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Associative Structure of Fear Memory After Basolateral Amygdala Lesions in Rats

Christine A. Rabinak and
Department of Psychology, University of Michigan

Stephen Maren
Department of Psychology and Neuroscience Program, University of Michigan

Abstract

We have recently demonstrated that rats with basolateral amygdala (BLA) lesions acquire Pavlovian fear conditioning after overtraining. However, it is not known whether the associative basis of Pavlovian fear memory acquired by rats with BLA lesions is similar to that of intact rats. Associations are typically formed between the conditional (CS) and unconditional (US) stimuli (stimulus-stimulus; S-S), although it is possible for stimuli to enter into association with the responses they produce (stimulus-response; S-R). Indeed, the central nucleus of the amygdala, which is essential for fear conditioning in rats with BLA lesions, may mediate S-R associations in some Pavlovian tasks. We therefore used a post-conditioning US inflation procedure (i.e., exposure to intense footshock USs) to assess the contribution of S-S associations to fear conditioning after overtraining in rats with BLA lesions. In Experiment 1, intact rats that were overtrained and later inflated displayed elevated freezing levels when tested, indicating that S-S associations contribute to overtrained fear memories. Interestingly, neither neurotoxic BLA lesions nor temporary inactivation of the BLA during overtraining prevented the inflation effect (Experiment 2 and 3, respectively). These results reveal that S-S associations support Pavlovian fear memories after overtraining in both intact rats and rats with BLA lesion, and imply that the central nucleus of the amygdala encodes CS-US associations during fear conditioning.

Keywords

Pavlovian conditioning; basolateral amygdala; associative structure; US inflation

Pavlovian fear conditioning is a behavioral model used to investigate the neurobiology underlying the development and maintenance of fear learning and memory (Bouton et al., 2001; Grillon et al., 1996; Kim & Jung, 2006; LeDoux, 1998; LeDoux, 2000; Maren, 2001b; Maren, 2005). In this paradigm an innocuous conditioned stimulus (CS), such as a tone, is paired with an aversive unconditioned stimulus (US), such as a footshock. After one or more pairings the rat learns that the CS predicts the US. As a consequence, CS presentations alone elicit a conditioned fear response (CR), which includes increases in heart rate, arterial blood

Correspondence concerning this article should be sent to: Stephen Maren, Department of Psychology, University of Michigan, 530 Church St., Ann Arbor, MI, 48109−1043. Email: maren@umich.edu..

pressure, hypoalgesia, potentiated acoustic startle, stress hormone release, and freezing (somatomotor immobility).

Many years of work have identified the critical brain structures involved in the formation, consolidation, and retrieval of fear memories (Davis & Whalen, 2001; Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001b; Maren & Quirk, 2004). Among them, the amygdala is a candidate region in which fear memories are encoded and stored. Within the amygdala there are two sub-regions that contribute to fear learning and the expression of learned fear responses. The basolateral complex of the amygdala (BLA; consisting of the lateral, basolateral, and basomedial nuclei) is where CS and US information converge and become associated (yielding the fear memory), and the central nucleus of the amygdala (CEA) translates this information into behavioral fear responses (Davis & Whalen, 2001; Fanselow & Gale, 2003; Fendt & Fanselow, 1999; LeDoux, 1998; LeDoux, 2000; Maren, 2001b; Schafe et al., 2001). In support of this view, many studies have shown that either neurotoxic lesions or pharmacological inactivation of the BLA or CEA prevent the acquisition and/or expression of fear memories (Campeau & Davis, 1995; Cousens & Otto, 1998; Fanselow & Gale, 2003; Gale et al., 2004; Goosens & Maren, 2001, 2003; Helmstetter, 1992; Helmstetter & Bellgowan, 1994; Killcross et al., 1997; Koo et al., 2004; Maren, 1998; Maren, 1999, 2001a, 2001b; Maren et al., 1996a; Maren et al., 1996b; Muller et al., 1997; Nader et al., 2001; Walker & Davis, 1997; Wilensky et al., 2006; Wilensky et al., 1999; Wilensky et al., 2000; Zimmerman et al., 2007).

However, rats with pre-training BLA lesions can acquire fear CRs if given sufficient training (Maren, 1999; Zimmerman et al., 2007). This suggests that another brain area is involved in forming fear associations in the absence of the BLA (Gale et al., 2004; Maren, 1998; Maren, 1999; Zimmerman et al., 2007). Recent studies implicate the CEA in the acquisition and consolidation of fear memories (Goosens & Maren, 2003; Pare et al., 2004; Samson & Pare, 2005; Wilensky et al., 2006; Zimmerman et al., 2007). For example, Wilensky and colleagues (2006) have shown that temporary inactivation of the CEA impairs the acquisition of fear responses. In addition, we have recently reported that neurotoxic CEA lesions completely eliminate both the acquisition and expression of conditioned freezing even in rats that have been overtrained (Zimmerman et al., 2007). Temporary inactivation of the CEA also prevents both the acquisition and expression of overtrained fear memories (Zimmerman et al., 2007). This suggests that the CEA may mediate fear memory in the absence of the BLA.

Interestingly, recent work in appetitive conditioning paradigms also suggests a role for the CEA in Pavlovian learning. In these paradigms, it has been proposed that the type of association mediated by the CEA might be quite different than that mediated by the BLA (Blair et al., 2005; Cardinal et al., 2002; Everitt et al., 2003; Holland & Gallagher, 2004; Holland & Rescorla, 1975; Parkinson et al., 2000; Pickens & Holland, 2004; Pickens et al., 2003; Rescorla, 1973, 1974). Everitt and colleagues suggest that while the BLA may represent associations between the CS and US (stimulus-stimulus associations; S-S) and code US value, CEA neurons may drive Pavlovian CRs through direct associations with the behavioral response (stimulus-response associations; S-R) (Everitt et al., 2003; Killcross et al., 1997). In addition, these associations are a function of the amount of training. In instrumental conditioning, for example, S-S associations mediate responding early in training, while S-R associations mediate performance in extensively trained animals (Dickinson et al., 1995; Holland, 2004; Holland & Gallagher, 2004). It is unknown whether the associative structure of Pavlovian fear memories changes as a function of training. However, BLA lesions disrupt acquisition of conditioned fear responses with limited training, but not overtraining (Maren, 1998; Maren, 1999; Zimmerman et al., 2007). This suggests that the associative basis of fear conditioning may also change as a function of training. Alternatively, it is possible that the associative basis of conditioned fear in rats with BLA lesions is different from that in intact rats. More specifically, S-S associations (mediated by the BLA) may underlie fear memory in intact rats, whereas S-

R associations (mediated by the CEA) may underlie memory in rats with BLA lesions. The following experiments addressed these possibilities by using a US inflation procedure (Rescorla, 1974) to probe the associative structure of fear memory in intact rats and rats with BLA lesions after overtraining.

General Methods

Subjects

The subjects were 192 adult male Long-Evans rats (60−90 days old; 200−224 grams; Blue Spruce) obtained from a commercial supplier (Harlan Sprague-Dawley, Indianapolis, IN). Upon arrival all rats were individually housed in conventional Plexiglas hanging cages and kept on a 14 hr light/10 hr dark cycle (lights on at 7:00am) with free access to food and tap water. To acclimate the rats to the experimenter they were handled daily (10−15 sec per rat) for 5 days following their arrival. All experimental procedures were conducted in accordance with the approved guidelines as stated by the University of Michigan Committee on Use and Care of Animals (UCUCA).

Behavioral Apparatus

All sessions were conducted in eight identical rodent conditioning chambers (30 × 24 × 21 cm; MED Associates, St. Albans, VT). The chambers were positioned inside sound-attenuating cabinets located in an isolated room. Each chamber was constructed of aluminum (2 side walls) and Plexiglas (rear wall, ceiling, and hinged front door); the floor consisted of 19 stainless-steel rods, (4 mm diameter) spaced apart 1.5 cm (center to center). The grid floor was connected to a shock source and solid-state grid scrambler (MED Associates), which delivered the footshock US. Mounted on one wall of the chamber was a speaker to provide a distinct auditory CS and on the opposite wall was a 15-W house light; a fan provided background noise (65dB).

