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Extinction of Fear-potentiated Startle: Blockade by Infusion of an NMDA Antagonist into the Amygdala

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Data derived from *in vitro* preparations indicate that NMDA receptors play a critical role in synaptic plasticity in the CNS. More recently, *in vivo* pharmacological manipulations have suggested that an NMDA-dependent process may be involved in specific forms of behavioral plasticity. All of the work thus far has focused on the possible role of NMDA receptors in the *acquisition* of responses. However, there are many examples in the behavioral literature of learning-induced changes that involve the *reduction* or *elimination* of a previously acquired response. Experimental extinction is a primary example of the elimination of a learned response. Experimental extinction is well described in the behavioral literature, but has not received the same attention in the neurobiological literature. As a result, the neural mechanisms that underlie this important form of learning are not at all understood. In the present experiments, the fear-potentiated startle paradigm was employed to begin to investigate neural mechanisms of extinction. The results show that infusion of the NMDA antagonist D,L-2-amino-5-phosphonovaleric acid (AP5) into the amygdala, a limbic structure known to be important for fear conditioning, dose-dependently blocked extinction of conditioned fear. Control experiments showed that the blockade of extinction was neither the result of the permanent disruption of amygdaloid function nor the result of decreased sensitivity of the animals to the conditioned stimulus. Infusion of AP5 into the interpositus nucleus of the cerebellum, a control site, did not block extinction. Finally, intra-amygdala infusion of a selected dose of the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione did not block extinction of conditioned fear. These results, together with a previous report from our laboratory (Miserendino et al., 1990), demonstrate the importance of the amygdala in the elaboration of conditioned fear and suggest that an NMDA-dependent process might underlie the extinction of conditioned fear.

The search for the biological basis of learning has largely focused on response acquisition. However, the behavioral literature is replete with examples of learning phenomena that involve response reduction or inhibition, and these phenomena have played a central role in contemporary learning theory. For example, in Pavlovian fear conditioning, response acquisition occurs by pairing a neutral conditioned stimulus with an aversive stimulus. This pairing leads to a variety of behavioral effects such as freezing, autonomic changes, and fear-potentiated startle that are used to define a state of conditioned fear. If, following this training, the conditioned stimulus is repeatedly presented in the absence of the aversive stimulus (i.e., the conditioned stimulus is nonreinforced), the conditioned stimulus loses its ability to produce these behavioral effects. The reduction in conditioned fear is referred to as experimental extinction and is thought to involve the formation of a new memory rather than the passive decay or erasure of the original memory (Konorski, 1948; Estes, 1955; Rescorla, 1979). Learning phenomena that involve response reduction have not received much attention in the neurobiological literature. Because response reduction is an integral part of learning, a complete understanding of the neurobiological basis of learning will require an understanding of the mechanisms of response reduction, including experimental extinction.

Over the past few years a number of studies have supported the role of NMDA receptors in behavioral plasticity (Morris, 1989; Robinson et al., 1989; Staubli et al., 1989; Flood et al., 1990; Kim and McGaugh, 1990; Shapiro and Carmanos, 1990). Recently, NMDA receptors within the amygdala, a limbic forebrain structure known to be involved in fear (Gloor, 1960; Goddard, 1964; Sarter and Markowitsch, 1985; Kapp and Pascoe, 1986; Davis et al., 1987; LeDoux, 1987; Mishkin and Appenzeller, 1987; Gray, 1989), have been implicated in the acquisition of conditioned fear (Kim and McGaugh, 1990; Liang and Davis, 1990; Miserendino et al., 1990). For example, intra-amygdala infusion of the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (AP5) dose-dependently blocked the acquisition of conditioned fear as assessed by potentiation of the acoustic startle response, a brainstem reflex with a neural pathway known to be directly connected with, and modulated by, the amygdala (Davis et al., 1982, 1987; Miserendino et al., 1990).

Extinction is thought to be a learning process and as such may involve NMDA receptors. Because the amygdala is critically involved in fear conditioning and because NMDA receptors within the amygdala appear to be involved in the acquisition of conditioned fear, we investigated the role of NMDA receptors within the amygdala in extinction of conditioned fear.

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Conditioned fear was assessed with the potentiated startle paradigm. In this paradigm, conditioned fear is operationally defined as an increase in the amplitude of the acoustically elicited startle response in the presence of a light-conditioned stimulus that was previously paired with shock. Fear-potentiated startle has proven to be a reliable measure of conditioned fear, as it is sensitive to anxiolytic drugs and is disrupted by anatomical lesions known to affect conditioned fear (Davis et al., 1987).

Materials and Methods

Animals

Male albino Sprague-Dawley rats (Charles River Co.) weighing between 330 and 430 gm were used. Nonoperated rats were housed in groups of five, and cannulated rats were housed individually. All rats were maintained on a 12 hr light/12 hr dark cycle (lights on at 7:00 AM) with food and water continuously available.

General behavioral procedures

Apparatus. Five identical aluminum and Plexiglas boxes (30 cm × 25 cm × 25 cm) located on two shelves within a sound attenuating chamber were used for potentiated startle training. The floor of each box consisted of 4.8 mm stainless steel bars spaced 19 mm apart. The conditioned stimulus was provided by an 8 W fluorescent light located on the outside back wall of each training box. The training box was completely dark except when the conditioned stimulus was illuminated. The unconditioned stimulus was a footshock generated by five Lehigh Valley constant current shock generators located outside of the isolation chamber. Shock intensity was measured with a 1 K Ω resistor across a differential channel of an oscilloscope in series with a 100 K Ω resistor connected between adjacent floor bars within each training box. Current was defined as root mean square (RMS) voltage across the 1 K Ω resistor where mA = 0.707 × 0.5 × peak-to-peak voltage. According to this method, the shock intensity was 0.4 mA. Background white noise was 55 dB. The apparatus used to measure startle has been described in detail (Cassella and Davis, 1986). Briefly, five stabilimeter devices were housed on two shelves of a sound-attenuating chamber. Each stabilimeter consisted of an 8 cm × 15 cm × 15 cm Plexiglas and wire mesh cage suspended between compression springs within a steel frame. An 8 W fluorescent bulb identical to that used in training was located behind each stabilimeter. Cage movement resulted in displacement of an accelerometer, with the resultant voltage being proportional to the velocity of displacement. Startle amplitude was defined as the maximum accelerometer voltage that occurred during a 200 msec period after the onset of the startle stimulus. The startle stimulus was a 50 msec burst of white noise provided through a high-frequency speaker (Radio Shack Super Tweeter) located 10 cm from the back of each stabilimeter. Background white noise was 55 dB.

