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The Nucleus Accumbens is not Critically Involved in Mediating the Effects of a Safety Signal on Behavior

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Although considerable progress has been made towards understanding the neural systems mediating conditioned fear, little is known about the neural mechanisms underlying conditioned inhibitors of fear (or safety signals). The present series of experiments examined the involvement of the nucleus accumbens (NAC) in mediating the effects of safety signals on behavior using a conditioned inhibition of fear-potentiated startle paradigm. Neither increasing dopaminergic nor decreasing glutamatergic function in the NAC altered the magnitude of conditioned fear or conditioned inhibition of fear in rats. Furthermore, large pre- or post-training electrolytic lesions of the NAC did not affect acquisition or expression of fear-potentiated startle or conditioned inhibition of fear-potentiated startle. Taken together, these data suggest that the NAC is not critically involved in the acquisition or expression of fear-potentiated startle or conditioned inhibition of fear-potentiated startle. Previous research has implicated the NAC in 'reward-attenuated startle' in which presentation of a stimulus paired with food decreased startle responding. The present results, therefore, indicate important neural dissociations between the processing of appetitive and safety signals, even though behavioral studies and learning theories have suggested that these two forms of learning share some commonalities.

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INTRODUCTION

Classical fear conditioning has been used extensively to delineate the neural systems mediating conditioned fear. In these experiments, a previously neutral stimulus (eg, light or tone; conditioned stimulus; CS) that is paired with an aversive stimulus (footshock; unconditioned stimulus; US) acquires the ability to elicit conditioned fear responses. Results from many labs using this task show that the acquisition and expression of Pavlovian fear conditioning critically involves the amygdala (for reviews, see Davis, 1992; LeDoux, 2000; Schafe *et al*, 2001; Wallace and Rosen, 2001; Fanselow and Gale, 2003; Maren, 2003). Once acquired, conditioned fear may last a lifetime (Fanselow and Gale, 2003; Gale *et al*, 2004). However, the expression of conditioned fear acquired under one set of circumstances may be inappropriate or even disadvantageous in another set of circumstances in which the CS no longer predicts a realistic threat. Therefore, it is also important to understand the neural mechanisms underlying the process of fear inhibition or reduction. The findings from these studies may provide therapeutic insights into clinical states associated with dysfunctions of fear inhibition such as anxiety disorders.

Fear may be reduced or inhibited in several ways. For example, extinction training, in which a stimulus that was previously paired with shock is presented repeatedly in the absence of the shock (CS-no shock), gradually reduces conditioned fear responses (Pavlov, 1927; Cain *et al*, 2003; Quirk and Gehlert, 2003). Conditioned inhibition of fear, in which a stimulus signals the absence of shock, is another procedure that reduces conditioned fear responses (Rescorla, 1969). In one example of this paradigm, a light is presented with a shock (Light-shock) but, on some trials, a noise is paired with the light in the absence of shock (Noise & Light-no shock). Following several pairings, the light acquires the ability to elicit conditioned fear responses and the noise acquires the ability to reduce the conditioned fear responses elicited by the light. In this way, the noise comes to predict the absence of shock and hence becomes a safety signal (Falls and Davis, 1995).

Little is known about the neural systems that mediate the fear-inhibiting effects of a safety signal. However, several

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lines of evidence converge to suggest that the nucleus accumbens (NAC) may be involved. First, the NAC receives inputs from many brain regions, including those involved in conditioned fear (McDonald, 1991; Brog *et al*, 1993). Indeed, descending glutamatergic projections from the amygdala, hippocampus and prefrontal cortex, and ascending dopaminergic fibers arising from the midbrain converge at the NAC (Groenewegen *et al*, 1999). Second, presentation of a safety signal is associated with an increase in the expression of immediate early genes in the NAC, among other areas (Campeau *et al*, 1997).

Finally, an extension of some learning theories implicates the involvement of the NAC. These theories hypothesize the existence of two mutually antagonistic motivational systems in the brain; the appetitive and aversive motivational systems (Konorski, 1967; Dickinson and Dearing, 1979). As these motivational systems would reciprocally inhibit one another, activation of the aversive system would inhibit the appetitive system. By extension, a safety signal that inhibits the aversive system may be perceived by the animal as motivationally equivalent to a stimulus that directly activates the appetitive motivational system. There is general agreement that the NAC is critically involved in mediating the motivational impact of appetitive Pavlovian cues on behavior (Robbins *et al*, 1989; Berridge and Robinson, 1998; Dayan and Balleine, 2002; de Borchgrave *et al*, 2002). For instance, a stimulus that was previously paired with sucrose availability enhances both instrumental responding for sucrose (an effect known as Pavlovian-to-instrumental transfer) and elicits an increase in locomotion (an effect known as autoshaping) (Brown and Jenkins, 1968). Importantly, both of these effects of are blocked by lesions of the NAC (Balleine and Killcross, 1994; Parkinson *et al*, 2000; Corbit *et al*, 2001; Hall *et al*, 2001) while enhancing dopaminergic transmission in the NAC increases Pavlovian-to-instrumental transfer (Wyvell and Berridge, 2000). Of interest is the recent finding that presentation of a safety signal increases locomotion (Rogan *et al*, 2003), perhaps by a mechanism similar to autoshaping. It is conceivable, therefore, that the NAC may also mediate the effects of a safety signal on behavior.