Three distinct contexts were created by manipulating multiple visual, olfactory and tactile cues: 1) Context A: 1% acetic acid odor in the chamber, houselights and room lights on, fans on in the cabinets, cabinet doors open, and grid floors; 2) Context B: 1% ammonium hydroxide odor in the chamber, red lights on in the room, houselights off, fans off in the cabinets, cabinet doors closed, and Plexiglas floors; 3) Context C: 70% ethanol odor in the chamber, house lights on, room lights off, fans off in the cabinets, cabinet doors open, and grid floors.

Each chamber rested on a load-cell platform, which was used to record chamber displacement in response to each rat's motor activity. The output from each load-cell was amplified to a level previously established to detect freezing responses. For each chamber, the load-cell amplifier output was digitized at 5 Hz (300 observations per minutes per rat) and acquired online using Threshold Activity software (MED Associates). Locomotor activity was quantified by the raw load cell values (range = 0 − 100) and freezing behavior was quantified by calculating the number of load cell values below the freezing threshold (threshold = 10). However, to prevent the inclusion of momentary bouts of inactivity as freezing, (i.e., < 1 sec) freezing was only scored after five or more contiguous observations below the freezing threshold (for details see Maren, 1998; Maren, 1999, 2001a). Freezing observations during each session were transformed into a percentage of total observations. In Experiments 2 & 3 sensitivity to the footshock US was measured by comparing the average locomotor activity over the 2-sec period prior to the first footshock presentation and the average locomotor activity during the first presentation of the footshock (2 sec).

Data Analysis

Freezing data were converted to a percentage of total observations, which is a probability estimate that is amenable to analysis with parametric statistics. These values were analyzed

using analysis of variance (ANOVA) and post hoc comparisons using Fishers LSD tests were performed after a significant overall *F* ratio was obtained. All data are represented as means ± SEMs.

Experiment 1

In order to characterize fear memory in rats with BLA lesions, an overtraining procedure must be used. However, overtraining itself may alter the associative basis of fear memory. Indeed, the associative structure of instrumental learning is a function of training insofar as S-S associations control performance early in learning and S-R associations dominates performance of well-trained responses (Dickinson et al., 1995; Holland, 2004). Therefore, the purpose of Experiment 1 was to determine whether overtraining itself alters the associative basis of Pavlovian fear conditioning, which normally depends on S-S associations (Rescorla, 1974).

Method

Subjects and design—Thirty-two rats were randomly assigned to one of three training groups. One group received 75 paired tone-shock trials (P75), while another group received 75 unsignaled shocks (U75); the third group did not receive training (NS). After overtraining rats in each group received a US inflation session (INF), in which several high-intensity shocks were delivered in a novel context. The U75-INF and NS-INF groups served as controls to assess the contribution of sensitization to conditioned responding to the CS. In addition, another group of rats that received 75 conditioning trials, but no inflation (P75-NoINF), served as a control for the magnitude of inflation in the P75-INF group. Conditioned freezing was measured during all phases of training to index fear to the conditioning context and the auditory CS. This yielded the following groups: P75-INF (n = 8), P75-NoINF (n =8), U75-INF (n=8), and NS-INF (n = 8).

Conditioning, inflation and test procedure—Fear conditioning was conducted using an overtraining procedure. Rats were transported from their home cages in squads of eight and placed in the conditioning chambers (Context A). Chamber position and experimental group were counterbalanced for each squad. Rats in the paired overtraining group (P75) received 75 paired presentations of a tone (10 seconds, 2kHz, 85dB) that co-terminated with a footshock (1.0mA, 2 seconds) beginning 3 minutes after being placed in the chambers. There was a 60 second intertrial interval (ITI) and the animals remained in the boxes 60 seconds after the last footshock presentation. Rats in the unsignaled overtraining group (U75) received a similar procedure except that they were given 75 unsignaled presentations of the same footshock. Rats in the no-training group were placed in the conditioning chambers for the same amount of time as the training groups, but did not receive tone or shock presentations. Twenty-four hours after conditioning all rats were placed in another, novel environment (Context C) for US inflation. The inflation session consisted of exposure to 5 high-intensity footshocks (3.0mA, 2 seconds) 3 minutes after placement in the chambers. There was a 60-sec ITI, and the animals remained in the boxes 60 seconds after the last footshock. Rats in the P75-NoINF group were placed in the chamber for the same duration as the rats in the inflation groups but did not receive footshocks. Forty-eight hours after conditioning, all rats were placed back into Context A for 10 minutes to assess contextual fear. Twenty-four hours after the context test, fear to the tone was tested by placing the rats into a third novel context (Context B) and presenting 30 tone alone presentations (10 seconds, 2kHz, 85dB, 60 sec ITI) 3 minutes after placement into the chambers. Freezing behavior was measured throughout all experimental sessions.

Results and Discussion

Behavior—An ANOVA of the average post-shock freezing during the conditioning session revealed a significant main effect of group [$F_{(2,28)} = 32.2$; $p < 0.0001$] (Figure 1). Not surprisingly, both groups of rats that received 75 footshocks (P75 and U75) acquired high levels of freezing, while the no-training group remained low. Rats in the no-training group exhibited some immobility late in the session that was not related to fear, but rather to quiescence late in the session. This significant difference in freezing between the groups that had received training versus the no-training group was confirmed by further post hoc comparisons ($p < 0.0001$).

Data from the inflation session are shown in Figure 2. An ANOVA calculated for the average freezing during the post-shock ITI periods of the inflation session (minutes 4−8) revealed a significant main effect of group [$F_{(3,27)} = 9.0$; $p < 0.0003$]. Post-hoc comparisons revealed that all rats receiving inflated shocks (P75-INF, NS-INF, and U75-INF) exhibited more freezing than rats not receiving shock (P75-NoINF) ($p < 0.0001$ for comparison to the P75-INF group; $p < 0.0002$ for comparison to the U75-INF group; and $p < 0.01$ for comparison to the NSINF group). There was no significant difference between any of the inflation groups.

Conditioned freezing during the context test is displayed in Figure 3a. During the context test, rats in the P75-INF and U75-INF groups exhibited significantly more freezing than rats in the NS-INF and P75-NoINF groups. This observation was confirmed in an ANOVA that revealed a significant main effect of group [$F_{(3,27)} = 24.7$; $p < 0.0001$]. Post hoc comparisons revealed that the groups that had received overtraining and inflation (P75-INF and U75-INF) displayed elevated levels of freezing that were significantly different from the other groups (NS-INF and P75-NoINF) (Figure 3a). Although both the NS-INF and P75-NoINF groups froze significantly less than the groups that had received overtraining and inflation, the NS-INF group displayed higher levels of freezing than the P75-NoINF group ($p = 0.01$). Elevated levels of freezing behavior in the NS-INF group is likely due to the generalization of fear from the inflation context to the test context. Overall, US inflation increased contextual fear in animals that had previously received 75 footshocks in that context, whether they were signaled or not. This was not simply the result of sensitization by intense footshocks, insofar as the NS-INF rats exhibited relatively low levels of freezing.

An ANOVA performed for the tone freezing data (Figure 3b) revealed that there was a significant main effect of group [$F_{(3,27)} = 4.8$; $p < 0.01$] and post-hoc tests revealed significantly higher levels of freezing in the P75-INF group when compared to all other groups throughout the entire session ($p < 0.02$ for comparison to the P75-NoINF group; $p < 0.005$ for comparison to the U75-INF group; and $p < 0.002$ for comparison to the NS-INF group). There was no significant difference between any of the other groups (P75-NoINF, U75-INF, and NS-INF). In contrast to the context test, only rats that received inflation after 75 tone-shock trials exhibited elevated freezing during the tone test (Figure 3b). These results indicate that elevated freezing in the P75-INF group was due to US revaluation rather than non-associative shock sensitization, because neither 75 conditioning shocks nor inflation shocks alone were sufficient to elevate freezing to the tone CS. During both the context and tone tests, the non-inflated group displayed low levels of freezing, which may be due to generalized extinction from exposure to similar chambers during the inflation session. Nonetheless, freezing in the inflation groups following overtraining was augmented by the inflation procedure, and this is due to an associative increase in fear. Overall these data indicate that overtrained fear memories are sensitive to US inflation, suggesting that S-S associations mediate fear memory even after extended training.

