Persistence of fear memory across time requires the basolateral amygdala complex

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Mammals evolved a potent fear-motivated defensive system capable of single-trial fear learning that shows no forgetting over the lifespan of the animal. The basolateral amygdala complex (BLA) is considered an essential component of this conditional fear learning system. However, recent studies challenge this view and suggest that plasticity within other brain regions (i.e., central nucleus of the amygdala) may be crucial for fear conditioning. In the present study, we examine the mnemonic limits of contextual fear conditioning in the absence of the BLA using overtraining and by measuring remote fear memories. After excitotoxic lesions of the BLA were created, animals underwent overtraining and were tested at recent and remote memory intervals. Here we show that animals with BLA lesions can learn normal levels of fear. However, this fear memory loses its adaptive features: it is acquired slowly and shows substantial forgetting when remote memory is tested. Collectively, these findings suggest that fear-related plasticity acquired by brain regions outside of the BLA, unlike those acquired in the intact animals, do so for a relatively time-limited period.

fear conditioning | forgetting | remote memory | savings

Failure to defend against an environmental threat such as predation exacts an extreme cost on adaptive fitness (1). Unlike a single missed feeding or mating opportunity, a single failure to defend means you will have no future opportunities to pass on your genes. As a consequence, mammals have evolved a potent fear-motivated defensive system that is capable of single-trial learning (2) and shows no forgetting over the lifespan of the animal (3). Prior studies suggest that such memories are normally established and permanently maintained within the basolateral complex of the amygdala (BLA) (3–7). Typically, damage to the BLA eliminates the acquisition and expression of Pavlovian fear memories across a wide spectrum of mammals, including humans (8) and rodents (5, 6, 9–13). Both electrophysiological and molecular markers of neural activity within the BLA reveal a learning- and retrieval-specific pattern of activation (14–18). Moreover, blockade of NMDA receptors or de novo protein synthesis within the BLA disrupts the acquisition and consolidation of fear memories (19–23). In addition, regardless of whether fear memories are 1 day or 1.5 years old, posttraining BLA lesions completely abolish the expression of fear (3). Collectively, such findings are consistent with a mnemonic role of the BLA in fear learning.

However, recent studies have challenged this view and suggest that plasticity in other brain regions are capable of supporting fear conditioning (24, 25). Indeed, there is growing evidence that fear conditioning can be established in the absence of the BLA. In particular, deficits resulting from either lesions or inactivations of the BLA can be overcome with extensive overtraining (7, 25–27). Furthermore, both Zimmerman et al. (25) and Wilensky et al. (24) have recently shown that disruption of central nucleus of the amygdala (CEA) function can prevent fear conditioning.

In light of these recent findings that suggest a role of BLA independent memory systems in fear conditioning, we wanted to investigate the mnemonic limits of contextual fear conditioning established in the absence of the BLA by measuring memory retention at recent (1 day) and remote (7 and 30 days) long-term memory intervals. In addition, we examined whether the absence of fear expression at these later retention intervals was due to either a retrieval or storage failure by testing for savings (experiment 2). Here we show that fear learning that occurs in the absence of the BLA, unlike BLA-based learning, reveals extreme deficits in the retrieval of remote memory and suggests that the storage of such memories rapidly decays as a function of time.

Results

Experiment 1: Context Fear Acquisition and the Retention of Recent and Remote Long-Term Memory in BLA Lesions Rats. The first experiment examined the acquisition of contextual fear conditioning and the retention of remote memory in BLA-lesioned animals. During surgery, animals received either pretraining excitotoxic (NMDA 20 mg/1 mL PBS; $n = 20$) or sham lesions ($n = 14$) of the BLA (Fig. 1). Seven animals from the BLA-lesioned group were excluded from the present analysis because of either unilateral damage to the BLA or bilateral damage that included the CEA. To examine the acquisition of fear, rats were overtrained with 76 presentations of a footshock (1 mA) in a novel context (26–28), during which time we used an online assessment of context conditioning as measured by postshock freezing (28). Animals were returned to the original training context (8 min) at either 1 or 30 days after acquisition to assess recent and remote memory retention, respectively (Fig. 1). Next, to examine the extent of the lesions, animals were overdosed (sodium pentobarbital), and their brains were fixed, extracted, and prepared for NeuN staining procedures (Fig. 2*A*).

