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Role of bed nucleus of the stria terminalis and amygdala AMPA receptors in the development and expression of context conditioning and sensitization of startle by prior shock

Michael Davis and David L. Walker

Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 954 Gatewood Rd NE (Yerkes Neuroscience Bldg), Rm. 5214, Atlanta, USA

David L. Walker: dlwalke@emory.edu

Abstract

A core symptom of post-traumatic stress disorder is hyper-arousal—manifest in part by increases in the amplitude of the acoustic startle reflex. Gewirtz et al. (*Prog Neuropsychopharmacol Biol Psychiatry* 22:625–648, 1998) found that, in rats, persistent shock-induced startle increases were prevented by pre-test electrolytic lesions of the bed nucleus of the stria terminalis (BNST). We used reversible inactivation to determine if similar effects reflect actions on (a) BNST neurons themselves versus fibers-of-passage, (b) the development versus expression of such increases, and (c) associative fear versus non-associative sensitization. Twenty-four hours after the last of three shock sessions, startle was markedly enhanced when rats were tested in a non-shock context. These increases decayed over the course of several days. Decay was unaffected by context exposure, and elevated startle was restored when rats were tested for the first time in the original shock context. Thus, both associative and non-associative components could be measured under different conditions. Pre-test intra-BNST infusions of the AMPA receptor antagonist NBQX (3 µg/side) blocked the non-associative (as did infusions into the basolateral amygdala) but not the associative component, whereas pre-shock infusions disrupted both. NBQX did not affect baseline startle or shock reactivity. These results indicate that AMPA receptors in or very near to the BNST are critical for the expression and development of non-associative shock-induced startle sensitization, and also for context fear conditioning, but not context fear expression. More generally, they suggest that treatments targeting the BNST may be clinically useful for treating trauma-related hyper-arousal and perhaps for retarding its development.

Keywords

Bed nucleus of the stria terminalis; Amygdala; Sensitization; Startle; Hyperarousal; Fear; Anxiety; AMPA

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Correspondence to: David L. Walker, dlwalke@emory.edu.

Conflict of interest The authors declare that they have no conflict of interest. Portions of these data have previously appeared in abstract form (Walker and Davis 2005) and in a review article (Walker et al. 2009).

Introduction

Traumatic experiences result in the formation of powerful associations between the trauma itself and various, often incidental, stimuli that may also be present. Subsequent exposure to these or similar stimuli can evoke vivid memories and various somatic and autonomic responses that, in extreme cases, may be infused with all the emotional intensity of the original experience. These same experiences can also lead to a less stimulus-bound, presumably non-associative sensitization (i.e., hyper-arousal) that lasts for days, weeks, or even years (e.g., up to 4 decades in Goldstein et al. 1987). In practice, it can be difficult to discriminate between associative fear and non-associative sensitization (e.g., Kamprath and Wotjak 2004), but because both contribute to the long-lasting and sometimes-debilitating after-effects of trauma (e.g., Pitman et al. 1993; Shalev et al. 1992), considerable resources have been directed towards identifying their neural substrates.

A voluminous body of pre-clinical findings, derived largely from Pavlovian fear-conditioning studies in rats, has implicated the amygdala in trauma-related associative fear (c.f., Walker and Davis 2002; Fanselow and LeDoux 1999; Maren 2001; Mahan and Ressler 2012) and results from imaging (c.f., Hughes and Shin 2011; Rauch et al. 2006) and lesion (Koenigs et al. 2008) studies in humans have generally supported this view. In contrast, the neural substrates of trauma-related sensitization are far less clear (c.f., Stam 2007a, b). Of special relevance to this study, Gewirtz et al. (1998) reported that pre-shock electrolytic lesions of the bed nucleus of the stria terminalis (BNST)—an area closely related to the amygdala (Alheid et al. 1995; Alheid and Heimer 1988; de Olmos and Heimer 1999) but one that may be more involved in longer-duration anxiety-like responses (c.f., Davis et al. 2010; Walker et al. 2009)—disrupted startle increases that developed over the course of a multi-day repeated footshock procedure. Because rats in that study were shocked and tested in the same chamber, it was unclear if the primary effect was on non-associative sensitization or associative fear to the context, although based on the lack of overshadowing effects when the shocks were paired with a discrete cue, the authors suggested that an effect on non-associative sensitization was more likely. It was also unclear if the effect should best be attributed to a disruption of acquisition or expression, or even whether the effect was attributable to damage to BNST neurons themselves or to fibers-of-passage.

Using a different design in which an 8-min noise was paired with footshock, we too have observed persistent post-shock increases in baseline startle. While these increases precluded any meaningful analysis of fear-potentiated startle to the 8-min noise (our original intent with this protocol), they did present an opportunity to revisit issues originally raised, but not settled, in Gewirtz et al. (1998). It is the analysis of those data that are presented below.

General methods

Animals

Male Sprague–Dawley rats (Charles River, Raleigh, NC) were group-housed (4 rats/cage) in 190 × 135 × 80-cm (length × width × height) polycarbonate cages and maintained on a 12-h light/dark cycle (lights on at 0800 hours) with food and water available ad libitum. All procedures were conducted in full accordance with USDA and Animal Welfare Act

regulations and with the approval of the Emory University Institutional Animal Care and Use Committee.

