

Double Dissociation between the Involvement of the Bed Nucleus of the Stria Terminalis and the Central Nucleus of the Amygdala in Startle Increases Produced by Conditioned versus Unconditioned Fear

David L. Walker and Michael Davis

Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06508

The amplitude of the acoustic startle response is reliably enhanced when elicited in the presence of bright light (light-enhanced startle) or in the presence of cues previously paired with shock (fear-potentiated startle). Light-enhanced startle appears to reflect an unconditioned response to an anxiogenic stimulus, whereas fear-potentiated startle reflects a conditioned response to a fear-eliciting stimulus. We examine the involvement of the basolateral nucleus of the amygdala, the central nucleus of the amygdala, and the bed nucleus of the stria terminalis in both phenomena. Immediately before light-enhanced or fear-potentiated startle testing, rats received intracranial infusions of the AMPA receptor antagonist 2,3-

dihydroxy-6-nitro-7-sulphamoylbenzo(F)-quinoxaline (3 μ g) or PBS. Infusions into the central nucleus of the amygdala blocked fear-potentiated but not light-enhanced startle, and infusions into the bed nucleus of the stria terminalis blocked light-enhanced but not fear-potentiated startle. Infusions into the basolateral amygdala disrupted both phenomena. These findings indicate that the neuroanatomical substrates of fear-potentiated and light-enhanced startle, and perhaps more generally of conditioned and unconditioned fear, may be anatomically dissociated.

Key words: amygdala; bed nucleus of the stria terminalis; glutamate; AMPA; fear; anxiety; memory

Fear can be expressed as either a learned reaction to stimuli that predict danger (conditioned fear) or as an innate reaction to stimuli with intrinsic fear-eliciting properties (unconditioned fear). In our laboratory, fear and anxiety have been studied using fear-potentiated startle (Davis et al., 1993), a paradigm in which the amplitude of acoustic startle is elevated when elicited in the presence of conditioned stimuli (CS) previously paired with shock (Brown et al., 1951).

Evidence from fear-potentiated startle and other paradigms has pointed to a key role for the amygdala in the expression of conditioned fear. Lesions of the basolateral amygdala disrupt a variety of conditioned fear behaviors (LeDoux et al., 1990; Lorenzini et al., 1991; Sananes and Davis, 1992; Campeau and Davis, 1995; Lee et al., 1996; Maren et al., 1996), and lesions of the central nucleus of the amygdala, which receives inputs from the basolateral amygdala and which projects in turn to other brain regions mediating individual fear behaviors (Davis, 1992), are similarly effective (Kapp et al., 1979; Gentile et al., 1986; Iwata et al., 1986; Zhang et al., 1986; Sananes and Campbell, 1989; Hitchcock and Davis, 1991; Helmstetter, 1992; Weisz et al., 1992; Kim and Davis, 1993; Falls and Davis, 1995).

In contrast, there is little agreement about the neurobiological basis of unconditioned fear. Although several studies have implicated the amygdala (e.g., Blanchard and Blanchard, 1972; Fox and Sorenson, 1994), contradictory findings have also been reported

(e.g., Watkins et al., 1993; Sananes and Campbell, 1989). Recently, we found that chemically induced lesions of the central nucleus of the amygdala completely eliminated fear-potentiated startle but left intact the increase in startle produced by intracerebroventricular corticotropin-releasing hormone (CRH) (Lee and Davis, 1997). Conversely, chemically induced lesions of the bed nucleus of the stria terminalis, which like the central nucleus of the amygdala also receives input from the basolateral amygdala and projects to brain regions mediating individual fear behaviors (Alheid et al., 1995), abolished CRH-enhanced startle but left intact the increase in startle produced by conditioned fear (Lee and Davis, 1997). Based on these data, we hypothesized that the central nucleus of the amygdala may be preferentially involved in the expression of conditioned fear, whereas the bed nucleus of the stria terminalis may be preferentially involved in the expression of unconditioned fear. The basolateral amygdala, a primary source of innervation for both structures, would be important for conditioned as well as unconditioned fear.

To test this hypothesis, we evaluated the involvement of all three structures in fear-potentiated startle (i.e., conditioned fear) and in an unconditioned but closely related phenomenon termed light-enhanced startle (Walker and Davis, 1997). This latter paradigm uses prolonged illumination as an unconditioned anxiogenic stimulus to increase startle amplitude in rats and is sensitive to the anxiolytic compounds buspirone (Walker and Davis, 1997) and chlordiazepoxide (D. L. Walker and M. Davis, unpublished observations). Thus, in the following experiments, we evaluated the effect of intracranial infusions of the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(F)-quinoxaline (NBQX) into the basolateral amygdala, the central nucleus of the amygdala, and the bed nucleus of the stria terminalis, on fear-potentiated startle and on light-enhanced startle.

Received June 17, 1997; revised Sept. 17, 1997; accepted Sept. 19, 1997.

This research was supported by National Institute of Mental Health Grant MH-47840, Research Scientist Development Award MH-00004, Air Force Office of Scientific Research Grant AFOSR F49620 DEF, and the State of Connecticut.

Correspondence should be addressed to Dr. David L. Walker, 34 Park Street, Connecticut Mental Health Center, 3rd Floor, New Haven, CT 06508.

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MATERIALS AND METHODS

Animals

Ninety-four male albino Sprague Dawley rats (Charles River, Portage, MI) were used, weighing 350–500 gm at the time of surgery. When not undergoing startle testing, the rats were individually housed in hanging wire cages (18 × 25 × 20 cm) and were maintained on a 12 hr light/dark cycle (lights on at 7:00 A.M.) with food and water available *ad libitum*.

