgery interaction \([F(1,27) = 0.40, p = 0.531]\). However, pairwise comparisons within each training condition revealed no differences between lesion and sham rats \((p > 0.05; \text{Figure 4B})\).

During the tone (averaged across the three presentations), there was a significant main effect of training \([F(1,27)= 16.17, p < 0.001]\), a significant main effect of surgery \([F(1,27)= 73.82, P < 0.001]\), but no training X surgery interaction \([F(1,27) = 0.04, p = 0.844]\). Delay conditioned animals froze significantly more than trace conditioned animals. Further, both trace and delay lesioned animals showed a significant deficit in freezing to tone compared to their corresponding sham controls \((p < 0.05; \text{Figure 4B})\).

During the trace interval (or trace interval equivalent), there was a significant main effect of surgery \([F(1,27) = 195.02, p < 0.001]\), but no main effect of training \([F(1,27) = 2.29, p = 0.142]\) and no training X surgery interaction \([F(1,27) = 0.04, p = 0.841]\). Following both trace and delay conditioning, lesion rats froze significantly less than shams during the trace period \((p < 0.05; \text{Figure 4B})\).

**Experiment 2: Basolateral amygdalar lesions disrupt simultaneously learned contextual fear memory in trace and delay conditioned rats.** The average percentage of time spent freezing over the entire 8 minutes of the context test was calculated \((\text{Figure 4C})\). There was a significant main effect of surgery \([F(1,27) = 11.41, p < 0.01]\), but no main effect of training \([F(1,27) = 0.82, p = 0.372]\) and no training X surgery interaction \([F(1,27) < 0.01, p = 0.975]\). Following both trace and delay conditioning, lesion rats froze significantly less than sham rats during the context test \((p < 0.05)\).

**Discussion**

The present data provide evidence for the involvement of the basolateral amygdala in trace fear conditioning. Post-training infusions of the protein synthesis inhibitor, cycloheximide, into the BA attenuate freezing to tone and context in trace, but not delay, conditioned animals. Additionally, pre-training lesions of the BLA disrupt freezing to tone and context in both trace and delay conditioned animals. Kwapis and colleagues (2011) demonstrated that infusions of another protein synthesis inhibitor, anisomycin, primarily targeting the lateral nucleus of the amygdala results in deficits in tone and context in both trace and delay conditioned animals. The present results, combined with the results of Kwapis et al., (2011), suggest that the lateral amygdala is important for both trace and delay fear conditioning, whereas the basal amygdala may be necessary for trace, but not delay, fear conditioning. However, it is important to note that the current study does not directly manipulate the LA, and therefore inferences we can draw about LA contributions to trace fear conditioning are limited. Explicit contributions of the LA to trace fear conditioning are not yet fully understood.
In the present study, ten acquisition trials were used for both trace and delay fear conditioning. While ten trials is typical for studies of trace fear conditioning in order to acquire a fear response to the tone, delay conditioning can be acquired using a single tone-footshock pairing. Thus, ten trials of delay conditioning yield very strong conditioning. It is possible that the lack of a deficit in cycloheximide-infused delay conditioned animals is a function of overtraining, rather than evidence of BA-independent delay conditioning. However, previous studies have shown that even animals given 75 overtraining trials using delay conditioning with an intact BLA subsequently display a significant deficit in freezing to the tone following BLA lesion or inactivation (Zimmerman et al., 2007; Ponnusamy et al., 2007). This suggests that in animals overtrained with intact basal and lateral nuclei of the amygdala, delay fear memory remains dependent upon those nuclei. Therefore, we believe that overtraining is an unlikely explanation for our pattern of results, and suggest that protein synthesis in the BA is not necessary for the consolidation of delay-conditioned fear.