Potentiated startle training and testing. In all experiments, fear-conditioning consisted of 10 presentations on each of two consecutive days of a 3.7 sec light-conditioned stimulus that coterminated with the presentation of a 500 msec 0.4 mA footshock. The mean intertrial interval (ITI) was 4 min (range, 3–5 min). Five days after training, rats were given a test of potentiated startle to assess their initial level of fear to the light. The rats were placed in the stabilimeter devices and, after a 5 min period during which no stimuli were administered, were given 20 initial 95 dB startle-eliciting noise bursts at a 30 sec ITI followed immediately by three presentations of the startle-eliciting noise burst alone and three presentations of the startle-eliciting noise burst 3.2 sec after the onset of the 3.7 sec light-conditioned stimulus (light & noise). The noise burst alone and light & noise trials were presented in a pseudorandom sequence. The mean of the last 10 initial noise-alone trials for each rat was taken as that rat's baseline startle amplitude (i.e., noise alone). Conditioned fear to the light was defined as greater startle amplitude in the presence of the light (i.e., on light & noise trials) than in the dark (i.e., the last 10 initial noise-alone trials).

Extinction. On each of the next 2 d following the initial test, the rats were returned to the stabilimeters, and after a 5 min period in which no stimuli were presented, they were given 30 presentations of the 3.7 sec light in the absence of either shock or the startle-eliciting noise burst (light-alone extinction trials). The ITI was 1 min. The context-alone control groups remained in the stabilimeters for an equivalent period

of time and no stimuli were given. The colony control group remained undisturbed in the colony room for this period of time. One day later the rats were returned to the stabilimeters and given a test for fear-potentiated startle similar to the initial test described above.

Surgery

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and stereotaxically implanted with bilateral guide cannulas (22 gauge; Plastic Products, C313G) aimed at the basolateral nucleus of the amygdala (AP -2.4, ML \pm 5.1, DV -8.5 relative to bregma) or the interpositus nucleus of the cerebellum (AP -11.4, ML \pm 2.4, DV -6.3 relative to bregma, skull flat orientation; Paxinos and Watson, 1986). Fear conditioning began 1 week after surgery.

Drugs

AP5 was dissolved in artificial cerebrospinal fluid (ACSF), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was dissolved in 100% dimethyl sulfoxide (DMSO). Both drugs were adjusted to a pH of 7.0. The drugs were administered bilaterally in a volume of 0.5 μ l via a 28 gauge inner cannula (Plastic Products, C3131) at a rate of 0.5 μ l/min.

Detailed methods

Evaluation of extinction using fear-potentiated startle: experiment 1. To evaluate experimental extinction within this paradigm, 26 rats were given potentiated startle training as described. Following an initial test of fear-potentiated startle, the rats were divided into three groups with equivalent noise-alone and light & noise amplitudes. On each of the next 2 consecutive days group context + lights-alone ($n = 8$) received 30 light-alone presentations in the testing apparatus, group context-alone ($n = 9$) was placed in the testing apparatus for an equivalent amount of time during which no stimuli were given, and group colony ($n = 9$) remained undisturbed in the colony room. One day later all groups were again tested for fear-potentiated startle.

The involvement of NMDA receptors within the amygdala in extinction of fear-potentiated startle: experiment 2. To assess the role of NMDA receptors within the amygdala in extinction of fear-potentiated startle, 29 rats were implanted with bilateral guide cannulas aimed at the basolateral nucleus of the amygdala. Following recovery, the rats were trained and given an initial test of fear-potentiated startle as described in the general methods. Based upon this initial test, the rats were divided into four groups having equivalent noise-alone and light & noise amplitudes. On each of the next 2 d, the rats were given bilateral intra-amygdala infusions of either 50 nmol AP5 or vehicle immediately before either 30 light-alone trials or equivalent exposure to the experimental context. One day later, fear-potentiated startle testing occurred in the absence of a prior infusion.

The dose dependency of the blockade of extinction by AP5: experiment 3. To assess whether the blockade of extinction by AP5 was dose related, 24 rats were implanted with bilateral guide cannulas aimed at the basolateral nucleus of the amygdala. Following recovery the rats were trained and given an initial test of fear-potentiated startle as described above. Based upon this initial test, the rats were divided into four groups having equivalent noise-alone and light & noise amplitudes. On each of the next 2 d, the rats were given bilateral intra-amygdala infusions of either 1.25, 6.25, 12.5, or 25 nmol AP5 immediately before 30 light-alone trials. One day later, fear-potentiated startle testing occurred in the absence of a drug infusion.

Anatomical specificity of AP5's blockade of extinction: experiment 4. To determine some anatomical specificity of AP5's blockade of extinction, 10 rats were implanted with bilateral guide cannulas aimed at the interpositus nucleus of the cerebellum. Following recovery the rats were trained and given an initial test of fear-potentiated startle as described. Based upon this initial test, the rats were divided into two groups having equivalent noise-alone and light & noise amplitudes. On each of the next 2 d, the rats were given bilateral intra-interpositus infusions of either 50 nmol AP5 or ACSF vehicle immediately before 30 light-alone trials. One day later, fear-potentiated startle testing occurred in the absence of a drug infusion.

Pharmacological specificity of AP5's blockade of extinction: experiment 5. To determine whether AP5 exerted its blockade of extinction through actions at non-NMDA receptors, eight rats were implanted with bilateral guide cannulas aimed at the basolateral nucleus of the amygdala. Following recovery the rats were trained and given an initial test

