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Measuring Pavlovian fear with conditioned freezing and conditioned suppression reveals different roles for the basolateral amygdala

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Abstract

In Pavlovian fear conditioning, pairing a neutral cue with aversive foot shock endows a cue with fear-eliciting properties. Studies of Pavlovian fear conditioning measuring freezing have demonstrated the basolateral amygdala (BLA) to be critical to both fear learning and memory. The nucleus accumbens core (NAc), while not important to freezing, is important to the enhancement of instrumental responding by cues paired with food reward. In the present study we investigated the role of the BLA and the NAc in another property of fear cues, the ability to suppress instrumental responding for food rewards (conditioned suppression). Sham, BLA and NAc-lesioned rats received a fear discrimination procedure in which one visual cue (CS+) predicted foot shock while a second cue (CS−) did not. Conditioning took place over a baseline of instrumental responding, allowing for concurrent measure of freezing and instrumental suppression. NAc lesions left fear conditioning fully intact. BLA lesions impaired acquisition and discrimination of fear when assessed with conditioned freezing. However, BLA lesions only altered fear acquisition and left discrimination completely intact when assessed with conditioned suppression. These findings suggest a critical role for the BLA in fear when assessed with conditioned freezing but a diminished role when assessed with conditioned suppression.

Keywords

conditioned freezing; conditioned suppression; nucleus accumbens; associative learning; amygdala; anxiety

1. Introduction

In Pavlovian fear conditioning a neutral cue is made to predict electric foot shock. As a result, the cue comes to elicit a variety of behavioral and autonomic responses (Bolles and Collier, 1976; Iwata et al., 1986; Kapp et al., 1979), one of which is freezing. Studies of freezing in Pavlovian fear conditioning have found a prominent role for the basolateral amygdala (BLA). Pharmacological manipulations or lesions of the BLA impair the normal acquisition of Pavlovian fear (Gale et al., 2004; Koo et al., 2004; Maren et al., 1996; Muller et al., 1997; Petrovich et al., 2009; Phillips and LeDoux, 1992). In addition to eliciting freezing a fearful cue will also strongly suppress instrumental responding for food rewards

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(Estes and Skinner, 1941), an effect termed conditioned suppression. Previous work suggests that conditioned freezing and conditioned suppression may be mediated, in part, by separate neural circuits. Lesions of the central nucleus of the amygdala (CeA) (Lee et al., 2005; McDannald, 2010) but see (Killcross et al., 1997) or ventrolateral periaqueductal grey (vlPAG) (Amorapanth et al., 1999; McDannald, 2010) that impair conditioned freezing leave conditioned suppression relatively or fully intact. Conditioned suppression may be especially relevant to human anxiety disorders in which anxious thoughts or feelings interfere with normal function.

The present study used a fear discrimination procedure to ask whether the BLA plays similar or different roles in acquiring fear through conditioned freezing and conditioned suppression. Additionally, we asked whether the nucleus accumbens core (NAc) might contribute to acquisition and discrimination in conditioned suppression that is independent of conditioned freezing. NAc lesions do not impair cued freezing (Jongen-Relo et al., 2003; Levita et al., 2002). A role for the NAc in conditioned suppression would be in accordance with its role in the enhancement of instrumental responding by rewarding cues (de Borchgrave et al., 2002; Hall et al., 2001) but see (Corbit et al., 2001) plus evidence from electrophysiology (Setlow et al., 2003) and immediate-early genes (Beck and Fibiger, 1995; Campeau et al., 1997; Thomas et al., 2002) showing the NAc to encode aversive cues. Rats with neurotoxic lesions of either the BLA or NAc received Pavlovian fear conditioning in which one visual cue (CS+) predicted foot shock while a second cue (CS−) did not. Conditioning took place over a baseline of instrumental responding, allowing for concurrent assessment of conditioned freezing and conditioned suppression. The roles of the BLA and NAc in Pavlovian fear conditioning are then discussed.