Surgery

Rats were anesthetized with Nembutal (sodium pentobarbital, 50 mg/kg, i.p.) and placed in a Kopf stereotaxic instrument. Stereotaxic coordinates were defined by the distance from the bregma [anteroposterior (AP), dorsoventral (DV), and mediolateral (ML)]. The skull was exposed, and 22 gauge guide cannulae (model C313G; Plastic Products, Roanoke, VA) were lowered bilaterally into either the basolateral amygdala (AP, -3.3; DV, -8.2; and ML, ±5.4), the central nucleus of the amygdala (AP, -2.2; DV, -8.4; and ML, ±4.2), or the bed nucleus of the stria terminalis (AP, -0.5; DV, -7.4; and ML, ±2.7). For placement into the bed nucleus of the stria terminalis, the close apposition of the two cannulae mandated an angled approach, such that the cannulae converged on the bed nucleus from either side, moving lateral to medial at an angle of 80° from the surface of the skull (i.e., 10° from vertical).

Size 0 insect pins (Carolina Biological Supply, Burlington, NC) were inserted into each cannula to prevent clogging. The tip of each extended ~1 mm past the end of the guide cannula. Jeweler screws were anchored to the skull, and the entire assembly was cemented in place using Cranioplastic Powder (Plastic Products). A minimum of 10 d elapsed between surgery and the behavioral procedures.

Infusion

Immediately before testing for either light-enhanced or fear-potentiated startle, rats were infused with either 3 µg of the AMPA receptor antagonist NBQX (dissolved in a 0.3 µl volume of PBS) or with PBS alone. For drug infusions, NBQX (generously supplied by Dr. W. Danysz, Merz & Co.) was dissolved in 1N NaOH and diluted with 0.1 M PBS to a final concentration of 10 µg/µl. The pH of the resulting solution was adjusted to 7.4.

Infusions (0.1 µl/min) were made through 28-gauge injection cannulae (model C313I, Plastic Products) attached by polyethylene tubing to a Hamilton microsyringe. After the infusion was completed, the injection cannulae were left in place for 60 sec before being withdrawn.

Light-enhanced startle testing

Apparatus. Animals were tested in two identical 8 × 15 × 15 cm Plexiglas and wire mesh cages, each suspended between compression springs within a steel frame and located within a sound-attenuating chamber (inside dimensions, 56 × 56 × 81 cm) (Industrial Acoustics Corp., Bronx, NY). Cage movement (e.g., produced by a startle response) resulted in displacement of a type 302 accelerometer (M. B. Electronics, New Haven, CT) affixed to the bottom of each cage. The voltage output of the accelerometer was amplified (NSO4, M. B. Electronics) and digitized on a scale from 0 to 4096 units by a MacADIOS II board (GW Instruments, Somerville, MA) interfaced to a Macintosh II computer. Startle amplitude was defined as the maximal peak-to-peak voltage occurring during the first 200 msec after onset of the startle-eliciting stimulus.

Background noise (55 dB) was produced by a speaker (model KFC 630; Kenwood, Long Beach, CA) located 18 cm from the rear of each cage, and connected to a white noise generator (model 15011; Lafayette, Lafayette, ID). Startle responses were elicited by 50 msec white noise bursts and were delivered through high-frequency speakers (Radio Shack Super Tweeter; Tandy, Fort Worth, TX) also located 18 cm from the rear of each cage. The noise bursts were produced by a white noise generator (model 455C; Grason-Stadler, West Concord, MA) with output amplified by a Realistic stereo amplifier (model MPA-80, Tandy).

Illumination was provided by a white fluorescent bulb (8 W), placed 18 cm behind and at floor level to the test cages. This light produced an illumination level of 700 footlamberts as measured from the middle of the test cage with a Telephotometer (model 2000; Gama Scientific Inc., New York, NY).

Behavioral procedures. Rats were infused with either drug or vehicle and immediately placed into the darkened test cage. After a 5 min stimulus-free acclimation period, 30 startle-eliciting noise bursts (10 each at 90, 95, and 105 dB, presented in a balanced, irregular order) were delivered at an interstimulus interval of 30 sec. These 30 stimuli consti-

tuted phase I. The animals were then removed from the test chamber and taken to an adjacent room for ~5 min before being returned for a second test (phase II). The sequence of events for phase II was identical to that for phase I. Together, these two phases constituted a single test session.

Using this procedure, each rat was tested on four separate occasions with individual tests separated by a minimum of 48 hr. For two of the four sessions, the test chamber was dark during phase I and also during phase II (dark → dark session type). For the other two sessions, the test chamber was also dark during phase I but was illuminated during phase II (dark → light session type). For both session types, animals were tested once after infusion of NBQX and once after infusion of PBS. Thus, each rat was tested under four conditions (i.e., PBS dark → light, NBQX dark → light, PBS dark → dark, and NBQX dark → dark). The ordering of session type and treatment was counterbalanced across animals.

Statistical analyses. For each animal, a difference score was calculated by subtracting the mean startle amplitude during phase I from the mean startle amplitude during phase II. These scores were subsequently compared using a two-way ANOVA with session type (i.e., dark → dark vs dark → light) and treatment (i.e., PBS vs NBQX) as within-subject factors. Follow-up comparisons were made using two-tailed *t* tests for pairwise comparisons.

Fear-potentiated startle training and testing

Apparatus. Animals were trained and tested in five 8 × 15 × 15 cm Plexiglas and wire mesh cages. The floor of each cage consisted of four stainless steel bars (6 mm diameter, 18 mm apart) through which scrambled foot shocks could be delivered. Each cage was located within a double-walled, ventilated, plywood isolation box (inner dimensions, 68.5 × 35.5 × 42 cm; outer dimensions, 76 × 47 × 51 cm), and together, the five double-walled boxes were further contained within a 2.5 × 2.5 × 2 m ventilated sound-attenuating chamber (Industrial Acoustics).

A 16.5 cm speaker (model 6267AX; Alpine Electronics, Torrance, CA) was located 36 cm from the rear of each cage. These speakers were connected to a white noise generator (model 15011, Lafayette) which provided 55 dB of background noise. Ventilation fans attached to the side walls of both the inner and outer plywood isolation boxes produced additional background noise, such that the overall noise level was 69 dB.

The 50 msec startle-eliciting noise bursts were generated by a second white noise generator (model 10B, Lafayette) and were amplified by a stereo amplifier. These noise bursts were delivered through Radio Shack Super Tweeter speakers, located 2 cm from the front of each cage. A 3.7 sec visual cue, produced by an 8 W fluorescent bulb, identical to that used in the light-enhanced startle experiments, was used as the conditioned fear stimulus.