The present study, therefore, examined the role of the NAC in mediating the effects of safety signals on behavior using conditioned inhibition of fear-potentiated startle. As dopamine- and glutamate-mediated mechanisms are key to NAC function (Choi *et al*, 2000; Everitt and Wolf, 2002; David *et al*, 2004), Experiments 1a and 1b examined the effects of enhancing dopaminergic function (via the dopamine agonist, amphetamine) or disrupting glutamatergic function (via the AMPA/kainate receptor antagonist, CNQX) in the NAC on the expression of conditioned inhibition of fear-potentiated startle. Previous research shows that infusion of amphetamine into the NAC disrupts prepulse inhibition of the acoustic startle response (eg, Swerdlow *et al*, 1986; Wan *et al*, 1995). As a positive control to ensure that our infusions produced adequate perfusion of the NAC, Experiment 1c examined the effects of infusing amphetamine into the NAC in the same rats on prepulse inhibition. Experiments 2a and 2b examined the effects of electrolytic lesions of the NAC on the acquisition and retention of conditioned inhibition of fear-potentiated startle, respectively.

METHODS AND MATERIALS

Animals

Male albino Sprague–Dawley rats (Charles, River, Kingston, NY, USA) weighing between 300–400 g were used (unless otherwise specified). Rats were housed in hanging wire cages (two rats per cage) and maintained on a 24-h light–dark cycle (lights on at 7 am) with *ad libitum* access to food and water. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States Public Health Service, with the experimental protocols approved by Yale University and the Northern Illinois University Institutional Animal Care and Use Committees. All efforts were made to minimize the number of animals used.

Cannulation Procedure and Infusions

Rats ($n = 15$) were anesthetized (sodium pentobarbital, 60 mg/kg, i.p.) and placed in a Kopf stereotaxic instrument. Bilateral chronic indwelling cannula (22 gauge, Plastic One, Roanoke, VA, USA) were implanted into the NAC (coordinates Anterior/Posterior $= +1.2$, Medial/Lateral $= \pm 1.2$, Dorsal/Ventral $= -7.4$ relative to bregma, Paxinos and Watson, 1998).

Infusions were performed immediately before testing. Injection cannulas (Plastics One, Model C3131; 28gauge) cut to extend 1 mm beyond the tip of the guide cannulas were attached by polyethylene tubing to Hamilton microsyringes controlled by an infusion pump (Harvard Apparatus, South Natick, MA, USA). The volume of all infusions was 0.5 μl (infused over 60 s). After the infusion was complete, the injection cannula was left in place an additional 60 s to increase diffusion of the drug. We chose to infuse sufficient volume of fluid to perfuse the entire NAC since we had no *a priori* prediction as to the involvement of a specific NAC subterritory (core, shell and rostral pole (Zahm, 2000), or combination thereof).

Amphetamine (Sigma Chemicals, St Louis, MO) was dissolved in distilled water to result in concentrations of 0, 10, and 20 μg/0.5 μl. Previous experiments show that this dose range of amphetamine increased the motivational impact of appetitive Pavlovian cues on behavior (eg, locomotion and responding for conditioned reinforcements) (Parkinson *et al*, 1999). The AMPA receptor antagonist, 6-cyanao-7-nitroquinoxaline-2,3-dione (CNQX) (Sigma Chemicals) was dissolved in 1 N NaOH and diluted with 0.1 M PBS. The pH of the resulting solution was adjusted to 7.4. Previous studies show that the present dose of CNQX (3 μg/0.5 μl) infused into the NAC blocked the increase in locomotor activity produced by a dopamine agonist (David *et al*, 2004).

Lesion Procedure

Rats (Sprague–Dawley, derived from Charles River, but purchased from Harlan Laboratories SD) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a Kopf stereotaxic instrument. The skin was retracted and holes were drilled in the skull above the NAC. Lesions were made

by passing anodal current (0.5 mA for 55 s) through electrodes (Kopf Model NE-300 electrodes (0.25 mm in diameter and insulated to within 0.5 mm of the tip)) at the following co-ordinates AP = +1.2, ML = ±1.2, DV = −7.6 and AP = +1.2, ML = ±1.6, DV = −7.8 relative to bregma. Sham rats received that same treatment except that no current was passed through the electrode.