Apparatus—Rats were tested in four identical 8 × 15 × 15-cm Plexiglas and wire mesh cages. The floor of each consisted of four 6.0-mm diameter stainless steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed 90 × 70 × 70-cm ventilated sound-attenuating chamber.

Background noise (60 dB wideband) was provided by a ACO Pacific Inc. (Belmont, CA) model 3024 noise generator and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (SPL) were made with a Brüel & Kjær (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (Type 4176) located 7 cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing.

Startle responses were evoked by 95 dB (and in Experiment 2, 110 dB as well) 50 ms white-noise bursts (5 ms rise-decay) generated by a Macintosh G3 computer soundfile, amplified by a Radio Shack amplifier (100 Watt; Model MPA-200; Tandy, Fort Worth, TX), and delivered through the same speakers as those used to provide background noise. Startle responses were quantified using an accelerometer (model U321AO2; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage that when displaced (e.g., by cage movement produced by the rats' startle or footshock response), produced a voltage output proportional to the velocity of cage movement. This output was amplified (PCB Piezotronics, Model 483B21) and digitized on a linear scale of 0–9.98 arbitrary units by an InstruNET device (GW Instruments, Model 100B; Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after onset of the startle-eliciting stimulus.

Scrambled shocks (0.5-s, 0.4-mA), produced by LeHigh Valley (Beltsville, MD) shock generators (model SGS-004), were delivered through the floorbars. Shock intensity was measured with a 1-kW resistor across a differential channel of an oscilloscope in series with a 100-kW resistor connected between adjacent floor bars within each cage. Current was defined as the root mean square voltage across the 1-kW resistor where $\text{mA} = 0.707 \times 0.5 \times \text{peak-to-peak voltage}$. Footshock reactions were measured in the same way as startle reactions (i.e., based on the accelerometers' output during shock), but the window during which accelerometer output was sampled was increased from 200 to 500 ms (i.e., the duration of shock).

The presentation of all stimuli was controlled by a Macintosh G3 computer using custom-designed software (The Experimenter, Glassbeads Inc.; Newton, CT).

Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intra-peritoneal) and placed in an ASI, inc. (Warren, MI) stereotaxic frame with the nosebar set to –3.6 mm (flat-skull position). The skull was exposed and guide cannulae (PlasticsOne, Roanoke, VA) were

lowered into either the basolateral amygdala or BNST. For amygdala placements, 22-gauge cannulae (model C313G) were lowered vertically to a position 3.3 mm caudal, 5.4 mm lateral, and 7.2 mm ventral to bregma. For BNST placements, 26 gauge cannulas (model C315G) were oriented at a 20 degree angle to vertical (i.e., in the same transverse plane) and then positioned 0.3 mm caudal and 3.8 mm lateral to bregma, after which they were lowered 5.8 mm. The smaller diameter cannula and the angled entry were used for BNST placements to decrease the incidence of cannula passing through or perforating the wall of the lateral ventricle. Stainless steel wires, cut so as to protrude 1 mm from the end of the guide cannula, were inserted into each cannula to maintain patency. Jeweler screws were anchored to the skull and the entire assembly cemented in place using Cranioplastic Powder (PlasticsOne, Roanoke, VA). A minimum of 8 days elapsed between surgery and the behavioral procedures.

Drugs and infusion procedure

Rats were infused with phosphate-buffered saline (PBS) or the AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3, 4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX-3 $\mu\text{g/side}$) disodium salt (Tocris Cookson Inc., Ellisville, MO). Infusions (0.25 $\mu\text{l/min}$, 0.5 μl total volume) were made either through 28- or 33-gauge injection cannula (PlasticsOne, Models C313I and C315I for amygdala and BNST infusions, respectively) that were attached by polyethylene tubing to a Hamilton microsyringe. Injection cannulas were left in place for 2 min after the infusion was completed.

Behavioral procedures

Acclimation session—Rats were placed into the test cages and after 5 min, presented with 30 95-dB startle-eliciting noise bursts (inter-stimulus interval [ISI] = 30 s). The response to these noise bursts was used to sort rats into different treatment groups such that each began with equivalent mean baseline startle levels. For acclimation, the floorbars were covered with a sandpaper insert (i.e., a piece of sandpaper, approximately $8 \times 15\text{-cm}$, mounted with adhesive onto a similarly sized piece of Plexiglas). The cage was dark and there were no explicit odors introduced into the test cage. We refer to this as Context A.

Pre-shock test—One to three days later, rats were returned to the test chamber (Context A) where they received, after 5 min, 48 95-dB startle-eliciting noise bursts (ISI = 30 s). A 60-dB low-frequency noise was presented during test trials 17–32. The low-frequency noise was included because it was originally our intent with this design to fear-condition rats to this stimulus. However, rather than seeing consistent startle increases in the presence of the noise, we saw large and more reliable startle increases, from the pre-conditioning to post-conditioning test, even before noise onset (i.e., test trials 1–16). Those increases precluded any meaningful analysis of noise-evoked fear but, as noted in the “Introduction”, presented a fortuitous opportunity to evaluate the BNST’s role in shock-induced baseline startle increases.

