Behavioral/Systems/Cognitive

Selective Neurotoxic Lesions of Basolateral and Central Nuclei of the Amygdala Produce Differential Effects on Fear Conditioning

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In the fear conditioning literature, it is generally hypothesized that neurons in the basolateral amygdalar complex (BLA) (lateral and basal nuclei) support the formation of conditioned fear memory and project to neurons in the central nucleus (CeA) for the expression of conditioned fear responses. According to this serial processing–transmission view, damage to either BLA or CeA would comparably disrupt the expression of a variety of conditioned fear responses. In the present study, we further investigated the roles of BLA and CeA in fear conditioning by concurrently assessing freezing and 22 kHz ultrasonic vocalization (USV) as dependent measures of fear in rats. Selective neurotoxins, NMDA for the BLA and ibotenic acid for the CeA, were used to destroy intrinsic neurons [evidenced by thionin dye and NeuN (neuronal nuclei) antibody stainings] without damaging the fibers of passage (confirmed by myelin staining). During the 10 tone–footshock paired training, postshock freezing and USV responses were significantly impaired in BLA-lesioned animals, whereas CeA-lesioned animals exhibited only mild deficits. Similarly, conditioned fear responses assessed 24 hr after training were severely reduced in BLA-lesioned animals but not in CeA-lesioned animals. In contrast to ibotenic lesions of the CeA, small electrolytic lesions of the CeA strongly affected both postshock and conditioned freezing and USV. Together, these results do not support the currently espoused BLA-to-CeA serial processing–transmission view of fear conditioning. Instead, the expression of conditioned fear appears to primarily involve BLA projections that course through the CeA en route to downstream fear response structures.

Key words: **emotion; ibotenic acid; learning; memory; NMDA; glutamate**

Introduction

Classical or pavlovian fear conditioning occurs when initially innocuous conditioned stimuli (CS) (e.g., tone and context) are contingently paired with aversive unconditioned stimuli (US) (e.g., footshock and loud noise) that reflexively activate unconditioned fear responses (Rescorla, 1967; Watson and Rayner, 2000). Through CS–US association formation, the CS comes to elicit various conditioned responses (CRs) that share similar characteristics to innate fear responses. In rats, typical fear CR measures include freezing (Blanchard and Blanchard, 1969; Kim and Fanselow, 1992), enhancement of musculature reflexes (Brown et al., 1951; Leaton et al., 1985; Davis, 1997), analgesia (Fanselow, 1986; Helmstetter, 1992), 22 kHz ultrasonic vocalization (USV) (a distress call) (Blanchard et al., 1991; Lee et al., 2001), and alterations in autonomic activities (e.g., increased blood pressure, heart rates, and respiration rates) (Kapp et al., 1979; Iwata et al., 1986, 1987; Stiedl and Spiess, 1997). Because fear conditioning occurs rapidly and is long lasting, it has become a popular behavioral assay for studying the anatomical, cellular, and molecular bases of learning and memory.

Converging lines of evidence point to the amygdala as the key neural system subserving fear conditioning (Kim et al., 1993; LeDoux, 1996; Davis, 1997; Fendt and Fanselow, 1999). The amygdala, one of the principal structures of the limbic system (Isaacson, 1982), has long been implicated as a crucial emotive brain center in monkey studies (Kluver and Bucy, 1937; MacLean and Delgado, 1953; Weiskrantz, 1956). Anatomically, the amygdala is positioned to receive sensory inputs from diverse areas of the brain (e.g., thalamus, neocortex, and olfactory cortex) and to send projections to various autonomic and somatomotor structures [e.g., bed nucleus of stria terminalis (BNST) for activating stress hormones, periaqueductal gray matter (PAG) for defensive behavior, and lateral (LA) hypothalamus for sympathetic activation] that mediate specific fear responses (LeDoux et al., 1988). It is generally accepted that sensory information enters the amygdala through its basal and lateral nuclei [basolateral amygdalar complex (BLA)] (Fanselow and LeDoux, 1999; Maren, 1999; Aggleton, 2000) in which CS–US association formation is believed to take place via long-term potentiation (LTP) or LTP-like processes (Chapman et al., 1990; Miserendino et al., 1990; Kim et

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Table 1. Coordinates relative to the skull surface at bregma (in millimeters)

	Anteroposterior	Mediolateral	Dorsoventral
CeA	2.0	±4.3	8.3
	2.6	±4.3	8.3
BLA	2.3	±5.0	8.4, 8.8
	3.1	±5.2	8.4, 8.8
	3.8	±5.3	8.4, 8.8

al., 1991). These nuclei are interconnected with the central nucleus (CeA), which is thought to be the main amygdaloid output structure sending projections to various autonomic and somatomotor centers involved in mediating specific fear responses (LeDoux, 2000; Medina et al., 2002). In support of this serial processing–transmission view, chemical lesions of CeA neurons have been reported to abolish the expression of conditioned freezing (Goosens and Maren, 2001) and fear-potentiated startle (Campeau and Davis, 1995). Neither study, however, performed fiber staining to confirm that chemical lesions were specific to CeA neurons and did not include fibers of passage. Moreover, Goosens and Maren (2001) performed chemical lesions on only one side of CeA, whereas the contralateral side was lesioned electrolytically. Thus, the present study revisited the role of BLA and CeA in fear conditioning by using bilateral chemical lesions, performing neuronal and fiber staining, and assessing two reliable measures of fear, freezing and USV, concurrently from the same animals.

Materials and Methods

Subjects. Experimentally naive male Charles River Laboratories (Wilmington, MA) Sprague Dawley rats (initially weighing 275–325 gm) were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility and maintained on a reverse 12 hr light/dark cycle (lights on at 7:00 P.M.) with *ad libitum* access to food and water. All experiments were conducted during the dark phase of the cycle and were in strict compliance with the Yale Animal Resource Center guidelines.