Apparatus

Experiment 1 was conducted at Yale University (apparatus as previously described by Cassella and Davis, 1986; Falls and Davis, 1997), while Experiment 2 was performed at the University of Northern Illinois (apparatus as previously described by Falls *et al*, 1997). In general, the apparatus used at both institutions had only minor differences (see below). Rats were trained and tested in stabilimeter devices. Each stabilimeter consisted of Plexiglas and wire-mesh cages (8 × 15 × 15 cm) suspended between compression springs within a steel frame housed in a sound-attenuated chamber (Industrial Acoustics Co., Model #105278, Bronx, NY). The sound-attenuated chamber was ventilated by a fan that also provided the background noise (55 dB sound pressure level (SPL A)). The floor of each stabilimeter consisted of four stainless steel bars through which a scrambled foot shock (0.5 s, 0.6 mA) could be delivered (generated by Lehigh Valley constant current shock generators, Model SGS-004, BRS/LVE, Beltsville, MD for Experiment 1 or Lafayette Instruments, Layfayette, IN, for Experiment 2). The startle-eliciting stimulus (a 105-dB, 50-ms burst of white noise with a rise/decay time of 4 ms) was provided by a white noise generator (Grasson-Stadler, Model 901B, West Concord, MA) connected to a 16.5 cm speaker (Alpine electronics, Model 6267 AX, Torrance, CA for Experiment 1, and Radio Shack Super Tweeter, Tandy Inc., Froth Worth, TX for Experiment 2) located behind each stabilimeter. The light CS was produced by an 8 W fluorescent light bulb (rise-decay time of 100 μs, 630 fL intensity) and controlled by a light control unit (Fintronics, Orange, CT). The noise CS (a 4-KHz, 70-dB band pass-filtered noise; 24 dB SPL per octave attenuation) was delivered through a full-range speaker (Radio Shack, #40-1286C) located 10 cm from the stabilimeter. In the Light-shock pairing condition of Experiment 1, the shock was presented 3.2 s after the onset of the 3.7-s light and in the Noise & Light-no shock condition, the 3.7-s noise was followed immediately by the 3.7-s light. During the test, the startle stimulus was presented 3.2 s after the onset of the light (eg when the shock previously occurred). For Experiment 2, the durations of the light CS and noise CS were 4 s. In the Light-shock pairing condition, the shock was presented 3.5 s after the onset of the light. During the test, the startle stimulus was presented 3.5 s after the onset of the light (eg when the shock previously occurred). Cage movement displaced an accelerometer (PCB Piezotronics, #321A, Depew, NY), which created a voltage change proportional to the velocity of the displacement. The accelerometer output was amplified (Fintronics Accelerometer, #FA 560, Orange, CT) and digitized (MacADIOS II Board, GW Instruments, Somerville, MA) on a 0-4096 unit scale. Startle amplitude was defined as the peak-to-peak accelerometer voltage that occurred during a 200-ms period after the onset of the startle stimulus. Data acquisition and stimulus presentations were controlled by a computer (Macintosh Power PC 7100/66).

Conditioned Inhibition of Fear-Potentiated Startle

Habituation. On each of 2 days before training, rats were placed in the startle chamber and 5 min later presented with 10 startle stimuli at each of 95, 100 and 105 dB intensities. The three intensities of startle stimuli were presented in a random order with an intertrial interval of 30 s.

Training. Training was conducted over two phases. During the first phase, the Light was paired with shock and during the second phase, the Noise & Light was presented with no shock. Phase 1 of training took place over 2 consecutive days during which animals were placed in the startle chamber and 5 min later received 10 Light-shock pairings with an overall intertrial interval of 2 min (range from 1–3 min). Phase 2 of training took place over 5 consecutive days during which rats were presented with five Light-shock trials intermixed with 15 Noise & Light-no shock trials. In these nonreinforced trials, the Noise CS was followed immediately by the Light and the shock was not presented. The two trial types (Light-shock and Noise & Light-no shock) were presented in a random sequence and the mean intertrial interval was 2 min (range between 1.5 and 2.5 min).

Test. To test for fear-potentiated startle and conditioned inhibition of fear-potentiated startle, rats were placed in the startle apparatus for 5 min and presented with 30 startle-eliciting stimuli alone (105 dB) followed by 15 startle stimuli (105 dB) in each of the three conditions: (1) in the dark with no explicit CS (embedded baseline startle trials), (2) in the presence of the Light (Light-startle trials to assess fear-potentiated startle), and (3) in the presence of the Noise & Light stimulus (Noise & Light-startle trial to assess conditioned inhibition of fear-potentiated startle). The order of the three trial types was randomized. All startle stimuli were presented with an interstimulus interval of 30 s.

Experiment 1

On tests days 1 and 2, rats were infused with amphetamine (0 and 20 μg/0.5 μl per side, in a counterbalanced order, $n = 15$). On test day 3, a subset of rats (five chosen at random) was infused with 10 μg/0.5 μl of amphetamine. Rats were infused with CNQX (0 and 3 μg/0.5 μl per side, in a counterbalanced order, $n = 10$) on test days 4 and 5. The tests were separated by at least 48 h (ie two training days).

Experiment 2

In Experiment 2a, rats received NAC lesion ($n = 9$) or sham surgery ($n = 10$) prior to training, while in Experiment 2b rats received post-training lesions of the NAC ($n = 14$) or

sham surgery ($n = 12$) 24–48 h after the last day of training. Rats were allowed to recover for at least 7 days.

Prepulse Inhibition of Startle

The same apparatus as above was used to assess prepulse inhibition of the acoustic startle response (except that the background noise was 70 dB). Following a 5-min acclimation period, rats were presented with 10 100-dB startle stimuli, followed by 10 100-dB startle stimuli presented alone or 100 ms after a tone (2 kHz, 20 ms) at each of three intensities (72, 74, 78 dB). In all, 10 of each trial type (no prepulse (NP), 72, 74, and 78 dB prepulse) were presented in a random order. Animals were infused with amphetamine (0, 10 μg/0.5 μl) immediately prior to prepulse inhibition testing. A second test in which animals received the other dose of amphetamine was conducted 72 h later.

Histological Assessment

Upon completion of the experiment, rats were overdosed with chloral hydrate and perfused through the heart with PBS followed by 10% formaldehyde. Brains were sectioned (40 μm) and stained with cresyl violet to assess infusion site or lesion size and placement.