Sham rats showed significant learning after one trial, and learning was asymptotic after the second trial (Fig. 3*A*). However, rats with excitotoxic lesions of the BLA acquired the freezing response at a significantly slower rate, reaching asymptotic levels close to those of the sham animals by the 20th trial. Such impressions were confirmed by a reliable 2-factor ANOVA that indicated a between group effect of lesion [$F(1, 150) = 7.43$; $P = 0.01$] and a repeated measure factor for trials [$F(6, 150) = 21.10$; $P < 0.0001$]. There was also a reliable lesion by trial interaction [$F(6, 150) = 2.28$; $P < 0.05$]. When memory was tested 24 h later, BLA- and sham-lesioned rats expressed nearly identical levels of fear (Fig. 3*B*). A separate group of sham-lesioned animals showed no evidence of forgetting, when tested 30 days later. However, BLA-lesioned animals expressed robust levels of freezing during overtraining and failed to express a significant level of contextual fear at this 30-day interval. This was confirmed by 2×2 factorial ANOVA revealing a reliable interaction between lesion and retention interval [$F(1, 24) = 6.20$; $P < 0.05$].

Experiment 2: Savings of Fear at Remote Memory Retention Intervals. Fear memories are relatively stable across time and show virtually no forgetting. However, animals that learned fear

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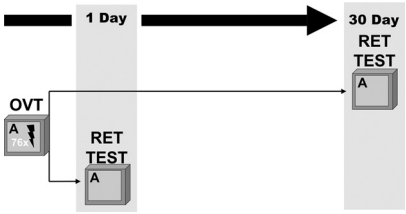

Fig. 1. Experimental design for experiment 1. Animals with sham or BLA lesions were overtrained for context fear and retention tested 1 or 30 days later.

without a BLA showed total forgetting within 30 days. Forgetting has been attributed to either a loss of stored information or a failure to retrieve stored information. In the second experiment, we determined whether the absence of fear at remote memory intervals in BLA-lesioned animals was due to either a retrieval or storage failure. As described in experiment 1, pretraining sham (n = 19) or BLA lesions (n = 88) were created (see Fig. 2*C*), and animals were presented with context fear overtraining. Animals with either unilateral BLA damage or bilateral damage that included the CEA were removed from the present analysis (n = 22). Remote fear memory was indexed at 7- and 30-day

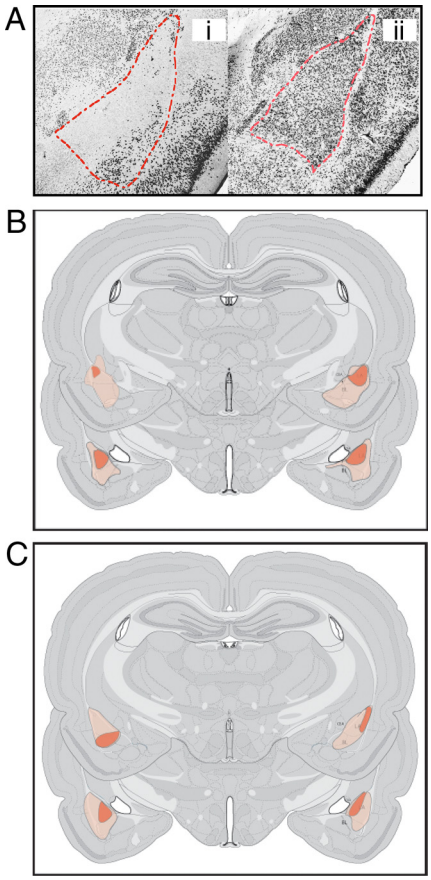

Fig. 2. Histological analysis of BLA or Sham lesions for Experiments 1 and 2. (*A*) Representative photomicrograph of coronal sections (4× magnification) through the amygdala stained with NeuN antibody: (i) lesion, (ii) sham. The dotted red line depicts the extent of the BLA (lateral and basal nuclei). (*B*) Experiment 1: Reconstruction of the minimal (red) and maximal (pink) excitotoxic lesion in the region of the BLA (AP 2.6 and 3.3 mm). Coronal section images of the brain taken and adapted from Swanson (40). (*C*) Experiment 2: Reconstruction of the minimal (red) and maximal (pink) excitotoxic lesion in the region of the BLA (AP 2.6 and 3.3 mm). Coronal section images of the brain taken and adapted from Swanson (40).