Surgery. Rats were anesthetized via intraperitoneal injection of 30 mg/kg ketamine and 2.5 mg/kg xylazine solution with supplemental injections administered as needed. Using aseptic procedures, a stereotaxic instrument with nonpuncture ear bars (Stoelting, Wood Dale, IL) was used to secure the animals for surgery. The scalp was incised, and small burr holes were made on the skull for lesioning. Bilateral chemical BLA lesions (cBLA) were made by injecting 0.1 μl (per hole) of NMDA (Sigma, St. Louis, MO) dissolved in PBS (20 μg/μl) at three sites in each hemisphere. The stereotaxic coordinates relative to the skull surface at bregma are given in Table 1. Injections were made at a rate of 0.1 μl/min with a Hamilton microsyringe (Hamilton, Reno, NV) equipped with a 30 gauge needle (cf. Pickens et al., 2003). Bilateral chemical CeA lesions (cCeA) were made by injecting 0.1 μl (per hole) of ibotenic acid (Regis Chemical, Morton Grove, IL) dissolved in PBS (10 μg/μl) at two sites in each hemisphere (Table 1). Injections were made at a rate of 0.1 μl/min with a pulled glass micropipette that was connected to a Picospritzer III (Parker Hannifin, Fairfield, NJ) (cf. Pickens et al., 2003). It should be noted that both NMDA and ibotenic acid are selective agonists for NMDA receptors and (at critical concentrations) produce similar excitatory effects on neurons with NMDA (glutamate) receptors. Generally, the NMDA is used to produce discrete BLA lesions, whereas the ibotenic acid is effective in producing relatively confined CeA lesions (Hatfield et al., 1996; Han et al., 1997). Bilateral electrolytic CeA lesions (eCeA) were made by passing constant current (0.5 mA, 15 sec) (Ugo Basile, Comerio, Italy) through an epoxy-coated insect pin (number 00) with ~0.3 mm of the tip exposed; the coordinates were the same as those used in the cCeA group (Table 1). The operated sham control rats received injections of PBS vehicle in CeA or BLA. After surgery, animals were allowed to recover for at least 10 d and acclimated to daily handling before commencement of behavioral procedures.

Fear conditioning apparatus. Training and testing took place in two modular operant test chambers, each equipped with speaker modules (Coulbourn Instruments, Allentown, PA) and located in a controlled acoustic room (Industrial Acoustics, New York, NY). The two chambers differed in several features. Chamber A was rectangular (27 cm width × 28 cm length × 30.5 cm height), whereas chamber B was octagonal (26.5 cm diameter × 25 cm height); chamber A had front and back walls made of clear Plexiglas and two side walls made of metal plates, whereas chamber B had all eight walls constructed of clear Plexiglas. Furthermore, chamber A was placed in a wooden isolation box (46 cm width × 53 cm length × 49 cm height) that was painted white, whereas chamber B was placed in a similar box that was painted black. The grid floor of chamber A was composed of 16 stainless-steel bars (4.5 mm diameter) spaced 17.5 mm center-to-center and wired to a Coulbourn Instruments (Allentown, PA) precision-regulated animal shocker. The floor of chamber B was made of smooth Plexiglas. The floor grid, the covering Plexiglas, and base pan of each chamber were washed thoroughly with tap water and dried completely before training and testing of each animal.

Procedure. The experiment took place on 3 consecutive days. On training day 1, rats were placed in chamber A, which was wiped with 5% ammonium hydroxide solution. The overhead room light was on. After 1 min, animals were presented with 10 coterminating tone–footshock pairings (tone, 2.9 kHz, 82 dB, 10 sec; footshock, 1 mA, 1 sec) with 1 min intertrial intervals (ITIs). Animals were removed 1 min after the last shock and returned to their home cages. On day 2, rats were placed back in the trained context for 8 min of context testing. On day 3, animals were given a tone-retention test, which consisted of 1 min of baseline, followed by 8 min of continuous tone in the novel chamber B (wiped with a 1% acetic acid solution, Plexiglas floor, the overhead light turned off).

Freezing and USV data collection and analysis. The stimulus presentations were controlled, and the freezing data were collected by an IBM (White Plains, NY) personal computer equipped with the Coulbourn Instruments LabLinc Habitest Universal Linc System. Although the collection of the USV and freezing data were fully automated, each session was recorded for video and audio analysis off-line, if necessary, using an infrared light source and miniature video camera (CB-21; Circuit Specialists, Mesa, AZ).

A 24 cell infrared activity monitor was mounted on top of each chamber and was used to assess freezing behavior. The monitor detects movement of emitted infrared (1300 nm) body heat images from the animals in the *x*, *y*, and *z* axes (cf. Lee and Kim, 1998; Lee et al., 2001; Choi and Brown, 2003; Frick et al., 2004; Scicli et al., 2004). In brief, the total time of inactivity exhibited by each animal was measured using a computer program, and freezing was defined as continuous inactivity lasting at least 3 sec. Any behavior that yielded an inactivity of <3 sec was recorded as general activity.

A heterodyne bat detector (Mini-3; Noldus Information Technology, Wageninge, The Netherlands) was used to transform high-frequency (22 ± 5 kHz) ultrasonic vocalizations into the audible range (cf. Lee et al., 2001; Frick et al., 2004; Lee and Kim, 2004). The output of the bat detector was fed through an audio amplitude filter (Noldus Information Technology), which filtered out signals falling below an amplitude range that was individually adjusted for each animal. The resulting signal was then sent to an IBM personal computer equipped with Noldus UltraVox vocalization analysis software.

Histology and immunohistochemistry. At the completion of behavioral testing, the subjects were overdosed with ketamine HCl and xylazine and perfused intracardially with 0.9% saline, followed by 10% buffered Formalin. The brains were removed, stored in 10% Formalin for at least 2 weeks, and kept in 30% sucrose until they sank. Adjacent series of transverse sections (50 μm) were taken through the extent of the lesions and stored at 4°C in a cryoprotective buffer containing 25% ethylene glycol, 25% glycerin, and 0.05 M phosphate buffer until the sections were processed for histology and immunohistochemistry (cf. Koo et al., 2003). Consecutive sections were (1) mounted onto gelatin-coated slides and stained with thionin to verify the neurotoxic lesion, (2) stained free-floatingly for myelin (Schmued, 1990) to verify whether the neurotoxins produced fiber damage at the lesion sites, or (3) immunohistochemically stained for NeuN (neuronal nuclei) to verify the neurotoxic lesion.