Statistical Analyses

An analysis of variance (ANOVA) was first conducted on the embedded baseline startle scores for each treatment group to determine if the treatment altered baseline startle responding. As there was no treatment effect on baseline startle scores in these experiments, these mean baseline startle scores were then subtracted from the mean Light-startle and mean Noise & Light-startle scores for each animal. The resulting difference scores reflect the magnitude of fear-potentiated startle (difference scores on Light-startle trials) and conditioned inhibition of fear-potentiated startle (difference scores on Noise & Light-startle trials). A second ANOVA, using Treatment (eg Lesion, Sham) as a between-subjects factor and Trial Type (Light, Noise & Light) as a within-subjects factor, was conducted on the difference scores to determine if the treatment affected fear-potentiated startle or conditioned inhibition of fear-potentiated startle.

RESULTS

Experiment 1a: Effects of Amphetamine Infusions into the NAC on Fear-Potentiated Startle and Conditioned Inhibition of Fear-Potentiated Startle

Histology. Figure 1a is a schematic representation of the infusion sites. As can be seen from this figure, all cannulae were placed within the boundaries of the NAC. Coronal sections are based on atlas plates from Paxinos and Watson (1998). Coordinates are in millimiter and relative to bregma.

Intra-NAC amphetamine: baseline startle. The mean (±SEM) baseline (embedded) startle amplitudes for rats receiving amphetamine (0, 10, 20 μg) into the NAC were 248.32 (30.97), 280.37 (50.18), and 268.95 (38.64), respectively. An ANOVA performed on baseline startle amplitudes for animals that received two doses of amphetamine (0, 20 μg) showed no effect of amphetamine on baseline startle amplitudes ($F(1,14) = 0.37$, $p > 0.05$). An additional ANOVA performed on the data from the animals that received all doses of amphetamine (0, 10, and 20 μg) similarly showed no effect of amphetamine ($F(2,8) = 1.52$, $p > 0.05$). Thus, infusion of amphetamine into the NAC did not significantly affect baseline startle responding.

Intra-NAC amphetamine: fear-potentiated startle and conditioned inhibition of fear-potentiated startle. Figure 1b shows the effect of intra-NAC amphetamine on fear-potentiated startle (Light trials) and conditioned inhibition of fear-potentiated startle (Noise & Light trials). All groups show robust fear-potentiated startle and conditioned inhibition of fear-potentiated startle. The results of a mixed ANOVA performed on the data from animals receiving 0 and 20 μg of amphetamine support this interpretation showing a significant effect of Trial Type ($F(1,14) = 23.18$, $p < 0.001$), but no significant effect of Drug ($F(1,14) = 1.11$, $p > 0.05$) or interaction involving Drug ($F(1,14) = 1.95$, $p > 0.05$). An additional ANOVA performed on the data from the subgroup of animals that received all Drug treatments (0, 10, and 20 μg amphetamine) similarly showed only a significant effect of Trial type ($F(1,4) = 9.39$, $p < 0.05$) but no effect of Drug ($F(2,8) = 0.15$, $p > 0.05$) or interaction of Drug × Trial Type ($F(2,8) = 0.14$, $p > 0.05$). Thus, neither the expression of fear-potentiated startle or conditioned inhibition of fear-potentiated startle was affected by intra-NAC infusion of amphetamine.

Experiment 1b: Effects of CNQX Infusions into the NAC on Fear-Potentiated Startle and Conditioned Inhibition of Fear-Potentiated Startle

Intra-NAC CNQX: baseline startle. The mean (±SEM) baseline startle scores for rats receiving intra-NAC infusions of CNQX or vehicle were 419.47 (65.99) and 528.97 (77.0), respectively. An ANOVA performed on these scores showed no effect of the AMPA receptor antagonist on baseline startle amplitudes ($F(1,9) = 2.72$, $p > 0.05$). Thus, infusion of CNQX into the NAC did not significantly affect baseline startle responding.

Intra-NAC CNQX: fear-potentiated startle and conditioned inhibition of fear-potentiated startle. Figure 1c shows the effects of intra-NAC infusion of CNQX on fear-potentiated startle (Light trials) and conditioned inhibition of fear-potentiated startle (Noise & Light trials). Both vehicle and CNQX groups showed robust fear-potentiated startle and conditioned inhibition of fear-potentiated startle. An ANOVA showed a significant effect of Trial Type ($F(1,9) = 36.3$, $p < 0.001$), but no significant effect of Drug ($F(1,9) = 3.26$, $p > 0.05$) or interaction involving Drug ($F(1,9) = 1.18$, $p > 0.05$). Thus, fear-potentiated startle and conditioned inhibition of fear-potentiated startle are not affected by intra-NAC infusions of CNQX, showing that the expression of these processes does not depend on AMPA/kainate glutamate receptors in the NAC.