A 0.6 mA foot shock, measured using the method described by Casella and Davis (1986), was used as the unconditioned stimulus. These foot shocks were produced by five (i.e., one for each cage) Lehigh Valley constant current shock generators (model SGS-004, BRS/LVE, Beltsville, MD).

Behavioral procedures. It would generally be preferable to counterbalance fear-potentiated startle and light-enhanced startle procedures. This was not possible in this study, because the light-shock pairings used during fear-potentiated startle training would have contaminated subsequent tests for unconditioned light effects (i.e., during light-enhanced startle testing). Therefore, fear-potentiated startle training began for all animals 7 d after the final light-enhanced startle test and took place on 2 consecutive days. Each of these two training sessions consisted of 10 light-shock pairings. The first pairing occurred 5 min after the rats had been placed into the startle chamber, and successive presentations occurred, on average, every 4 min (range, 3–5 min). For each pairing, the 0.5 sec shock was delivered 3.2 sec after onset of the 3.7 sec visual CS.

On the following day, rats received a brief infusion-free test to assess the effectiveness of fear conditioning. For this procedure, animals were placed in the startle chamber for a 5 min acclimation period and then presented with 20 startle-eliciting noise bursts. These initial noise bursts allowed the startle response to habituate and to become more stable in amplitude before collection of the test data. Six test trials were then performed. For three of these test trials, the 50 msec noise burst was presented 3.2 sec after onset of the light CS (i.e., light CS plus startle stimulus trials). For the other three test trials, the startle-eliciting noise bursts were presented in the dark (i.e., startle stimulus-alone trials). The two trial types were presented in a balanced, mixed order. Difference scores were determined by subtracting the mean startle amplitude on startle stimulus-alone trials from the mean startle amplitude on light CS

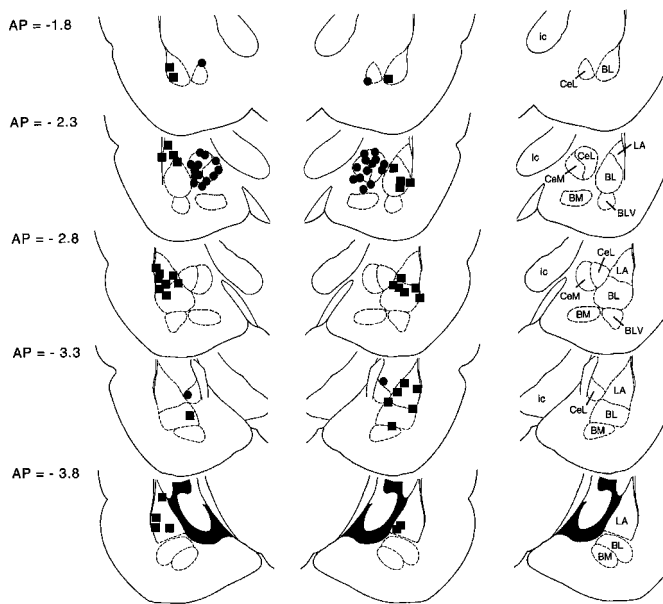


Figure 1. Cannula tip placements for animals included in the basolateral amygdala group (filled squares) and for animals included in the central nucleus of the amygdala group (filled circles), as transcribed onto atlas plates adapted from Paxinos and Watson (1986). The distance from bregma is indicated to the left; the various nuclei and their subdivisions are identified to the right. AP, Distance from bregma in millimeters; BM, basomedial amygdaloid nucleus; BL, basolateral amygdaloid nucleus; BLV, basolateral amygdaloid nucleus, ventral part; CeM, central amygdaloid nucleus, medial division; CeL, central amygdaloid nucleus, lateral division; ic, internal capsule; LA, lateral amygdaloid nucleus.

plus startle stimulus trials. Based on these results, rats were divided into two groups with equivalent mean difference scores.

Twenty-four hours later, rats from one of the two matched groups were infused with NBQX, and rats from the other matched group were infused with PBS. The animals were placed immediately into the test cages and after 5 min presented with 30 startle-eliciting noise bursts followed by 15 startle stimulus-alone test trials and 15 intermixed light CS plus startle stimulus test trials. Throughout these procedures, noise bursts were presented at an interstimulus interval of 30 sec and at an intensity of 95 dB.

Statistical analyses. Startle amplitude during light CS plus startle stimulus trials and during startle stimulus-alone trials were compared, within groups, using paired *t* tests. Difference scores (i.e., startle amplitude on light CS plus startle stimulus trials minus startle amplitude on startle stimulus-alone trials) of drug-infused and vehicle-infused rats were compared using two-tailed *t* tests for independent samples.

Histology

Animals were killed by chloral hydrate overdose and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and immersed in a 30% sucrose–formalin solution for at least 3 d, after which 40 μ m coronal sections were cut through the area of interest. Every fourth section was mounted and stained with cresyl violet.

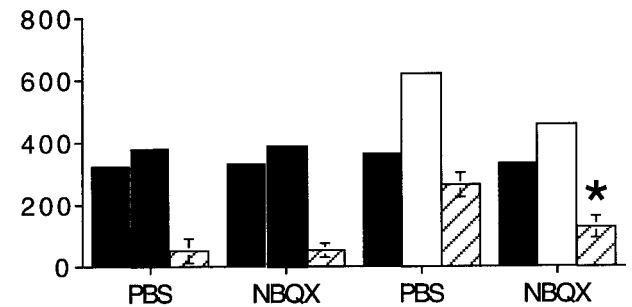
RESULTS

Basolateral amygdala

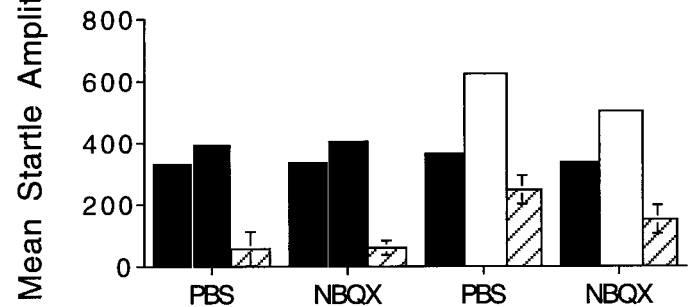
Histology

Figure 1 (filled squares) shows the cannula placements for animals included in the basolateral amygdala group. Cannulae were located in both the lateral and basolateral nuclei of the amygdala and included placements at rostral and caudal levels of each. Six of the 25 animals originally assigned to this group did not have bilateral cannulations of the basolateral amygdala, and these data were excluded from the statistical analyses that follow. One ad-