Raybuck and Lattal (2011) demonstrated that muscimol inactivation of the amygdala impaired delay, but not trace, fear conditioning in mice. This discrepancy can possibly be explained by a number of differences in our approach. The present study used rats rather than mice. Additionally, we used a stronger fear conditioning procedure (10 pairings) than did Raybuck and Lattal (2011), who used 1, 2, or 4 trials. Kwapis and colleagues were able to demonstrate deficits in both delay and contextual fear conditioning using 4 or 6 trials (2011). However, this does not seem a sufficient explanation as animals in all studies froze to the CS during testing, demonstrating associative learning. The type of drug used may also contribute to the disparate results observed across studies. Raybuck and Lattal (2011) inactivated the amygdala using muscimol, while, in a similar approach to our own, Kwapis and colleagues (2011) inhibited protein synthesis using anisomycin. The present study also utilized inhibition of amygdalar protein synthesis, but we chose to use cycloheximide rather than anisomycin. As noted by Kwapis and colleagues (2011), under some conditions protein synthesis and reconsolidation can take place in an inactivated amygdala (e.g., Ben Mamou et al., 2006). As such, inactivation via muscimol may fail to prevent the consolidation of trace fear memory where protein synthesis inhibitors are effective. Additionally, it is possible that alternative mechanisms are able to compensate for the amygdala in trace fear conditioning that occurs when the amygdala is inactivated, as trace conditioning critically depends upon a number of other structures such as the hippocampus. Under some conditions, learning that is normally hippocampus-dependent can be acquired via alternative mechanisms if the hippocampus has been inactivated (e.g., Rudy and O’Reilly, 1999; Wiltgen et al., 2006). It is possible that the inactivation procedure used by Raybuck and Lattal (2011) facilitated the use of extra-amygdalar compensatory mechanisms, while protein synthesis inhibition employed in the present experiment and by Kwapis and colleagues did not. Thus, amygdalar protein
synthesis inhibition results in deficits in trace fear conditioning, while muscimol inactivation may not.

Some studies suggest that the BA is important in conditioned fear (e.g., Sananes and Davis, 1992) and, more specifically, delay fear conditioning (Goosens and Maren, 2001; Amano et al., 2011). However, other sources suggest that the BA does not play a role in delay fear conditioning (e.g., Killcross et al., 1997; Amorapanth et al., 2000; Nader et al., 2001). Similarly, we observe no deficit in delay fear conditioning as a result of post-training administration of cycloheximide into the BA. Differences in procedure may account for discrepant results. Goosens and Maren (2001) utilized a pre-training lesion procedure in which rats received a large electrolytic lesion of the amygdala on one side, and a nucleus-specific neurotoxic lesion on the contralateral side. Lesions targeting the BA resulted in deficits to delay fear conditioning, but had no effect if the anterior portion of the BA was spared. It is possible that our cycloheximide infusions similarly spared the most anterior portion of the BA. Alternatively, it is also possible that while lesions of the BA disrupt delay conditioning, the formation of this association does not depend upon de novo protein synthesis in the BA. Finally, fibers of passage that would be destroyed with an electrolytic lesion of the BA are spared during cycloheximide infusion, which may account for differences in the two manipulations. However, it is important to mention that Amano and colleagues (2011) found that a substantial portion of BA neurons acquire excitatory responses to the CS during delay fear conditioning. Specifically, basomedial responses persist long after CS-offset, suggesting that they are not merely passive relays of rapidly adapting LA input. Additionally, they demonstrated that pre-testing muscimol inactivation of the entire BA (including medial and lateral portions) attenuated freezing to the tone. This result strengthens the possibility that our cycloheximide infusions may have partially spared the BA.

Post-training BA infusions of cycloheximide produced a deficit in contextual fear conditioning in trace animals. This was an expected result, as there is strong evidence that the BA is critical for contextual fear conditioning (Muller et al., 1997; Goosens and Maren, 2001; Vlachos et al., 2011). However, no deficits were observed in contextual fear conditioning in delay animals. This is most likely due to a floor effect, as both vehicle and cycloheximide infused delay animals froze at relatively low levels during the context test (see Figure 2C). In a 10-trial delay conditioning procedure, it is reasonable to expect that conditioning to the context would be relatively weak.