2. Results

Histology

Neurotoxic lesions were targeted to the lateral and basal subregions of the BLA (n = 11) or the core subregion of the nucleus accumbens (n = 11). The BLA and NAc borders were defined using Nissl stained sections in combination with a brain atlas (Swanson, 2003). Photomicrographs showing representative intact, lesioned BLA and lesioned NAc are shown in Figure 1. BLA lesions were rejected if bilateral damage was less than 75% or confined to one hemisphere. Nine of eleven rats were determined to have successful BLA lesions. Damage was quantified at four anterior-poster levels and was as follows: bregma ***−2.12***, 89.7 ± 5.1%; ***−2.56***, 94.85 ± 2.1%; ***−3.14***, 86.9 ± 4.7%; and ***−3.60***, 83.9 ± 5.7%. The average lesion comprised 88.8% ± 3.2 of the total BLA area. Little or no damage was observed within the adjacent, central nucleus of the amygdala. NAc lesions were rejected if bilateral core damage was less than 50% or was confined to one hemisphere. Eight of eleven rats were determined to have successful NAc core lesions. Damage was quantified at four anterior-poster levels and was as follows: bregma ***+2.20***, 41.7 ± 11.4%; ***+1.70***, 50.5 ± 12.2%; ***+1.20***, 76.9 ± 5.0%; and ***+0.70***, 85.6 ± 2.9%. The average lesion comprised 64.6% ± 6.5 of the total NAc core with greatest damage evident in the posterior. NAc damage can be seen in the Neu-N stained sections. NAc lesions were also typically accompanied by shrinkage of the area and enlargement of the adjacent ventricle. Damage to the adjacent shell subregion was less than 10% on average. No tissue damage was observed in either Sham-BLA (n = 5) or Sham-NAc (n = 5) lesions.

Freezing

BLA lesions impaired the acquisition of CS+ freezing. Figures 2A-C show mean ± SEM percent time freezing during pre-CS, CS− and CS+ over the course of fear conditioning; discrimination is shown in six, two-day blocks. ANOVA (day x cue x lesion) for mean

percent time freezing during pre-CS and CS+ over all days of conditioning found significant effects of cue ($F_{(1,24)} = 65.02$, $p < 0.01$), day ($F_{(8,192)} = 8.82$, $p < 0.01$), and the cue x day interaction ($F_{(8,192)} = 9.07$, $p < 0.01$). Of greater interest, were the significant effects of lesion ($F_{(2,24)} = 4.75$, $p < 0.05$) and cue x lesion interaction ($F_{(2,24)} = 4.47$, $p < 0.05$). Across all days of conditioning Sham ($p < 0.01$) and NAc ($p < 0.01$) rats, but not BLA rats ($p > 0.1$) froze significantly more during CS+ than pre-CS. Also across all days Sham ($p < 0.05$) and NAc rats ($p < 0.01$) froze at significantly higher levels during the CS+ than BLA rats.

BLA lesions impaired discriminative freezing between CS− and CS+. ANOVA (day x cue x lesion) for mean percent time freezing during pre-CS, CS− and CS+ over the six blocks of discrimination revealed significant effects of cue ($F_{(2,48)} = 48.58$, $p < 0.01$), lesion ($F_{(2,24)} = 4.65$, $p < 0.05$), and the cue x lesion interaction ($F_{(4,48)} = 2.99$, $p < 0.05$). Post-hoc comparisons found that Sham rats acquired significant discriminative freezing ($p < 0.05$) between CS− and CS+ by discrimination block d5 (Figure 2A), NAc rats by d4 (Figure 2B) while BLA rats (Figure 2C) never acquired significant discrimination.

Instrumental Suppression

BLA lesions altered the pattern of acquisition of CS+ suppression and reduced the absolute level of CS+ suppression. Figures 2D-F show the mean ± SEM Kamin ratios for CS− and CS+ over the course of fear conditioning. ANOVA (day x lesion) for mean CS+ Kamin ratios on all days of conditioning found significant effects of day ($F_{(14,336)} = 13.59$, $p < 0.01$) and the day x lesion interaction ($F_{(28,336)} = 1.68$, $p < 0.05$). Both Sham and NAc showed highly significant differences in CS+ Kamin ratios from f1 to d6 (ps < 0.01) while BLA rats showed no differences ($p > 0.1$). ANOVA (day x lesion) for CS+ Kamin ratios for only the six discrimination blocks revealed a significant lesion effect ($F_{(2,24)} = 5.17$, $p < 0.05$). Post-hoc comparisons found that Sham and NAc rats had significantly lower CS+ Kamin ratios than BLA rats (ps < 0.05). However, all rats showed Kamin ratios significantly lower than 0.5 indicating all rats acquired conditioned suppression. Mean ± SEM CS+ Kamin ratios for discrimination block d6 were: Sham 0.05 ± 0.02; NAc 0.04 ± 0.02 and BLA 0.16 ± 0.05.

BLA lesions left discriminative suppression between CS+ and CS− fully intact. ANOVA (day x cue x lesion) for mean CS+ and CS− Kamin ratios over the six blocks of discrimination revealed a significant effect of cue ($F_{(1,24)} = 60.89$, $p < 0.01$) lesion ($F_{(2,24)} = 4.46$, $p < 0.05$) but no cue x lesion interaction ($F_{(1,24)} = 0.14$, $p = 0.86$). Post-hoc comparisons found that Sham (Figure 2D) and BLA rats (Figure 2F) acquired significant discriminative suppression by discrimination block d3, while NAc (Figure 2E) rats acquired by d4.