For myelin staining, free-floating sections were rinsed three times for 10 min in 0.02 M phosphate buffer with 0.6% NaCl and incubated for 1 hr in 0.2% gold chloride trihydrate (Sigma) dissolved in PBS containing 0.012% H_2O_2. The reaction was terminated by rinsing the tissues for 10 min in PBS. Sections were then fixed for 5 min in 5% sodium thiosulfate, rinsed three times for 5 min in PBS, and mounted on slides.

For NeuN immunostaining, free-floating sections were treated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase. After the tissues were washed, they were preincubated in PBS containing 0.3% Triton X-100 and 2% bovine serum albumin (blocking solution) for 1 hr and incubated (with shaking) overnight at 4°C in mouse monoclonal anti-NeuN antibody (1:200 in PBS; Chemicon, Temecula, CA). Next, the sections were incubated for 1 hr with biotinylated secondary anti-mouse IgG antibodies (1:200; Vectastain Elite ABC; Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Then, the sections were incubated for 1 hr in avidin–biotin–peroxidase complex (Vectastain Elite ABC; Vector Laboratories), followed by peroxidase detection with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma) as a chromogen (0.05% DAB and 0.01% H_2O_2 in PBS). Afterward, sections were mounted on slides, dehydrated, cleared, and coverslipped.

All stained sections were examined with a light microscope (Zeiss, Oberkochen, Germany). Lesions were verified by reconstructing the damage on stereotaxic atlas templates (Paxinos and Watson, 1998). Acceptable lesions for statistical analysis include bilateral damage of the area investigated throughout most of its extent with minor damage of surrounding areas. Specifically, neurotoxic and electrolytic lesions of CeA had to include the medial part of the CeA, whereas BLA neurotoxic lesions had to include the lateral nucleus of the BLA with additional but variable damage to basolateral (BL) and basomedial (BM) nuclei (Campeau and Davis, 1995).

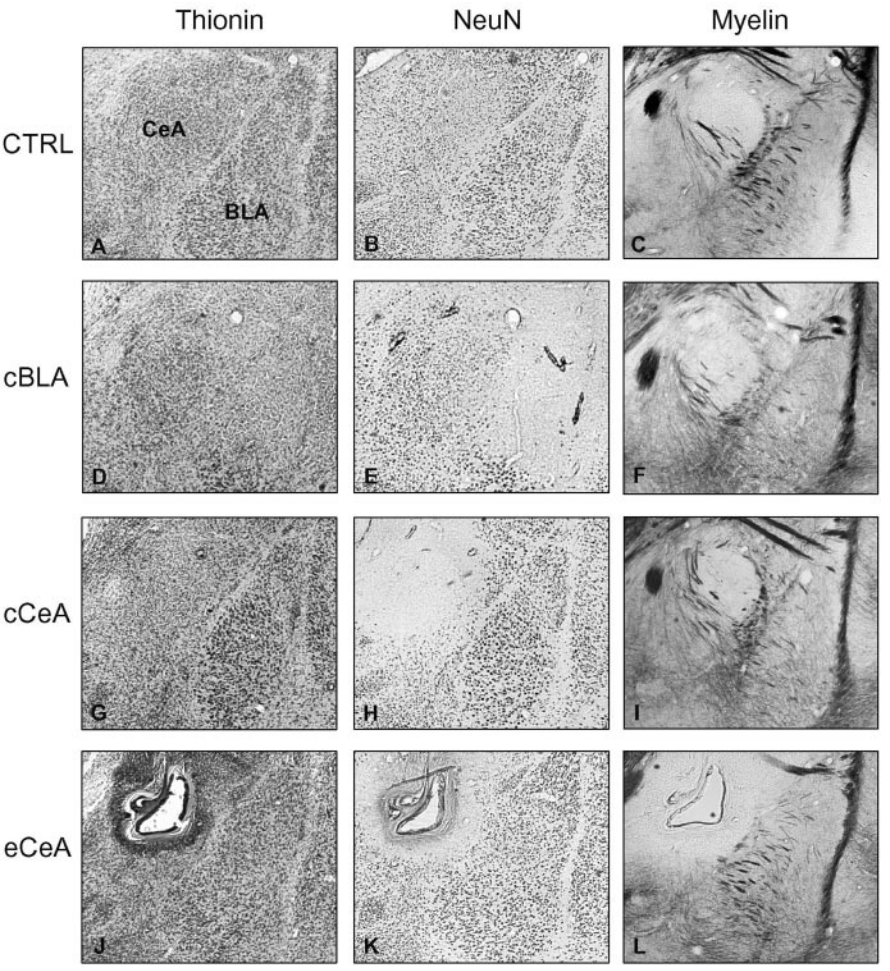

Figure 1. Photomicrographs of thionin-stained (left), NeuN-immunopositive (middle), and gold chloride myelin-stained (right) coronal sections from a vehicle-injected control (*A–C*), an NMDA-induced BLA-lesioned (*D–F*), an ibotenic acid-induced CeA-lesioned (*G–I*), and an electrolytic CeA-lesioned (*J–L*) brain. Note that the neurotoxic lesions of the BLA and the CeA show heavy gliosis (*D*, *G*) and neuron loss (*E*, *H*) in the lesion area. Spared neurons are evident in parts of the target nucleus distant from the cannula and micropipette tip. However, myelin is intact in the lesion area (*F*, *I*). In contrast, the electrolytic lesion of the CeA induces damage to fibers of passage (*L*), as well as neuron loss (*K*) in the lesion area.