Shock sessions—On each of the three following days, rats were returned to the same chamber in which they received the pre-shock test. The contextual cues were modified, however (i.e., in relation to the test context), by removing the sandpaper inserts, turning on

an 8-W fluorescent bulb located 10 cm behind each test cage, and placing an alcohol-wetted gauze pad as an olfactory stimulus just outside the test cage. We refer to this as Context B. Five minutes after being placed in the cage, rats received the first of two 8-min bouts of 7 footshocks each. Within each bout, the average inter-shock interval was 60 s (range, 6–196 s), and the two bouts were themselves separated by an 8-min shock-free period. Overall then, rats received 14 shocks on each of 3 consecutive days. For all but a subset of animals from Experiment 1A, a continuous 60-dB low-frequency filtered noise was present during the 8-min periods during which shocks were delivered. Rats were removed from the cages 8 min after the final footshock.

Post-shock test—Procedurally, the post-shock test was identical to the pre-shock test and, for most animals, occurred 24–48 h after the final shock session. For a subset of animals from Experiment 3, however, the first post-shock test occurred 6 days after the final shock session. All rats received post-shock tests in Context A (i.e., which contained contextual elements different from those that were present during conditioning). As indicated in the “Results” for specific experiments, some rats received an additional test in Context B (i.e., the shock context).

Statistical analyses

For each rat, mean startle amplitude during the first 16 trials (i.e., prior to onset of the 8-min noise) of the pre- and post-shock tests was determined, and a percent change score calculated (i.e., mean startle amplitude during the post-shock test divided by mean startle amplitude during the pre-shock test $\times 100 - 100$). Percent change rather than difference scores are used because we have found them to be less sensitive to between-subject variability in baseline startle amplitude (Walker and Davis 2002). Between- and within-subjects comparisons were made using ANOVA and *t*-tests. The criterion for significance was $p < 0.05$ (two-tailed). Analyses were performed using SPSS 13.0.0 and Graphpad Prism 6.0b software.

Histology and inclusion criteria

Rats were euthanized by chloral hydrate overdose and perfused intracardially with 0.9 % saline (wt/vol) followed by 10 % formalin (vol/vol). The brains were removed and immersed in a 30 % sucrose-formalin solution (wt/vol) for at least 3 days, after which 40 μ m coronal sections were cut through the area of interest. Every fourth section was mounted and stained with cresyl violet. Cannula placements, and the determination as to whether the cannula was within or sufficiently near the intended target to be scored as a hit, were judged by a scorer blind to the animal’s group assignment and behavioral data. Results from animals which did not have both cannulas within 0.5 mm of the intended target were excluded from the statistical analyses as were data from animals in which one or both cannulas passed through a ventricle.

Results

Histology

Representative placements are shown in Fig. 1. Of 157 implanted animals, data from 107 were included in the final analyses (others were excluded based on placement criteria or, less commonly, headcap loss prior to the completion of behavioral testing). For BNST placements, the cannula tips were located in the anterolateral BNST, in most cases just dorsal or ventral to the anterior commissure or just medial to the internal capsule. For amygdala placements, the cannula tips were located almost exclusively in the lateral or basolateral nucleus, although some were sufficiently near to the central nucleus of the amygdala that AMPA receptors in this area may have been affected as well.

Experiments 1A and 1B: effect of pre-test NBQX infusions on shock-induced startle increases

Experiment 1A—48 hours after the final shock session, rats were infused with vehicle or NBQX and immediately thereafter tested in Context A (i.e. with different contextual elements than those that were present during shock exposure). The results are shown in Fig. 2. ANOVA indicated a significant Treatment (i.e., PBS vs. NBQX) effect ($F_{(1,63)} = 7.83$, $p = 0.007$) without a significant Placement effect ($F_{(1,63)} = 0.22$, $p = 0.64$) or a significant Treatment X Placement interaction ($F_{(1,63)} = 0.15$, $p = 0.7$). Included in these analyses are data from 16 BNST-cannulated rats (of which 7 received PBS and 9 NBQX infusions) that did not receive noise presentations during shock. The effect of NBQX on shock-induced startle increases was comparable irrespective of whether the noise was included or not, as assessed with a separate ANOVA using Design (with or without noise) and Treatment (PBS or NBQX) as between-subject factors. Thus, there was a significant effect of Treatment ($F_{(1,42)} = 9.51$, $p = 0.004$) but not of Design ($F_{(1,42)} = 0.67$, $p = 0.418$), and there was not a significant interaction ($F_{(1,42)} = 1.94$, $p = 0.171$).

Experiment 1B—The effect of intra-BNST NBQX infusions was also evaluated in a different group of rats that were shocked and then tested in the same context (Context B). These results are shown in the right panel of Fig. 2. In contrast to the results of Experiment 1A, where potentiation scores in rats shocked and tested in the same context were completely abolished by intra-BNST NBQX infusions ($t_{(44)} = 2.68$, $p = 0.01$), there was very little evidence of an NBQX effect in these animals. Thus, the potentiation scores of NBQX-infused animals (50 ± 23 %, mean $\pm$ SEM) were only slightly less than those of PBS-infused rats (67 ± 33 %), and not significantly different ($t_{(15)} = 0.46$, $p = 0.65$). As discussed later, we believe these data reflect a critical involvement of BNST AMPA receptors in the expression of sensitization, but not context fear.