Unconditioned responses to foot shock

Neither BLA nor NAc lesions impaired unconditioned responses to foot shock as assessed by general activity or lever-pressing. Figure 3A shows the mean ± SEM activity/min for pre-CS and foot shock on all days of fear conditioning. ANOVA (day x period x lesion) for mean pre-CS and foot shock activity found significant effects of day ($F_{8,192} = 2.12$, $p < 0.05$) and period ($F_{1,24} = 68.88$, $p < 0.01$). Post-hoc comparisons found greater activity to the foot shock, compared to baseline in all groups (ps < 0.01). Figure 3B shows the mean ± SEM lever-press/min for pre-CS and foot shock on all days of fear conditioning. ANOVA (day x period x lesion) for mean pre-CS and foot shock lever- pressing found significant effects of day ($F_{8,192} = 3.07$, $p < 0.01$), period ($F_{1,24} = 102.4$, $p < 0.01$) and the period x day x lesion interaction ($F_{16,192} = 1.72$, $p < 0.05$). The latter effect was driven by a lack of difference between pre-CS and shock lever-pressing by NAc rats on the final day of

discrimination. Post-hoc comparisons found significantly lower lever-press rates during the foot shock, compared to baseline in all groups (ps < 0.01).

3. Discussion

Sham rats quickly acquired freezing and came to suppress instrumental responding during the CS+. Initially, the CS− also produced significant freezing and suppressed instrumental responding. By the end of conditioning excellent discrimination between CS+ and CS− was observed in both measures: Sham rats selectively froze during the CS+ and performed instrumental actions during the CS−. The acquisition of conditioned behavior was accompanied by normal unconditioned behavior to the foot shock, which included a burst in activity upon foot shock presentation and suppression of instrumental responding. Important to the interpretation of lesion findings was the observation that discrimination occurred at similar, but not identical, rates in freezing and instrumental suppression. Significant discrimination in conditioned freezing was not present until the 5^{th} block while discrimination in conditioned suppression occurred by the 3^{rd} block. Thus, while ending at comparable levels, the rate of discrimination did differ between these two measures.

As a final note, the present study differed from other Pavlovian fear conditioning studies in that no final extinction test was given. In the present study lesions took place before any conditioning. Thus, performance in a final probe test would offer little insight into BLA or NAc function since any deficit present could be attributed to a deficit in acquisition. For this reason we focus on a detailed account of fear acquisition in Sham, NAc and BLA rats.

Effects of NAc lesions

The present results suggest that the NAc is not necessary for the acquisition of either conditioned freezing or conditioned suppression. Like Shams, NAc rats quickly came to suppress lever-pressing and also demonstrated robust freezing during the CS+. Initially the CS− elicited the same responses as the CS+ but as discrimination continued the CS− ceased to suppress lever-pressing and also produced lesser freezing. Thus, NAc lesions had no effect on discrimination between CS+ and CS− when assessed with conditioned freezing or conditioned suppression. The lack of an effect of NAc lesions on conditioned freezing is consistent with previous reports (Jongen-Relo et al., 2003; Levita et al., 2002) although the finding of no effect on conditioned suppression is novel. The present findings should be interpreted with some caution as they do not preclude a role for the NAc in Pavlovian fear. In a recent experiment (McDannald et al, under review) we trained Sham and NAc-lesioned rats with appetitive conditioning procedures in which a low-value cue predicted one food reward and a high-value cue predicted three food rewards. Sham rats acquired greater conditioned responding to the high-value cue than the low-value cue while NAc rats showed a trend towards impairment in this discrimination. Thus, NAc might be critical for acquiring relative value but not absolute value. A similar role for the NAc in Pavlovian fear may be revealed by using procedures in which different cues predict different intensities or quantities of foot shock.

The lack of effect of NAc lesions on the suppression of instrumental responding by fear cues is in contrast to its established role in the enhancement of instrumental responding by appetitive cues (de Borchgrave et al., 2002; Hall et al., 2001). At least two forms of instrumental enhancement by appetitive cues have been identified: one relying on specific features of food rewards and another on general features (Corbit and Balleine, 2005). Here we chose to investigate the NA core because it has been implicated in enhancement driven by general features (Hall et al., 2001) – possibly making it more similar to conditioned suppression. Despite this outward similarity no role for the NAc was observed in conditioned suppression. Thus, the NAc does not serve as a general output for the

augmentation of instrumental responding by Pavlovian cues. It is possible that selective NA shell lesions would have impaired conditioned suppression. This would be consistent with the previous finding the shell contributes the enhancement of instrumental responding by appetitive cues (Corbit et al., 2001).