Experiment 2

Experiment 1 reveals that S-S associations contribute to the expression of fear in overtrained rats. It has been argued in previous work that BLA damage impairs both the encoding of S-S associations and interferes with US revaluation (Blundell et al., 2001; Hatfield et al., 1996; Holland & Gallagher, 2004; Killcross et al., 1997; Pickens et al., 2003). Because rats with BLA damage acquire conditioned fear after overtraining (Maren, 1999; Zimmerman et al., 2007), it is therefore possible that S-R associations mediate this memory. If so, the fear memory in rats with BLA lesions may be insensitive to US revaluation procedures. Experiment 2 used the inflation procedure to examine this possibility in rats in which neurotoxic BLA lesions were made prior to overtraining.

Method

Subjects and design—The subjects were 64 rats housed and handled as described in Experiment 1. Prior to overtraining they were divided into two equal groups: one group that received bilateral neurotoxic lesions in the basolateral complex of the amygdala (BLA) and a second group that underwent sham surgery (SHAM). Following overtraining each surgery group was further divided into two groups: one that received US inflation (INF) after overtraining or a group that did not undergo US inflation (NoINF).

Surgery—One week prior to training, each rat was anesthetized with an intraperitoneal (i.p.) injection of a Nembutal (sodium pentobarbital; 65 mg/kg body weight) and atropine methyl nitrate (0.4 mg/kg body weight) cocktail. Ocular lubricant was used to moisten the eyes. The scalp was shaved, cleaned with antiseptic (Betadine) and the rat was mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). After the scalp was incised and retracted, the skull was positioned so that bregma and lambda were in the same horizontal plane. Small burr holes were drilled bilaterally in the skull to allow placement of 28-gauge injectors in the BLA (3.3 mm posterior to bregma and 5.0 mm lateral to the midline). Injectors were attached to polyethylene tubing and connected to 10 μL syringes mounted on an infusion pump (Harvard Apparatus, South Natick, MA). *N*-methyl-D-aspartate (NMDA; 20 mg/mL dissolved in 100 mM PBS, pH 7.4; Sigma, St. Louis, MO) was infused (0.1 μL/min) at two sites for each BLA lesion: 8.0 mm ventral to the brain surface (0.2 μL) and 7.5 mm ventral to the brain surface (0.1 μL) ventral to the brain surface. Five minutes were allowed for diffusion of the drug into the target structure before the injectors were removed. SHAM rats received a similar surgery except that the injectors were not lowered into the brain. After surgery the incision was closed with stainless steel wound clips and the rats were kept on a heating pad until they recovered from anesthesia before returning to their home cages. The rats were allowed one week of recovery prior to overtraining.

Conditioning, inflation and test procedure—One week after surgery, all groups received overtraining, US inflation, and retention testing as described in Experiment 1.

Histology—After behavioral testing, rats were euthanized with an overdose of sodium pentobarbital (i.p. 100 mg/kg) and were transcardially perfused with physiological saline followed by 10 % formalin. Brains were removed and post-fixed in 10% formalin followed by 10% formalin/30% sucrose solution until sectioning. Coronal brain sections (45 μm) were cut on a cryostat and wet-mounted with 70% ethanol on glass microscope slides. Once dry, the sections were stained with 0.25% thionin to visualize neuronal cell bodies and identify lesion sites.

Results and Discussion

Histology—Eight rats were excluded from the analyses because their lesions were either larger than intended, misplaced, or unilateral. This yielded the following groups designated by lesion type and inflation condition: BLA-INF (*n* = 12), BLA-NoINF (*n* =11), SHAM-INF (*n* = 16) and SHAM-NoINF (*n* = 16). Successful lesions were generally confined to the targeted structure, although some rats in the BLA group had damage to the rostral endopiriform nucleus and caudate putamen (Figure 4). NMDA infusions into the BLA spared the central nucleus of the amygdala.

Behavior—A two-way ANOVA of the average post-shock freezing during the overtraining session revealed a significant main effect of lesion [$F_{(1, 53)} = 7.7$; $p < 0.01$], a main effect of time (5 minute blocks) [$F_{(15, 795)} = 92.5$; $p < 0.0001$], and an interaction between lesion and time [$F_{(15, 795)} = 5.5$; $p < 0.0001$] (Figure 5a). These results indicate that freezing levels differed among the groups across the session. Rats with BLA lesions displayed freezing levels that were slightly below those in the SHAM group. However, a post hoc comparison of average freezing during the last 10 trials of the overtraining session revealed that rats with BLA lesions acquired a level of freezing comparable to that of SHAM rats ($p = 0.05$; Figure 5b). In addition, BLA lesions did not affect sensitivity to the footshock US [$F_{(1, 53)} = 0.69$; $p = 0.41$], which suggests that BLA lesions impaired conditional freezing compared to controls during the beginning of the overtraining session (data not shown).

Post-shock freezing during the inflation session is shown in Figure 6. A two-way ANOVA calculated for the average freezing during minutes 4–8 of the session revealed a significant main effect of inflation condition [$F_{(1, 51)} = 72.3$; $p < 0.0001$], and an interaction between lesion and inflation condition [$F_{(1, 51)} = 8.4$; $p < 0.01$]. It is apparent that rats in the SHAM-INF group displayed more freezing behavior relative to their no-inflation controls than those in the BLA-INF group ($p < 0.005$ for comparison with BLA-INF and $p < 0.001$ for all other comparisons). Rats in the BLA-INF group displayed lower levels of freezing than rats in the SHAM-INF group. Importantly, BLA lesions did not alter sensitivity to the intense footshocks used during the inflation session [$F_{(1, 26)} = 2.56$; $p = 0.12$] (data not shown).

Conditioned freezing during the context is displayed in Figure 7a. Rats in both the SHAM-INF and BLA-INF groups exhibited more freezing than non-inflated controls, and this effect appear more robust in the SHAM rats. These impressions were confirmed by a two-way ANOVA that revealed a significant main effect of lesion [$F_{(1,51)} = 17.6$; $p < 0.0001$], a main effect of inflation condition [$F_{(1,51)} = 136.6$; $p < 0.0001$], and an interaction between lesion and inflation condition [$F_{(1,51)} = 18.5$; $p < 0.0001$]. Although rats with BLA lesions exhibited less inflation than SHAM controls, rats in the BLA-INF group did display significantly higher levels of freezing than those in the BLA-NoINF group ($p < 0.0001$).

A two-way ANOVA of the tone test data (Figure 7b) revealed a significant main effect of lesion [$F_{(1,51)} = 16.7$; $p < 0.0005$] and a main effect of inflation condition [$F_{(1,51)} = 19.4$; $p < 0.0001$], however the interaction between lesion and inflation condition was not significant [$F_{(1,51)} = 2.4$; $p = 0.125$]. These data indicate that although BLA lesions generally blunted freezing, rats in both conditions exhibited inflation. Indeed, planned comparisons revealed that the BLA-INF group exhibited higher levels of freezing than the BLA-NoINF group [$t_{(21)} = 2.3$; $p < 0.05$]. These data reveal that rats with BLA lesions exhibit inflation despite the fact that amygdala damage produces a general reduction in freezing. In addition, these data suggest that S-S associations underlie overtrained fear in both intact rats and rats with BLA lesions.

Experiment 3

Experiment 2 indicates that S-S associations mediate overtrained fear memories acquired by rats with BLA lesions. Nonetheless, the inflation effect was attenuated in rats with BLA lesion (at least during the context test). Amygdala damage may have produced a general deficit in freezing that could have obscured US inflation. Moreover, the BLA itself has been implicated in US revaluation (Blundell et al., 2001; Everitt et al., 2003; Hatfield et al., 1996; Holland, 2004; Killcross et al., 1997; Pickens et al., 2003), and BLA lesions may have therefore disrupted US inflation independently of the nature of the association underlying fear memory. To address this issue, Experiment 3 used temporary pharmacological inactivation of the BLA during overtraining to mimic the conditions of acquiring fear in the absence of the BLA. Rats then received the inflation procedure with a functional BLA. Hence, any deficits in US inflation in rats trained under BLA inactivation cannot be attributed to either lesion-induced performance deficits on test or impaired US revaluation.

Method

Subjects and design—The subjects were 32 rats housed and handled as described in Experiment 1. The rats were divided into two equal groups: one group that received pre-training bilateral infusions of muscimol (MUS), a $GABA_A$ agonist, and a second group that received bilateral infusions of a vehicle control (VEH; artificial cerebral spinal fluid) prior to overtraining. Muscimol has long-lasting effects that have been shown to impair fear acquisition during overtraining when infused into the central nucleus of the amygdala (Zimmerman et al., 2007). After overtraining, each drug group was further divided into two groups: one that received the US inflation procedure (INF) and a group that did not undergo US inflation (NoINF).