(A) All Basolateral Placements



(B) Rostral Basolateral Placements



(C) Caudal Basolateral Placements

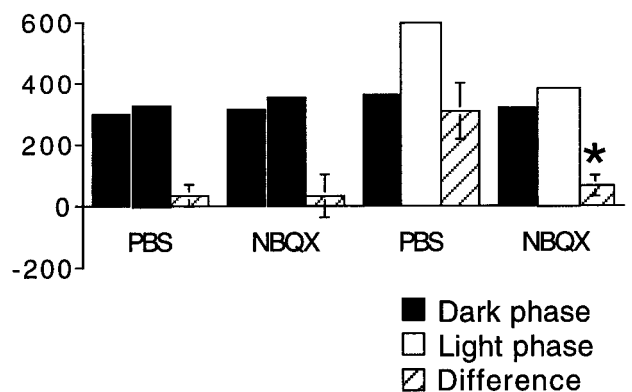


Figure 2. Light-enhanced startle for animals with bilateral cannulations in the basolateral amygdala. Data for all placements are shown in *A*. These same results were reanalyzed as a function of placement and are shown in *B* (rostral basolateral placements) and *C* (caudal basolateral placements). Asterisks indicate significance ($p < 0.05$, paired *t* test) of NBQX versus PBS difference scores within a given session (dark \rightarrow dark session types shown on left; dark \rightarrow light session types shown on right).

ditional animal was dropped from the study before testing for fear-potentiated startle because of a lost head cap.

Light-enhanced startle

As reported previously (Walker and Davis, 1997), the amplitude of acoustic startle was significantly greater when elicited in the presence of bright light than when elicited in the dark (Fig. 2*A*). This was reflected statistically by a significant effect of session type ($F_{(1,18)} = 18.87$; $p < 0.05$). The new finding here is that light-enhanced startle was significantly disrupted by infusions of

NBQX into the basolateral amygdala, as indicated by a significant session type \times treatment interaction ($F_{(1,18)} = 7.06$; $p < 0.05$).

Because the basolateral amygdala is a large and heterogeneous group of neurons with regional variations in chemical composition (Ben-Ari, 1981; Fallon and Ciofi, 1992), intrinsic connectivity (Krettek and Price, 1978; Pitkänen et al., 1995; Savander et al., 1995), and extrinsic connectivity (Mascagni et al., 1993; Shi and Cassell, 1997), it is likely that some subdivisions are more involved than others. To assess with greater precision the anatomical specificity of these effects, the behavioral results were also evaluated as a function of cannula placement.

An initial comparison between the histological and behavioral results suggested that placements into the caudal amygdala were particularly effective. Specifically, placements at, or caudal to, AP = -2.8 seemed to produce marked deficits, whereas more rostral placements were relatively ineffective. To statistically support this impression, rats were sorted into two groups: those with bilateral cannulations at, or caudal to, AP = -2.8 ($n = 12$) and those with one or both cannulae placed rostral to AP = -2.8 ($n = 7$). These data, shown in Figures 2B (rostral placements) and 2C (caudal placements), were analyzed as before, with the additional inclusion of a between-group placement factor (i.e., rostral vs caudal). The statistical results confirmed that the effectiveness of NBQX varied with cannula placement. Specifically, there was a significant session type \times treatment \times placement interaction ($F_{(1,17)} = 10.76$; $p < 0.05$).

In view of previous findings that projections from area Te2 (i.e., the primary source of modality specific visual information to the amygdala) are distributed within the basolateral amygdala almost exclusively at, or caudal to, AP = -2.8 (Mascagni et al., 1993; Shi and Cassell, 1997), these results are not surprising. These findings reinforce the view that the role of the basolateral amygdala in light-enhanced startle is to serve as an initial sensory way station, receiving and perhaps processing information that is subsequently routed to downstream target areas (i.e., to the bed nucleus of the stria terminalis, as discussed later).

Fear-potentiated startle

Startle amplitude during light CS plus startle stimulus trials was significantly greater than startle amplitude during startle stimulus-alone trials for PBS-infused rats ($t_{(8)} = 2.73$; $p < 0.05$). As shown in Figure 3, the difference scores of animals infused with NBQX were significantly lower than the difference scores of animals infused with PBS ($t_{(16)} = 2.63$; $p < 0.05$). These results are consistent with previous findings indicating that electrolytic (Campeau and Davis, 1995) or chemical (Sananes and Davis, 1992; Campeau and Davis, 1995) lesions of the basolateral amygdala, or inactivation of the basolateral amygdala with the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Kim et al., 1993), also block the expression of fear-potentiated startle. Because individual animals were assigned to only one of the two treatment conditions, further division into PBS-rostral, PBS-caudal, NBQX-rostral, and NBQX-caudal groups produced group numbers that did not allow for meaningful statistical evaluation of the effect of placement.