The rank order of lesion size was determined on the basis of the lesion volume estimated from three (cCeA and eCeA) or four (cBLA) thionin-stained sections per rat. The lesion volume was calculated from average of bilateral lesioned areas on each section and the distance between coronal sections (600 μm). For that purpose, each border of the bilateral lesions was marked under a 100× magnification. Each marked area was measured using NIH ImageJ software, and then average of the areas from left and right lesions on each section was calculated. The average areas of each section were summed up and multiplied by the distance between sections (600 μm) (cf. Brandt et al., 2003).

Statistical analyses. Results are presented as mean ± SE. All statistical analyses were performed with Statistical Program for the Social Sciences version 11.0 (SPSS, Chicago, IL). For comparisons of freezing and USV during fear conditioning (training and retention) tests, one-way ANOVA with repeated measures was performed, followed by Tukey's test as *post hoc* analysis for additional examination of group differences. Furthermore, Spearman rank order correlations were performed to examine the relationship between lesion size and either freezing or USV.

Results

Histology

Figure 1 shows photomicrographs of thionin-stained (left), NeuN-stained (middle), and gold chloride myelin-stained (right) sections from typical control (CTRL), cBLA, cCeA, and eCeA animals. The thionin-stained section from cBLA (Fig. 1*D*) shows gliosis in the BLA. The NeuN-immunostained section shows that the majority of neurons in the BLA were destroyed (Fig. 1*E*). Myelin staining for fibers at the lesion site, however, is intact (Fig. 1*F*). Similarly, the cCeA damaged the intrinsic neurons but not the fibers of passage in the CeA (Fig. 1*G–I*). On the other hand, the eCeAs caused damage to both the intrinsic neurons and the fibers of passage (Fig. 1*J–L*).

Based on the histological results, 27 of the 68 rats were excluded from the analysis resulting in the following sample size per group: cBLA ($n = 9$), cCeA ($n = 10$), eCeA ($n = 10$), and CTRL ($n = 12$). The extent of the amygdala lesions for animals included in the analyses is depicted in Figure 2. As can be seen, lesions were generally confined to the target nucleus of the amygdala. NMDA BLA lesions typically included the LA (dorsolateral, ventrolateral, and ventromedial divisions) nuclei of the amygdala, with variable damage to the BL and BM subnuclei. Some BLA lesions extended superficially into the piriform cortex. Ibotenic acid and electrolytic CeA lesions included most of the lateral division of the CeA and a large portion of the medial division of the CeA. A few lesions extended slightly into the BL and the substantia innominata. Overall, the extents of BLA and CeA lesion sizes appeared to be comparable

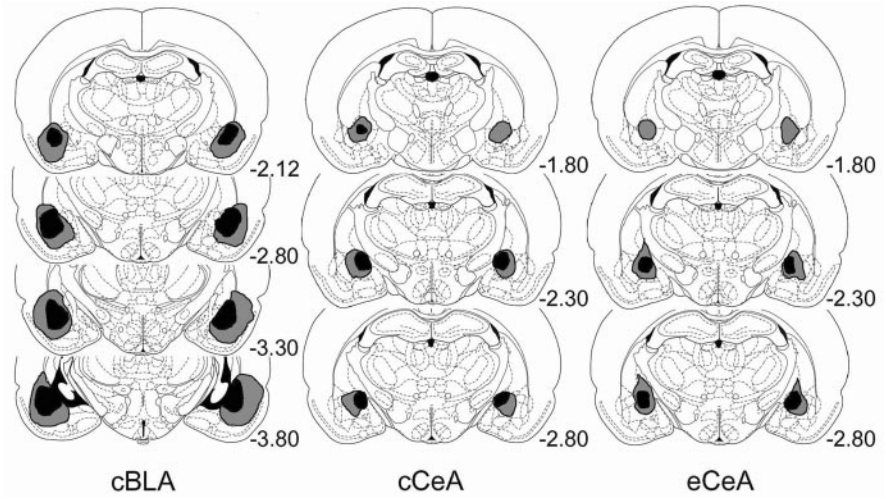

Figure 2. Schematic representation of the minimum (black) and maximum (gray) extent of damage from an NMDA-induced BLA-lesioned (left), an ibotenate-induced CeA-lesioned (middle), and an electrolytic CeA-lesioned (right) brain. Numbers represent distance in millimeters posterior to bregma. Coronal brain section images adapted from Paxinos and Watson (1998).

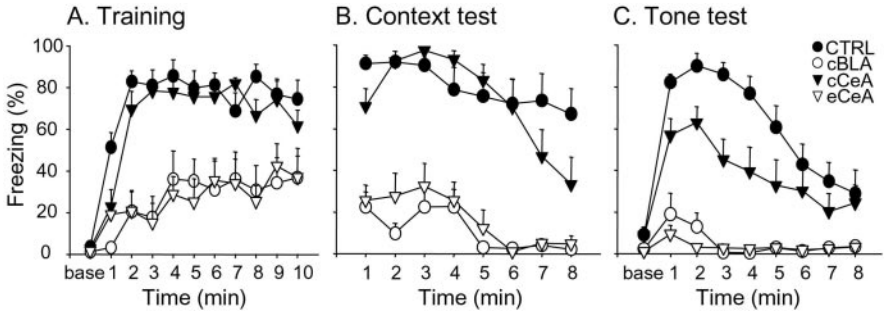

Figure 3. Effects of neurotoxic and electrolytic lesions of the amygdala on conditioned freezing. *A*, Mean ± SE (error bars) percentage of freezing during the 1 min baseline and during the intervening 10 tone–shock pairings of the 1 min ITIs in context A from sham control ($n = 12$), NMDA-induced BLA lesion ($n = 9$), ibotenate-induced CeA lesion ($n = 10$), and eCeA ($n = 10$) animals. *B*, Mean ± SE percentage of freezing during the 8 min context-retention testing in context A. *C*, Mean ± SE percentage of freezing during the 1 min baseline and the 8 min tone-retention testing in context B.