Surgery—One week prior to training, the rats were anesthetized and prepared for surgery as described in Experiment 2. Small burr holes were drilled bilaterally in the skull to allow for the placement of 26-gauge guide cannulae (Plastics One, Roanoke, VA) in the BLA (3.3 mm posterior to bregma, 5.0 mm lateral to the midline, and 6.5 mm ventral to the brain surface), along with holes for 3 small jeweler's screws. Dental acrylic was applied to the cannulae, screws, and skull surface to hold the guide cannulae in place. After surgery, 33-gauge dummy cannulae (16 mm; Plastics One) were inserted into the guide cannulae and the rats were allowed to recover as described in Experiment 2. Dummy cannulae were replaced daily during the week of recovery.

Procedure—Prior to overtraining, rats were transported to the infusion room in squads of eight from their home cages in white 5-gallon buckets. Hamilton syringes (10 μl; Harvard Apparatus) were mounted in two infusion pumps (10 syringes/pump; Harvard Apparatus) and connected to 33-gauge internal cannula (1.0 mm longer than the implanted guide cannulae) with polyethylene tubing (A-M Systems). Dummy cannulae were removed from each rat and internal cannulae were inserted into each guide cannula. Either muscimol (1 mg/mL dissolved in ACSF, pH 7.4; Sigma) or vehicle (same volume and rate) was infused bilaterally into the BLA (0.25 μl/side; 0.1 μl/min). One minute was allowed for diffusion of the drug into the target structure before the injectors were removed. Dummy cannulae were inserted into the guide cannulae once the injectors were removed and the rats were immediately taken to the conditioning chambers for overtraining. All groups received overtraining, US inflation, and retention testing as described in Experiment 1.

Histology—Histology was conducted as described in Experiment 2.

Muscimol-TMR-X Intracranial Microinfusion—After behavioral testing, six rats that had previously received muscimol infusions prior to overtraining were given bilateral infusions of fluorescent muscimol (muscimol-TMR-X conjugate; Invitrogen, Carlsbad, CA) to map the spread of muscimol infused into the BLA (Allen et al., 2008). Prior to the infusion, the rats were anesthetized with Nembutal (i.p. injection; 65 mg/kg body weight). Muscimol-TMR-X (1 mg/mL dissolved in 0.01M PBS) was infused bilaterally into the BLA (0.25μl/side; 0.1 μl/min). One minute was allowed for drug diffusion into the target structure before the injectors were removed. Rats were sacrificed 80 minutes after the infusion (comparable to the duration of the overtraining session). The rats were transcardially perfused with physiological saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde/30% sucrose solution until sectioning. Coronal brain slices (45 μm) were cut on a cryostat in the dark and wet-mounted with 70% ethanol on glass microscope slides. Sections were re-hydrated with 0.01M PBS and pictures were taken under a light field as a reference for cytoarchitecture and under a 543/569 nm fluorescent filter to visualize muscimol-TMR-X spread. Sections were later stained with 0.25% thionin to identify cannulae track placements.

Results and Discussion

Histology—Four rats were excluded from the analyses because their cannulae placements were not targeted at the BLA (Figure 8a). This yielded the following groups designated by drug type and inflation condition: MUS-INF ($n = 6$), MUS-NoINF ($n = 7$), VEH-INF ($n = 7$), and VEH-NoINF ($n = 8$). Terminal injections of fluorescent muscimol revealed that muscimol infusions were generally confined to the BLA, although some rats had spread to the rostral endopiriform nucleus (Figure 8b).

Behavior—As shown in Figure 9, muscimol slowed, but did not prevent, the acquisition of conditioned freezing during the overtraining session. An ANOVA of the average post-shock freezing during the conditioning session revealed a significant main effect of time (5 minute blocks) [$F_{(15, 390)} = 31.6$; $p < 0.0001$], and an interaction between drug and time [$F_{(15, 390)} = 5.9$; $p < 0.0001$] (Figure 9). The effect of muscimol on freezing during overtraining was similar to that of BLA damage (see Figure 5). Muscimol in the BLA did not affect sensitivity to the footshock US [$F_{(1, 26)} = 0.09$; $p = 0.76$] (data not shown).

Post-shock freezing during the inflation session is shown in Figure 10. It is apparent that rats receiving inflation shocks displayed increased freezing relative to their no-inflation controls ($p < 0.0001$ for comparison between INF and NoINF and $p = 0.576$ for comparison between MUS and VEH). Prior drug experience did not have a significant effect on freezing levels during inflation. These observations were confirmed by a two-way ANOVA on average freezing over minutes 4−8 that revealed a significant main effect of inflation condition [$F_{(1,24)} = 48.9$; $p < 0.0001$].

Conditioned freezing during the context test is displayed in Figure 11a. Rats in the VEH-INF and MUS-INF groups exhibited significantly more freezing than rats in the non-inflated controls. This observation was confirmed by a two-way ANOVA that revealed a significant main effect of inflation condition [$F_{(1,24)} = 81.7$; $p < 0.0001$], however there was no significant interaction between the inflation condition and drug [$F_{(1,24)} = 0.07$; $p = 0.8$]. These data indicate that although muscimol blunted freezing during overtraining, rats in both conditions exhibited similar degrees of inflation when inflation and testing occurred with a functional amygdala.

A two-way ANOVA of the tone test data (Figure 11b) revealed a significant main effect of drug type [$F_{(1,24)} = 6.4$; $p < 0.02$] and a main effect of inflation condition [$F_{(1,24)} = 14.8$; $p < 0.0001$], but the interaction between the inflation condition and drug type was not significant [$F_{(1,24)} = 0.5$; $p = 0.475$]. Both inflation groups (MUS-INF and VEH-INF) displayed significantly higher levels of freezing when compared to the non-inflated groups (MUS-NoINF

and VEH-NoINF; $p < 0.05$ for all comparisons). Although both non-inflated groups displayed lower levels of freezing than the inflated groups, the VEH-NoINF group displayed significantly higher levels of freezing than the MUS-NoINF group ($p < 0.05$). Previous reports suggest that remote memories acquired by rats with BLA inactivation are weaker than BLA-dependent memories, which could explain the difference in freezing between the non-inflated groups (Poulos et al., 2006). Overall these data suggest that S-S associations mediate overtrained fear memories in rats with temporary BLA lesions and that the BLA is not necessary for US revaluation.

General Discussion

The present experiments used a post-conditioning manipulation of US value (an inflation procedure) to assess the associative structure of overtrained fear in both intact rats and rats with amygdala lesions. We found that despite being overtrained, the fear memory of intact rats is sensitive to the inflation procedure, which suggests it is mediated by an S-S association (Experiment 1). Moreover, neurotoxic lesions of the BLA (Experiment 2) or temporary inactivation of the BLA during overtraining (Experiment 3) did not prevent inflation of fear memory. These results reveal that S-S associations mediate conditional fear not only in intact rats, but also in rats with BLA lesions. These data suggest that brain structures, such as the CEA, that mediate fear in the absence of the BLA encode CS-US associations during fear conditioning.

Unlike previous studies in instrumental conditioning tasks, we found no evidence that the associative structure of fear conditioning changes as a function of training. That is, early in training instrumental responding relies upon action-outcome (A-O) representations, but late in training these responses come to depend on S-R associations (Dickinson et al., 1995; Holland, 2004). The emergence of S-R associations in instrumental conditioning drives the transition of instrumental responding from goal-directed actions to outcome-independent habits. In our experiments, overtrained fear responses remain sensitive to inflation procedures, suggesting continued involvement of S-S associations in their expression. Pavlovian fear responses are notoriously insensitive to instrumental contingencies (Bolles et al., 1974), and appear to require US representations for expression in behavior even after overtraining.

An important aim of these experiments was to determine whether rats with BLA lesions that acquire fear during overtraining use the same underlying associative structure as intact rats. We found that rats with BLA lesions exhibited US inflation, suggesting that S-S associations underline fear in both intact rats and rats with BLA lesions. However, the inflation effect was blunted in rats with BLA lesions; amygdala damage may have produced a general deficit in freezing that could have obscured US inflation. To address this issue, we used temporary pharmacological inactivation of the BLA during overtraining to mimic the conditions of acquiring fear in the absence of the BLA. Hence, any deficits in US inflation in rats trained under BLA inactivation could not be attributed to either lesion-induced performance deficits on test or impaired performance. Fear memories acquired in the absence of the BLA during overtraining were still sensitive to inflation, which suggests that these memories are mediated by S-S associations.