Mean baseline startle (i.e., the pre-shock startle levels from which potentiation scores are derived) for the six groups that comprised Experiment 1 ranged from between 0.69 and 0.96 units and were not significantly different based on one-way ANOVA with group as a factor ($F_{(5,78)} = 0.58$, $p = 0.715$).

Experiment 2: effect of intra-BNST NBQX infusions on startle amplitude in non-shocked rats

To determine if the effect of pre-test NBQX was attributable to a specific disruption of shock-induced increases, or if it instead reflected a more general decrease of startle that subtracted from and, therefore, masked the shock-induced increases, the effect of intra-BNST NBQX was also evaluated in rats that had not been shocked. These rats received an initial acclimation session and then, 24 and 72 h later, a pre-test infusion of either NBQX (3 μ g/side) or PBS (counterbalanced within-subjects). Immediately after the infusions, they were placed into the test chamber and presented with 8 95-dB and 8 110-dB noise bursts. The 110-dB noise bursts were included to control for the possibility that NBQX effects might be baseline dependent (i.e., only apparent at higher startle amplitudes such as those characteristic of previously shocked rats).

As indicated in Fig. 3, however, NBQX did not significantly influence startle amplitude in non-shocked rats at either intensity. Mixed-model (i.e., with between- and within-subjects factors) ANOVA indicated a significant effect of Noise Burst Intensity ($F_{(1,5)} = 73.8, p < 0.001$) but not of Treatment (PBS vs. NBQX) ($F_{(1,5)} = 0.67, p = 0.45$) or Order (PBS or NBQX first) ($F_{(1,5)} = 0.06, p = 0.824$). There were no significant interactions ($p > 0.68$).

Experiment 3: effect of shock-free context exposures on shock-induced startle increases

The shock-induced startle increases observed in Experiment 1 might reflect non-associative sensitization or, if our attempt to provide distinctive contexts was insufficient, associative fear that had generalized to context A. Previous studies indicate that conditioned fear responses are very stable—showing little if any decrement over a 40-day period for fear-potentiated startle to a light (i.e., the longest interval looked at in Lee et al. 1996) and for more than a year as assessed with freezing to a shock-associated context (Gale et al. 2004). However, conditioned fear responses can be extinguished when the conditioned stimulus (CS) is repeatedly presented without the aversive stimulus. To help determine if the startle increases which we observed reflected (1) conditioned fear that had generalized from Context B to Context A or (2) non-associative sensitization, we evaluated in Experiment 3 the stability of these increases across time, and their response to Context A exposure without shock. If the previously observed startle increases were due to conditioned fear, we would expect these increases to be reduced by context exposure without shock (i.e., by extinction), but to be stable in the absence of such exposures. Alternatively, if the increases were due to non-associative sensitization, we would expect that the post-shock startle increases would decay with the passage of time and that this decay would not be accelerated by extinction.

Rats received a pre-shock test and three shock sessions as previously described. One group (Repeated Test Group) received their first post-shock test 24 h later in Context A and additional tests every 24 h thereafter until startle amplitude had decayed to the pre-shock baseline (note that because these tests occur without shock, they also constitute, at least nominally, extinction training). For a 2nd group, the first test was delayed until startle amplitude in the Repeated Test group had returned to baseline. Both groups then received a final test in the shock context (Context B).

As shown in Fig. 4, rats tested 1 day after the final shock session showed the expected increase in startle amplitude ($t_{(9)} = 4.28, p = 0.002$ versus pre-shock). This increase gradually faded across the next several sessions ($t_{(9)} = 3.44, p = 0.007$; $t_{(9)} = 5.05, p = 0.001$; and $t_{(9)} = 4.36, p = 0.002$; for tests on the 2nd, 3rd, and 4th days post-shock, respectively), arriving at pre-shock levels by the 5th post-shock day ($t_{(9)} = 0.48, p = 0.641$) and dipping slightly below the pre-shock baseline on the 6th day ($t_{(9)} = 3.44, p = 0.007$). When tested for the first time 6 days after the final shock session, mean startle amplitude in the Delayed Test group was not higher than, but comparable to, pre-shock levels ($t_{(9)} = 0.14, p = 0.889$), even though these animals had not received context exposures (i.e., no extinction). Had we measured startle 24 h after the final shock session (we did not do so since this might have constituted an extinction session if the animals were not discriminating between Contexts A and B, thereby muddying the distinction between the Delayed Test and Repeated Test groups), we assume that startle would have been elevated, as it was with all other non-NBQX groups in this study. We conclude, therefore, that the shock-induced startle increases that are normally observed in this paradigm decay passively and are not accelerated by unreinforced context exposure as would be expected if startle increases in Context A were due to associative fear generalized from Context B.

Twenty-four hours after the final test in the safe context (Context A), rats were tested once more, this time in the shock context (Context B). Startle amplitude in both groups increased (i.e., from day 6), suggesting that they had indeed formed an association between shock and the shock context and that this association could be expressed in the presence of appropriate context cues. Statistically, paired t -tests indicated significant startle increases from the Day 6 non-shock-context to the Day 7 shock-context tests for the Repeated Test ($t_{(9)} = 3.02, p = 0.015$) as well as the Delayed Test ($t_{(9)} = 2.33, p = 0.045$) groups. ANOVA indicated a significant effect of Context ($F_{(1,18)} = 11.13, p = 0.004$) but not of Group ($F_{(1,18)} = 11.13, p = 0.113$). The Group $\times$ Context interaction was not significant ($F_{(1,18)} = 0.88, p = 0.360$).