Effects of BLA lesions

Consistent with many previous findings, BLA lesions impaired the acquisition of CS+ freezing (Gale et al., 2004; Koo et al., 2004; Maren et al., 1996; Muller et al., 1997; Petrovich et al., 2009; Phillips and LeDoux, 1992). More novel was the finding that BLA lesions impaired discriminative freezing between CS+ and CS−. BLA rats froze just as much to a CS− that never predicted shock than to a CS+ that always predicted shock. This deficit persisted throughout discrimination. The lack of discriminative freezing between CS+ and CS− highlights the critical contribution of the BLA to normal Pavlovian fear conditioning. By contrast the BLA is not necessary for the acquisition of conditioned suppression, but does contribute to the absolute level of conditioned suppression that may be achieved. Most novel was the finding that BLA-lesioned rats demonstrated excellent discriminative suppression between CS+ and CS−. Discrimination emerged at the same rate as Sham rats and did not differ significantly from Shams by the end of training. This is in stark contrast to discriminative freezing in which BLA rats never demonstrated significant differences. The differential effects of BLA lesions on discrimination in conditioned freezing and conditioned suppression cannot be explained by deficits in unconditioned responding, which could reflect reduced processing of the foot shock. Sham and BLA-lesioned rats showed an identical activity burst upon foot shock presentation and both showed strong suppression of instrumental responding by the foot shock itself.

A diminished role for the BLA in conditioned suppression is consistent with previous studies employing different fear conditioning procedures (Killcross et al., 1997; Lee et al., 2005). The lack of an effect of BLA lesions in these previous studies cannot be attributed to an incomplete lesion as BLA-lesioned rats were found to be insensitive to conditioned punishment (Killcross et al., 1997) or had persistent deficits in conditioned freezing (Lee et al., 2005). These findings in combination with the present results suggest the BLA normally contributes to the acquisition of Pavlovian fear but plays a minimal role in displaying this fear specifically through conditioned suppression.

One could argue that, as behavioral measures, conditioned freezing and conditioned suppression have different sensitivities. If freezing is a more sensitive measure of fear then effects of lesions on freezing should appear before other less sensitive measures like conditioned suppression. The finding that BLA lesions impair one measure but not another would then reveal more about those measures than it would about BLA function. Under this interpretation it is unclear what would make one behavioral measure more or less sensitive than another.

An account we find more plausible is that conditioned freezing is but one behavioral mechanism with its own underlying neural circuitry. Conditioned suppression is normally mediated by multiple behavioral mechanisms, each with their own underlying neural circuitries. A fear cue may suppress instrumental responding through freezing, avoidance of the area containing the rewarded response (Bevins and Ayres, 1994) or through reduction of the hunger drive (Estes and Skinner, 1941; Petrovich et al., 2009). The BLA is necessary for the acquisition of active avoidance (Choi et al., 2010; Lazaro-Munoz et al., 2010) but is not necessary for a fear cue to suppress feeding (Petrovich et al., 2009). Under this interpretation, BLA lesions impaired conditioned freezing because it is a critical component of the neural circuitry responsible. BLA lesions left conditioned suppression intact because

behavioral mechanisms such as the reduction of hunger by a fear cue do not depend on the BLA.

The neural mechanisms underlying Pavlovian fear

Evidence suggests that the BLA, CeA and ventrolateral periaqueductal grey (vlPAG) normally work in a serial manner to produce conditioned freezing (Jimenez and Maren, 2009; Wilensky et al., 2006). This same circuit does not appear to underlie conditioned suppression – suggesting that conditioned suppression is not solely the product of conditioned freezing but is likely to be mediated by several behavioral mechanisms. So far no role for the vlPAG in conditioned suppression has been found (Amorapanth et al., 1999; McDannald, 2010) unless higher-order conditioning procedures are used (McDannald, 2010). CeA lesions transiently impair the acquisition of, but not discrimination in, conditioned suppression; however, CeA-lesioned rats go on to show normal conditioned suppression (Lee et al., 2005; McDannald, 2010). Here we report that BLA lesions reduce the absolute level of suppression observed but leave discrimination fully intact. Parallel neural circuitries through the BLA and CeA may normally mediate conditioned suppression (Balleine and Killcross, 2006; Pare et al., 2004; Wilensky et al., 2006). These circuitries may support different behavioral mechanisms, for example, a CeA circuitry for the reduction of hunger by fear cues and a BLA circuitry for the acquisition of avoidance. Thus, lesions of either the BLA or CeA alone are not sufficient to produce deficits in conditioned suppression. However, combined lesions of the BLA+CeA impair the acquisition of conditioned suppression (Lee et al., 2005). Based on the current results the NAc does not appear to serve as the amygdalar output of conditioned suppression. Future studies will address the hypothesis of parallel amygdalar processing and determine what amygdalar outputs are involved in the expression of conditioned suppression.