Central nucleus of the amygdala

Histology

The location of cannula tips for animals included in the central nucleus group are shown as *filled circles* in Figure 1. These cannulae were located in both the lateral and medial divisions of the central nucleus, predominantly but not exclusively at AP =

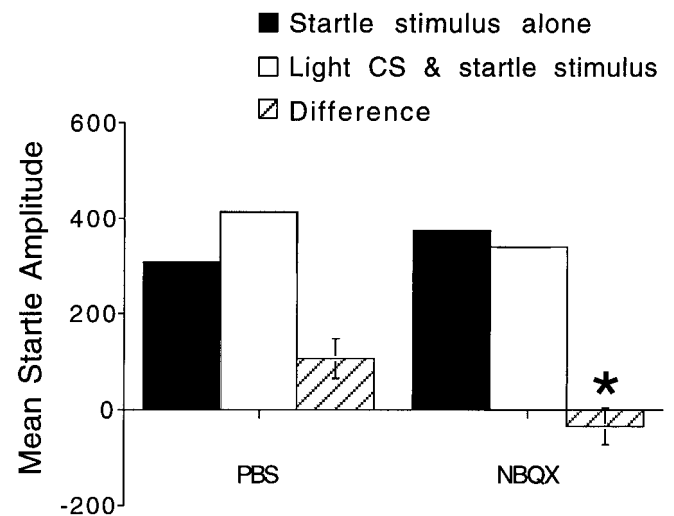


Figure 3. Fear-potentiated startle for animals with bilateral cannulations in the basolateral amygdala. The asterisk indicates significance ($p < 0.05$, t test for independent samples) of NBQX versus PBS difference score.

-2.3. Seven of the 22 animals originally assigned to this group did not have bilateral cannulations of the central nucleus of the amygdala, and these data are excluded from the statistical analyses that follow.

Light-enhanced startle

The increase in startle amplitude, from phase I to phase II, was significantly greater during dark \rightarrow light sessions than during dark \rightarrow dark sessions, as indicated by a significant session type effect ($F_{(1,14)} = 7.63$; $p < 0.05$). As shown in Figure 4A, the same dose of NBQX that disrupted light-enhanced startle when infused into the basolateral amygdala had no effect on light-enhanced startle when infused into the central nucleus of the amygdala. The treatment \times session type interaction was not statistically significant ($p > 0.1$).

Fear-potentiated startle

Startle amplitude during light CS plus startle stimulus trials was significantly greater than startle amplitude during startle stimulus-alone trials for PBS-infused rats ($t_{(6)} = 3.47$; $p < 0.05$). As expected, and as shown in Figure 4B, infusions of NBQX into the central nucleus of the amygdala completely blocked fear-potentiated startle, compared with PBS-infused rats ($t_{(13)} = 3.75$; $p < 0.05$). These data are consistent with previous results of a blockade of fear-potentiated startle after electrolytic (Campeau and Davis, 1995; Falls and Davis, 1995) or chemical (Campeau and Davis, 1995; Lee and Davis, 1997) lesions of the central nucleus of the amygdala.

Bed nucleus of the stria terminalis

Histology

Cannula tips were located throughout the bed nucleus of the stria terminalis in the dorsal and ventral aspects of the medial and lateral divisions. These placements are illustrated in Figure 5. Ten of the 36 animals originally assigned to this group did not have bilateral cannulations of the bed nucleus of the stria terminalis, and three other animals were noted to have CSF leaking from the cannulae during the infusion procedure. Data from these animals were excluded from the following statistical analyses. One additional animal was dropped from the study before

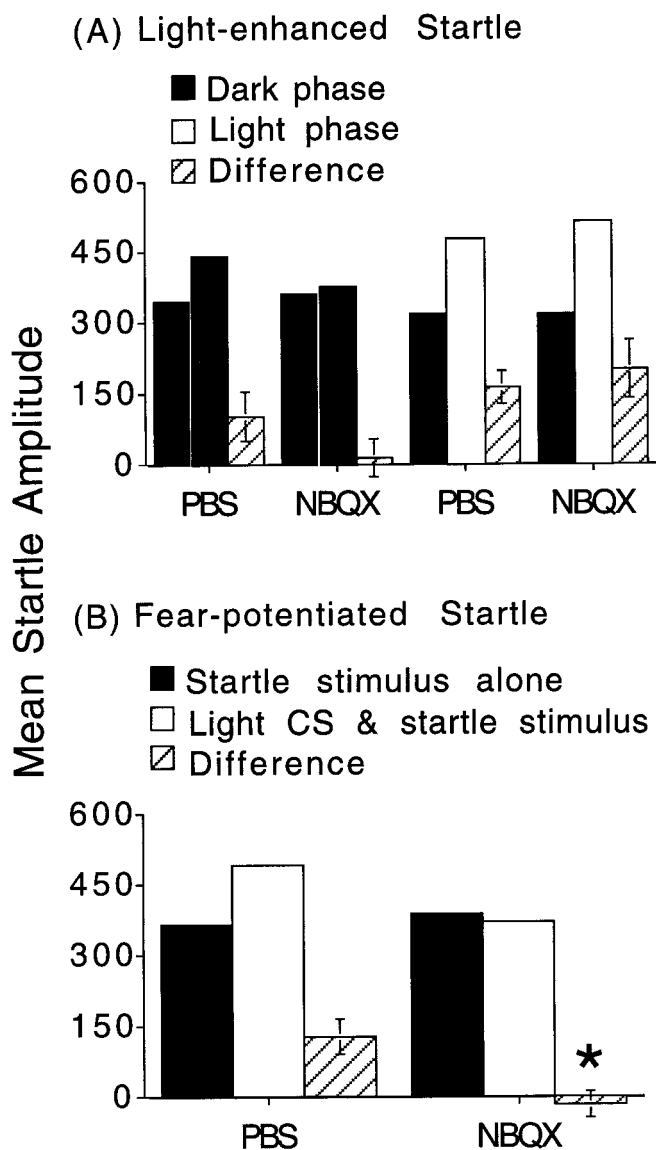


Figure 4. Light-enhanced (*A*) and fear-potentiated (*B*) startle for animals with cannula placements in the central nucleus of the amygdala. NBQX did not significantly influence light-enhanced startle, but abolished fear-potentiated startle. The asterisk indicates significance ($p < 0.05$, t test for independent samples) of NBQX versus PBS difference score.

testing for fear-potentiated startle because of a lost head cap. Also, a supplementary analysis was performed on a separate group of animals (the delayed test procedure described below; $n = 11$). These placements are also shown in Figure 5. There was no attrition from this group.