Protein synthesis inhibitors are sometimes criticized for their non-specific effects, such as cell death and catecholamine synthesis inhibition (Flexner and Goodman, 1975; Radulovic and Tronson, 2008; Rudy, 2008). However, there is an established history of experiments examining amygdalar contributions to delay fear conditioning using protein synthesis inhibitors as amnesic agents, (e.g., Bailey et al., 1999; Schafe and LeDoux, 2000; Maren et al., 2003; Kwapis et al., 2011). As little is known about amygdalar
contributions to trace fear conditioning, it is a sound practice to employ a broad approach rather than attempting to target a more specific signaling cascade. Cycloheximide is a less commonly used protein synthesis inhibitor than anisomycin, but it is sometimes preferred as it is easier to keep in solution. There is no evidence to suggest that it is less effective than other protein synthesis inhibitors (e.g., Lai et al., 2008; Milekic et al., 2006), and it has been successfully used in the amygdala as an amnesic agent in a number of studies (e.g., Berman et al., 1978; Duvarci et al., 2005; Pedroso et al., 2013), including the present study.

Though the diffusion extent of cycloheximide was not measured for the present experiments, evidence suggests diffusion was confined to the BA. A labeling study carried out by Parsons and colleagues (2006) demonstrated that another protein synthesis inhibitor, anisomycin, remained within the boundaries of the amygdala using a similar infusion size (0.5µl). Similarly, Amano and colleagues (2011) administered 0.3µl of 0.5mM fluorescent muscimol dissolved in aCSF targeting the lateral or medial portion of the BA. Imaging revealed that infusions targeting the individual basal nuclei were reasonably well-contained 10 minutes after infusion time. While inactivation of either subnucleus alone had no effect, combined inactivation of the basal medial and basal lateral nuclei resulted in a deficit in delay fear conditioning learning. Additionally, the pattern of behavioral results suggests that the LA was spared by our infusions, as pre-training lesions (e.g., Nader et al., 2001) and pre-training inactivation (Wilensky et al., 2006) of the LA produce deficits in delay conditioning. Additionally, protein synthesis in the lateral amygdala has been shown to be critical for the consolidation of delay fear conditioning (e.g., Schafe and LeDoux, 2000; Kwapis et al., 2011). In addition, the CeA was likely unaffected by infusion extent, as manipulations targeting this region also produce deficits in delay fear conditioning (e.g., Wilensky et al., 2006).

In conclusion, we believe the present data support a theory of distributed processing of trace conditioning throughout the BLA. Delay conditioning is disrupted by complete BLA lesions but is spared by protein synthesis inhibition of the BA, suggesting that delay conditioning does not critically depend upon the BA. However, trace conditioning is disrupted by both complete BLA lesions and BA protein synthesis inhibition. Taken together with previous work demonstrating that protein synthesis inhibition of the lateral amygdala disrupts the consolidation of trace fear conditioning (Kwapis et al., 2011), it appears that trace conditioning may be more widely distributed within the amygdala than delay fear conditioning. However, this interpretation should be taken with caution as we did not directly manipulate the LA in this series of experiments.

Additional work is needed to more completely assess the contributions of individual amygdalar nuclei to trace and delay fear conditioning. A future study will address the contributions of the LA to trace and delay fear conditioning by following an identical approach to that of Experiment 1, except that coordinates will target the LA rather than the BA. It is hypothesized that inhibition of LA protein synthesis during
memory consolidation will produce deficits in freezing to tone and context for both delay and trace conditioned animals. This result would support the currently proposed theory of more widely distributed processing of trace fear memories.