Conditioned suppression may be especially relevant to human anxiety disorders in which anxious thoughts or feelings interfere with necessary and pleasurable activities. Uncovering the neural mechanisms responsible for conditioned suppression may greatly aid our understanding of human anxiety and its disorders – potentially reducing its impact on society.

4. Experimental Procedure

Experimental Animals

The subjects were 32 male Long-Evans rats, 2 months old on arrival (Charles River Laboratory, Raleigh, NC) housed in individual cages under a 12h light/dark cycle (lights on at 6:00 AM). After arrival, rats were given *ad libitum* access to food for 1 week and then placed on food deprivation until reaching 85% of their free feeding body weight. Rats had free access to water in their home cage for the duration of the experiment. The following week rats received instrumental training after which they were returned to *ad libitum* food access and given neurotoxic lesions of BLA (n=11), NAc (n=11), Sham-BLA (n=5) or Sham-NAc (n=5). Sham-BLA and Sham-NAc rats showed no differences in behavior and were subsequently analyzed together. A postoperative recovery interval of ~2 weeks occurred before behavioral procedures resumed. To account for weight gained during the surgery period, 5% of their post-surgical weight was added to their 85% weight. All experiments were conducted according to the NIH guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Johns Hopkins Institutional Animal Care and Use Committee.

Apparatus

The behavioral training apparatus consisted of four individual chambers (22.9 20.3 20.3 cm) with aluminum front and back walls, clear acrylic sides and top, and a floor made of 0.48-cm stainless steel rods spaced 1.9 cm apart. The steel rods were connected to a shock generator (Coulbourn Instruments, Allentown, PA, USA). A dimly illuminated food cup was recessed in the center of one end wall. To the left of the recessed food cup a retractable lever 1.5 cm wide protruded from the wall. A 6-W lamp was mounted behind a jeweled lens on the front panel, 10 cm above the food cup; illumination of this lamp served as one CS. A second light mounted to the rear wall served as the second CS. Ventilation fans provided masking noise (70 dB). A TV camera was mounted within each shell to provide a view of the chamber; the output from each camera was digitized, merged into a single image of all four chambers and recorded on videotape. General activity was sampled using a ceiling mount infrared activity monitor (Coulbourn Instruments, Allentown, PA, USA).

Surgery

All surgeries were performed under aseptic conditions using isoflurane gas for induction and maintenance of anesthesia, using a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Neurotoxic, bilateral lesions of BLA were made with a 2.0 μl Hamilton syringe using N-methyl-D-aspartic acid (NMDA) (Sigma Aldrich, St. Louis, MO, USA) in a 0.1 M phosphate buffer solution. Each BLA rat received a total of four injections, one site in each hemisphere. Injection sites were 2.8 mm posterior to bregma and 5.1 mm lateral from the midline. The volumes of NMDA injected were 0.16 μl, 8.7 mm ventral from the skull surface and 0.08 μl at 8.4 mm. Neurotoxic, bilateral lesions of the NAc were made with a 10 μl Hamilton syringe using quinolinic acid (Sigma Aldrich, St. Louis, MO, USA). Injection sites were 1.9 mm anterior to bregma and 1.7 mm lateral from the midline. A single injection of 0.24 μl quinolinic acid was made 7.0 mm ventral to the skull surface. Pilot work found this procedure produced maximal damage to the posterior portion of the core, sparing the more anterior portion. Sham lesions were made by lowering the syringe to the same coordinates, NAc (n=5) and BLA (n=5), only no solution was injected. After surgery, all rats received a single subcutaneous injection of 0.01 mg/kg buprenorphine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) for amelioration of pain.

Behavioral procedures

Rats were first trained to lever-press for food reward. Prior to instrumental acquisition rats were placed in the experimental chamber where 30, 45 mg food pellets were delivered over the course of 30 min (mean ITI - 1 min). In the following 3 days the lever was introduced and reinforced on a continuous basis (1 lever press = 1 pellet). On the first day only, a mixture of water, sucrose and food pellets (termed 600P) was applied to the lever to encourage investigation. Rats remained in the experimental chamber until 50 presses were reached or 30 min expired. Those that failed to reach 50 responses in 30 min received additional training. Following lever press acquisition rats were gradually brought up to a variable interval 90s (VI90) schedule. On this schedule lever-pressing was reinforced every 90s, on average, with reinforcement occurring no sooner than 60s and no later than 120s. Once stable instrumental responding was achieved rats were given lesions. Rats were assigned to lesion groups in order to equate for body weight and lever-press rates.