Light-enhanced startle

As indicated by a significant session type effect ($F_{(1,22)} = 11.1$; $p < 0.05$), illumination increased startle amplitude. There was also an overall effect of treatment ($F_{(1,22)} = 9.06$; $p < 0.05$), indicating that NBQX disrupted phase I to phase II increases (Fig. 6*A*). There was not, however, a significant session type \times treatment interaction ($p > 0.1$). Thus, NBQX attenuated the phase I to phase II increase irrespective of whether that increase occurred on dark \rightarrow dark or on dark \rightarrow light sessions. In fact, startle

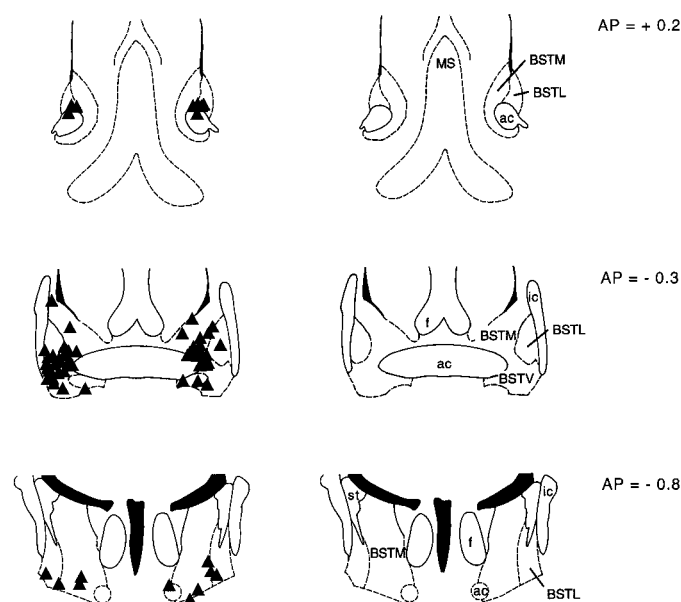


Figure 5. Cannula tip placements for the bed nucleus of the stria terminalis group, as transcribed onto atlas plates adapted from Paxinos and Watson (1986). The various nuclei and their subdivisions, along with the distance from bregma, are identified to the right. AP, Distance from bregma in millimeters; ac, anterior commissure; BSTL, bed nucleus of the stria terminalis, lateral division; BSTM, bed nucleus of the stria terminalis, medial division; BSTV, bed nucleus of the stria terminalis, ventral division; f, fornix; ic, internal capsule; MS, medial septum; st, stria terminalis.

amplitude did show an atypically large increase from phase I to phase II, even with dark \rightarrow dark sessions.

If the phase I to phase II increase on dark \rightarrow dark sessions was a meaningful and reproducible phenomena, similar in nature to that seen with dark \rightarrow light sessions (i.e., an unusually strong unconditioned anxiety response to the incidental handling that occurs between phases), then the similar effects of NBQX on both session types would be consistent with a role for the bed nucleus in anxiety. However, it is also possible that NBQX was exerting a less specific influence on startle amplitude, which became more pronounced as the session progressed (i.e., as the concentration of NBQX increased at the relevant receptor areas), and which was not, therefore, specific to the phase II increase. In other words, NBQX may have had a delayed depressant effect on startle amplitude, which simply subtracted from the phase I to phase II increase but did not disrupt its underlying cause (e.g., anxiety).

To allow for a more confident attribution of the effects of NBQX to anxiolytic influences, the above experiment was repeated in an additional group of animals. In this second group, however, testing was delayed for 20 min such that phase I began at the same time, with respect to infusion, as phase II had begun for animals in the previous experiment. If NBQX was producing a general decrease in startle amplitude that was not readily apparent until at least 20 min after drug infusion, then the phase I startle amplitude of NBQX-infused rats in this second experiment should be significantly lower than the phase I startle amplitude of PBS-infused rats.

The results of this experiment are shown in Figure 6*B*. Phase I startle amplitudes of NBQX- and PBS-infused rats were comparable, suggesting that the effects of NBQX observed in the previous experiment were not attributable to

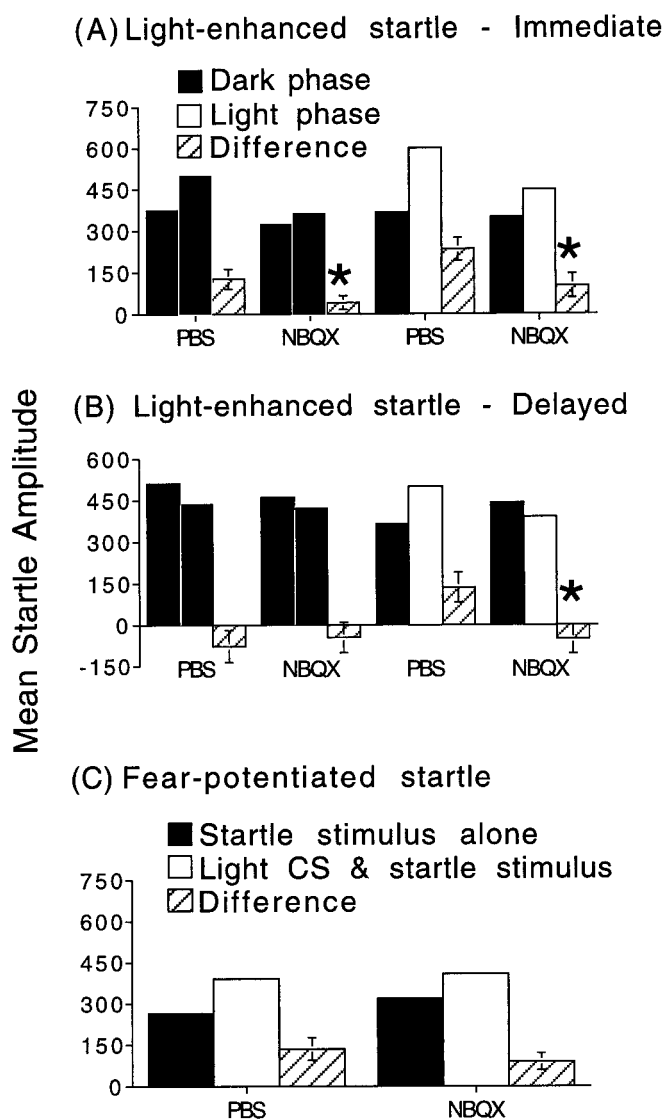


Figure 6. Light-enhanced and fear-potentiated startle for animals with cannula placements in the bed nucleus of the stria terminalis. The effect of NBQX on light-enhanced startle is shown in *A* for animals tested immediately after NBQX or PBS infusion, and in *B* for animals tested 20 min after NBQX or PBS infusion. Infusions of NBQX did not disrupt fear-potentiated startle (*C*). Asterisks indicate significance ($p < 0.05$, paired t tests) of NBQX versus PBS difference scores within a given session type (dark \rightarrow dark sessions shown on *left*; dark \rightarrow light sessions shown on *right*).