Experiment 4: effect of BNST inactivation on the development of sensitization and context fear conditioning

For this experiment, rats were infused with either PBS ($N = 19$) or NBQX ($N = 16$) immediately *prior to* each of the three shock sessions. These data were collected in two replications. For both, rats were tested in the safe context (Context A) 24 h after the final footshock session. For the 2nd replication only, rats (PBS $N = 10$, NBQX $N = 9$) were also tested 12 days after the final footshock session in the safe context (Context A) and once more on the following day in the shock context (Context B).

As shown in Fig. 5 (left panel), intra-BNST NBQX infusions completely blocked the development of shock-induced sensitization ($t_{(33)} = 2.87, p = 0.007$ versus PBS-infused rats). This did not appear to be secondary to a reduction in shock perception because footshock reactions in the PBS- (3.51 ± 0.28 , mean $\pm$ SEM) and NBQX- (4.06 ± 0.3) infused rats were highly similar ($t_{(33)} = 1.37, p = 0.18$). For the subset of animals that received all three tests (i.e., Day 1 and Day 12 tests in the non-shock context and a final test in the shock context; right panel), the Group (PBS vs. NBQX) effect approached but did not reach significance ($F_{(1,17)} = 3.55, p = 0.077$), and there was not a significant Session effect ($F_{(2,34)}$

= 0.2, $p = 0.817$). Most importantly, however, the Group X Session interaction *was* significant ($F_{(2,34)} = 3.89$, $p = 0.03$) and significantly fit to a quadratic trend ($F_{(1,17)} = 6.50$, $p = 0.021$). This reflects the observation (see Fig. 5, right panel) that percent potentiation scores converged as sensitization decayed and then diverged during the test for context fear (post-shock Day 13). Thus, pre-training NBQX infusions into the BNST disrupted the development of sensitization as well as context fear conditioning.

Discussion

The main findings are that AMPA receptors in or very near to the BNST participate in the expression as well as the development of non-associative sensitization (i.e., as assessed by startle reflex amplitude) and that they also participate in the acquisition of context fear.

Experiment 1 showed that pre-test intra-BNST infusions of an AMPA receptor antagonist blocked the increase in startle that otherwise occurs when startle is later measured in a neutral context. These results are broadly consistent with those of Gewirtz et al. (1998) who found that pre-shock electrolytic BNST lesions also prevented shock-induced startle increases. Our results expand on those findings by discriminating between effects on (a) BNST neurons themselves versus fibers-of-passage, (b) the development versus expression of shock-induced startle increases, and (c) associative fear versus non-associative sensitization. Our results also identify AMPA receptors as a key player.

We did not find an effect of pre-test intra-BNST NBQX infusions when rats were tested in the same context in which shocks occurred. The simplest explanation is that startle increases observed in the shock context had both an associative (i.e., conditioned fear to the context) and non-associative component and that the associative component was not susceptible, or at least *as* susceptible, to AMPA receptor blockade. Thus, by measuring startle in different contexts at different times, it was possible to independently assess the effect of NBQX infusions on these two processes.

The relation between hyper-arousal and associative fear is of some interest clinically as both are core components of PTSD. Previous findings have suggested some degree of independence. In particular, hyper-arousal has been shown to persist even when context fear conditioning is prevented (i.e., by pre-shock intra-hippocampal infusions of the NMDA receptor antagonist AP5 or by “immediate shock” training (Siegmund and Wotjak 2007; Sauerhofer et al. 2012) or suppressed as a result of extinction (Golub et al. 2009). Our results showing a block of sensitization, but not context fear, complement those by demonstrating the inverse pattern (i.e., intact context fear without evidence of hyper-arousal following pre-test intra-BNST NBQX).

Because we targeted a particular receptor system (i.e., AMPA receptors), our results should not be over-interpreted to indicate that the BNST, more broadly, is not involved in context fear expression. Indeed, results from several studies (Hott et al. 2012; Luyten et al. 2011; Resstel et al. 2008; Sullivan et al. 2004) that have used lesion or other inactivation procedures to disrupt BNST function, and a variety of behavioral and autonomic measures to assess fear, indicate that it *is* involved. Even NBQX was found to disrupt freezing to a

shock-associated context in Zimmerman and Maren (2011). Although those results might at first seem inconsistent with our own, data in that study were collected from rats that had previously received basolateral amygdala lesions—a manipulation known to produce compensatory changes that alter the BNST's involvement in fear expression (Poulos et al. 2010). Taken together, the available evidence suggests that the BNST *does* participate in the expression of long-term context fear memories but that this involvement does not always require the participation of AMPA receptors.