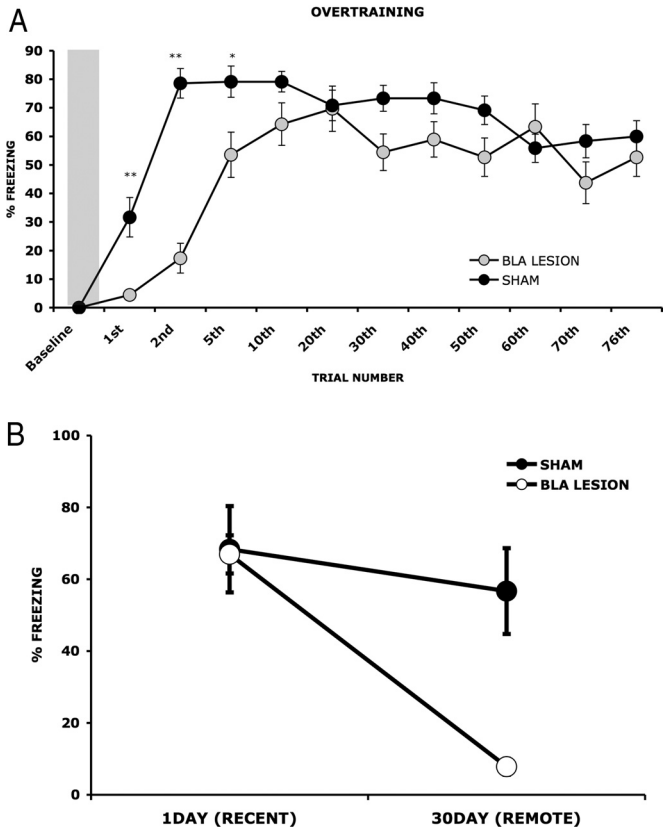

Fig. 3. Behavioral measurements of Fear Conditioning in sham or BLA lesioned animals, during 76 trial fear acquisition and a test context fear memory retention at 1 or 30 days after overtraining.(*A*) Acquisition of conditional fear as indexed by percent time observed freezing. Mean (±SEM) percentage of observations spent freezing during a 76-footshock contextual fear conditioning session. (*B*) Total mean (±SEM) percentage of observations spent freezing during an 8-min test session for conditional fear.

retention intervals in different animals. Next, to assess the savings of fear, animals were returned to the original training context, presented with a single footshock (1 mA) savings trial, and tested for freezing the next day. Such a test of savings, where memory is restored after a minimal amount of retraining, has been shown to be a very powerful assay of retrieval failure (29).

As a control for nonassociative sensitization to the repeated footshocks (30), we included another group of BLA-lesioned animals overtrained, and 7 and 30 days later given a single footshock—this time in a novel context where sensitized freezing was measured the next day. As indicated in experiment 1, animals with BLA lesions failed to exhibit contextual fear after a single trial (see Fig. 3*A*). To provide a baseline measure of freezing in animals incapable of acquiring fear conditioning, another group of BLA-lesioned animals, instead of receiving 76-trial overtraining, were presented single-trial context fear training with memory retention tested at 1- or 30-day intervals followed by savings tests (Fig. 4).

For each animal, we calculated a savings score, which was the difference between the savings test (1 day after savings trial) and the original retention test (at either 7 or 30 days after training). To distinguish between freezing based on a context-shock association and any nonassociative freezing caused by shock exposure, we also calculated a sensitization score for each rat. This sensitization score was the difference between freezing in a novel chamber 7 or 30 days after training and the original retention test.