Subsequent to recovery all rats were given at least one additional day of instrumental training to ensure VI90 responding was stable. For the remainder of the procedure the lever was always present in the experimental chamber and reinforced on a VI90 schedule. All conditioning sessions were 60 min in duration. In order to overcome unconditioned suppression, 3 days of pretesting were administered during which both visual stimuli, an intermittent panel light and a constant house light 20 s in duration, were presented 4 times.

The first 3 days of conditioning were composed only of CS+ trials. The panel light served as CS+ for half of the rats in each group, while the house light was the CS+ for the remaining half. CS+ trials began with a 20-s empty period followed by 20-s CS presentation and terminating with electric foot shock delivery (1 mA, 0.5 s). Thus, the CS+ always predicted the occurrence of foot shock. Only 2 CS+ trials occurred on these days (mean ITI - 20 min). The final 12 days consisted of discrimination, 2 CS+ and 6 CS− trials occurred on these days (mean ITI – 6 min). On discrimination days, CS+ trials occurred exactly as above, fully predicting the occurrence of foot shock. The CS− for each rat was the visual cue not used as CS+. CS− trials began with a 20-s empty period followed by 20-s CS presentation which not followed by any event. Thus, the CS− never predicted the occurrence of shock.

Behavioral measures

Freezing, orienting and lever-pressing were sampled on every trial. Sessions were recorded on videotape and scored for freezing and orienting. Freezing was defined as the absence of movement accompanied by a stiff, rigid posture. To be counted as freezing, all forelimbs had to remain on the floor. Head movements that did not interfere with the stiff posture were also counted as freezing. These head movements mostly consisted of slow scanning back and forth. While not quantified, hyperventilation and piloerection were also used as evidence that the rat was freezing and not merely quiescent. Whisker movements or sniffing while in the stiff posture did not count as freezing. Our measure of orienting was rearing (see Figure S1 for orienting results). Rats were considered to be rearing when both forelimbs were off the floor, excluding when the rat was grooming. Rat's behavior was sampled at 1.25-s intervals during pre-CS, CS+ and CS− periods. The pre-CS was the average of both pre-CS+ and pre-CS− trials. During each sample the rat was determined to either be freezing, rearing or quiescent. Percent time freezing and orienting for both CS+ and CS− were calculated by dividing the number of instances of behavior by the number of samples and multiplying by 100.

Lever-presses were recorded automatically during pre-CS, CS+ and CS− periods for all trials. The lever-press rate was corrected to account for orienting and freezing. This was done by dividing the number of presses by the time spent not freezing or orienting. For example, if a rat made 5 presses during the 20-s CS presentation (15 response/min), but spent 25% of his time freezing (5 s) and 25% of his time orienting (5 s), his lever-press rate was 5 presses per 10 s (30 response/min). This ensured that differences in freezing and/or orienting between groups did not bias our report of conditioned suppression. From these lever-press rates a Kamin ratio was constructed by taking: (CS rate)/(CS rate + pre-CS rate). A Kamin ratio of 0.0 would indicate complete suppression while 0.5 would indicate no suppression.

Activity counts were detected by the overhead infrared monitor and were sampled during pre-CS and foot shock periods. Activity/min was calculated by taking the number of activity counts in a sampled time period divided by the time period.

Histological procedures

After completion of the behavioral procedures, rats were deeply anesthetized with isoflurane gas and perfused with 0.9% saline. Sham and neurotoxic lesioned NAc rats were then perfused with 4% paraformaldehyde in 0.1 m PB while sham and neurotoxic BLA lesioned rats were perfused with 4% formalin. Brains were removed, postfixed overnight in the same solution used for perfusion plus 12% sucrose, and frozen the next day for storage at −80°C. Brains were sliced on a freezing microtome and 40 μm coronal sections through NAc and BLA were collected in four series. One series from each brain was mounted and stained for nissl.

Immunohistochemical procedures

For sham and neurotoxic NAc lesioned rats an adjacent series was processed for the antibody Neu-N. This was done because previous studies have found Neu-N to be superior to Nissl in assessing lesions of rats with long post-surgery survival (Jongen-Relo et al., 2003). The Neu-N antibody selectively reacts with neuronal nuclei. Loss of neurons in a brain region results in no Neu-N staining in that region. Endogenous peroxidase within the tissue was blocked by washing free-floating sections in 0.3% H2O2 in 0.1 M PB containing 0.9% saline (PBS) for 30 min. After several rinses in PBS, tissues were incubated for 2 h in PBS containing 0.3% Triton X-100 (PBST) and 3% normal horse serum (Vector Laboratories, Burlingame, CA). Sections were then incubated in mouse Neu-N antibody (1:3000 dilution; AB153; Chemicon, Temecula, CA) in PBST containing 3% normal horse serum for 72 h at 4°C. After the primary antibody incubation, sections were rinsed in PBS, incubated in the biotinylated goat anti-mouse IgG (Vector Laboratories) for 90 min, rinsed in PBS, and then incubated in avidin–biotin peroxidase conjugate (PK-6100; Vector Laboratories) for 1 h. After several rinses in PBS, tissues were reacted using a Vector SG substrate kit for peroxidase (SK-4700; Vector Laboratories). Tissues then were mounted on slides, dehydrated in ascending concentrations of alcohol, defatted in xylene, and coverslipped with Permount.