Recent evidence indicates that the CEA mediates conditional fear in rats with BLA lesions (Ponnusamy et al., 2007; Wilensky et al., 2006; Zimmerman et al., 2007). The present experiments suggest that the CEA may encode such memories in the form of S-S associations. This contrasts with work indicating that the CEA may mediate Pavlovian S-R associations. For example, CEA lesions disrupt conditioned suppression, conditioned orienting, conditioned locomotor approach, and Pavlovian-instrumental-transfer, all of which are thought to depend on S-R associations (Gallagher et al., 1990; Hall et al., 2001; Hatfield et al., 1996; Killcross et

al., 1997; Parkinson et al., 2000). In these cases, associations formed by the CEA are sensorimotor associations that do not incorporate US value, which supports the idea that the CEA mediates learning through S-R associations under some conditions (Cardinal et al., 2002). However, other work has posited that the CEA does form associations with the US, although with motivational properties of the US which are apparently insensitive to US revaluation procedures (Balleine et al., 2003; Balleine & Killcross, 2006; Blundell et al., 2001, 2003; Konorski, 1967). The present data indicate that the CEA represents properties of the US that are sensitive to inflation.

In contrast to previous work in appetitive paradigms, the present data indicate that the BLA is not essential for US revaluation (Balleine et al., 2003; Blundell et al., 2001; Everitt et al., 2003; Hatfield et al., 1996; Holland, 2004; Holland & Gallagher, 2004; Killcross et al., 1997; Pickens et al., 2003). Surprisingly, we found that rats with BLA lesions are sensitive to inflation procedures, suggesting that the BLA is not necessary for coding the value of aversive USs. (but see Fanselow & Gale, 2003). There are many differences between the appetitive paradigms and the present study that could account for differences in whether the BLA is involved in US revaluation. For example, the nature of the USs (food versus shock) and the motivational systems they engage are different in the two paradigms. In addition, devaluation procedures in appetitive conditioning typically rely upon an instrumental component. For example, US devaluation through selective satiety depends on the animal approaching a food pellet and consuming it until the animal is sated. Perhaps BLA dysfunction only impairs “instrumental” devaluation. Another major difference is the direction in which the US is revalued. In appetitive studies the US experiences a decrease in value, whereas in our experiments the US experiences an increase in value. Studies of attention have revealed differential involvement of neural structures that depends on whether there are increases or decreases in attention (Baxter et al., 1997; Holland & Gallagher, 1999). For example, the hippocampus mediates decrements and not increases in attention (Baxter et al., 1999; Holland & Gallagher, 1993). Similar to the role of the hippocampus in attention, the BLA may be differentially responsible for US revaluation depending on which direction the US is revalued.

Together, these experiments provide important information regarding the neural substrates involved in maintaining and updating the representation of aversive stimuli. Our experiments suggest that the CEA may encode fear associations in a manner similar to that observed in the BLA. Indeed common cellular mechanisms appear to underlie fear conditioning in both structures (Goosens & Maren, 2003; Wilensky et al., 2006; Zimmerman et al., 2007). Elucidating the mechanisms by which the amygdala encodes fear memory is critical for developing effective treatments for anxiety disorders, including Post-Traumatic Stress Disorder (PTSD) (Davey, 1989; Hosoba et al., 2001; Unger et al., 2003; White & Davey, 1989).

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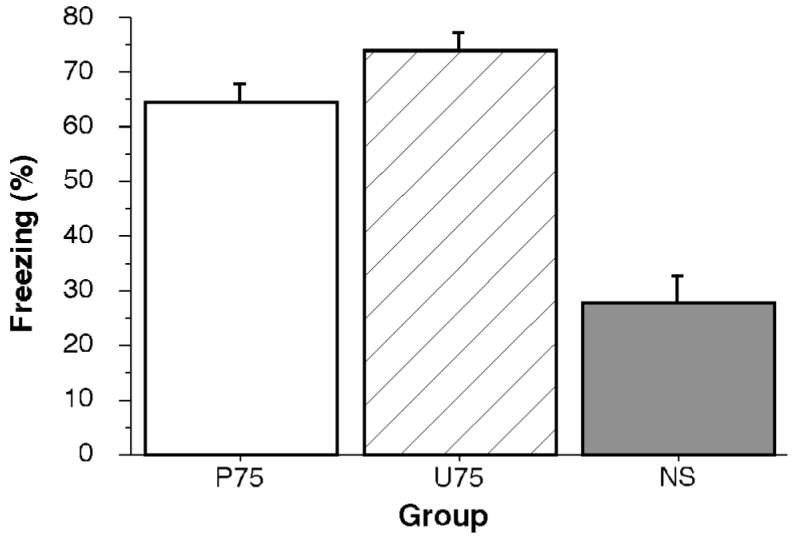


Figure 1.
Post-shock freezing during the overtraining session (Experiment 1). Mean percentage of freezing (± SEM) during the 75-trial session in Context A are displayed for each of the three groups.

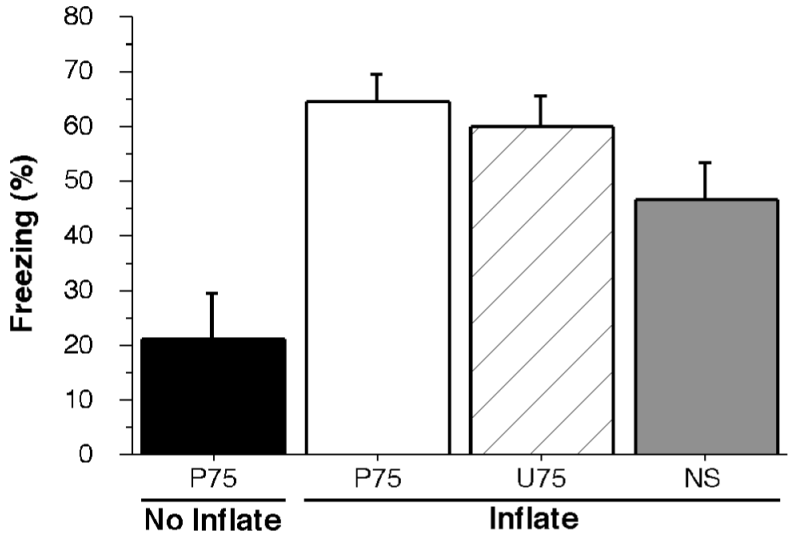


Figure 2.

Post-shock freezing during the inflation session (Experiment 1). Mean percentage of freezing (± SEM) during the five 60 sec ITI periods between the inflation shocks. Data are shown for NS-INF (gray bar), U75-INF (hatched bar), P75-INF (white bar) and P75-NoINF (black bar).