We also evaluated the effect of pre-test NBQX infusions into the basolateral and basomedial amygdala nuclei, which both project heavily to the BNST (c.f., Dong et al. 2001). The effects were statistically indistinguishable from that of intra-BNST infusions. Surprisingly, few studies have documented an involvement of the amygdala in either the development or expression of persistent stress-induced hyperarousal. In rats, Adamec et al. (1999) reported that intra-amygdala infusions of an NMDA receptor antagonist shortly prior to cat exposure prevented sensitization as assessed by increased startle and decreased risk assessment behavior, but had no effect on persistent increases in anxiety as assessed in the plus-maze. Also, analyses of PTSD rates in combat veterans with penetrating brain injuries found the occurrence of PTSD to be significantly lower in those with damage to either the ventromedial prefrontal cortex or amygdala compared with those who sustained damage to other brain areas or to no-damage controls (Koenigs et al. 2008). In fact, PTSD was entirely absent in the veterans with amygdala damage—an effect that was equally apparent across each of the three primary symptom categories of re-experiencing, avoidance/numbing, and hyper-arousal.

An important issue bearing on the interpretation of our data concerns the locus of NBQX's action. Based on our previous experience with this drug, we believe that its effects in the present study were mediated by actions very near to the site of infusion. We have now found in several different studies that the ability of NBQX (at the same dose as that used here) to disrupt fear/anxiety-potentiated startle is site-specific even to individual amygdala nuclei. Thus, bilateral infusions into the caudal basolateral, but not central amygdala nucleus, disrupted unconditioned light-enhanced startle in Walker and Davis (1997), unilateral infusions into the central but not medial amygdala nucleus disrupted conditioned fear-potentiated startle in Walker et al. (2005), and infusion of NBQX into the deep layers of the superior colliculus/deep mesencephalic nucleus blocked expression of fear-potentiated startle to a visual cue, whereas infusion just 1 mm away did not in Zhao and Davis (2004). These findings strongly suggest that the behavioral effects described herein are due to the blockade of AMPA receptors at or very near to the intended target.

Experiments 2 and 3 attempted to verify two additional premises upon which interpretation of the preceding data hinge. Experiment 2 asked if NBQX was specifically disrupting the facilitatory influence on startle of prior shock, or if NBQX was instead disrupting startle itself. Given the results of Experiment 1B, in which NBQX reduced post-shock startle amplitude only in rats that were shocked and tested in different contexts, we thought it likely that BNST inactivation was disrupting the facilitatory influence of prior shock rather than startle. Experiment 2 confirmed this by evaluating the effect of intra-BNST NBQX on non-shocked rats using 90 as well as 105 dB startle-eliciting noise bursts. The latter were used to

evoked a startle response similar in amplitude to that which occurs in sensitized rats, in case an NBQX disruption of startle was baseline-dependent. At neither intensity, however, was an effect on startle apparent. Although we did not also examine the effect of intra-amygdala NBQX infusions on baseline startle, we have done so previously using the same doses and stereotaxic coordinates used in the present study, and also found no effect (i.e., Walker et al. 2005). Thus, we are confident that NBQX specifically disrupted the facilitating influence of prior shock, and not startle itself.

More difficult to determine was whether these effects should be attributed to an interference with non-associative sensitization or conditioned fear (i.e., to the context). To minimize the latter possibility, different contextual elements (referred to collectively as the “A” vs “B” contexts) were used for the shock versus test sessions. However, it is possible that the generalization curve was sufficiently broad so as to include both. It is also possible that other aspects of the test procedure, such as being transported to the test room or being placed into a small enclosure, evoked an associative fear response which was independent of the test context, defined more narrowly as the physical apparatus within which shocks occurred. Nonetheless, we believe that the overall pattern of results is more consistent with a non-associative rather than an associative account.

First, the results of Experiment 3 indicated that the large increases in startle that were reliably present 24 h after conditioning decayed over the course of several days (see data for the Repeated Test group in Fig. 4). In contrast, numerous studies have shown that the effect of conditioned fear on startle and freezing is very stable, even over the lifespan of laboratory rats and even in rats that receive far fewer shocks (Gale et al. 2004; Kim and Davis 1993; Lee et al. 1996; Maren et al. 1996). The decay that we observed could not readily be attributed to extinction (which would assume that the animals were generalizing across contexts A and B) because the same decay was observed in the Delayed Test control group (which did not receive non-reinforced context exposures). Note also that when rats were retested in the context in which shocks did occur, startle increased once more, suggesting that the shock procedure did result in associative learning and that these two processes (i.e., sensitization and context fear) could be evaluated independently under different experimental conditions.

We do note that on the final two test days, startle was nominally lower in the Repeated Test compared with Delayed Test group. This could be viewed as evidence for a partial contribution of generalized context fear that was extinguished in the Repeated Test group. The difference between groups was not statistically significant but, if real, could alternatively be attributed to habituation in the Repeated Test group (e.g., Leaton 1981; Marlin and Miller 1981). Indeed, with repeated testing, some degree of habituation is expected and would also account for the slight dip below baseline in the Repeated Test group. Yet another interpretation is that the startle increases we observed *were* attributable to generalized context fear and that the decay of these increases reflected a time-dependent sharpening of the generalization gradient. However, this is an even less satisfactory account in that numerous previous studies have shown just the opposite, namely that conditioned fear generalization gradients actually broaden with time (c.f., Riccio et al. 1992). We cannot conclude from any of these data that our rats did not experience some degree of associative

fear. If such fear existed, however, it appears to have contributed only modestly, if at all, to the post-shock startle increases we observed. The most defensible position, in our view, is that the dominant influence on startle was non-associative.