Statistical Methods

Data were analyzed with ANOVA using software by Statistica and post-hoc comparisons made with Tukey's Honestly Significant Difference. In all cases $p < 0.05$ was considered significant. Mean percent time freezing and mean Kamin ratio during the twelve days of discrimination were analyzed in six, two-day blocks. For example, the four CS+ trials occurring on discrimination days 1 and 2 were averaged to give the d1 CS+ mean; the twelve CS− trials were averaged to give the d1 CS− mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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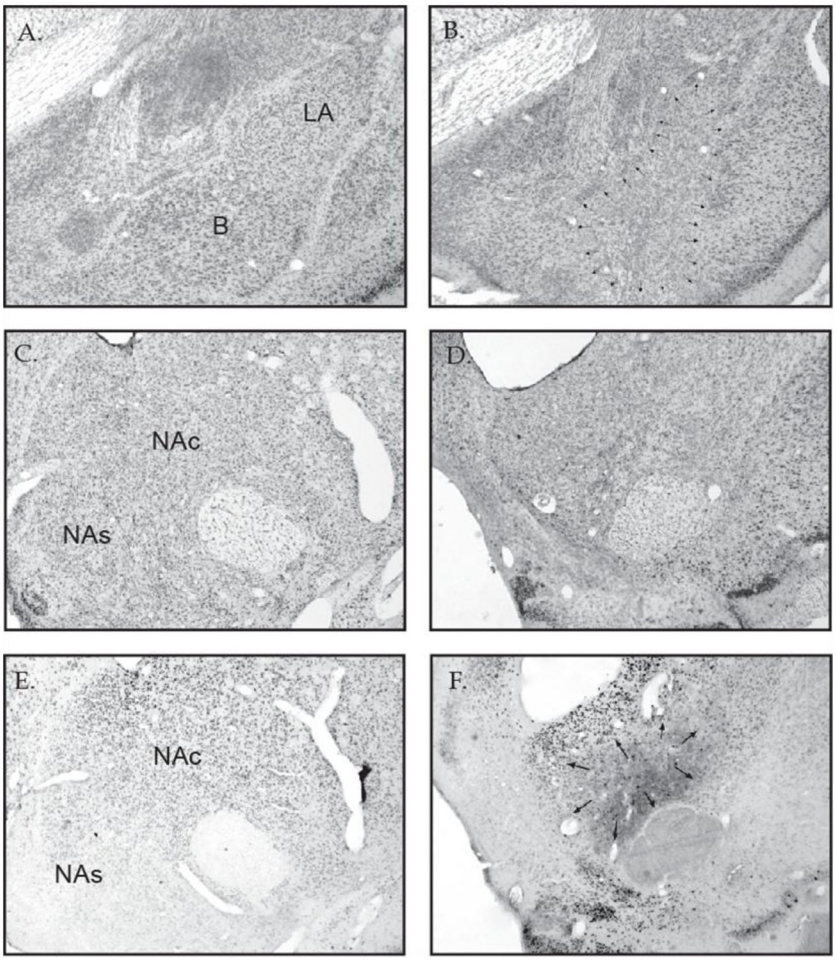

Figure 1.
Histology. Photomicrographs showing representative brain sections of the BLA and NAc of rats that received Sham, BLA or NAc lesions. ***A, B,*** Sections stained with Nissl in the intact and lesioned BLA, respectively. ***C, D,*** Sections stained with Nissl in the intact and lesioned NAc, respectively. ***E, F,*** Sections subjected to immunohistochemistry with Neu-N in the intact and lesioned NAc, respectively. LA, Lateral amygdala; B, Basal amygdala NAc, Nucleus accumbens core; NAs, Nucleus accumbens shell.