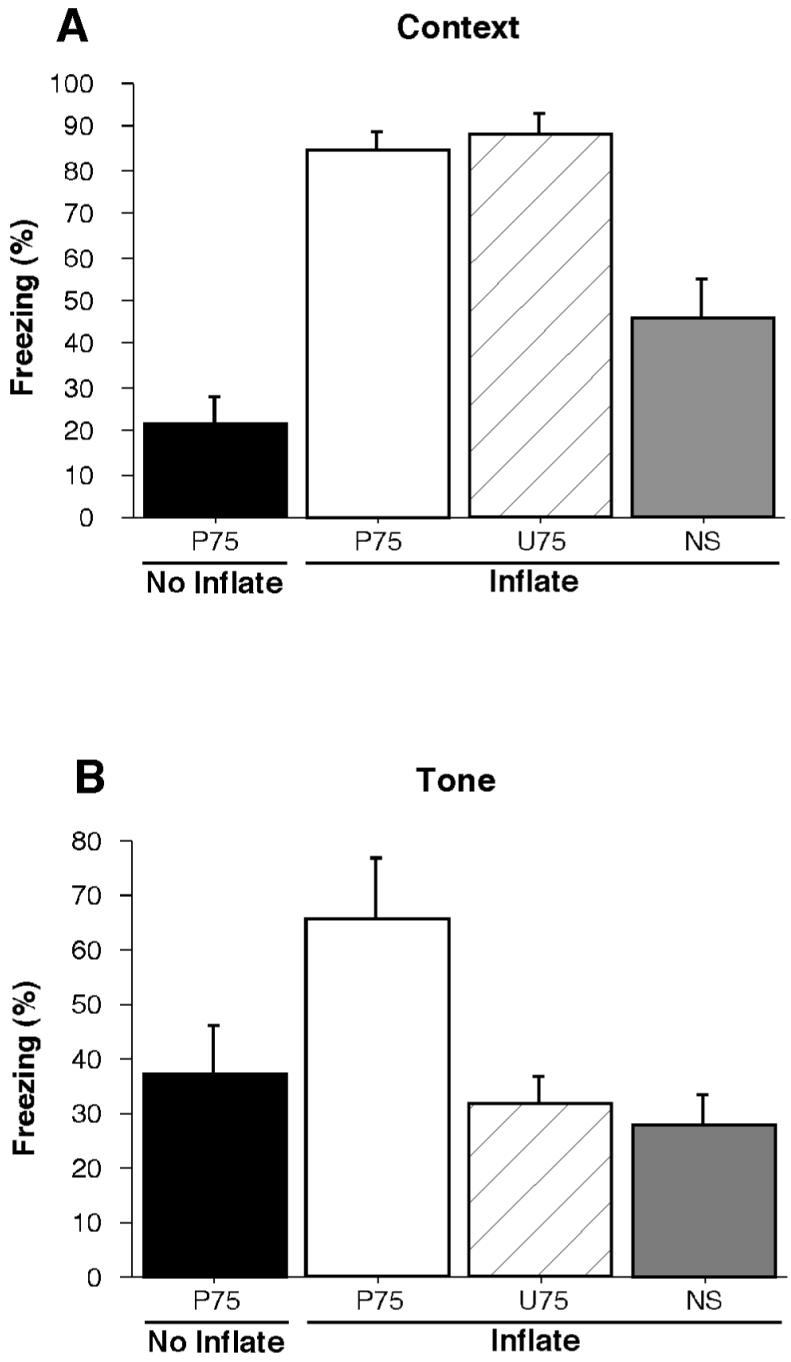


Figure 3.

Conditioned freezing during the context and tone tests (Experiment 1). *A*, Mean percentage of freezing (± SEM) across the 10-minute context test. *B*, Mean percentage of freezing (± SEM) during the 10-trial tone test. Data are an average of freezing during the ITI periods. Data are shown for NS-INF (gray bar), U75-INF (hatched bar), P75-INF (white bar) and P75-NoINF (black bar).

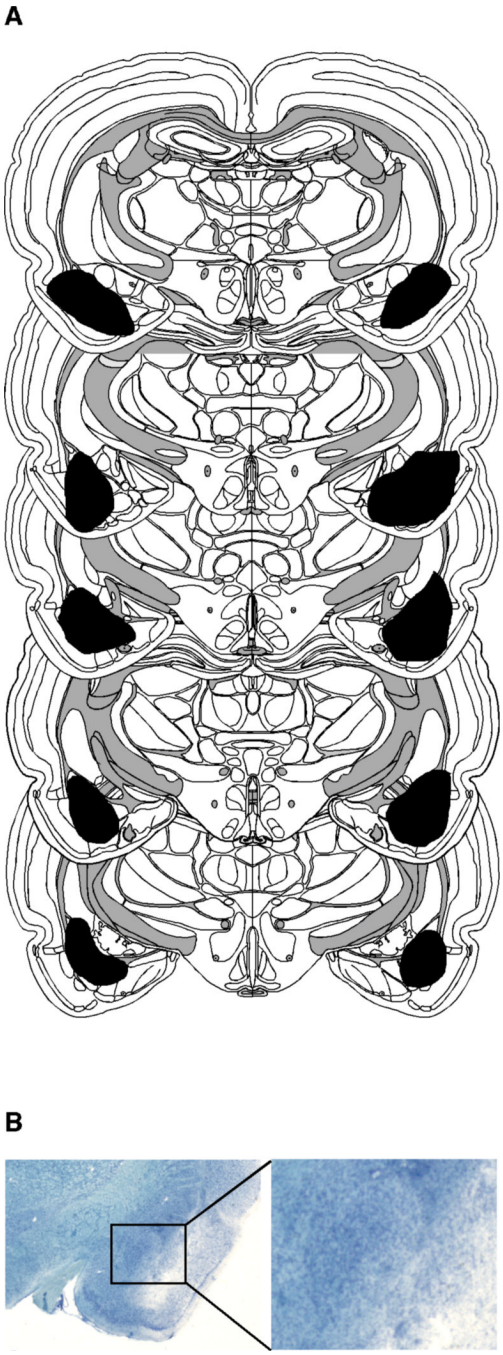


Figure 4.
A, Schematic representation of the extent of pre-training NMDA lesions in the BLA for Experiment 2. Coronal brain images were adapted from (Swanson, 1992). *B*, Representative thionin-stained section from rats that received lesions of the BLA.

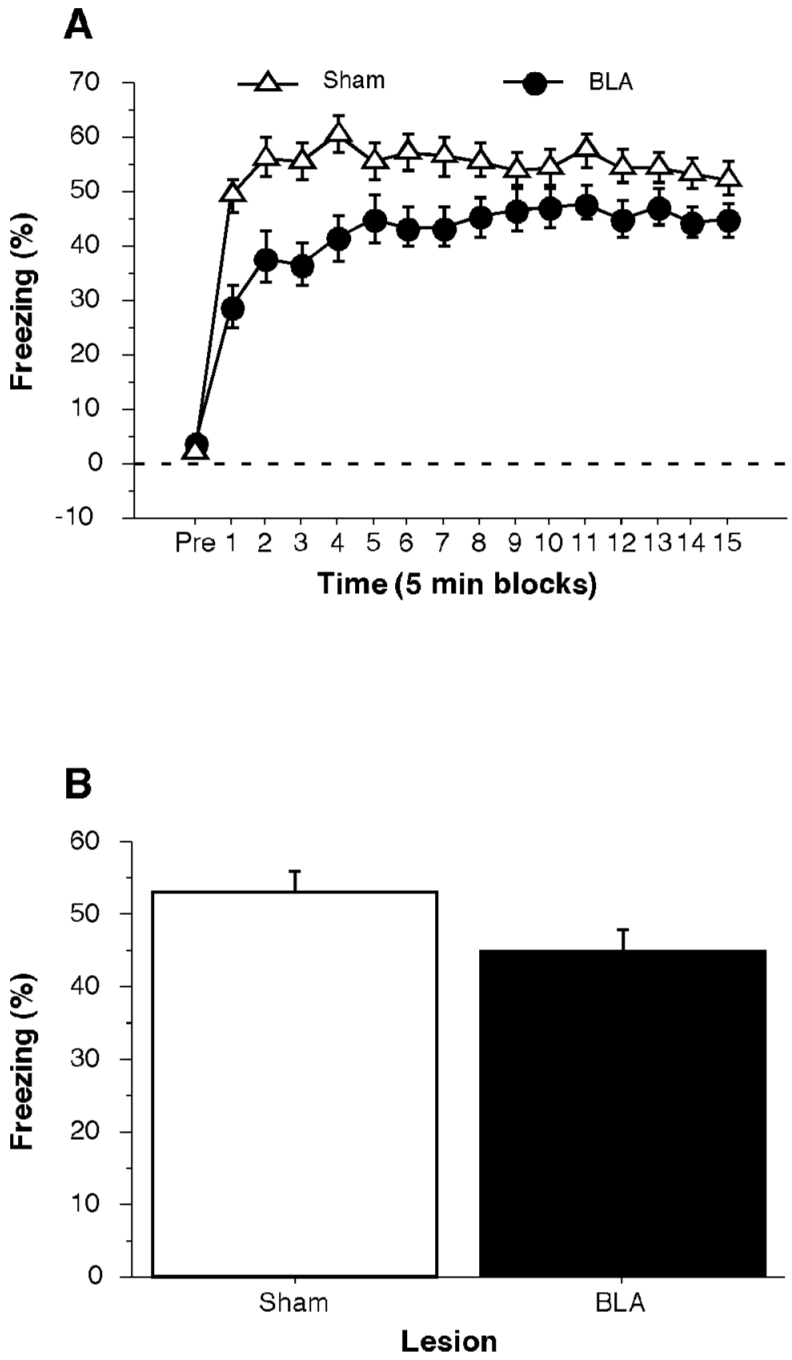


Figure 5.