As shown in Fig. 5, sham animals when tested 7 and 30 days after overtraining expressed robust levels of contextual fear,

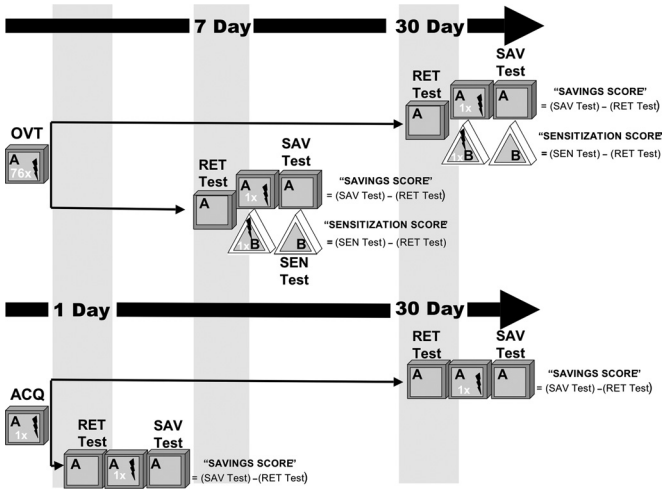

Fig. 4. Experimental design for experiment 2. Animals with sham or BLA lesions were overtrained or single-trial trained with context fear tested 7 and 30 days later. BLA-lesioned animals were given an additional footshock in the original overtrained context (*A*) or in a novel context (*B*), and the next day were returned to the same context to measure savings or sensitization, respectively.

whereas animals with BLA lesions at matched intervals failed to exhibit significant levels of fear [$F(3, 53) = 51.675$; $P < 0.001$]. BLA-lesioned animals given an additional shock in context A following a 7-day, but not 30-day, retention test, showed a significant increase in freezing (savings test [$F(1, 22) = 10.56$; $P < 0.01$]). However, the same shock in a different context failed to increase freezing following either a 7- or 30-day retention test (sensitization test [$F(1, 15) = 1.30$; $P > 0.05$]).

Overall, savings as indicated by savings scores were highest in animals with BLA lesions, overtrained, and tested at the 7-day interval [see Fig. 6; $F(5, 33) = 3.406$; $P < 0.05$]. When savings in a similar group was tested at a 30-day interval, savings scores were significantly lower, as confirmed by an LSD post hoc analysis ($P = 0.01$). This lower level of savings was also similar to BLA-lesioned animals given single-trial conditioning and tested at 1 ($P = 0.01$) or 30 days ($P < 0.01$). A similar trend was found in BLA-lesioned sensitization control animals, which produced similarly low sensitization scores as a result of single footshock in a novel context whether tested at 7 ($P = 0.001$) or 30 days ($P < 0.01$). Finally, comparison of raw freezing percentages revealed significantly greater freezing during the savings vs. sensitization test at the 7-day (*t* test: $P < 0.02$) but not the 30-day

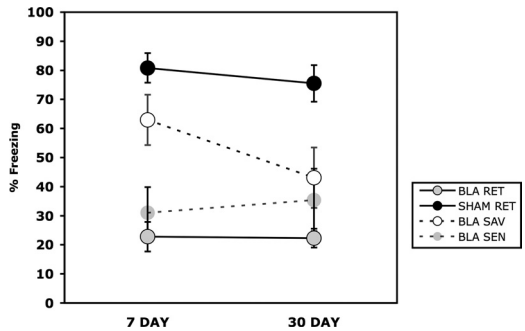

Fig. 5. Total mean (±SEM) percentage of observations spent freezing during an 8-min test session for conditional fear. BLA RET, BLA-lesioned group with retention test at 7 or 30 days; SHAM RET, sham group with retention test at 7 or 30 days; BLA SAV, BLA-lesioned group with saving tested 2 days following retention test at 7 or 30 days; BLA SENS, BLA-lesioned group with sensitization tested 2 days following retention test at 7 or 30 days.)