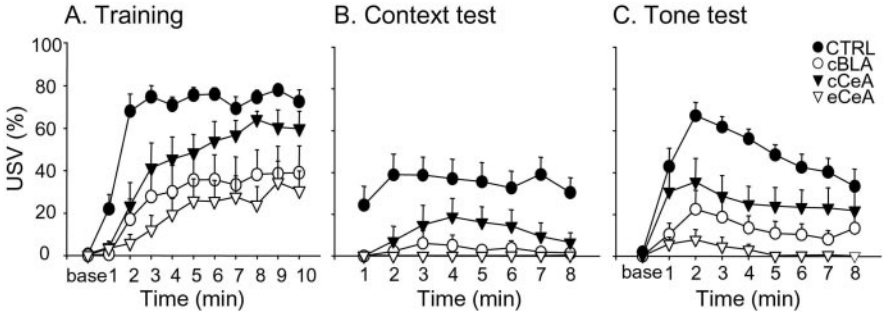

Figure 4. Effects of neurotoxic and electrolytic lesions of the amygdala on conditioned USV. *A*, Mean ± SE (error bars) percentage of USV during the 1 min baseline and during the intervening 10 tone–shock pairings of the 1 min ITIs in context A from CTRL, cBLA, cCeA, and eCeA animals ($n = 10$). *B*, Mean ± SE percentage of USV during the 8 min context-retention testing in context A. *C*, Mean ± SE percentage of USV during the 1 min baseline and the 8 min tone-retention testing in context B.

between the present study and those of Campeau and Davis (1995) and Goosens and Maren (2001).

Conditioned freezing

Rats received a context and tone extinction test 24 and 48 hr after conditioning, respectively. Figure 3*A* depicts the mean percentage of freezing displayed by cBLA ($n = 9$), cCeA ($n = 10$), eCeA ($n = 10$), and CTRL ($n = 12$) groups during 1 min baseline and 10 1 min ITIs on training day 1. The groups exhibited negligible freezing during 1 min baseline (mean freezing, 3.66% for CTRL, 1.35% for cBLA, 2.85% for cCeA, and 1.63% for eCeA) that did not differ reliably. This observation was confirmed by a one-way ANOVA on freezing during the 1 min preshock period (baseline, $F_{(3,37)} < 1.0$). During tone–footshock presentations, however, the groups showed a difference in the magnitude of postshock freezing ($F_{(3,24)} = 18.78$; $p < 0.01$; one-way ANOVA with repeated measures). Tukey's *post hoc* test revealed that rats in both CTRL and cCeA groups exhibited robust postshock freezing that did not differ statistically ($p > 0.05$). In contrast, cBLA and eCeA rats exhibited impaired freezing compared with the CTRL and cCeA rats ($p < 0.01$).

On day 2, the context test (Fig. 3*B*) indicated that both CTRL and cCeA rats exhibited comparably high levels of freezing. In contrast, cBLA and eCeA rats displayed significant deficits in context freezing compared with CTRL. These observations were confirmed by a significant group effect in the one-way ANOVA with repeated measures ($F_{(3,24)} = 66.44$; $p < 0.01$). *Post hoc* comparisons demonstrated that cBLA and eCeA rats froze significantly less than rats in the CTRL and cCeA groups (p values < 0.01). *Post hoc* comparisons also revealed that cCeA rats did not significantly differ from CTRL rats.

On day 3, the tone test (Fig. 3*C*) revealed that cBLA and eCeA rats exhibited robust deficits in conditioned freezing to the tone CS relative to CTRL rats, whereas the cCeA rats exhibited only mild deficits. One-way ANOVA with repeated measures revealed a significant main effect of group ($F_{(3,24)} = 24.40$; $p < 0.01$). *Post hoc* comparisons demonstrated that cBLA and eCeA rats froze significantly less than CTRL and cCeA rats (p values < 0.01). The cCeA rats also exhibited significantly less freezing than CTRL rats ($p < 0.05$).

Conditioned USV

Conditioned USV was another measure of fear responses during fear conditioning and extinction tests. Figure 4*A* depicts the mean duration of USV from tone–shock-trained animals in cBLA, cCeA, eCeA, and CTRL groups. As shown, none of the groups exhibited USV behavior during the 1 min preshock period (0% for all groups). With successive tone–footshock presentations, however, cBLA, cCeA, and eCeA rats exhibited deficits in postshock USV duration compared with CTRL rats. This observation was confirmed by significant group

differences ($F_{(3,24)} = 8.11$; $p < 0.01$) in the one-way ANOVA with repeated measures. *Post hoc* comparisons revealed that cBLA and eCeA rats exhibited deficits in postshock USV duration compared with CTRL rats (p values < 0.01). Although cCeA animals showed trends of exhibiting less USV behavior than CTRL and more than cBLA and eCeA animals, the differences did not reach statistical significance.

In the context test, cCeA, eCeA, and cBLA rats showed impaired USV (Fig. 4*B*). One-way ANOVA with repeated measures confirmed that there was a significant group difference in USV duration ($F_{(3,24)} = 8.46$; $p < 0.01$). *Post hoc* comparisons demonstrated that cBLA and eCeA rats produced significantly fewer vocalizations than CTRL rats (p values < 0.01) and that cCeA rats also produced significantly attenuated USV compared with CTRL rats ($p < 0.05$). Although cCeA animals showed trends of exhibiting greater USV behavior than cBLA and eCeA animals, the differences did not reach statistical significance. The tone test (Fig. 4*C*) also showed that there was a significant group difference in USV duration ($F_{(3,24)} = 12.48$; $p < 0.01$). *Post hoc* comparisons demonstrated that cBLA and eCeA rats exhibited significantly fewer vocalizations than CTRL rats (p values < 0.01) and that cCeA rats also exhibited significantly attenuated USV compared with CTRL rats ($p < 0.05$). Conversely, cCeA rats showed significantly greater USV behavior than eCeA rats ($p < 0.05$).