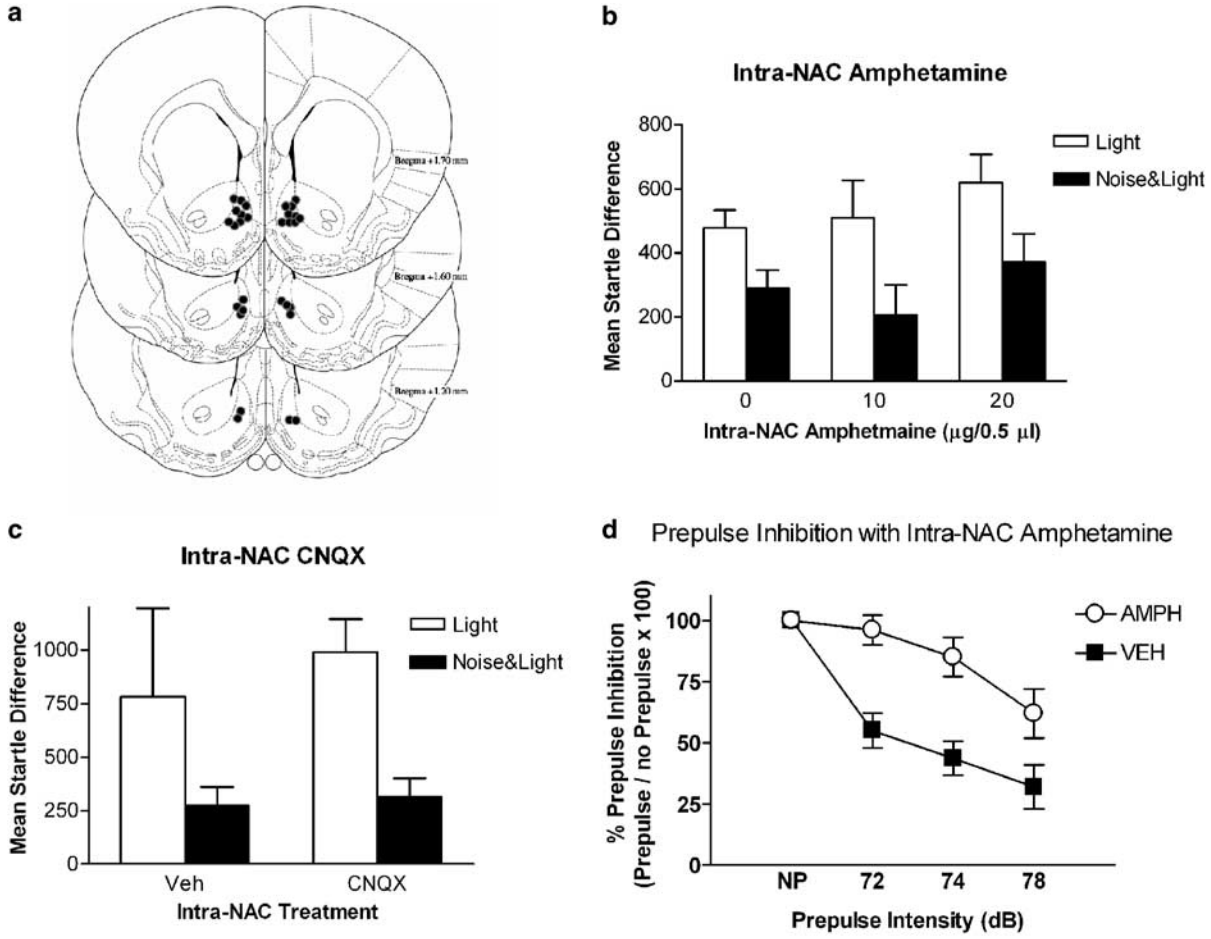

Figure 1 Effects of increasing dopaminergic function or decreasing glutamatergic function on the acoustic startle modulation. (a) Drawings depicting the location of the microinjection cannula tips. Coronal sections are based on atlas plates from Paxinos and Watson (1998). Coordinates are in millimiter and relative to bregma. (b) Effects of intra-NAC infusions of amphetamine (0, 10 μg) on the expression of fear-potentiated startle and conditioned inhibition of fear-potentiated startle. The data shown are the mean (±SEM) difference scores computed by subtracting the mean baseline startle amplitude in startle alone test trials from the mean startle amplitude on Light trials (white bars) and Noise & Light trials (black bars). (c) Effects of intra-NAC infusions of CNQX (0, 3 μg) on the expression of fear-potentiated startle and conditioned inhibition of fear-potentiated startle. (d) Effects of intra-NAC infusions of amphetamine (0, 10 μg) on the expression of prepulse inhibition of startle. The data shown are the mean (±SEM) percent prepulse computed as ((amplitudes on prepulse trials/startle amplitudes on no prepulse trials) × 100%).

Experiment 1c: Effects of Amphetamine Infusions into the NAC on Prepulse Inhibition of the Acoustic Startle Response

The mean (±SEM) startle amplitude on baseline and prepulse (NP, 72, 74, and 78 dB) trials following infusion of amphetamine (0 or 10 μg) were 625.92 (112.16), 338.52 (45.52), 322.89 (53.19), and 237.30 (38.49) for vehicle and 520.20 (110.58), 469.02 (102.87), 442.46 (82.44), and 260.82 (59.63), for amphetamine, respectively.

For the sake of clarity, the prepulse inhibition scores are presented as percent prepulse inhibition ((startle amplitude on prepulse trials/startle amplitude on baseline trials) × 100%) in Figure 1d. Prepulse inhibition using prepulses of lower intensity was significantly disrupted by intra-NAC infusions of amphetamine. An ANOVA using Drug (amphetamine *vs* vehicle) as a between-subjects factor and Trial Type (NP, 72, 74, 78 dB prepulse intensity) as a within-subjects factors shows a significant Drug × Trial Type interaction ($F(3,27) = 12.45$, $p < 0.001$) and significant effects of Trial Type ($F(3,27) = 65.24$, $p < 0.001$) and Drug ($F(1,9) = 26.59$, $p < 0.001$). *Post hoc* Newman–Keul comparisons show that the percentage of prepulse inhibition on all prepulse trials (72, 74, and 78 dB) was significantly lower ($p < 0.05$) than on startle alone (no prepulse trials) for vehicle-infused rats. However, in amphetamine-treated rats, prepulse inhibition was significantly different from startle alone trials following only the most intense prepulse stimulus (78 dB). Thus, in accordance with previous studies, intra-NAC infusions of amphetamine disrupt prepulse inhibition of the acoustic startle response. It is important to point out, however, that this dose of amphetamine, in the same anatomical region in the same rats, had no effect on fear-potentiated startle or conditioned inhibition of fear-potentiated startle.