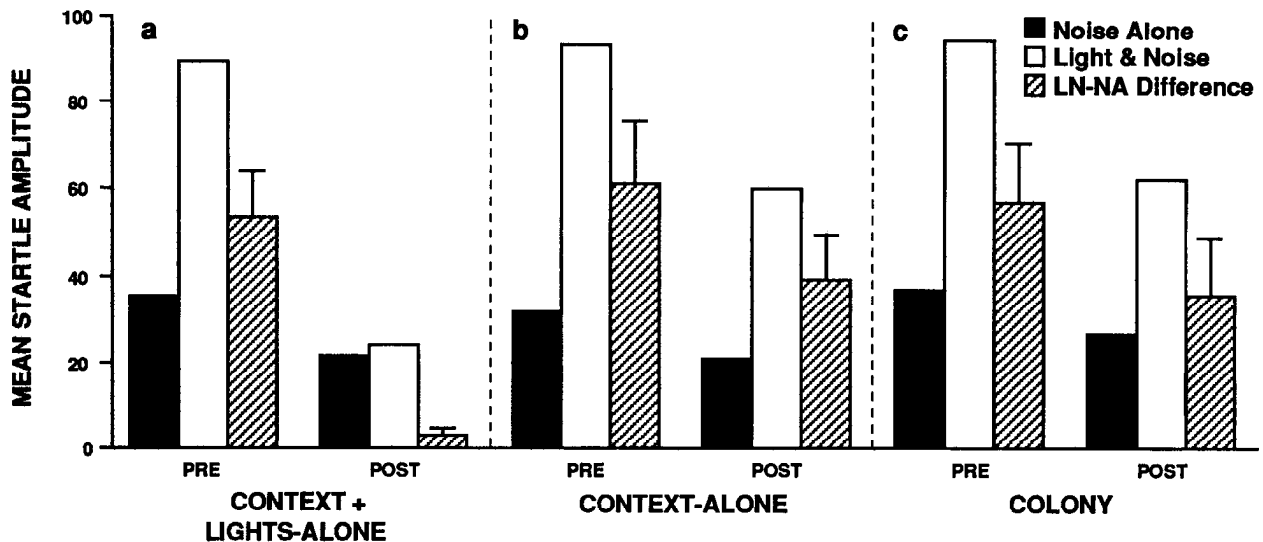


Figure 1. The amplitude of fear-potentiated startle before and after manipulations designed to reveal the relative contributions of light-alone presentations, context exposure, and the passage of time on the extinction of conditioned fear. Shown is the amplitude of startle in the presence [light & noise (LN)] and absence [noise-alone (NA)] of the light-conditioned stimulus and the difference between the two trial types (LN-NA Difference, + SEM). The LN-NA difference score represents the magnitude of conditioned fear. The group that received context + lights-alone (a) showed a significant reduction in the magnitude of fear-potentiated startle from the pretest to the posttest. In contrast, neither exposure to the context alone (b) nor remaining in the colony room (c) significantly reduced subsequently tested fear-potentiated startle. Hence, these groups showed significant fear-potentiated startle during both the preextinction and the postextinction tests.

of fear-potentiated startle. On each of the next 2 d, the rats were given bilateral intra-amygdala infusions of 12.5 nmol CNQX immediately before 30 light-alone trials. One day later, fear-potentiated startle testing occurred in the absence of a drug infusion.

Histology

At the end of each experiment, cannulated rats were killed with an overdose of chloral hydrate and perfused intracardially first with saline and then with 10% formalin in a phosphate buffer. Brains remained in a 30% sucrose formalin solution for at least 48 hr before 40 μ m coronal sections were taken. Sections were stained with cresyl violet, and the locations of the injection cannulas were transcribed onto atlas plates (Paxinos and Watson, 1986).

Statistical analysis

Mean startle amplitude in the presence of the light (light & noise), the absence of the light (noise-alone) and the difference between the two (light & noise minus noise-alone) are presented. Statistical comparisons were made with a mixed-model analysis of variance with groups as a between-subjects factor and test (preextinction, postextinction) and trial type (noise-alone, light & noise) as within-subjects factors. To evaluate any reduction in fear-potentiated startle resulting from group treatments, simple test \times trial type interactions were analyzed separately for each of the experimental groups. Comparisons of significant simple interactions were made with *t* tests.

Results

Evaluation of extinction using fear-potentiated startle: experiment 1

Following fear conditioning, but before light-alone presentations, the rats showed fear of the light as assessed by greater amplitude of startle in the presence versus the absence of the light (Fig. 1a, PRE). However, following 60 light-alone presentations in which both the footshock and startle stimuli were omitted, there was a significant reduction (i.e., extinction) in the amplitude of fear-potentiated startle relative to the preextinction level (Fig. 1a, POST). This reduction in the magnitude of fear-potentiated startle from the pretest to the posttest was supported

by a significant test by trial-type interaction [$F(1,23) = 12.67$; $p < 0.005$]. Subsequent dependent *t* tests revealed significant fear-potentiated startle at the preextinction test [$t(7) = 3.99$; $p < 0.01$] but not at the postextinction test [$t(7) < 1$]. To test the contribution of exposure to the experimental context and the passage of time to the observed reduction in fear-potentiated startle, two additional groups were run. Figure 1, b and c, shows that reexposure to the experimental context alone, without light-conditioned stimulus presentations, or remaining in the colony room contributes very little to the reduction in fear-potentiated startle from the preextinction to the postextinction tests [interaction $F(1,23) = 2.65$ and 2.51 ($p > 0.05$), respectively]. Subsequent *t* tests revealed that both groups showed significant fear-potentiated startle during the preextinction test [dependent $t(8) = 4.82$ and 4.50 ($p < 0.01$), respectively] and the postextinction test [dependent $t(8) = 3.96$ and 3.60 ($p < 0.01$), respectively]. Therefore, the observed reduction in fear-potentiated startle resulted from the repeated presentation of the light in the absence of shock, rather than from exposure to the experimental context or the passage of time.

AP5 infused into the amygdala blocks extinction of fear-potentiated startle: experiment 2

Rats receiving intra-amygdala infusion of ACSF vehicle ($n = 10$) immediately before light-alone trials showed a significant decrease in the amount of fear-potentiated startle relative to their preextinction level [interaction $F(1,15) = 10.16$; $p < 0.01$; Fig. 2a]. Subsequent dependent *t* tests revealed significant fear-potentiated startle at the preextinction test [$t(9) = 3.14$; $p < 0.025$] but not at the postextinction test [$t(9) = 1.23$; $p > 0.05$]. In contrast, rats receiving intra-amygdala infusion of 50 nmol AP5 ($n = 7$) did not show a significant reduction in fear-potentiated startle (interaction $F < 1$). In the AP5 group there was still significant fear-potentiated startle following light-alone trials [dependent $t(6) = 3.29$; $p < 0.025$; Fig. 2a]. Therefore, AP5