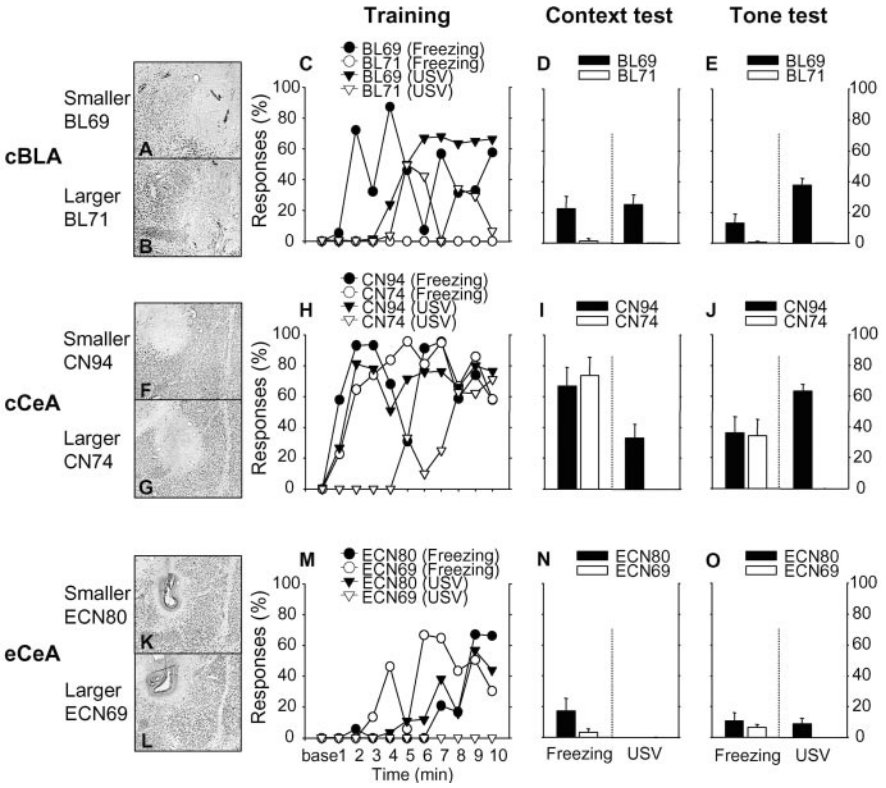

Figure 5. Representative samples of conditioned freezing for cBLA (*A–E*), cCeA (*F–J*), and eCeA (*K–O*) rats with large or small lesions. Photomicrographs of NeuN-immunopositive sections from representative animals with small lesion (*A*, *F*, *K*) or large lesion (*B*, *G*, *L*). *C*, *H*, *M*, Typical changes in percentage of freezing and USV during the 1 min baseline and during the intervening 10 tone–shock pairings of the 1 min ITIs. *D*, *I*, *N*, Mean ± SE (error bars) percentage of freezing and USV during the 8 min context-retention testing. *E*, *J*, *O*, Mean ± SE percentage of freezing and USV during the 8 min tone-retention testing. Filled circles, triangles, and bars indicate representative rats with small lesions, and open circles, triangles, and bars indicate rats with large lesions from each group.

Examples of individual sample data

To better appreciate BLA and CeA lesion effects on fear conditioning, photomicrographs, freezing, and USV data from a representative cBLA, cCeA, and eCeA animal with small or large lesions are provided in Figure 5.

The mean ± SE lesion size of cBLA rats was 3.57 ± 0.31 mm^3. A cBLA rat with the smallest lesion (3.14 mm^3) had damage restricted to the LA and a part of the BL (Fig. 5*A*), whereas a cBLA rat with the largest lesion (4.09 mm^3) had damage that extends into the BM and slightly into the piriform cortex (Fig. 5*B*). Both the small and large cBLA lesion rats showed impairments in freezing and USV levels (Fig. 5*C–E*). With regard to freezing, Spearman rank correlation analyses revealed that there was no reliable correlation between rank order of lesion size and average freezing levels in the cBLA group (postshock training, $r_s = 0.60$, $p > 0.05$; context test, $r_s = 0.53$, $p > 0.05$; tone test, $r_s = 0.54$, $p > 0.05$). With regard to USV, however, Spearman rank correlation analysis revealed positive correlations between rank order of lesion size and average USV levels in postshock training ($r_s = 0.87$; $p < 0.01$) and tone test ($r_s = 0.83$; $p < 0.01$) that did not reach the level of statistical significance in context test ($r_s = 0.46$; $p > 0.05$).

The mean ± SE lesion size of cCeA rats was 1.51 ± 0.18 mm^3 (range, 1.23–1.92 mm^3). Freezing and USV data from representative cCeA rats with the smallest (including medial and lateral parts of the CeA) (Fig. 5*F*) and the largest (extending slightly into the BL) (Fig. 5*G*) lesions are presented in Figure 5*H–J*. As shown, there were no differences between smallest- and largest-lesioned animals in the levels of freezing during training (Fig. 5*H*), context (Fig. 5*I*), and tone (Fig. 5*J*) tests. These observations were confirmed by Spearman rank correlation analysis, which revealed that there was no correlation between rank order of lesion size and average freezing levels in the cCeA group (postshock training, $r_s = -0.27$, $p > 0.05$; context test, $r_s = -0.20$, $p > 0.05$; tone test, $r_s = 0.28$, $p > 0.05$). However, in terms of USV, there was a reliable positive correlation between rank order of lesion size and average USV levels in tone test ($r_s = 0.67$; $p < 0.05$) that did not reach the level of statistical significance in postshock training ($r_s = 0.45$; $p > 0.05$) and context ($r_s = 0.50$; $p > 0.05$) tests.