Experiment 2a: Effect of Pretraining NAC Lesions on the Acquisition of Fear-Potentiated Startle and Conditioned Inhibition of Fear-Potentiated Startle

Pre-training electrolytic lesion of the NAC: lesion assessment. Figure 2a shows a schematic representation of the

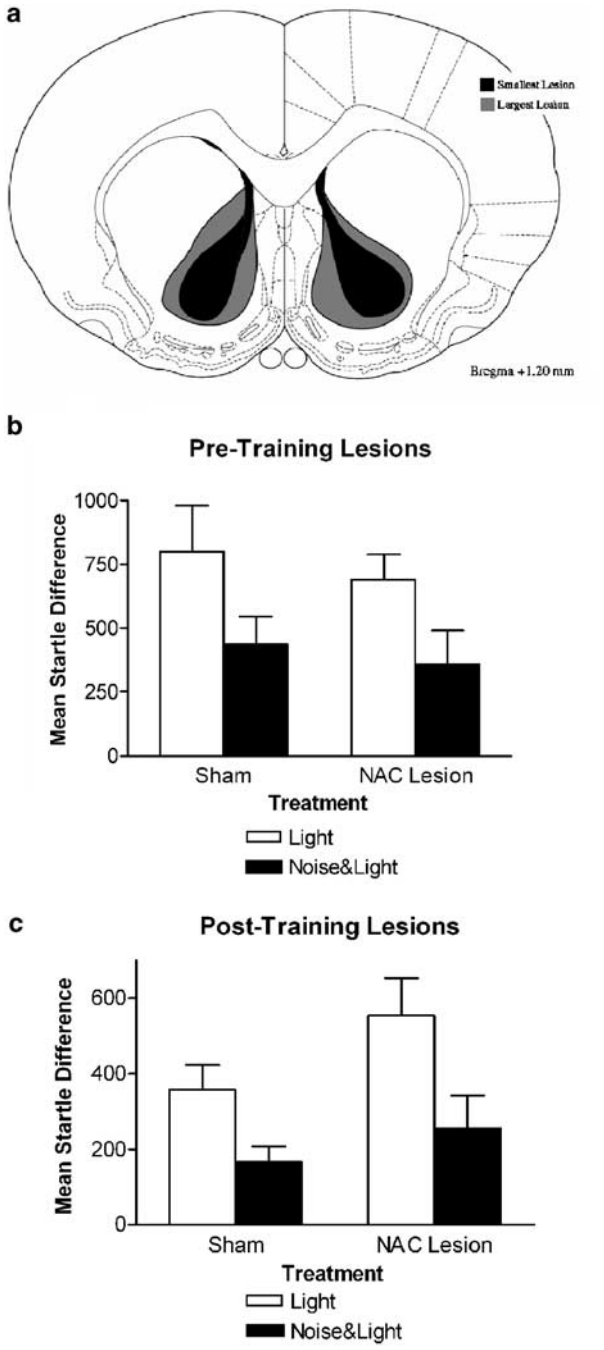

Figure 2 Effects of pretraining (b) and post-training (c) electrolytic lesions of the NAC on the acquisition and expression of fear-potentiated startle and conditioned inhibition of fear-potentiated startle. (a) Representation of the minimum (black) and maximum (gray) damage produced by electrolytic lesions to the NAC in Experiment 1. The coronal section is based on atlas plates from Paxinos and Watson (1998). Coordinates are in millimiter and relative to bregma. (b) Effects of pretraining electrolytic lesions of the NAC on the acquisition of fear potentiated startle and conditioned inhibition of fear-potentiated startle. The data shown are the mean ($\pm$SEM) difference scores computed by subtracting the mean baseline startle amplitude in startle alone test trials from the mean startle amplitude on Light trials (white bars) and Noise & Light trials (black bars). (c) Effects of post-training electrolytic lesions of the NAC on the expression of fear-potentiated startle and conditioned inhibition of fear-potentiated startle.

damage produced by the electrolytic lesions. Representative boundaries of the largest (gray) and the smallest (black) lesions of the NAC are depicted. Lesioned rats sustained extensive bilateral damage to the NAC, involving both the core and shells regions over the entire rostral-caudal extent of the NAC. These lesions produced limited damage to adjacent regions, including the ventral pallidum, lateral septal nucleus, and the medial forebrain bundle. Sham rats showed a small amount of cortical damage caused by lowering the electrode but there was no evidence of damage to the NAC.

Pre-training electrolytic lesion of NAC: baseline startle. The mean ($\pm$SEM) baseline startle scores for Lesion (872.81 ± 163.21) and Sham (1000.55 ± 148.10) treatment groups did not differ ($F(1,17) = 0.34$, $p > 0.05$). Thus, pretraining electrolytic lesions of the NAC do not significantly affect baseline startle amplitude.