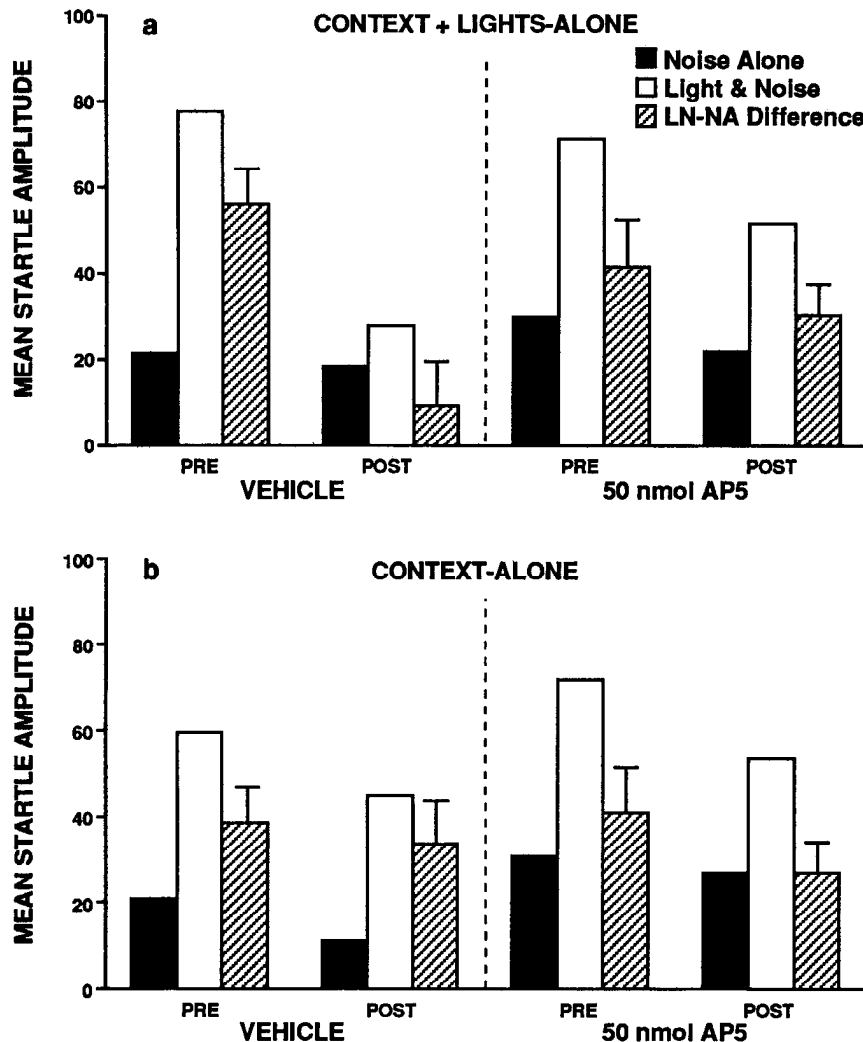


Figure 2. Effects of 50 nmol AP5 on extinction of fear-potentiated startle. Shown is the amplitude of startle in the presence [light and noise (LN)] and absence [noise alone (NA)] of the light conditioned stimulus and the difference between the two trial types (LN-NA difference; + SEM). *a*, Intra-amygdala infusion of ACSF vehicle or 50 nmol AP5 immediately prior to context + light-alone presentations. Rats that received vehicle immediately before light-alone presentations showed a significant reduction in fear-potentiated startle from the preextinction test. In contrast, rats that received AP5 did not show a significant reduction in fear-potentiated startle and had significant fear-potentiated startle during the postextinction test. *b*, Intra-amygdala infusion of ACSF vehicle or 50 nmol AP5 immediately prior to context-alone exposure. Neither the vehicle nor the AP5 group showed a statistically significant reduction in fear-potentiated startle from the preextinction to the postextinction tests, and both groups had significant fear-potentiated startle during both the preextinction and the postextinction tests.

blocked the extinction of fear-potentiated startle that normally occurs with light-alone presentations. Moreover, the blockade appears to be complete because the modest reduction of fear-potentiated startle observed in rats infused with AP5 is comparable to the reduction observed by exposure to the experimental context alone (Fig. 1*b*) or to the reduction observed in rats infused with ACSF ($n = 5$) or AP5 (50 nmol; $n = 7$) immediately before exposure to the experimental context alone (Fig. 2*b*). Neither the vehicle group nor the AP5 context-alone group showed a statistically significant reduction in fear-potentiated startle from the preextinction to the postextinction tests [interaction $F_s(1,10) = 0.163$ and 1.76 ($p > 0.05$), respectively]. Subsequent dependent t tests revealed statistically significant fear-potentiated startle during both the preextinction [$t(4) = 3.46$, $p < 0.05$; $t(6) = 4.37$, $p < 0.01$] and the postextinction tests [$t(4) = 3.62$, $p < 0.05$; $t(6) = 3.44$, $p < 0.025$] in both the vehicle and AP5 context-alone groups, respectively.

The blockade of extinction by intra-amygdala AP5 is dose dependent: experiment 3

Figure 3 shows that the blockade of extinction by intra-amygdala infusion of AP5 was dose dependent. Specifically, doses of 25 ($n = 5$) and 12.5 ($n = 4$) nmol were more effective in blocking extinction than the lower doses of 6.25 ($n = 10$) and 1.5 ($n =$

5) nmol. A linear contrast conducted on the mean light & noise minus noise-alone difference scores during the postextinction test revealed a statistically reliable linear trend [$F(1,20) = 4.52$; $p < 0.05$].

Anatomical specificity of AP5's blockade of extinction: experiment 4

Morris et al. (1989) have shown that the area of brain tissue affected by locally infused NMDA antagonists remains confined to a rather specific anatomical locus. To assess the anatomical specificity of AP5's blockade of extinction, rats were implanted with bilateral guide cannulas aimed at the interpositus nucleus of the cerebellum. The cerebellum is known to be important for motor learning and for fear conditioning in some situations (Supple et al., 1987; Supple and Leaton, 1990; Thompson, 1990). AP5 (50 nmol; $n = 5$) or ACSF ($n = 5$) infused into the interpositus nucleus of the cerebellum immediately prior to light-alone trials did not block extinction (Fig. 4). Analysis of the simple interaction revealed a significant test (pre-, postextinction) by trial type (noise-alone, lights & noise) interaction [$F(1,8) = 10.39$; $p < 0.025$]. Consistent with normal extinction, both the ACSF and AP5 groups displayed significant fear-potentiated startle during the preextinction test [$t_s(4) = 2.77$ and 2.84 ($p < 0.05$), respectively], but neither group displayed statistically sig-