Post-shock freezing in rats with pre-training amygdala lesions during the paired overtraining session (Experiment 2). *A*, Mean percentage of freezing (± SEM) during the 75-trial session. Data are shown for rats with BLA lesions (closed circles) and SHAM rats (open triangles). *B*, Mean percentage of freezing (± SEM) during the last 10 conditioning trials in rats with BLA lesions (black bar) and SHAM rats (white bar).

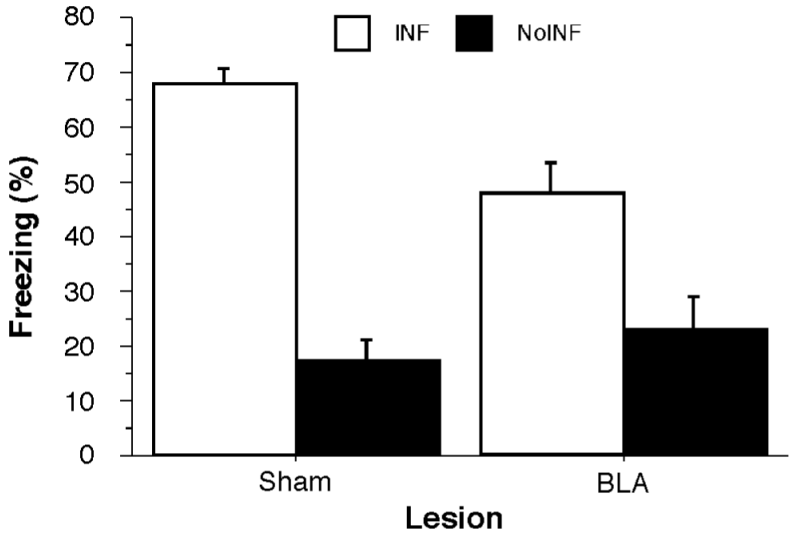


Figure 6.
Post-shock freezing in rats with pre-training amygdala lesions during the inflation session (Experiment 2). Mean percentage of freezing (± SEM) during the post-shock ITIs for the INF groups (white bar) and the NoINF groups (black bar) within each lesion type.

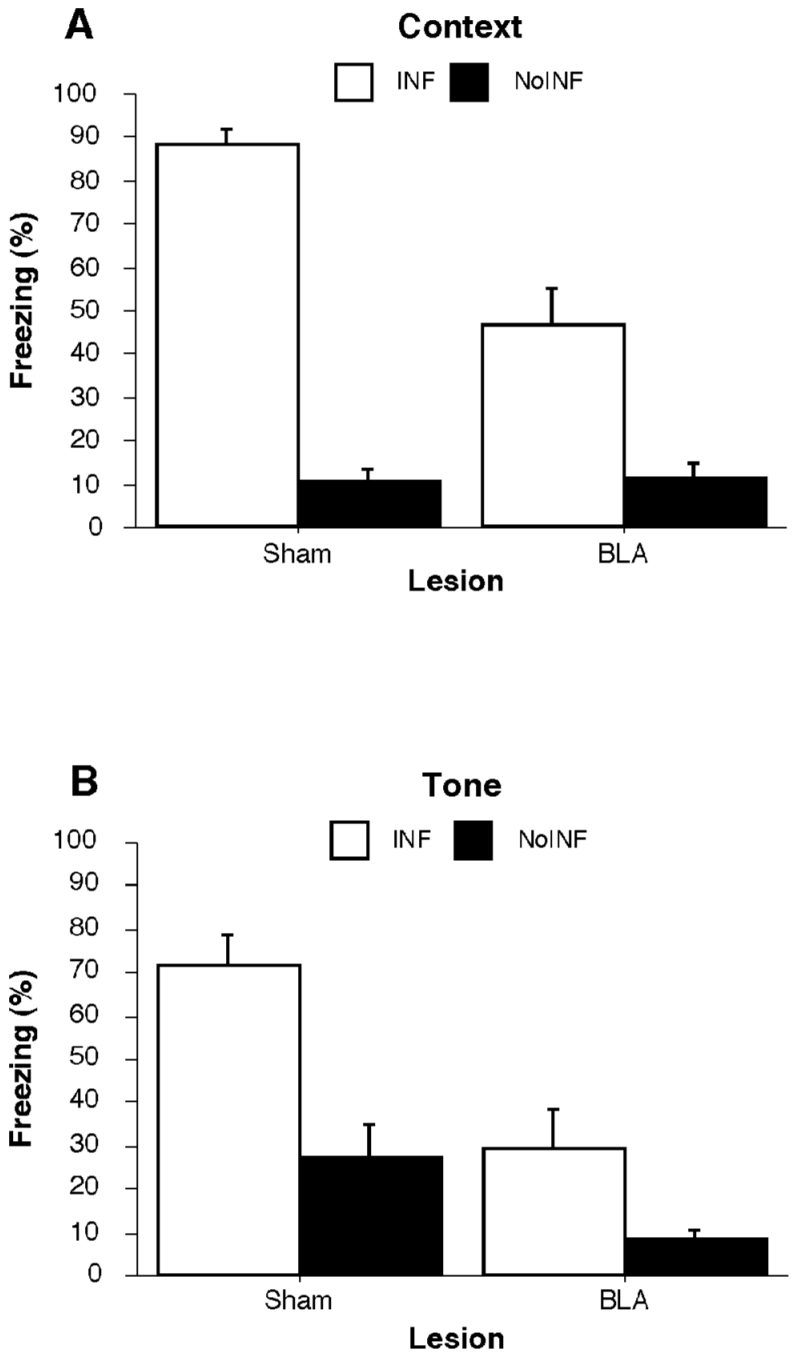
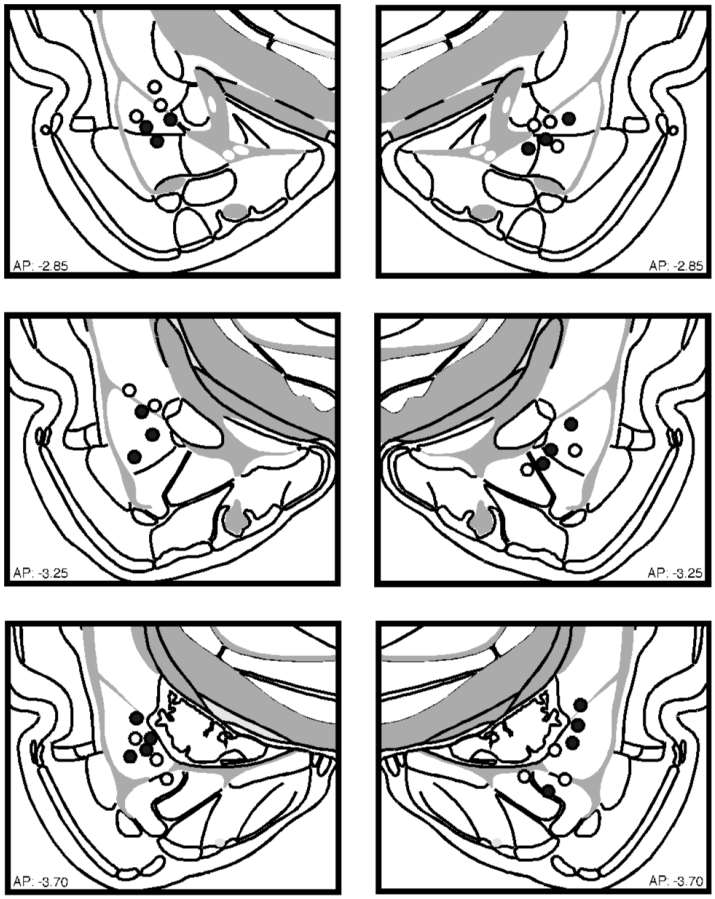


Figure 7.

Conditioned freezing during the context and tone tests (Experiment 2). *A*, Mean percentage of freezing (± SEM) during the 10-minute context test. *B*, Mean percentage of freezing (± SEM) during the 10-trial tone test for the INF groups (white bars) and for the NoINF groups (black bar) within each lesion type.