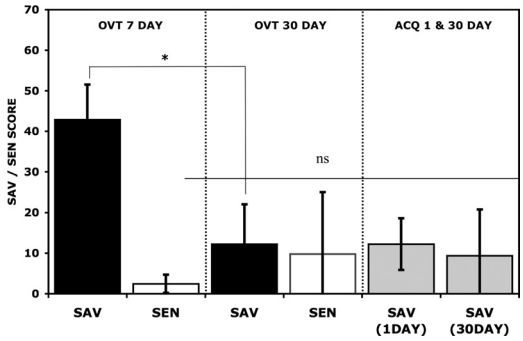

Fig. 6. Savings (SAV) and sensitization (SEN) mean scores as derived from subtracting the percent freezing between retention test and savings or sensitization test.

retention test interval [$F(2, 19) = 0.838$; $P > 0.05$], including savings in single-trial conditioning group.

Discussion

Previous work in our laboratory has tested remote fear memory at a large number of retention intervals (7, 41, 42). In all of this work, we have never seen a failure of fear memory regardless of the length of the retention interval (1 day to 1.5 years). This key adaptive feature of fear—a mammal never fails to remember a threat that it has learned about—also means that maladaptive fears can have a lifelong negative impact. The unique finding in this study is that fear memories are rapidly forgotten if they were formed without the BLA. Furthermore, this demonstrates a dissociation between long-term memory for fear mediated by the BLA and fear established independently of the BLA. In particular, fear established independent of the BLA, which can be expressed at least 1 day later, is completely absent in animals tested at 7 and 30 days. Thus, it is not only very difficult to learn fear without the BLA, but that fear loses one of its key features—longevity.

Particularly striking here is the absence of savings in overtrained animals with BLA lesions and tested 30 days later. Savings is a powerful test of retrieval because it is a reminder of all of the original training components (i.e., conditional stimulus [CS], unconditional stimulus [US], and contingency), but it is also a very liberal test because it allows for new learning. Despite this we detected no savings at 30 days, which supports the idea that encoded information was lost from storage. Normally the BLA allows for more efficient learning to occur and is the final place of memory storage. However, without the BLA, other brain regions support learning (though at a less efficient rate), and are incapable of maintaining permanent memory stores. Given this, there was evidence of savings at 7 days. The residual performance at this retention interval may have resulted from a loss in retrievability or a partial loss of encoded information. In any case, over the course of 30 days, the storage of fear memories rapidly decayed to levels roughly similar to naïve animals.

The savings evident at the 7-day intervals could not be attributed to the sensitization (nonassociative component) of fear resulting from the overtraining procedure, given that the same number of footshocks produced significantly lower sensitization scores at 7- or 30-day time points. These findings strongly suggest that fear acquired by brain regions outside of the BLA, unlike that acquired in intact animals, is relatively time limited, and that the relative permanence of conditional fear maybe an exclusive property of the BLA. These findings also refute a strictly modulatory role for the BLA in memory consolidation given that both BLA- and sham-lesioned animals expressed nearly identical levels of freezing when tested 24 h later (31). Together, these findings argue against a long-term mnemonic role for fear learning established by neural

structures outside of the BLA, as required by memory modulation of BLA function.

Given the present findings, recent studies have implicated a mnemonic role for the CEA similar to the BLA in the development and storage of fear memories. In both studies by Zimmerman et al. (25) and Wilensky et al. (24), either reversible inactivations or protein synthesis inhibition of the CEA disrupted the acquisition of auditory fear conditioning. In addition, Maren and colleagues (25) demonstrated that permanent or reversible lesions of the CEA disrupted the development of contextual fear conditioning, despite use of an overtraining procedure similar to the one used in the present study. Moreover, a recent paper by Kolber et al. (32) showed that the specific deletion CEA glucocorticoid receptors disrupted fear acquisition and could be subsequently rescued by intracranial ventricular infusions of corticotrophin releasing hormone (CRH). The findings presented here, and previous studies indicating a role of the CEA, suggest a reevaluation of the present serial model of fear conditioning and a possible dissociation of the contributions of the CEA and BLA in the acquisition and storage of fear memories. Further investigations of interactions between the BLA and CEA as well as other fear-related circuitry, such as the bed nuclei of the stria terminalis and ventral medial hypothalamus, could provide a more detailed analysis of neural circuits underlying fear conditioning. Interestingly, all 4 of these regions, which receive afferents from the hippocampus and send projections to the ventral periaqueductal gray (the final output in the expression of freezing), express CRH receptors and, if lesioned, attenuate fear responding (32–36). Our data are consistent with the suggestion that plasticity in regions outside of the BLA can support the acquisition of fear (24, 25). However, these forms of plasticity do not appear to be able to sustain a memory for the extraordinary long periods that plasticity in the BLA can support (3).