Materials and Methods

Animals

Fifty-one experimentally naïve male Long-Evans rats were purchased from Harlan Laboratories (Indianapolis, IN) for use in Experiment 1. Additionally, 31 female Long-Evans rats were bred in-house for use in Experiment 2. All rats were pair-housed in standard colony caging on a 12:12hr light:dark cycle and given *ad libitum* access to food and water. The rats were handled for 1 minute per day for 5 consecutive days prior to surgery. All procedures were performed during the light cycle and were approved by the Miami University Institutional Animal Care and Use Committee in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

Cannulation Surgery

Rats were anesthetized with 5% isoflurane (Vedco, St. Joseph, MO) in an induction chamber. They were placed in a standard stereotaxic instrument and maintained on 2-3% isoflurane at 2L/min. Body temperature was maintained on a heating pad located under the rat throughout surgery. The scalp was shaved, incised, and retracted. The head was leveled by equating bregma and lambda in the horizontal plane. Guide cannulae (22 gauge; Plastics One, Roanoke, VA) were lowered into the brain bilaterally targeting the basolateral amygdala using the following coordinates: AP -3.0 mm, ML ± 5.3 mm, DV -7.9 mm relative to bregma (Paxinos and Watson, 1998). Four skull screws and dental acrylic were used to secure the guide cannulae within the skull. Obturators were placed into the guide cannulae to prevent debris from entering. At the end of surgery, the rats were given two subcutaneous injections: 3 ml of 0.9% saline and 5 mg/kg/ml of Rimadyl to reduce pain and inflammation. Following surgery, the rats were placed into a recovery cage on a heating pad until they fully awoke from anesthesia. Post-operative care was performed for five consecutive days after surgery. Rimadyl (5 mg/kg/ml; s.c.) was administered at 24 and 48 hours post-surgery. Saline (0.9%; 3 ml; s.c.) was given as needed for signs of dehydration.

Lesion Surgery

Rats were anesthetized and leveled as described previously. Stainless steel tubing (28 gauge; Plastics One, Roanoke, VA) connected to 5 µl Hamilton syringes using clear polyethylene tubing (PE20) were lowered into the brain bilaterally targeting the basolateral amygdala. For coordinates, see table 1. N-Methyl-D-aspartate (NMDA; 20µg/µl; Sigma-Aldrich) was infused into each site, followed by a one minute diffusion time. Sham surgery consisted of the incision, retraction, and closing of the skin above the skull; no infusions of any kind were administered.
Following surgery, post-operative care was administered as described previously.

Behavioral Apparatus

Animals were fear conditioned and context tested in four identical Context A chambers (32.4 x 25.4 x 21.6 cm; MED-Associates, Inc., Georgia, VT). The ceiling and front door of the chamber were made of clear Plexiglas, the back wall was white Plexiglas and the two side walls were aluminum. The floor consisted of 19 equally spaced stainless steel rods. The grid floor in each chamber was wired to a shock generator and scrambler (MED-Associates, Inc.). The conditioning chambers were wiped down with an odorless 5% sodium hydroxide solution and scented with 50% vanilla flavor (Meijer) solution. The chamber was brightly lit (125 lux) by a light box located on top of the conditioning chamber.

Animals were tested for freezing to tone in Context B. These chambers (32.4 x 25.4 x 21.6 cm; MED-Associates, Inc.) were located in a different experimental room and were distinct from Context A. They consisted of a Plexiglas floor and a Plexiglas equilateral triangular insert. The context was cleaned and scented with a 1% glacial acetic acid solution. The light box above the chamber provided near-infrared lighting (0 lux).

The rats were continuously monitored by a progressive scan video camera with a visible light filter (VID-CAM-MONO-2A; MED-Associates, Inc.) connected to a computer in the experimental room running Video-Freeze software (MED-Associates, Inc.) designed for automated assessment of defensive freezing (see Anagnostaras et al., 2010).

Drugs and Infusions

Injectors (28 gauge) were connected to 10 µl Hamilton syringes using clear polyethylene tubing (PE20). The injectors were inserted into the cannulae so that they extended 1 mm below the guide. All infusions were delivered via an infusion pump (KD Scientific, Inc., Holliston, MA) at a rate of 0.1 µl/min for 5 min. Rats were placed in plastic bins with approximately 3 cm standard bedding during infusions, and were left for 4 min following infusion to allow for diffusion. In the first experiment, the protein synthesis inhibitor, cycloheximide (Sigma-Aldrich, Inc.), was dissolved in 50%DMSO/50%aCSF and infused into each site. In control rats, the vehicle was infused into the same location at the same rate and duration.