Pretraining electrolytic lesion of NAC: fear-potentiated startle and conditioned inhibition of fear-potentiated startle. Figure 2b shows the effects of pretraining lesions of the NAC on the acquisition of fear-potentiated startle (Light trials) and conditioned inhibition of fear-potentiated startle (Noise & Light trials). Both Lesion and Sham treatment groups show high levels of fear-potentiated startle and substantially lower responding on conditioned inhibition trials. The results of a mixed ANOVA support this interpretation, showing a significant effect of Trial Type ($F(1,17) = 16.57$, $p < 0.001$) but no significant effect of Lesion ($F(1,17) = 0.31$, $p > 0.05$) or interaction between Trial Type and Lesion ($F(1,17) = 0.28$, $p > 0.05$). Therefore, pretraining electrolytic lesions of the NAC had no effect on the acquisition of fear-potentiated startle or conditioned inhibition of fear-potentiated startle.

Experiment 2b: Effect of Post-Training NAC Lesions on the Expression of Fear-Potentiated Startle and Conditioned Inhibition of Fear-Potentiated Startle

Post-training electrolytic lesion of the NAC: lesion assessment. Similar to above, electrolytic lesions of the NAC produced substantial bilateral damage to the core and shell regions of the NAC. The sham control animals showed no damage to the NAC.

Post-training electrolytic lesion of the NAC: baseline startle. The mean ($\pm$SEM) baseline startle scores for Lesioned (648.56 ± 54.1) and Sham (586.22 ± 84.95) animals did not differ ($F(1,24) = 0.42$, $p > 0.05$). Therefore, NAC lesions performed either before (above) or after (present) training do not significantly affect the amplitude of baseline startle responses.

Post-training electrolytic lesion of the NAC: fear-potentiated startle and conditioned inhibition of fear-potentiated startle. Figure 2c shows the effects of post-training NAC lesions on the performance of fear-potentiated startle (Light trials) and conditioned inhibition of fear-potentiated startle (Noise & Light trials). Both Lesion and Sham treatment groups show high responding on the Light trials,

indicating robust fear-potentiated startle, and substantially lower responding on the Noise & Light trials, indicated conditioned inhibition of fear-potentiated startle. The results of a mixed ANOVA support this interpretation, showing a significant effect of Trial Type ($F(1,24) = 32.02$, $p < 0.001$), but no significant effect of Lesion ($F(1,24) = 1.87$, $p > 0.05$) or interaction between Trial Type and Lesion ($F(1,24) = 1.48$, $p > 0.05$). Therefore, lesions of the NAC do not affect the retrieval or performance of fear-potentiated startle or conditioned inhibition of fear-potentiated startle.

DISCUSSION

The acoustic startle reflex is sensitive to the emotional and/or motivational state of the subject. The startle reflex in humans is increased in the presence of cues signaling danger and decreased if elicited in a pleasant or rewarding emotional context (Vrana *et al*, 1988; Grillon *et al*, 1993). Likewise rodents show increased startle in the presence of cues paired with shock (Davis, 1992) and drugs that increase anxiety (Frankland *et al*, 1997) and decreased startle in the presence of cues paired with food (Schmid *et al*, 1995) or rewarding brain stimulation (Steidl *et al*, 2001).

The present series of experiments took advantage of the affective modulation of the acoustic startle response to investigate the role of the NAC in mediating cues signaling danger (shock) and safety (no shock). In this study, we paired a Light stimulus with shock and a Noise & Light stimulus with no shock. Previous experiments using this procedure (Falls and Davis, 1997) show that the Noise inhibits conditioned fear through Pavlovian conditioned inhibition rather than through other nonassociative mechanisms such as generalization decrement or 'external' inhibition (Pavlov, 1927). One advantage of this type of conditioned inhibition procedure is that the effects of NAC manipulations on fear excitation and inhibition can be assessed simultaneously.

The present study examined the role of the NAC in mediating the behavioral effect of a safety signal using acute drug manipulations and permanent lesions of the NAC. As previous research suggests that dopamine- and glutamate-mediated mechanisms are key to NAC function (Choi *et al*, 2000; David *et al*, 2004), we examined the effects of disrupting AMPA/kainate receptor function and potentiating dopamine function in the NAC. Neither manipulation altered the expression of fear-potentiated startle or conditioned inhibition of fear-potentiated startle. However, intra-NAC infusion of amphetamine did disrupt prepulse inhibition of startle in the same animals. These positive control data are consistent with previous reports (Swerdlow *et al*, 1986; Wan *et al*, 1995) and show that our treatment produced adequate perfusion of the NAC. In addition, large pre- or post-training lesions of the NAC had no impact on the acquisition or retention of fear-potentiated startle or conditioned inhibition of fear-potentiated startle. Taken together, the present results clearly show that the NAC is not critical for the acquisition or expression of fear-potentiated startle or conditioned inhibition of fear-potentiated startle.

These negative findings are surprising for two reasons. First, some learning theories postulate the existence of two mutually antagonistic motivational systems in the brain: the appetitive motivational system (that directs behavior towards an attractive stimulus such as food) and aversive motivational system (that directs behavior away from an aversive stimulus such as shock) (Konorski, 1967; Dickinson and Dearing, 1979). As these motivational systems would reciprocally inhibit one another, activation of the aversive system would inhibit the appetitive motivational system. By extension, a safety signal that inhibits the aversive system may be perceived by the animal as motivationally equivalent to a CS that activates the appetitive motivational system. Indeed, there is experimental evidence supporting this opponent process model of appetitive-aversive interactions (Denny, 1971; Dinsmoor, 2001; Pineno, 2004). For instance, the omission of food (an appetitive US) and a CS associated with such an omission shows aversive properties (Amsel, 1958; Leitenberg, 1965; Coughlin, 1972). On the other hand, a CS associated with the omission of an expected shock (a safety signal) shows appetitive properties such as the ability to support lever pressing (Hendry, 1967; DeVito and Fowler, 1986, 1994; Fowler *et al*, 1977). It follows, therefore, that the neural structures that mediate the behavioral effects of safety signals may overlap with those that mediate appetitive conditioning. As previous data shows that the NAC is critically involved in mediating conditioned appetitive states (Balleine and Killcross, 1994; Parkinson *et al*, 2000; Corbit *et al*, 2001; Hall *et al*, 2001), it is surprising that similar manipulations of NAC function did not affect conditioned inhibition of fear. It should be pointed out, however, that we did not directly assess whether our safety signal manipulation produced an appetitive state.