The present data indicate that the BLA is a crucial component of remote contextual fear memory. Recently there has been much attention to prefrontal cortical structures that are involved in remote contextual fear memories (37–39). The genetic modification of the alpha-CaMKII that affected remote memory was also not only present in the cortex but in the BLA as well (38). The data presented here indicates that the BLA is necessary for the establishment and/or maintenance of remote memory. Knowledge of the molecular events that mediate permanent memory remains elusive. Contrasting differences in the mechanisms required for the plasticity supporting similar fear memories established with and without the BLA may provide insight into these mechanisms. In conclusion, the present findings suggest that an important adaptive quality of fear memory—its persistence across time—may be an exclusive property of the BLA.

Methods

Subjects. Subjects were 141 male Long-Evans hooded rats (300–450 g; Harlan) were individually housed on a 12-h light/dark cycle in hanging cages and provided ad lib access to food and water. All rats were cared for in accordance with guidelines established by the University of California, Los Angeles, and approved by the UCLA Chancellor's Animal Research Committee.

Surgery. Rats were anesthetized with an i.p. injection of sodium pentobarbital (65 mg/kg) and atropine methyl nitrate 0.04 mL (0.1 mg/kg). An incision was made across the midline of the scalp in which the tissue and skin were retracted, exposing the skull. A craniotomy was performed over the site of cannula insertion. The orientation of the skull was adjusted so that lambda and bregma aligned in the same horizontal plane. Infusion cannulae (33 gauge) were positioned 2.6 and 3.3 mm posterior, 5.0 mm lateral, and 8.0 and 8.2 mm ventral to bregma. Infusions of NMDA (20 mg/mL of PBS) were made via 5-μL microsyringe (Hamilton Instruments) connected to polyethylene (PE20) tubing attached to infusion cannula. Compression of the syringe was produced by a syringe pump (Harvard Apparatus) at a rate of 0.1 μL/min. Total volume infused at each of the 4 sites was 0.2 μL. At the termination of the infusion, cannulae were maintained at the site of infusion for an additional 2 min to allow for the diffusion of NMDA from the tip of the cannulae. Stainless steel wound clips were applied along the extent of the incision. Thereafter, all animals were given the antibiotic baytril in their drinking water for the next 5 days, and i.p. injections of the analgesic/anti-inflammatory ketoprofen (2 mg/kg) for 2 days after surgery. Rats were allowed a total of 10 days to recover from surgery.

Apparatus. Context A consisted of 6 clear plastic observation chambers (28 $\times$ 21 $\times$ 21 cm; Lafayette Instruments). The floor of each chamber consisted of 18 stainless steel rods spaced 1.5 cm apart. The rods were wired to a shock generator and scrambler, which were controlled by a programmable stimulus controller (MED Associates). An electrical fan provided background noise during all training sessions. Before and after each training session a 5% solution of sodium hydroxide was used to wipe clean the interior of each chamber. In addition, below the rod floor of each chamber, a stainless steel pan was coated with benzaldehyde solution (5 μL/2 mL of 100% ethanol) as a background odor before each session.

Context B consisted of 6 distinct plastic observation chambers with the sidewalls collapsed at the top to form an A-shaped interior. The floor of context B chamber consisted of 18 stainless rods vertically staggered to be spaced 1.5 cm apart. A white noise generator provided background noise during the use of context B. Before and after each training session a 2% solution of acetic acid was used to wipe clean the interior of each chamber. In addition, below the rod floor of each chamber a stainless steel pan was coated with this same solution as a background odor before each session. The chamber activities were recorded via video cameras positioned on the wall opposite to each pair of chambers. Rats were recorded continuously during overtraining and test sessions.