Experiment 4 evaluated the involvement of the BNST in the development of shock-induced startle increases by infusing NBQX prior to each of the three shock sessions. The results were unambiguous. Startle potentiation was completely absent 24 h after the final shock session in the group that had received pre-training NBQX infusions. We do note that startle increased in this group from the Day 1 to Day 12 post-shock test, although the increase just missed statistical significance ($t_{(8)} = 2.254, p = 0.054$). This pattern is not unprecedented with startle, having previously been observed with respect to shock-induced startle increases in rats (Servatius et al. 1995), and trauma-induced startle increases in human (Shalev et al. 2000). Indeed, the gradual growth of fear-like responses has been noted in many paradigms and interpreted in most cases as fear “incubation” (Kumar 1970; Pamplona et al. 2011; Pickens et al. 2009; Schreurs et al. 2011). However, we believe that this would be a poor interpretation for the present results given that startle returned to baseline just 24 h later, during the Day 13 startle test in the shock context. We suggest instead that startle may have been transiently elevated on Day 12 due to dishabituation insofar as the rats had not been tested, exposed to experimental stimuli, or handled for the previous 10 days (presumably, the PBS-infused rats’ Day 12 scores would have been elevated by an equal amount as they were treated identically).

In any case, the different levels of startle potentiation observed during each of the post-shock tests for the NBQX-compared with PBS-infused rats could not be attributed to effects on shock perception during training, insofar as footshock reactions were similar in the two groups. To the best of our knowledge, this is the first evidence of an involvement of the BNST in the development of stress-induced hyper-arousal, although an involvement of the BNST in the development of other non-associative stress effects has been reported. Most notably, Christianson et al. (2011) found that infusions of the sodium-channel blocker tetrodotoxin (TTX) into the lateral ventral BNST prior to inescapable tailshock prevented the reduction of social exploration observed in vehicle-infused rats. One possibility is that BNST neurons are themselves modified by repeated footshock and that these modifications promote hyper-arousal and an anxiogenic phenotype. In fact, the BNST *is* modified by repeated stress as indicated, for example, by changes to corticotropin-releasing factor (CRF) (Lebow et al. 2012; Santibanez et al. 2006), PACAP (Hammack et al. 2009), and 5-HT (Hazra et al. 2012) signaling systems. Particularly striking are the gross morphological changes first reported by Vyas et al. (2003). In that study, chronic immobilization stress was found to produce significant increases in dendritic branching in BNST neurons. The changes were anatomically specific in that they were not also found in the closely related (Alheid et al. 1995; Alheid and Heimer 1988; de Olmos and Heimer 1999) central nucleus of the amygdala (CeA). A similar effect was reported by Pego et al. (2008), who showed also that dendritic hypertrophy in the BNST (but again, not in the CeA) was associated with increased startle and increased anxiety in the plus-maze, but not with increased fear-potentiated startle to a brief light cue that predicted shock. Note also that fear conditioning to a 3.7-second light was not disrupted by pre-training BNST lesions in Gewirtz et al. (1998). The results of

each of these studies are consistent with our general hypothesis that the BNST is more involved in persistent anxiety-like responses, whereas the CeA is more involved in shorter-duration fear responses to discrete stimuli (c.f., Davis et al. 2010; Walker et al. 2009).

Pre-shock NBQX infusions into the BNST not only disrupted sensitization (i.e., tested 48 h after shock in a neutral context) but also disrupted context conditioning assessed 13 days later in the shock context. Recall that pre-test infusions did not block context fear expression (i.e., Experiment 1B). The results suggest that AMPA receptors in the BNST play a unique and critical role in the induction of synaptic plasticity, but a non-critical role in the neuro-transmission of context-related information to these same neurons. One possibility is that NBQX interferes directly with the formation of context-shock associations. However, given that pre-test NBQX infusions also disrupted non-associative sensitization, a more parsimonious interpretation is that BNST AMPA receptors are necessary for processing the emotional consequences of shock itself (and perhaps other aversive stimuli).

As already noted, Gewirtz et al. (1998) found that fear conditioning to a 3.7-second light was not disrupted by pre-training BNST lesions, and we have found more recently (unpublished results) that pre-shock intra-BNST NBQX infusions do not disrupt fear-conditioning to a 3.7-s tone (i.e., 157 ± 58.8 vs. 157 ± 55.5 , for PBS and NBQX-infused rats, respectively). In addition to providing further support for the view that the BNST is more involved in longer-duration anxiety-like responses than in brief cue-specific fear responses (Walker et al. 2009), these data also suggest that the disruptive effect of pre-shock NBQX on both sensitization and context conditioning is not attributable to very general effects such as gross sensory disturbances which would likely also affect fear conditioning to a discrete cue.

Overall, these results point to a key role of the BNST in both the development and expression of non-associative shock-induced startle increases, and in the acquisition of associative fear to the context in which shock occurred. Shock-induced startle increases in rats mimic elements of post-trauma anxiety in humans (e.g., Garrick et al. 2001; Servatius et al. 1995) and indeed, exaggerated startle responses are explicitly noted in DSM-V as a diagnostic criterion for both acute- and post-traumatic stress disorder. Findings from Marshall et al. (2006) and from Schell et al. (2004) indicate that trauma-induced hyper-arousal is among the most persistent of symptoms and generally the most informative predictor (i.e., versus avoidance/numbing or re-experiencing) of PTSD development and remission. Assuming that the neural substrates of trauma-induced hyper-arousal are similar in rats and humans, the BNST would seem then to be a potentially useful target for the treatment of these symptoms and perhaps also their development (especially if post-trauma BNST manipulations were found to be effective in disrupting the “consolidation” of sensitization). In fact, results from a recent deep brain stimulation in patients with severe and otherwise intractable obsessive-compulsive disorder (OCD) found a dramatic reduction not only in OCD symptoms following stimulation of the “ventral internal capsule/ventral striatum” (which corresponds to the area of the BNST that was cannulated in our study), but also in co-morbid depression and non-OCD anxiety, which was common in these patients (Greenberg et al. 2010).