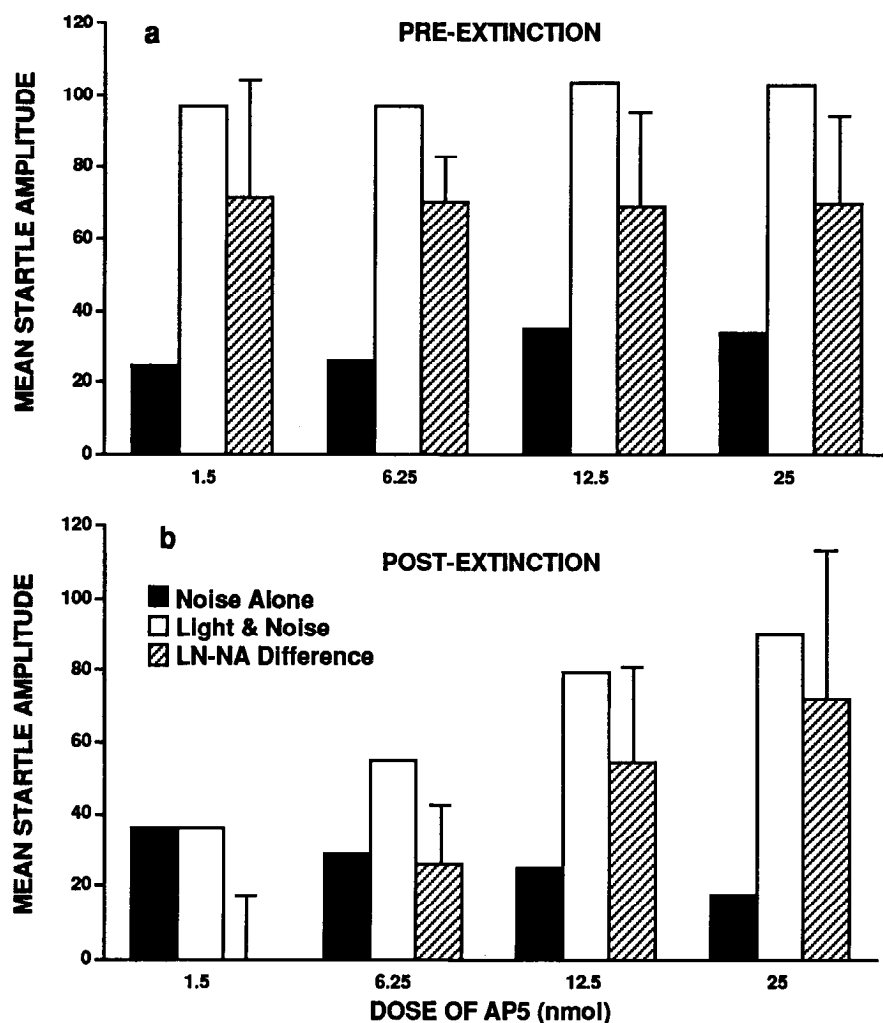


Figure 3. AP5 dose-dependently blocks extinction of fear-potentiated startle. Shown is the amplitude of startle in the presence [light and noise (LN)] and absence [noise alone (NA)] of the light conditioned stimulus and the difference between the two trial types (LN-NA difference; + SEM). *a*, Preextinction levels of fear-potentiated startle. *b*, Postextinction levels of fear-potentiated startle.

nificant fear-potentiated startle during the postextinction test [dependent $t_s(4) < 1$]. Hence, in this case, both the rats infused with AP5 and the rats infused with ACSF showed a significant reduction in fear-potentiated startle relative to their preextinction levels, indicating some anatomical specificity of the blockade of extinction by AP5.

Pharmacological specificity of the blockade of extinction by AP5: experiment 5

Because AP5 can antagonize non-NMDA receptors in addition to NMDA receptors (Honoré et al., 1988), it is unclear whether AP5 exerts its blockade of extinction through the selective blockade of NMDA receptors. To test this, rats were given intra-amygdala infusions of the selective non-NMDA receptor antagonist CNQX (12.5 nmol; $n = 8$) immediately before light-alone trials (Fig. 5). Intra-amygdala CNQX did not block extinction of fear-potentiated startle, unlike an equivalent nanomolar dose of AP5. For comparison, data from the 12.5 nmol dose of AP5 described in Figure 3 are included in the left panel. Rats given intra-amygdala infusion of CNQX showed a statistically significant reduction in fear-potentiated startle from the preextinction to the postextinction tests as revealed by a reliable test by trial-type interaction [$F(1,7) = 11.53$; $p < 0.05$]. Subsequent dependent t tests revealed significant fear-potentiated startle during the preextinction test [$t(7) = 4.24$; $p < 0.01$] but not during the postextinction test [$t(7) < 1$]. Because CNQX

is more than 1500 times more effective than AP5 at inhibiting binding at the AMPA (i.e., non-NMDA) receptor subtype (Honoré et al., 1988), it is unlikely that the blockade of extinction by AP5 results from a blockade of non-NMDA receptors.

Because damage of intrinsic amygdaloid cells is known to block the expression of fear-potentiated startle, an effect that behaviorally resembles extinction, we assessed whether intra-amygdala CNQX would prevent the expression of fear-potentiated startle when administered in the absence of extinction. To test this, rats were implanted with bilateral guide cannulas aimed at the basolateral nucleus of the amygdala. Following fear conditioning and an initial test of fear-potentiated startle, the rats were infused with CNQX dissolved in 100% DMSO (12.5 nmol; $n = 6$) in the colony room on each of the next 2 consecutive days. CNQX administered in the colony room did not disrupt fear-potentiated startle assessed at the same time after infusion as in the previous extinction experiment [interaction $F(1,5) = 1.51$; $p > 0.05$; data not shown]. Therefore, it is very unlikely that extinction of fear-potentiated startle under CNQX can be accounted for by a long-term disruption of amygdala function by CNQX or the DMSO vehicle.