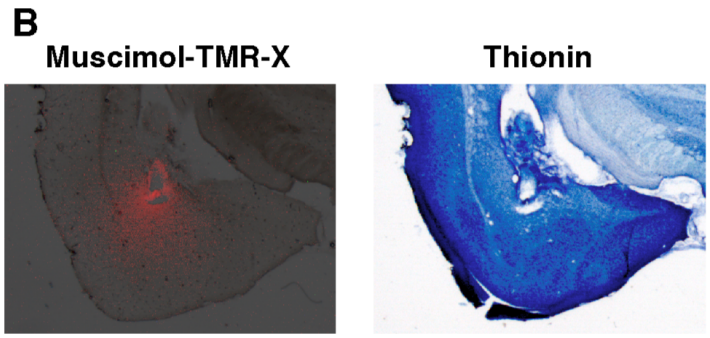


Figure 8.

A, Schematic representation of the locations of included cannula placements for the infusion of MUS (closed circles) or VEH (open circles) in the BLA for Experiment 3. A magnification of the amgydala is shown adjacent to the coronal brain sections. Coronal brain images were adapted from (Swanson, 1992). *B*, Representative images of muscimol-TMR-X spread 80 minutes following drug infusion (left) and a corresponding thionin-stained section (right).

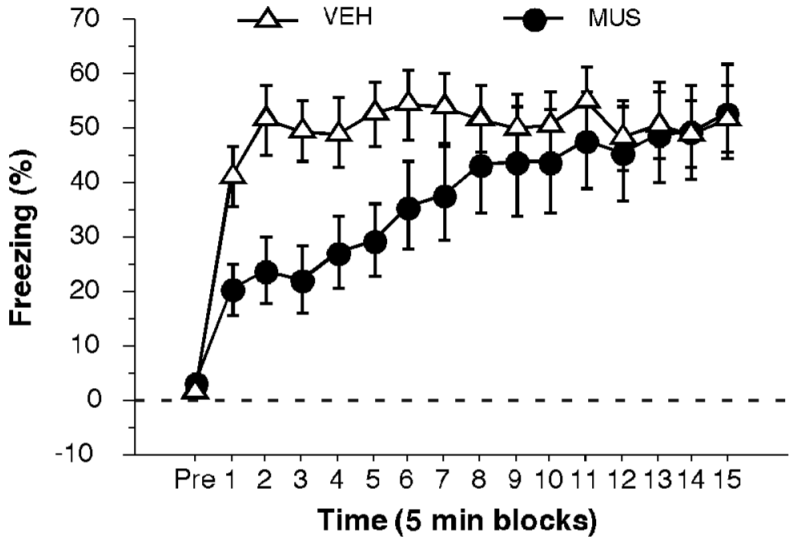


Figure 9.

Post-shock freezing in rats with pre-training muscimol infusions during the paired overtraining session (Experiment 3). *A*, Mean percentage of freezing (± SEM) during the 75-trial session. Data are shown for rats that received MUS (closed circles) and rats that received VEH (open triangles).

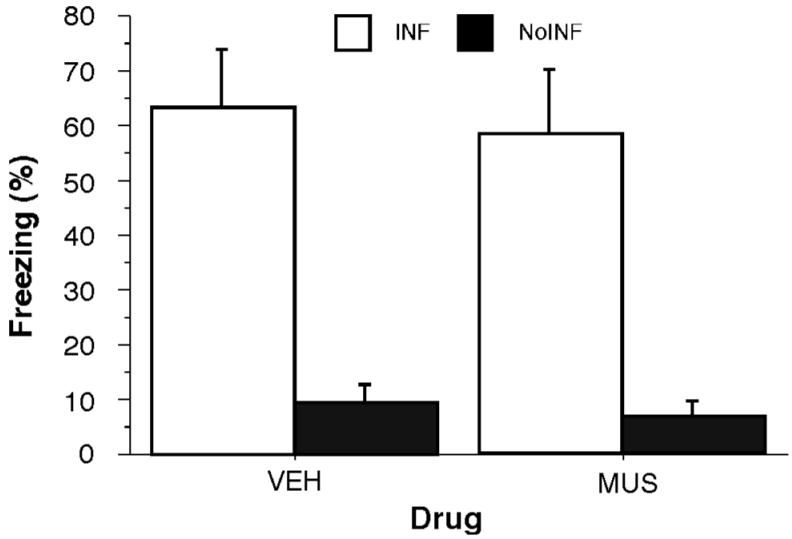


Figure 10.
Post-shock freezing during the inflation session (Experiment 3). Mean percentage of freezing (± SEM) during the post-shock ITIs for the INF groups (white bar) and the NoINF groups (black bar) within each drug group.

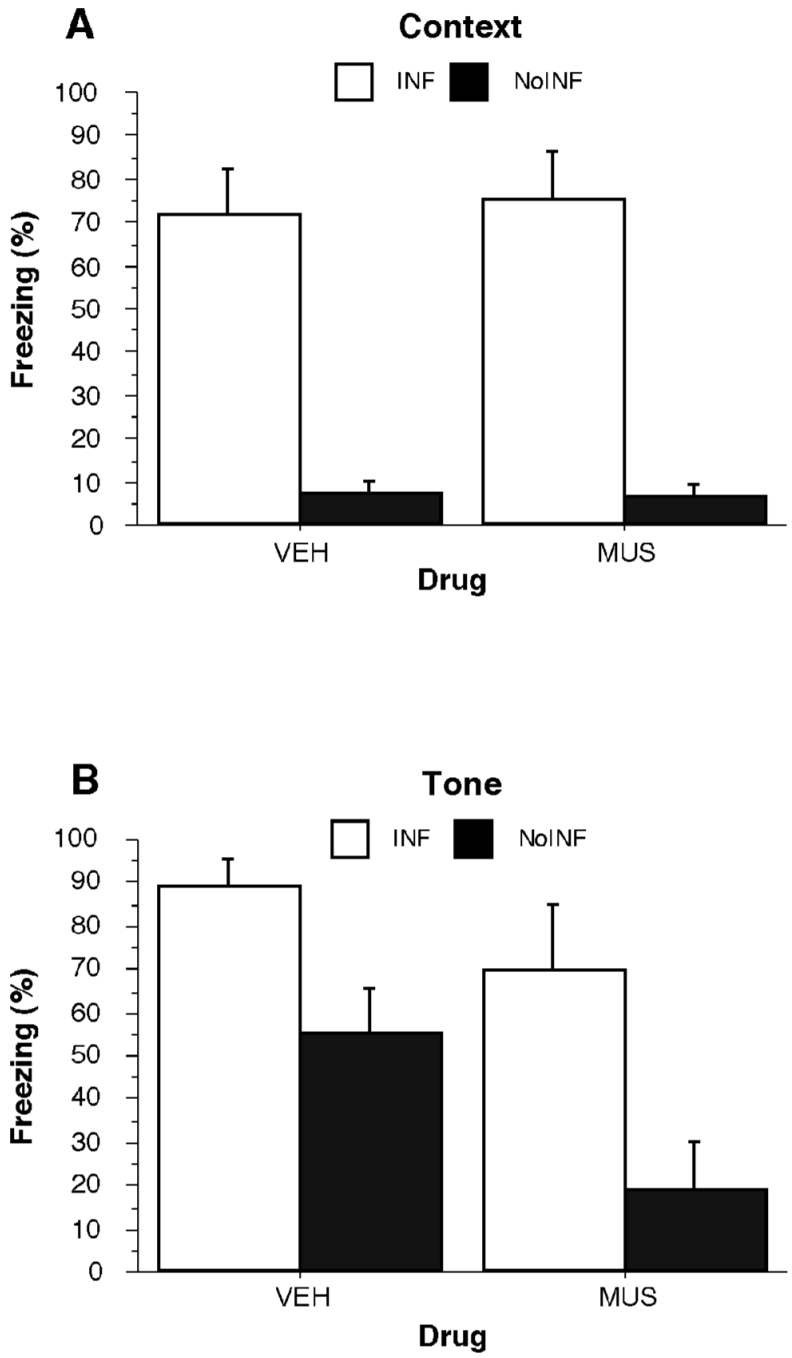


Figure 11.

Conditioned freezing during the context and tone tests (Experiment 3). *A*, Mean percentage of freezing (± SEM) during the 10-minute context test. *B*, Mean percentage of freezing (± SEM) during the 5-trial tone test for the INF groups (white bars) and for the NoINF groups (black bar) within each drug group.