nonspecific delayed influences on startle. Moreover, the increase in startle amplitude previously observed during dark \rightarrow dark sessions did not replicate, allowing for a more specific attribution of the effects of NBQX to a disruption of light-enhanced startle. There was a statistically significant session type effect ($F_{(1,10)} = 5.13$; $p < 0.050$) and also a significant session type \times treatment interaction ($F_{(1,10)} = 5.72$; $p < 0.05$). Thus, NBQX infusions into the bed nucleus of the stria terminalis completely blocked light-enhanced startle and did so independent of any general effects on baseline reactivity. For both protocols, there was no apparent relation between cannula placement and the behavioral data.

Fear-potentiated startle

Animals that had previously received PBS or NBQX just before testing for light-enhanced startle were subsequently trained and tested for fear-potentiated startle. Consistent with previous findings that neither electrolytic (Hitchcock and Davis, 1991) nor chemical (Lee and Davis, 1997) lesions of the bed nucleus of the stria terminalis block fear-potentiated startle, there was also no effect with NBQX (see Fig. 6*C*). Thus, for both PBS- and NBQX-infused rats, startle amplitude was significantly greater on light than on startle stimulus-alone trials ($t_{(9)} = 2.70$; $p < 0.05$) and ($t_{(11)} = 2.94$; $p < 0.05$), respectively, and difference scores for both groups were comparable ($p > 0.1$).

Double dissociation

Results from the central nucleus of the amygdala and bed nucleus of the stria terminalis groups suggested that these two structures could be functionally dissociated. To confirm this statistically, we compared the effect of NBQX on fear-potentiated versus light-enhanced startle using standardized behavioral data from the subset of animals that received NBQX during both procedures.

For central nucleus implants, fear-potentiated startle difference scores of NBQX-infused animals were divided by the mean difference score of the PBS-infused group to obtain a proportion-of-control score for each NBQX-infused rat. The effect of NBQX on light-enhanced startle was similarly calculated by dividing the dark \rightarrow light difference scores of rats that received NBQX during both procedures (thereby allowing for a within-subject comparison vis-à-vis fear-potentiated startle) by the mean dark \rightarrow light difference score for rats infused with PBS during both procedures. These calculations were repeated for animals with implants in the bed nucleus of the stria terminalis. These scores were then entered into a single ANOVA, using placement as a between-subject factor and paradigm as a within-subject factor.

The results of this analysis indicated a significant interaction between placement and paradigm ($F_{(1,17)} = 5.32$; $p < 0.05$), confirming a double dissociation between the central nucleus of the amygdala and the bed nucleus of the stria terminalis with respect to fear-potentiated and light-enhanced startle.

DISCUSSION

These findings indicate similarities as well as differences in the neuroanatomical substrates of fear-potentiated and light-enhanced startle. Both behaviors were disrupted by infusions of NBQX into the basolateral amygdala. Fear-potentiated, but not light-enhanced, startle was blocked by infusions into the central nucleus of the amygdala. Light-enhanced, but not fear-potentiated, startle was blocked by infusions into the bed nucleus of the stria terminalis.

Given that the basolateral amygdala receives input from several visual areas (Mascagni et al., 1993; McDonald and Mascagni, 1996; Shi and Cassell, 1997) and projects to both the central nucleus of the amygdala and to the bed nucleus of the stria terminalis (Alheid et al., 1995; Pitkänen et al., 1995; Savander et al., 1995), it seems likely that the processing of visual stimuli with fear-evoking properties proceeds serially, initially activating the basolateral amygdala and subsequently activating the central nucleus of the amygdala (e.g., for fear-potentiated startle), the bed nucleus of the stria terminalis (e.g., for light-enhanced startle), or both. Consistent with this serial organization, Lee and Davis (1997) reported that the startle-enhancing effect of intracerebroventricular CRH, which appeared to stimulate CRH receptors

within the bed nucleus of the stria terminalis directly, was not affected by excitotoxic lesions of the basolateral amygdala.

Lee and Davis (1997) also reported an anatomical double dissociation similar to that reported here, using fear-potentiated startle (dependent on the central nucleus of the amygdala) and CRH-enhanced startle (dependent on the bed nucleus of the stria terminalis) as behavioral measures. Our results provide additional evidence that the bed nucleus of the stria terminalis and the central nucleus of the amygdala can be functionally dissociated and implicate these two structures in natural reactions to fear-evoking stimuli.

It is perhaps surprising that fear-potentiated and light-enhanced startle would respond differently to inactivation of these structures. Both procedures use elevations in startle amplitude as the behavioral measure of fear, and in both cases these increases are evoked by the same visual stimulus. What is the fundamental difference between these two paradigms that would account for the dissociable involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala?

Perhaps the most obvious difference is that the fear-potentiated startle is a conditioned response to a previously neutral cue, whereas light-enhanced startle is an unconditioned response to a stimulus with intrinsically anxiogenic properties. Thus, the central nucleus of the amygdala may preferentially mediate the expression of conditioned fear, whereas the bed nucleus of the stria terminalis may preferentially mediate the expression of unconditioned fear. Results from a number of studies are consistent with this view. For example, lesions of the central nucleus of the amygdala disrupt conditioned freezing (Iwata et al., 1986), conditioned increases in arterial pressure (Iwata et al., 1986), fear-potentiated startle (Campeau and Davis, 1995), and other conditioned responses (e.g., Gentile et al., 1986; Helmstetter, 1992) but have minimal or inconsistent effects on unconditioned responses (e.g., Gentile et al., 1986; Sananes and Campbell, 1989; Watkins et al., 1993; Pesold and Treit, 1995).