Procedure Experiment 1

Rats were randomly assigned to one of five conditions: 1) unpaired controls that received vehicle post-training infusions; 2) trace conditioned rats that received vehicle post-training infusions; 3) trace conditioned rats that received cycloheximide post-training infusions; 4) delay conditioned rats that received vehicle post-training infusions; 5) delay conditioned rats that received cycloheximide post-training infusions. All infusions targeted the basal nucleus of the amygdala. The trace conditioned rats were
given a 120 sec acclimation period, followed by 10 trials consisting of a 16 sec tone (2 kHz), followed by a 28 sec trace interval and then a 2 sec footshock (0.9 mA). Delay conditioned rats were given a 120 sec acclimation period, followed by 10 trials consisting of a 16 sec tone (2 kHz), co-terminating with a 2 sec footshock (0.9 mA). The intertrial interval (ITI) was 166 sec (tone onset to tone onset). The unpaired conditioned rats were given a 120 sec acclimation period, followed by 10 tones and then 10 footshocks, or vice versa. The interstimulus interval (ISI) was 83 sec (stimulus onset to stimulus onset). The session durations for trace, delay, and unpaired conditioning were equal. Immediately following the training procedure, rats received infusions of vehicle or cycloheximide. On day 2, all rats were tested for context freezing in context A during an 8 min session. Freezing is defined as the absence of all movement except that necessary for respiration (e.g., Fanselow, 1980), with significant muscle tone. On day 3, rats were tested for freezing during the baseline period in a novel context (Context B), during the 3 discrete tone presentations, and 3 trace intervals (or equivalent periods).

Procedure Experiment 2

Rats were randomly assigned to one of four conditions: 1) trace conditioned rats that received pre-training sham surgeries; 2) trace conditioned rats that received pre-training lesions; 3) delay conditioned rats that received vehicle pre-training sham surgeries; 4) delay conditioned rats that received pre-training lesions. The procedure used in experiment 2 is identical to that of experiment 1, except no unpaired group was included and post-training infusions were delivered.

Histology

*Cresyl Violet Staining*

At the end of all behavioral testing, the rats were anesthetized with 0.2 ml Euthasol i.p. (Virbac Animal Health, Inc., Fort Worth, TX; 390 mg pentobarbital sodium + 50 mg phenytoin sodium per ml). To visualize infusion locations in Experiment 1, rats were administered 0.5 μl of Cresyl Violet acetate (10% in distilled water; Sigma-Aldrich, Inc.) into each site using the same rate and duration of drug infusions. The rats were perfused intracardially with 0.9% saline followed by 10% formalin. Brains were removed and placed into 10% formalin. One day later, each brain was transferred into a 10% formalin/30% sucrose solution. Brains were frozen and sliced on a cryostat in 50 μm coronal sections. Every fourth slice through the amygdala was collected and mounted onto microscope slides. The brain slices were stained with 0.05% thionin (Sigma-Aldrich, Inc.), coverslipped, and infusion sights were verified using a light microscope.

*GFAP and NeuN Immunofluorescence Staining*

At the end of all behavioral testing in Experiment 2, the rats were anesthetized with 0.2 ml Euthasol i.p. (Virbac Animal Health, Inc., Fort Worth, TX; 390 mg pentobarbital sodium + 50 mg phenytoin sodium per ml). The rats were perfused intracardially with a phosphate buffered saline solution followed by 0.4%
paraformaldehyde. Brains were removed and placed into 0.4% paraformaldehyde. One day later, each brain was transferred into a 30% glycerol in phosphate buffered saline solution. Brains were frozen and sliced on a cryostat in 40 μm coronal sections stored in 0.1% sodium azide in well plates until immunohistochemical staining. Antibodies were directed against: 1) the astrocyte marker glial fibrillary acidic protein (GFAP) and 2) the neuronal nuclei marker NeuN.