Second, the NAC has been implicated in mediating the effects of an appetitive Pavlovian cue on startle (Koch *et al*, 2000). In this 'reward-attenuated startle' paradigm, presentation of a CS previously paired with food availability reduced baseline startle responding (Schmid *et al*, 1995; Koch *et al*, 2000). Importantly, pretraining 6-hydroxydopamine lesions of the NAC block this effect (Koch *et al*, 1996). Therefore, the NAC is important for the inhibition of startle by a cue signaling food availability but not by a cue signaling safety, indicating that the neural circuits mediating these processes are dissociable. In this sense, brain areas responsible for processing a safety signal are not identical to those responsible for processing appetitive Pavlovian cues. The present results, therefore, indicate important neural dissociations between the processing of an appetitive signal and a safety signal.

The NAC has also been implicated in another type of inhibitory learning, latent-inhibition. Latent inhibition is a phenomenon in which repeated exposure to a stimulus without reinforcement retards subsequent conditioning to that stimulus (Lubow, 1973; Lubow and Gewirtz, 1995). Importantly, lesions of the shell region of the NAC attenuate the effect of stimulus pre-exposure (the latent inhibition effect) in a fear conditioning paradigm (Weiner *et al*, 1996; Murphy *et al*, 2000). Therefore, while both latent inhibition and conditioned inhibition of fear reduce conditioned fear responses, these two processes depend on different anatomical systems.

In addition to the finding that the NAC is not critically involved in conditioned inhibition of fear-potentiated startle, our study examined the role of the NAC in fear-potentiated startle. Previous findings on the role of the NAC in fear conditioning are mixed. While some studies show that stimuli previously paired with shock are associated with an increase NAC DA release (Wilkinson *et al*, 1998; Young *et al*, 1998; Pezze *et al*, 2001) and the expression of plasticity-associated immediate early genes (Beck and Fibiger, 1995; Thomas *et al*, 2002), other studies have failed to find change in DA release (Levita *et al*, 2002). These discrepancies may be due to procedural differences and the heterogeneity of the NAC. The present findings show that manipulations of the NAC (including large electrolytic lesions) do not affect acquisition or retention of conditioned fear as measured by fear-potentiated startle. Our results support growing evidence that lesions (Riedel *et al*, 1997; Levita *et al*, 2002; Jongen-Relo *et al*, 2003), temporary inactivation (Haralambous and Westbrook, 1999), or morphine infusions into the NAC (Westbrook *et al*, 1997) do not disrupt conditioned fear to discrete cues, such as tones or lights, regardless of how conditioned fear is measured (fear-potentiated startle or conditioned freezing). The NAC has been implicated, however, in contextual fear conditioning (without a discrete cue such as a light or a tone) (Westbrook *et al*, 1997; Haralambous and Westbrook, 1999; Levita *et al*, 2002), perhaps through hippocampal efferents via the ventral subiculum (Groenewegen *et al*, 1987). It has been suggested that the locomotor exploration required for processing contextual stimuli may be mediated by interactions between the NAC and hippocampus (Maren *et al*, 1997; Fanselow, 2000; Thomas *et al*, 2002).

The neural substrates that mediate safety signals are unclear. Although the amygdala has been shown to be critically involved in the acquisition and expression of conditioned fear, lesions of the central nucleus of the amygdala do not disrupt the effects of a safety signal on conditioned fear (Falls and Davis, 1995). While picrotoxin injections into the dorsal periaqueductal gray decrease the expression of conditioned inhibition of fear-potentiated startle (Fendt, 1998), lesions of prefrontal cortex (Gewirtz *et al*, 1997), perirhinal cortex (Falls *et al*, 1997), and auditory thalamus (Heldt and Falls, 1998) fail to disrupt it. Lesions of the hippocampus performed after training specifically disrupted the effects of the safety signal on conditioned fear responses without disrupting conditioned fear responses themselves (Heldt *et al*, 2002). However, the findings that further training overcomes the effect of the lesions and that similar lesions performed before training failed to affect the impact of the safety signal suggests that additional brain regions are also critically involved. Although the hippocampal–NAC axis has been emphasized in the flow of information (Grace, 2000), the present findings indicate that the NAC is not critically involved in conditioned inhibition of fear-potentiated startle.

A reduced ability to inhibit conditioned fear associations or responses when the threat is no longer relevant might contribute to the persistence of maladaptive fear. Indeed, failure of these inhibitory mechanisms may lead to clinical conditions such as pathological anxiety (Rosen and Schulkin, 1998; Bouton *et al*, 2001; Myers and Davis, 2002). Therefore, a greater understanding of the brain regions involved in the inhibition of fear, and the relationships between these regions, will enhance our understanding of fear processes and have important clinical implications for the treatment of disorders that associated with dysfunction of the inhibition of fear such as anxiety disorders.

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