The mean ± SE lesion size of eCeA rats was 1.31 ± 0.08 mm^3 (range, 0.98–1.72 mm^3). In contrast to cCeA animals, eCeA animals showed robust deficits in both freezing and USV, regardless of lesion size (Fig. 5*M–O*). Indeed, there was no significant correlation between rank order of lesion size and average freezing levels in the eCeA group (postshock training, $r_s = 0.13$, $p > 0.05$; context test, $r_s = 0.26$, $p > 0.05$; tone test, $r_s = 0.40$, $p > 0.05$). With regard to USV, we found no significant correlations between rank order of lesion size and average USV levels during postshock training ($r_s = 0.00$; $p < 0.05$), context test ($r_s = -0.14$; $p < 0.05$), and tone test ($r_s = 0.13$; $p < 0.05$).

Discussion

To examine the role of BLA and CeA neurons in pavlovian fear conditioning, we made bilateral neurotoxic lesions of either BLA or CeA and assessed two reliable measures of fear, freezing and USV, concurrently in the same rats. Whereas CeA neurotoxic lesions produced only mild deficits, BLA neurotoxic lesions significantly impaired both freezing and USV. CeA electrolytic lesions also induced robust deficits in freezing and USV. None of the amygdala lesions affected motor and vocal activities during the preshock period in the training. Therefore, the low levels of conditioned freezing and USV in rats with neurotoxic BLA and electrolytic CeA lesions cannot be ascribed to alterations in locomotor and vocalization behaviors.

The present data are in agreement with other lesion studies using different amygdalar-dependent behavioral tasks (Holland and Gallagher, 1999; Everitt et al., 2003). For example, fiber-sparing neurotoxic BLA, but not CeA, lesions impair taste-potentiated odor aversion learning (Hatfield et al., 1992; Ferry et al., 1995), second-order conditioning–reinforcer devaluation effects in appetitive learning (Hatfield et al., 1996), and conditioned taste aversion (Morris et al., 1999). Fiber-damaging electrolytic CeA lesions, however, disrupt these tasks (Lasiter and Glanzman, 1985). The fact that electrolytic, but not neurotoxic, lesions impair learning suggests that the deficit observed after electrolytic lesions is the result of incidental damage to the fibers coursing through the target structure (Dunn and Everitt, 1988; Jarrard, 1989, 2002; Frey et al., 1997; Morris et al., 1999). Similarly, in the present fear conditioning experiment, electrolytic CeA lesions robustly disrupted freezing and USV, in contrast to fiber-sparing neurotoxic CeA lesions. These results strongly suggest that the conditioned fear responses primarily depend on fibers passing through CeA rather than neurons localized within CeA.

Our data indicating that BLA functions do not necessitate BLA projections to CeA neurons conflict with a widely held hypothesis indicating that CeA is the obligatory output structure for fear conditioning (LeDoux, 1996; Aggleton, 2000). According to this hypothesis, BLA (primarily the lateral nucleus) acts as the associative site for CS–US representations, and CeA provides the output gateway through which these associations gain access to conditioned fear responses (Fanselow and LeDoux, 1999; LeDoux, 2000). Consistent with this serial model of BLA–CeA function, restricted electrolytic lesions to either BLA or CeA have been shown to abolish conditioned fear (Amorapanth et al., 2000; Nader et al., 2001; Choi and Brown, 2003). Because electrolytic lesions interrupt functions mediated by axons coursing through the lesion site, positive lesion effects must be cautiously interpreted (Dunn and Everitt, 1988).

Neurotoxic lesions, however, can also damage fibers of passage in various areas of brain (Coffey et al., 1988; Jarrard, 1989). In particular, Frey et al. (1997) observed that a high dose of ibotenic acid injected into CeA leads to demyelination, indicating damage to passing fibers. It must, therefore, not be assumed a priori that ibotenic acid injections into CeA will selectively destroy neurons and spare fibers of passage. Nonetheless, recent studies demonstrating that neurotoxic CeA lesions prevent the expression of conditioned fear (Campeau and Davis, 1995; Goosens and Maren, 2001) concluded that the behavioral deficits are caused not by damage to fibers of passage but by the destruction of neurons within CeA, in absence of verifying with fiber staining.

In contrast, the neurotoxic lesions in this study substantiated that lesions were specific to cell bodies in CeA without disruption of fibers. Nevertheless, one may argue that neurotoxic CeA lesions were not sufficiently destructive and thus the spared fear responses were attributable to remaining intact neurons in CeA. This is unlikely, however, because small electrolytic CeA lesions robustly impaired freezing and USV, whereas large neurotoxic CeA lesions did not. These results reaffirm the important role of fibers passing through CeA in fear conditioning.

Certain procedural differences between the studies merit mentioning. Goosens and Maren (2001) applied chemical lesions on only one side of CeA and electrolytically lesioned the entire contralateral amygdala. In contrast, both Campeau and Davis (1995) and this study made bilateral CeA neurotoxic lesions. However, Campeau and Davis infused ibotenic acid (10 μg/μl concentration; from Sigma) through a cannula at a single site (0.2 μl volume per hole or hemisphere) and assessed fear-potentiated startle, whereas we infused the neurotoxin (10 μg/μl; from Regis Chemical) via a pulled glass micropipette at two sites in each hemisphere (0.1 μl per hole or 0.2 μl per hemisphere) (cf. Pickens et al., 2003) and assessed freezing and USV. Thus, the discrepancies in findings might be a result of differences in lesion and/or fear response factors.