Histology

Histological verification of the cannula placements revealed that all rats had cannulas located within 0.5 mm of the basolateral nucleus of the amygdala. Figure 6 is a composite drawing of

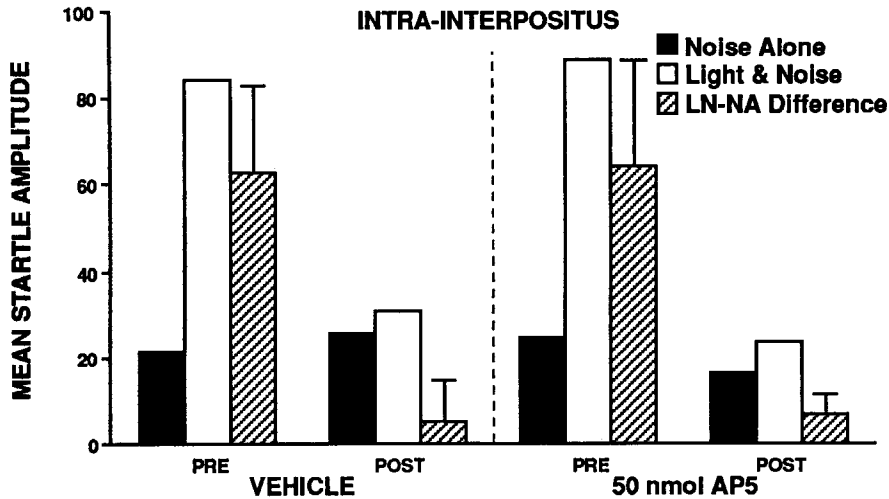


Figure 4. Effect of intra-interpositus infusion of 50 nmol AP5 on extinction of fear-potentiated startle. Shown is the amplitude of startle in the presence [light and noise (LN)] and absence [noise alone (NA)] of the light conditioned stimulus and the difference between the two trial types (LN-NA difference; + SEM). Both the vehicle and AP5 groups showed a statistically significant reduction in fear-potentiated startle from the pretest to the posttest. Although both the ACSF and AP5 groups displayed significant fear-potentiated startle during the pre-extinction test, neither group displayed statistically significant fear-potentiated startle during the postextinction test.

cannula tip locations. To characterize further the relationship between cannula placements and the blockade of extinction by AP5, the cannula placements from the marginally effective dose of 6.25 nmol AP5 (Fig. 4) were analyzed in more detail. Rats that were judged to have one or both cannulas within the 0.5 mm criterion, but lateral to the basolateral nucleus of the amygdala, had virtually no blockade of extinction [90% reduction in fear-potentiated startle from the preextinction to the postextinction test; interaction $F(1,8) = 9.99; p < 0.025$]. In contrast, rats with cannulas located within the basolateral nucleus of the amygdala showed a tendency toward a blockade of extinction [66% reduction in fear-potentiated startle; interaction $F(1,8) = 5.05; p > 0.05$]. The incomplete blockade of extinction in the rats with cannulas located within the basolateral nucleus of the amygdala is consistent with reduced efficacy of the lower (6.25 nmol) dose of AP5. The lack of a blockade in rats having cannulas lateral to the basolateral nucleus of the amygdala suggests that AP5 exerts its effect on extinction of conditioned fear within the basolateral nucleus of the amygdala.

Controls for the possible disruptive effects of intra-amygdala infusion of AP5

Amygdala damage is known to result in a complete blockade of fear-potentiated startle (Hitchcock and Davis, 1986). How-

ever, the blockade of extinction by AP5 in the present experiments (e.g., Figs. 2a, 3) cannot be attributed to damage produced by the intra-amygdala infusion because rats receiving intra-amygdala AP5 continued to demonstrate reliable fear-potentiated startle (i.e., a blockade of extinction). To test whether intra-amygdala AP5 damaged cells that were otherwise responsible for extinction, rats that had previously shown a blockade of extinction by AP5 were administered an extended test for potentiated startle in the absence of drug infusion. Because the shock is omitted in testing for fear-potentiated startle, the extended test is similar to an extinction session. Rats that had previously received ACSF displayed no potentiated startle (i.e., extinction) at the outset of testing, whereas rats that had previously received AP5 showed substantial potentiated startle (a blockade of extinction; e.g., see Fig. 2a). However, by the end of the 30 trial test session, the rats that had previously received AP5 no longer showed fear-potentiated startle (mean light & noise minus light-alone difference score of the final three light & noise trials = $-10.02; t(6) = 1.51; p > 0.05$). Therefore, the blockade of extinction by AP5 cannot be attributed to damage to cells that are otherwise responsible for extinction.

The rats' ability to detect visual stimuli was not impaired by AP5. Rats given pretest intra-amygdala infusions of AP5 (50 nmol; $n = 4$) did not differ from vehicle controls ($n = 4$) in a

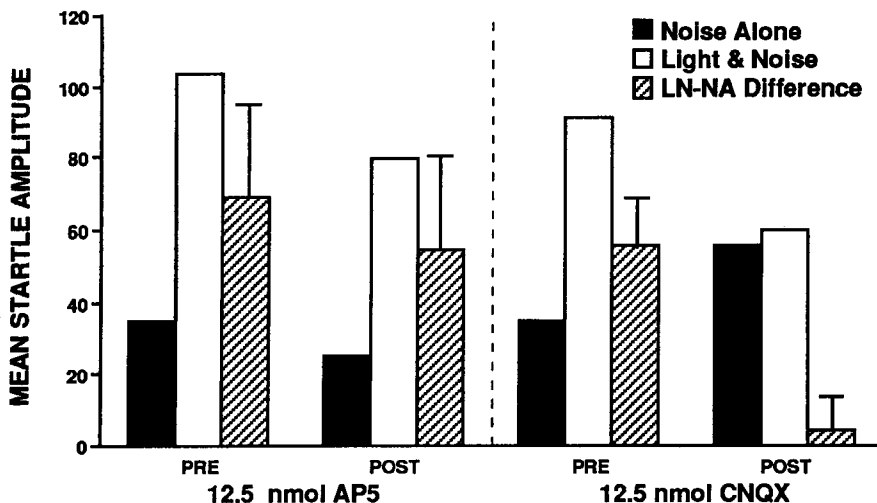


Figure 5. Intra-amygdala CNQX does not block extinction of fear-potentiated startle. Shown is the amplitude of startle in the presence [light and noise (LN)] and absence [noise alone (NA)] of the light conditioned stimulus and the difference between the two trial types (LN-NA difference; + SEM). For comparison purposes, data from the 12.5 nmol dose of AP5 described in Figure 3 are included in the left panel. In the CNQX group there was a statistically significant reduction in fear-potentiated startle from the preextinction to the postextinction tests, with significant fear-potentiated startle during the pre-extinction test but not during the postextinction test.