Behavioral Training. All animals were transported to observation chambers in their home cages. Overtraining consisted of the delivery of 76 footshocks (1 mA; 2 s) spaced 64 s apart. Two minutes following placement in the observation chamber, the first footshock was delivered. Rats in experiment 1 were then assigned to 1 of 4 groups: sham/1 day, sham/30 day, lesion/1 day, and lesion/30 day. Sham/1-day and lesion/1-day animals were returned to the observation chamber to measure context fear at 24 h, and sham/30-day and lesion/30-day animals were returned to observation chambers at 30 days following the overtraining session. Rats in experiment 2 were assigned to 1 of 8 groups: savings (sham/7 day, sham/30 day, lesion/7 day, lesion/30 day), sensitization (lesion/7 day, lesion/30 day), and single trial (lesion/1 day, lesion/30 day). Savings and sensitization groups received overtraining followed by 7- and 30-day test of context fear as described in experiment 1.

Twenty-four hours following context testing, savings animals were returned to the original observation chamber, and sensitization animals were placed in a novel observation chamber (odor: acetic acid; background noise: white noise; background illumination: red fluorescent lighting; chamber floor: vertically staggered rods; and chamber walls: A-shaped white opaque plastic) and after 2 min received a single footshock (1 mA; 2 sec). The next day, all animals were returned to the same observation chamber for a savings or sensitization test. Single-trial animals, instead of receiving overtraining, were placed in observation chambers and, following 2 min, received a single footshock and were returned 1 day or 30 days later to measure context fear. Twenty-four hours following context testing, animals were returned to the original observation chamber and, following 2 min, received a single footshock (1 mA; 2 sec). The next day, all animals were returned to the same observation chamber for a savings test. All test sessions consisted of animals being placed in the observation for a total of 8 min and 32 s.

Behavioral Measures. Observation of freezing behavior was used as an index of conditional fear. Freezing was defined as the lack of movement, with the exception of those related to respiration. Measurements of freezing were taken from videotaped recording of each overtraining and test session by an observer blind to the experimental condition of each rat. Freezing responses were transcribed in 64-s epochs every 8 s for each rat.

During overtraining, postshock freezing behaviors were scored after the first, second, fifth, 10th, 20th, 30th, 40th, 50th, 60th, 70th, and 76th footshocks, whereas during test sessions, freezing was scored throughout the entire session.

Histology and Immunohistochemistry. Following training procedures, all rats were deeply anesthetized with sodium pentobarbital and intracardially perfused with 0.9% saline solution followed by 4% paraformaldehyde solution. The brain was extracted from the skull, placed in a 4% solution of paraformaldehyde for 1–2 days, and then placed in a 30% solution of sucrose for another 1–2 days. Brains were then placed in a cryostat at -18 °C and cut into 50-μm coronal sections. Sections that included the amygdala were placed in holding wells that contained 1$\times$ PBS.

Immunohistochemistry. Brain sections were washed in PBS for 5 min (3 times) on a mechanical shaker and placed in a blocking solution (10% goat serum, PBS, and 0.5% Triton) for 1 h. Sections were then placed in NeuN antibody (1:4,000) prepared in 2% goat serum solution (PBS and 0.25% Triton) at 4 °C for 24 h. Tissues were then rinsed in PBS (0.25% Triton) for 15 min (4 times) and transferred to mouse antibody IgG at room temperature for 1 h and 15 min. After the sections were rinsed in PBS for 10 min (4×), they were placed in a solution of avidin-biotin-peroxidase complex on a shaker for 1 h. Sections were rinsed once more in PBS (6 times) and transferred into wells containing 3–3′ diaminobenzidine tetrahydrochloride (Sigma) as a chromagen. Sections were free floated onto subbed microscope slide and coverslipped.

Verification of lesions was made using NeuN-stained tissue viewed under a microscope (Carl Zeiss Inc.) magnified at 1.25× and 4×. The region of the amygdala was outlined and examined for NeuN staining. The lack of staining in this region was used as an indication of neuronal loss due to lesions performed during surgery.

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