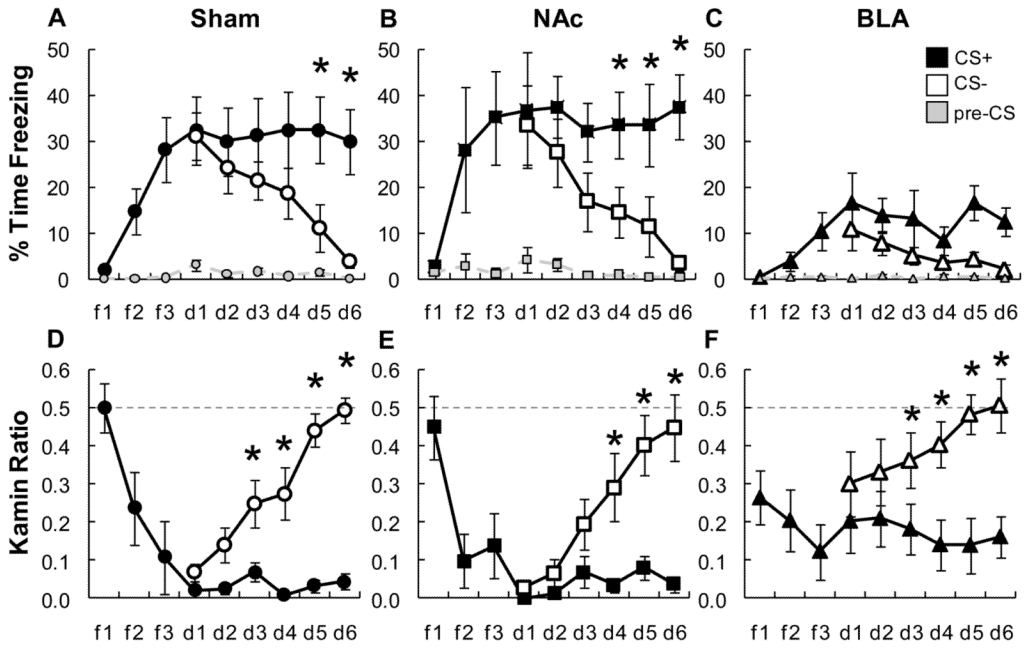

Figure 2.

Freezing and instrumental suppression in Pavlovian fear conditioning. ***A-C,*** Mean ± SEM % Time freezing over the 3 fear conditioning days (f1-3) and 6 fear discrimination blocks (d1-d6) during CS+ (closed symbols), CS− (open symbols) and pre-CS (grey symbols) for ***A,*** Sham, ***B,*** NAc and ***C,*** BLA rats. ***D-F,*** Mean ± SEM Kamin ratios over the 3 fear conditioning days (f1-3) and 6 fear discrimination blocks (d1-d6) during CS+ (closed symbols) and CS− (open symbols) for ***D,*** Sham, ***E,*** NAc and ***F,*** BLA rats. A Kamin ratio of 0.5 (dotted line) indicates the absence of instrumental suppression. (*$p < 0.05$, Tukey's Honestly Significant Difference)

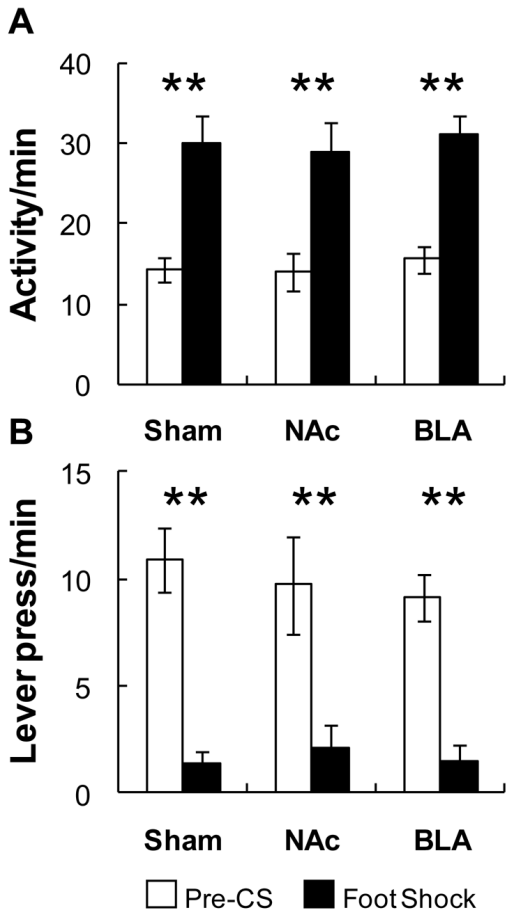

Figure 3.

Unconditioned responses to foot shock. ***A,*** Mean ± SEM activity/min during pre-CS (white bars) and foot shock (black bars) over all days of conditioning for Sham, NAc and BLA rats. ***B,*** Mean ± SEM lever-press/min during pre-CS (white bars) and foot shock (black bars) over all days of conditioning for Sham, NAc and BLA rats. (**$p < 0.01$, Tukey's Honestly Significant Difference)