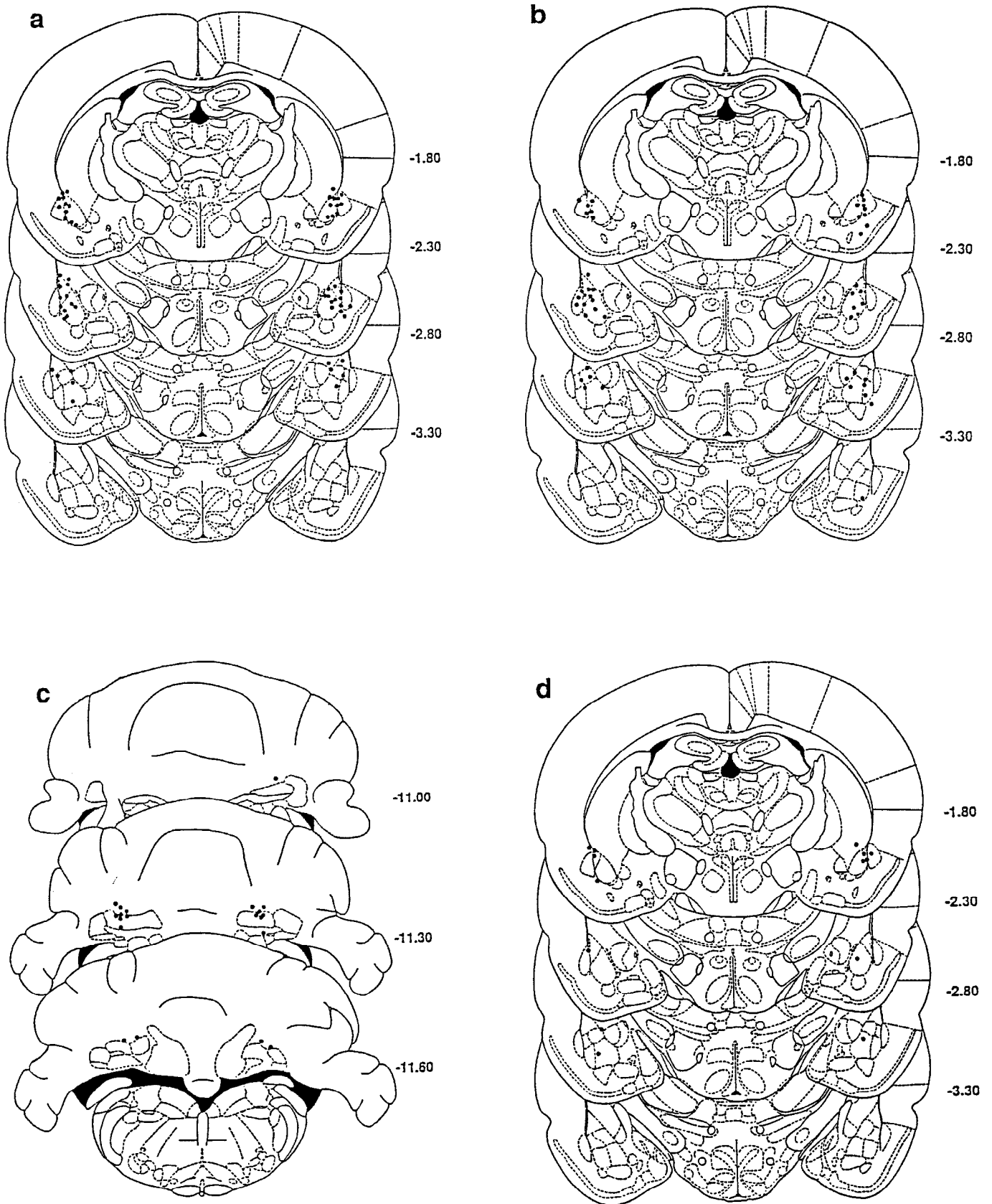


Figure 6. Serial reconstruction of cannula tip locations from experiments 2, 3, 4, and 5 (panels *a*, *b*, *c*, and *d*, respectively). Coordinates are relative to bregma.

test of visual prepulse inhibition in which a brief flash of light occurring just prior to a startle-eliciting noise burst inhibits the acoustic startle response [$F(1,7) = 3.47$; $p > 0.05$; data not shown; Ison and Hammond, 1971]. More importantly, Miserendino et al. (1990) have shown that intra-amygdala AP5 administered at a dose that blocked extinction in the present experiments did not block the performance of fear-potentiated startle, suggesting that AP5 does not block visual transmission at the level of the amygdala.

Discussion

The purpose of the present series of experiments was to investigate extinction of conditioned fear by first evaluating the conditions under which extinction occurs and then by assessing the role of NMDA receptors in the vicinity of the amygdala in this form of behavioral plasticity. The results show that extinction of conditioned fear required the presentation of the fear-eliciting conditioned stimulus and could be dose-dependently blocked by intra-amygdala infusion of the NMDA antagonist AP5. The blockade of extinction by AP5 showed some anatomical specificity because infusion of AP5 into the interpositus nucleus of the cerebellum failed to block extinction. However, many more infusion sites will have to be tested to determine the anatomical specificity of the amygdala in the blockade of extinction.

In contrast to AP5, intra-amygdala infusion of the competitive non-NMDA antagonist CNQX failed to block extinction. Because CNQX is over 1500 times more effective at blocking non-NMDA receptors than AP5 (Honore et al., 1988), it is unlikely that the blockade of extinction by AP5 resulted from a blockade of non-NMDA receptors. Moreover, a separate control experiment showed that the extinction observed in rats given intra-amygdala CNQX did not result from a disruption of amygdala function by CNQX or the DMSO vehicle. However, because of solubility constraints only one dose of CNQX was used. Therefore, the possible role of non-NMDA receptors in extinction remains to be determined.

In a previous experiment, our laboratory has shown that intra-amygdala infusion of AP5 does not disrupt performance of fear-potentiated startle using a visual conditioned stimulus. Therefore, the rat's ability to detect the visual stimulus, which would be required for extinction, was probably not impaired by intra-amygdala AP5.

Together, these results suggest that some NMDA-dependent process in the vicinity of the amygdala may be responsible for the extinction of conditioned fear.

State-dependent extinction

Learning that occurs in the presence of a drug may not be evident in the undrugged state (Overton, 1985). Drug-induced state-dependent acquisition has been observed in some situations but not in others (e.g., Overton, 1966; Henrickson and Jarbe, 1971; Ziskind and Amit, 1974; Cahill et al., 1986; Kim et al., 1991). State-dependent extinction has also been reported (e.g., Cunningham, 1979; Bouton et al., 1990). Thus, it is possible that in the present experiment, the apparent blockade of extinction by intra-amygdala AP5 did not result from a blockade of learning but from a failure to transfer the extinction memory from the AP5 drugged state to the nondrugged state (i.e., from extinction to the postextinction test). Although state-dependent extinction cannot be ruled out, it should be noted that such an effect would be the result of a state induced through the blockade of NMDA receptors in the vicinity of the amygdala. Further experiments

are needed to determine whether state-dependent learning can account for the present data.