Following a series of washes in 0.1M PBS, sections were incubated overnight in 0.1 M PBS-0.2% Triton-X solution, blocked with normal donkey serum, and then incubated for 48 h at 4 °C in primary antibody: Mouse anti-NeuN (Millipore MAB377) and chicken anti-GFAP (Abcam AB64674) diluted in 0.1 M PBS. Following a series of rinses, sections were incubated for 2 h in AlexaFluor conjugated antibodies directed toward the primary host antibody (Alexa Fluor 555 donkey anti-mouse, Life Technologies A-31570; Alexa Fluor 488 Donkey Anti-Chicken, Jackson Immuno 703-545-155). Sections then were rinsed, mounted on slides, and coverslipped using fluorescent mounting medium with DAPI (Vectashield, Vector Labs H-1200). Images were captured using an Olympus AX-70 Research System microscope.

Data Analysis

All statistics were calculated using SPSS version 20.0. Factorial (training and infusion or training and surgery) and repeated measures (tone number and trace interval number) analyses of variance (ANOVAs) were conducted to analyze the percentage of time spent freezing during the baseline, tone, trace interval, and context periods, with critical value α = 0.05. A priori planned comparisons between groups were performed using Fisher's LSD with α = 0.05.
References


Gilmartin MR, Kwapis J, Helmstetter F. 2012. Trace and contextual fear conditioning are impaired following unilateral microinjection of muscimol in the ventral hippocampus or amygdala, but not the medial prefrontal cortex. *Neurobiol Learn Mem* **97**: 452–464.


**Figure Captions**

**Table 1**: Lesion coordinates used in Experiment 2. All measurements are relative to bregma. Infusion volumes were 0.1μL per site with a 1 minute diffusion time.

**Table 2**: Group sizes for all experiments.

**Figure 1**: Cannula placement for all animals included in Experiment 1. Atlas images taken and modified from Paxinos and Watson (1998) (with permission from Elsevier ©1998). The number of animals in each group were as follows: unpaired veh, n = 9; trace veh, n = 11; trace cyclo, n = 11; delay veh, n = 10; delay cyclo, n = 10; N = 51.

**Figure 2**: (A) Timeline for Experiment 1. (B) The percentage of time spent freezing during the average baseline period (first 3 min), average of three test tones, and average of three trace intervals (TI) or trace interval equivalents (TIE) during the tone test. (C) Simultaneously learned contextual fear expressed during the context test.

**Figure 3**: The minimum (black) and maximum (gray) extent of bilateral lesions in BLA. Atlas images taken and modified from Paxinos and Watson (1998) (with permission from Elsevier ©1998). The number of animals in each group were as follows: trace sham, n = 7; trace lesion, n = 7; delay sham, n = 8; delay lesion, n = 9; N = 31.

**Figure 4**: (A) Timeline for Experiment 2. (B) The percentage of time spent freezing during the average baseline period (first 3 min), average of three test tones, and average of three trace intervals or trace interval equivalents during the tone test. (C) Simultaneously learned contextual fear expressed during the context test.
<table>
<thead>
<tr>
<th>Anterior/Posterior</th>
<th>Medial/Lateral</th>
<th>Dorsal/Ventral</th>
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Table 1

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<th>Experiment 1</th>
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<th>Experiment 2</th>
<th>N</th>
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<td>Trace Sham</td>
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<tr>
<td>Trace Veh</td>
<td>11</td>
<td>Trace Lesion</td>
<td>7</td>
</tr>
<tr>
<td>Trace Cyclo</td>
<td>11</td>
<td>Delay Sham</td>
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<tr>
<td>Delay Veh</td>
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<td>Delay Lesion</td>
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<td>Delay Cyclo</td>
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<tr>
<td>Total</td>
<td>51</td>
<td>Total</td>
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</tbody>
</table>
Figure 1
Figure 2

A

B

C

Context Test

Vehicle or Cyclo Infusion

Freezing (%)

Freezing (%)

Unpaired Trace Delay

Unpaired Trace Delay
Figure 3
Figure 4

A

Lesion Surgery → Training → 1 day → Context Test → Tone Test

B

Tone Test

C

Context Test

Figure 4