Although the second half of this hypothesis, that the bed nucleus of the stria terminalis is preferentially involved in unconditioned responses, is more difficult to evaluate given the paucity of relevant studies, the available data are, in most cases, consistent with this view as well. Silveira et al. (1993) reported that rats exposed for 15 min to the elevated plus maze showed marked increases of *c-fos* labeling in the bed nucleus of the stria terminalis. Within the amygdala, *c-fos* labeling also was noted in the basolateral, cortical, and medial nuclei but not, interestingly, in the central nucleus of the amygdala. In another study, Sorenson et al. (1994) reported that the unconditioned hypoalgesia produced in rats by exposure to a cat was blocked by chemical lesions of the bed nucleus of the stria terminalis. Although this group reported a similar effect of central nucleus lesions (Fox and Sorenson, 1994), the use in that study of electrolytic lesions may have compromised function of the bed nucleus of the stria terminalis by damaging inputs from the basolateral amygdala, which pass close to the central nucleus of the amygdala (Shi, 1995).

In contrast, there is relatively little evidence that the bed nucleus of the stria terminalis is involved in conditioned fear. In fact, LeDoux et al. (1988) found no effect of bed nucleus lesions on either conditioned freezing or on conditioned increases in arterial pressure, and, as previously noted, lesions of the bed nucleus also have no effect on fear-potentiated startle (Hitchcock and Davis, 1991). However, we did observe recently that lesions of the bed nucleus of the stria terminalis block the presumably nonassociative sensitization of startle that gradually develops in

rats receiving daily foot shock (K. A. McNish, J. C. Gewirtz, and M. Davis, unpublished observations). Provisionally, then, the conditioned versus unconditioned hypothesis seems tenable in view of the existing literature.

The second major difference between fear-potentiated and light-enhanced startle is stimulus duration. Whereas the light used for fear-potentiated startle is quite brief, the light used for light-enhanced startle is on for the duration of the 20 min experiment. Perhaps the central nucleus of the amygdala responds preferentially to brief stimuli or to stimulus onset (e.g., as a result of rapidly accommodating neurons), whereas the bed nucleus of the stria terminalis is more readily driven by sustained input. We are currently evaluating this hypothesis using context as a long-duration stimulus. Initial findings using electrolytic lesions of the bed nucleus of the stria terminalis suggest that stimulus duration may not be an important variable (McNish et al., 1996). However, results from experiments using NBQX infusions (which may mitigate recovery of function problems sometimes associated with lesion studies) are still being evaluated.

In this regard, it may also be informative to assess the effects of bed nucleus inactivation on dark pulse facilitation of startle, as described by Ison et al. (1991). In this paradigm, a brief dark pulse delivered just before startle elicitation increases startle amplitude and this increase is disrupted by the anxiolytic compound diazepam. Indeed, these authors have speculated that dark-onset potentiation may reflect an adaptive fear-related response to shadow-casting predators. As such, evidence that bed nucleus inactivation disrupts dark pulse facilitation would support the view that this structure plays a special role in unconditioned fear irrespective of stimulus duration. If, however, bed nucleus inactivation did not disrupt dark pulse facilitation, but inactivation of the central nucleus did, then the hypothesis that the former plays a special role in processing brief- versus long-duration anxiogenic stimuli would be strengthened.

The possible relevance of another long-duration startle-enhancing stimulus, moderate-intensity background noise (Hoffman and Searle, 1965; Ison and Hammond, 1971; Davis, 1974), also should be considered. Kellog et al. (1991) reported that noise-enhanced startle could be disrupted by the benzodiazepine receptor agonist diazepam and suggested that this phenomenon might also reflect an influence of anxiety. On the other hand, buspirone, which blocks fear-potentiated startle (Kehne et al., 1988; Mansbach and Geyer, 1988) and also light-enhanced startle (Walker and Davis, 1997), has no effect on noise-enhanced startle (Walker and Davis, unpublished observations). Moreover, amygdala lesions, which as noted previously, block numerous behaviors associated with fear and anxiety, are similarly ineffective (Shanbacher et al., 1996). Thus, it seems questionable whether the effects of background noise on startle can truly be attributed to unconditioned fear. It is interesting to note that noise-enhanced startle also survives electrolytic lesions of the bed nucleus of the stria terminalis (Davis and Walker, unpublished observations). Although it will be important to determine with greater certainty the underlying nature of noise-enhanced startle, these initial data suggest that the bed nucleus of the stria terminalis does not mediate the effects on startle of all unconditioned or long-duration stimuli, but only those that modulate startle through influences on fear or anxiety.

Although the interpretations advanced above appear to be consistent with much of the available data, they may not be categorically applicable in all instances. In particular, the effects of central nucleus and bed nucleus lesions on hormonal responses

to several stressors are complex (Van de Kar et al., 1991; Roozendaal et al., 1992; Gray et al., 1993) and are not readily predicted by any single theory. In general, however, it is clear that the central nucleus of the amygdala and the bed nucleus of the stria terminalis can, under many circumstances, be functionally dissociated, perhaps as a function of conditioned versus unconditioned fear or short- versus long-duration stimuli.

Importantly, these functional distinctions may have broader implications for the understanding and treatment of clinical disorders in humans. Although the experimental paradigms most commonly used in animal research have emphasized conditioned behaviors, an understanding of unconditioned fear is at least equally significant insofar as many clinical disorders do not involve learned fear but are manifest instead as a generalized and often long-lasting apprehension, for which a previous conditioning history can rarely be identified. As such, a closer examination of the biological similarities and differences between these two types of behaviors may provide useful information in developing therapeutic agents individually tailored for the treatment of specific types of disorders.

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