Mechanisms of extinction: behavioral considerations

The form of learning that occurs during extinction is a matter of debate within the behavioral literature. Although several different theoretical mechanisms have been proposed, two general classes of theory have emerged (cf. Wagner and Rescorla, 1972). Extinction has been explained in terms of either an "erasure" of the original associations that led to the production of the conditioned response (e.g., Estes, 1955) or the acquisition of new associations that compete with or "mask" the expression of the still-intact response-producing associations (e.g., Konorski, 1948). Both of these hypotheses hold that new learning occurs as a result of nonreinforcement. However, they make very different predictions regarding the fate of the conditioned response producing associations. The erasure hypothesis predicts that following nonreinforcement, the response-producing associations no longer exist and therefore the conditioned response can no longer be performed. On the other hand, the masking hypothesis predicts that the response-producing associations remain after nonreinforcement and therefore, if it were possible to temporarily remove the masking associations, the conditioned response could be performed. Behavioral experiments have attempted to evaluate these alternatives by assessing whether the conditioned response remains after extinction.

Several lines of evidence suggest that the original associations are intact following extinction. For example, Bouton and colleagues (Bouton and King, 1983, 1986; Bouton and Bolles, 1985) have shown that the expression of extinction is specific to the stimulus context in which nonreinforcement occurred. Therefore, placing the animal into a context different from the one in which nonreinforcement occurred results in a return of the conditioned response. In addition, several experiments have shown that the simple re-presentation of the unconditioned stimulus following extinction is sufficient for reinstating extinguished responding to some preextinction level (Pavlov, 1927; Rescorla and Heth, 1975; Bouton and Bolles, 1979). We have replicated this effect using fear-potentiated startle (W. A. Falls and M. Davis, unpublished observations).

Mechanisms of extinction: neurobiological considerations

The results of the present experiments suggest that an NMDA-dependent mechanism in the vicinity of the amygdala underlies extinction of conditioned fear, a different form of behavioral plasticity than has previously been examined (see introductory remarks). Because a great deal of emphasis has been placed on the possibility that mechanisms derived *in vitro* may underlie behavioral plasticity, it is useful to begin to consider how mechanisms derived from *in vitro* experimentation may help to generate hypotheses concerning this different form of behavioral plasticity. One of the most promising models of learning in vertebrates is long-term potentiation (LTP). LTP is an activity-dependent increase in synaptic efficacy that in certain hippocampal synapses is dependent upon NMDA receptor activation (Collingridge and Bliss, 1987; Brown et al., 1988; Nicoll et al., 1988). LTP has been observed in the amygdala both *in vitro* (Chapman et al., 1990) and *in vivo* (Clugnet and LeDoux, 1990). Because NMDA antagonists infused into the amygdala prevent extinction, it is possible that an LTP-like process in the vicinity of the amygdala may underlie extinction of conditioned fear.

Given this, the interesting question becomes how an increase in synaptic efficacy (i.e., LTP) can lead to a *reduction* in conditioned fear.

One of the very first behavioral theories of extinction proposed that extinction resulted from the development of inhibition that acted to oppose the existing excitation (Konorski, 1948). The plasticity underlying extinction may involve an activity-dependent (i.e., LTP-like) increase in synaptic efficacy of *inhibitory* interneurons in the vicinity of the amygdala that serve to inhibit cells that are otherwise responsible for the performance of conditioned fear. In fact, recent evidence has shown that NMDA-dependent LTP of IPSPs does occur (Stevens and Cotman, 1991). The amygdala is known to contain a high density of GABAergic interneurons (Le Gal La Salle et al., 1978; Carlsen, 1988) that can be activated by excitatory amino acids (Rainnie et al., 1991). However, it remains to be seen whether their responsiveness to afferent input can undergo potentiation and whether inactivating the putative inhibition, perhaps through intra-amygdala infusion of GABAergic antagonists, will reversibly block the expression of extinction.

Alternatively, the plasticity underlying extinction may involve an activity-dependent increase in synaptic efficacy of *excitatory* neurons in the vicinity of the amygdala. These neurons in turn may initiate an extra-amygdaloid circuit that inhibits cells that are otherwise responsible for the performance of conditioned fear. In fact, recent work has suggested that visual and auditory cortex may be important components of a neural circuit responsible for extinction of conditioned fear (LeDoux et al., 1989; Teich et al., 1989). Work is now underway in our laboratory to determine whether circuits extrinsic to the amygdala mediate extinction of fear-potentiated startle.

A decrease in synaptic efficacy is an alternative candidate mechanism for extinction. Long-term depression (LTD) of synaptic responses has been observed in the hippocampus and cortex (Abraham and Goddard, 1983; Levy and Steward, 1983; Bear and Cooper, 1989; Chattarji et al., 1989; Stanton and Sejnowski, 1989; Artola et al., 1990), and several authors have proposed that LTD may be the cellular mechanism for weakening behavioral responses (Teyler and Discenna, 1984; Artola et al., 1990; Goldman et al., 1990). However, because extinction does not seem to result from an erasure of the original associations, it is difficult to understand how extinction could result from a depression of *potentiated synapses* (i.e., reversal of LTP). Alternatively, extinction might result from a depression of non-potentiated synapses that might normally carry conditioned stimulus information to the same postsynaptic cell that underwent LTP during excitatory conditioning. If the strength of the conditioned response reflected the net result of both depressed and potentiated synaptic inputs to this postsynaptic cell, LTD would compete with LTP to reduce the production of the conditioned response. Importantly, however, the original potentiation would remain intact, even though the overall output of the system would be reduced. Hence, treatments that temporarily reversed LTD would result in a reinstatement of the original associations. LTD in the hippocampus is blocked by AP5 in some situations (Abraham and Wickens, 1991; Desmond et al., 1991) but not in others (Chattarji et al., 1989; Stanton and Sejnowski, 1989; Stanton et al., 1991). Therefore, it remains to be seen whether LTD can be induced in the amygdala and whether it can be blocked by AP5.

In summary, extinction is an integral part of learning. Therefore, a complete understanding of the neurobiological basis of

learning requires an understanding of the mechanisms of extinction. The present results indicate that extinction of conditioned fear is a learning process that appears to involve an NMDA-dependent mechanism in the vicinity of the amygdala. Although it is not yet clear whether or not this mechanism is similar to LTP or LTD, the present results provide further evidence that excitatory amino acids of the NMDA subtype contribute importantly to behavioral plasticity.

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