Pharmacological manipulation is of course more feasible for most patients. Although direct acting AMPA receptor antagonists such as NBQX would be poor candidates for clinical use due to widespread side-effects following systemic administration, modulators of glutamate function such as Group II metabotropic agonists, which moderate glutamate release in the BNST and elsewhere (e.g., Grueter and Winder 2005; Schoepp et al. 2003; Muly et al. 2007) and disrupt fear-potentiated startle and other stress-associated behaviors in rats (e.g., Walker et al. 2002; Helton et al. 1998; Bruijnzeel et al. 2001) as well as humans (Grillon et al. 2003), may be more useful. The BNST is also notable for its rich and varied population of neuro-active peptides (Arluison et al. 1994; Gray and Magnuson 1992; Ju et al. 1989; Shimada et al. 1989a, b; Walter et al. 1991; Woodhams et al. 1983) in cell bodies and terminals, and these too present numerous opportunities for modulating BNST function. CRF (Lee and Davis 1997), calcitonin gene-related peptide (CGRP) (Sink et al. 2011, 2013), and pituitary adenylate cyclase-activating peptide (PACAP) (Hammack et al. 2009) each increase startle when infused into the BNST and produce other hyper-arousal/anxiety-like effects as well (e.g., Lee et al. 2008; Ciccocioppo et al. 2003; Sahuque et al. 2006; Hammack et al. 2010; Sink et al. 2013). Research by Morilak and colleagues (c.f., Morilak et al. 2003) has pointed to a possible role for galanin, and still others have identified a role for norepinephrine (Deyama et al. 2008, 2009; Fendt et al. 2005; Hott et al. 2012; Schweimer et al. 2005), which is found in the BNST at concentrations higher than almost any other brain area (Brownstein et al. 1974; Kilts and Anderson 1986). These particular systems are likely to be only the tip of a very large iceberg, with the unique neurochemical makeup of the BNST and its afferents providing as-of-yet unmined opportunities to target with greater precision the most devastating symptoms of acute- and post-traumatic stress disorder, and possibly other anxiety conditions.

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Fig. 1. Representative cannula tracks into the BNST (*top*) and amygdala (*bottom*) in nissl-stained sections

Fig. 2.

Effect of NBQX infusions on the BNST and basolateral amygdala on pre- to post-shock startle increases. Infusions into both structures disrupted shock-induced startle increases when rats were tested in a context different from that in which they received shocks (Context A), as indicated by a significant Treatment effect without a significant Placement or Treatment $\times$ Placement interaction (left and center bars). The effect of NBQX was not as evident when rats were tested in the same context (Context B) in which they had received shocks (rightmost bars). In fact, the mean level of startle potentiation in NBQX-infused rats in that condition was comparable to all, and not significantly different from any, of the three control groups

Fig. 3.

Effect of intra-BNST NBQX infusions on startle amplitude in non-shocked rats. The startle response to 95-dB and 105-dB noise bursts was tested in rats that received PBS and NBQX infusions (counterbalanced within-subjects design). NBQX did not significantly influence startle amplitude to either intensity

Fig. 4.

Stability of post-shock startle increases and their response to non-reinforced context exposures. In this experiment, post-shock startle increases decayed to pre-shock levels (indicated by the first set of bars, and extended across the graph by the *dashed line*) within 6 days of the final shock session, with or without repeated testing in the non-shock context. When tested in the presence of cues that *were* present during shock (i.e., in Context B), rats in both groups showed an increase in startle, suggesting that they *had* formed an association between the context and shock, and that this association could be expressed under appropriate circumstances. Thus, rats show non-associative sensitization when tested within several days of shock in a neutral context, and conditioned fear when tested at longer intervals in the shock context. The abscissa indicates the number of days since the last shock session

Fig. 5.

Effect of pre-shock intra-BNST NBQX infusions on the development of non-associative (sensitization) and associative (context conditioning) startle increases. Pre-shock NBQX infusions prevented the development of non-associative shock-induced sensitization (*left panel*). Data for the subset of rats that received additional tests are shown in the *right panel*. This subset received a 2nd test in the neutral context (Context A) 12 days after the final shock session and one more test in the shock context (Context B) on the following day. By the 12th day after the final shock session, the two groups performed similarly when tested in the neutral context. When tested in the shock context on the following day, the groups diverged, with rats that received pre-shock PBS infusions showing startle levels approximately 120 % greater than their pre-shock baseline, and rats that had received pre-shock NBQX infusions showing startle levels nearly identical to the pre-shock baseline. Thus, intra-BNST NBQX infusions prevented the development not only of non-associative sensitization, but also of fear conditioning to the shock-associated context