It has been hypothesized that CeA receives inputs from all components of BLA complex [LA and basal nucleus (B)] and then projects directly to brainstem areas controlling the expression of fear responses (e.g., PAG for freezing) (LeDoux et al., 1988; Bellgowan and Helmstetter, 1996; Pitkänen et al., 1997, 2000; Medina et al., 2002; Comoli et al., 2003). Anatomical studies, however, indicate that fibers originating from B (BL and BM) pass through CeA (Smith and Millhouse, 1985; Dong et al., 2001; Walker et al., 2003). For instance, axons arising from BL travel through CeA toward the stria terminalis (ST) (Smith and Millhouse, 1985; Petrovich et al., 1996; Dong et al., 2001; Canteras, 2002). The BL innervations are relayed by the BNST to the medial hypothalamic zone, of which a main brainstem target is PAG (Dong et al., 2001; Canteras, 2002; Walker et al., 2003). These anatomical data suggest that BLA projections to BNST play an important role in the expression of fear CRs. A recent study showed that the BNST is critically involved in trimethylthiazoline (a component of fox feces)-induced freezing (Fendt et al., 2003). Nevertheless, previous attempts to implicate BNST in conditioned fear (to discrete CSs) were primarily unsuccessful (Walker et al., 2003). This failure can be explained by the existence of direct BM–medial hypothalamic zone projections that go through CeA and ST (Petrovich et al., 1996; Dong et al., 2001; Canteras, 2002). The role of BNST in the expression of conditioned fear to context remains to be investigated.

These anatomically distinctive output systems for fear conditioning can be differentially activated according to CS modalities. We found that neurotoxic CeA lesions produced moderate deficits in conditioned freezing to tone but not to context. In contrast, electrolytic CeA lesions disrupted freezing to both tone and context. These results imply that complex polymodal (context) CS information primarily involves a pathway that passes through CeA via BL–BM projections. In contrast, simple modality-specific (tone) CS information engages two parallel pathways, one relayed to CeA neurons and another coursing through CeA from LA and/or B (Fig. 6), in which each pathway is capable of supporting tone fear if the other is damaged. It follows that a combined neurotoxic lesion of both B and CeA (sparing LA) should abolish tone fear, a prediction consistent with the findings that neither fiber-sparing neurotoxic CeA lesions (this study) nor electrolytic lesions to B (Amorapanth et al., 2000) abolish tone

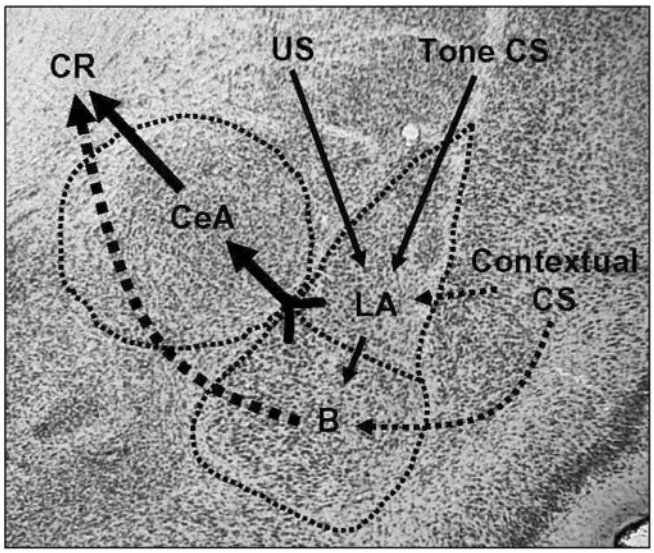

Figure 6. Anatomical model of the amygdala circuitry for fear conditioning. Contextual CS converges with footshock US in LA and/or B (dotted thin lines). Contextual CS–US information courses through CeA en route to downstream fear response structures by way of B for freezing (dotted thick line). Auditory CS comes into association with the US in LA (solid thin lines). Auditory CS–US information is relayed to CeA directly (solid thick lines), as well as to the pathway that travels through CeA via B. The direct pathway to CeA neurons from LA partially mediates USV responses regardless of CS modality.

fear. Our model is in accordance with evidence that context CS information and auditory CS information are transmitted primarily to B (Canteras and Swanson, 1992; Maren and Fanselow, 1995) and LA (Romanski and LeDoux, 1992; Doron and Ledoux, 1999), respectively. The differential activation of these distinctive output systems according to CS modalities might be controlled by the intercalated cell masses in the amygdala via generating and modifying their feedforward inhibition of CeA neurons depending on the distribution of BL activity in space and time (Royer and Paré, 2002; Paré et al., 2003).

Our results also conflict with another theory in the fear conditioning literature that suggests that the CeA output pathway governs pavlovian fear responses, whereas the B output pathway governs instrumental fear responses (Killcross et al., 1997; Amorapanth et al., 2000). However, results from this study and aforementioned anatomical studies implicate that B is not exclusively involved in instrumental fear but is also important for conditioned fear. It is possible that previous studies misattributed the contribution of the B pathway to conditioned fear to CeA because the fibers of passage go through CeA and can be damaged by CeA lesions.

Another interesting outcome of the present study relates to USV. The rat 22 kHz USV is thought to reflect an aversive behavioral state and has proven useful in the study of the neural mechanisms of anxiety and fear states (Knapp and Pohorecky, 1995). Thus, USV represents an additional measure of conditioned fear in conjunction with freezing (Lee et al., 2001; Frick et al., 2004; Lee and Kim 2004). In accordance, neurotoxic BLA and electrolytic CeA lesions disrupted USV and freezing in a similar manner. Interestingly, neurotoxic CeA lesions significantly impaired USV, but not freezing, during postshock training and context test. Moreover, larger neurotoxic CeA lesions produced bigger impairments in USV, but not freezing, in tone test. These data suggest that conditioned fear is not unitary and that distinctive output systems of the amygdala are involved in the expression of different fear CRs (i.e., CeA pathway relates to USV; non-CeA pathway relates to freezing). To our knowledge, the present study is the first to examine selective neurotoxic BLA and CeA lesion effects on conditioned USV to contextual and tone CSs.

In summary, our results suggest that the expression of conditioned fear primarily involves BLA projections that course through the CeA en route to downstream fear response structures. This notion differs from a currently espoused BLA–CeA serial model whereby CS–US information converges in LA and B and is relayed to CeA for the generation of fear CRs. By using neurotoxic lesions along with electrolytic lesions of CeA, we can be certain that the pattern of deficits reported is primarily caused by damage to fibers of passage rather than by the destruction of neurons within CeA. In addition, our data suggest that conditioned fear is mediated by different amygdaloidcircuits depending on the complexity of CS and the types of CR.

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