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Acquisition of Fear Extinction Requires Activation of NR2B-Containing NMDA Receptors in the Lateral Amygdala

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N-methyl-D-aspartate receptors (NMDARs) contribute to synaptic plasticity underlying learning in a variety of brain systems. Fear extinction, which involves learning to suppress the expression of previously learned fear, appears to require NMDAR activation in the amygdala. However, it is unclear whether amygdala NMDARs are required for the acquisition of extinction learning, and it is unknown whether NR2B-containing NMDARs are required in fear extinction. Here, we assessed the effects of selective NR2B blockade with ifenprodil on fear extinction learning, and found that both systemic and intra-amygdala ifenprodil treatment, given before extinction training, impaired the initial acquisition, and subsequent retrieval of fear extinction. These results confirm previous evidence showing that NMDARs in the amygdala are involved in fear extinction, and additionally show that NR2B-containing NMDARs are required. Contrary to the conclusion of previous studies, our findings demonstrate NMDARs are required for the initial acquisition, rather than only the retention, of fear extinction learning. Thus, our results support a previously not known role for NMDA-dependent plasticity in the lateral amygdala during the acquisition of fear extinction.
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INTRODUCTION

Fear conditioning involves the formation of an association between an initially neutral conditioned stimulus (CS), such as a tone, and an aversive unconditioned stimulus (US), such as a footshock. After fear conditioning, the CS elicits a complex pattern of fear-related behavioral responses, including freezing and potentiated startle (McAllister and McAllister, 1971; Fanselow, 1994). Fear extinction occurs when a fear CS is repeatedly experienced in the absence of aversive consequences, resulting in a reduction of CS-elicited fear. Similar to fear conditioning, fear extinction involves both learning and memory (Bouton, 2002; Myers and Davis, 2002). Thus, during extinction training, the subject *learns* that the CS is no longer predictive of the US, and as extinction proceeds, the conditioned response decreases in magnitude. The *memory* of this learning experience is then assessed some time later by again presenting the CS without the US in a retrieval test to determine whether the decrease in responding is retained.

Considerable evidence implicates the amygdala as a crucial component of the neural circuitry that underlies the acquisition and storage of fear conditioning (LeDoux, 2000; Maren, 2001). Although fear extinction also involves the amygdala (Davis *et al*, 2003), its role is less clear than in fear conditioning (Maren and Quirk, 2004; Sotres-Bayon *et al*, 2004). Much of the evidence implicating the amygdala in fear extinction has involved studies showing that blockade of *N*-methyl-D-aspartate receptors (NMDARs) in the lateral and basal amygdala, using APV (D,L-2-amino-5-phosphonovaleric acid), prevents the decrease in responding that normally occurs during extinction learning, as assessed in a later test (Falls *et al*, 1992; Lin *et al*, 2003). For technical reasons related to the behavioral paradigm used in these studies (fear-potentiated startle), it was not possible to assess the effects of NMDAR blockade during extinction learning. Given the role of NMDARs in the induction of synaptic plasticity (Martin *et al*, 2000; Riedel *et al*, 2003), the most likely interpretation of these results is that the synaptic plasticity required for the acquisition of fear extinction was disrupted. However, two studies (Santini *et al*, 2001; Suzuki *et al*, 2004), using a different measure of fear (freezing rather than potentiated startle) and systemic infusions of the non-subunit selective NMDAR antagonist CPP ((±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid), found that whereas the retention of extinction was impaired, there was no apparent impairment during the acquisition of extinction.

Taken together, these previous results suggested that NMDARs in the amygdala are involved in the retention/

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consolidation of the long-term extinction memory, but not its initial acquisition (Maren and Quirk, 2004). However, the results are inconclusive, because side effects of the two drugs used to block NMDARs may have interfered with assessment of extinction acquisition during the training session. APV disrupts synaptic transmission in the amygdala (Li *et al*, 1995; Maren, 1996; Weisskopf and LeDoux, 1999), and APV and CPP both interfere with the expression of conditioned freezing (Maren *et al*, 1996; Lee and Kim, 1998; Lee *et al*, 2001).

In the present study, we revisited the question of whether NMDARs, particularly those in the amygdala, are involved in the acquisition of fear extinction. We measured freezing behavior, which allows assessment of fear responses during extinction training as well as during a retrieval test. Moreover, we used ifenprodil, an antagonist that selectively blocks the NR2B subunit of NMDARs (Williams, 2001). Unlike the NMDA antagonists used previously in studies of extinction, ifenprodil disrupts the acquisition of fear conditioning without affecting fear expression or synaptic transmission (Rodrigues *et al*, 2001; Bauer *et al*, 2002; Blair *et al*, 2005). Further, it is now apparent that specific NMDAR channel properties depend on their subunit composition (Cull-Candy and Leszkiewicz, 2004), and the NR2B subunit is specifically involved in synaptic plasticity (Barria and Malinow, 2005). Ifenprodil may therefore constitute a relatively selective tool for studying the contribution of NMDAR-mediated plasticity in the amygdala to extinction. Thus, we first determined whether systemic treatment with ifenprodil affected fear extinction, and then examined the effects of local blockade in the amygdala.

MATERIALS AND METHODS

Subjects

Adult male Sprague–Dawley rats (Hilltop Lab Animals Inc., Scottdale, PA), weighing 325–350 g upon arrival, were individually housed in transparent polyethylene cages and maintained on a 12 h light/dark cycle (lights on at 0700 hours) within a temperature- and humidity-controlled environment. Food and water were available *ad libitum* throughout the duration of the experiments. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by the New York University Animal Care and Use Committee.

Behavioral Procedures

Apparatus and stimuli. Rats underwent habituation, fear conditioning and fear extinction in one of four identical chambers constructed of aluminum and Plexiglas walls (Rat Test Cage, Coulbourn Instruments, Allentown, PA), with metal stainless steel rod flooring that was attached to a shock generator (Model H13–15; Coulbourn Instruments). The chambers were lit with a single house light, and each chamber was enclosed within a sound-isolation cubicle (Model H10–24A; Coulbourn Instruments). An infrared digital camera, mounted on top of each chamber, allowed videotaping during behavioral procedures for later behavioral scoring. In addition, an overhead 24-cell, three-dimensional infrared activity sensor continuously monitored (temporal resolution of 20 ms) all movement in the chamber, and the data were recorded on a computer equipped with Coulbourn Instruments LabLinc Habitest Universal Linc System. The computer also controlled stimulus presentation with Graphic State 2 software (Coulbourn Instruments). Chamber grid floors, trays, and walls were thoroughly cleaned with water and dried between sessions. Rats were allowed to explore freely the chamber for 4 min before each behavioral procedure (ie habituation, fear conditioning, and extinction training/testing sessions).

Fear conditioning procedure. Conditioning was conducted in groups of four rats at a time, each in a different chamber (see above). All rats were first exposed to five habituation trials (CS-alone presentations) on day 0, followed by seven conditioning trials (CS–US pairings) on day 1. The CS was a 30-s, 5 kHz, 80 dB SPL sine wave tone, which co-terminated with a 1-s, 0.7 mA footshock US during fear conditioning. Mean intertrial interval was 4 min (2–6 min range) throughout habituation and fear conditioning. Freezing, a measure of conditioned fear, was continuously recorded during the conditioning session and later scored to determine the degree to which the rats acquired the conditioned association (see Measurement of freezing behavior below). After conditioning, rats were returned to their home cages and to the colony room.

Extinction procedures. Rats that showed $\leqslant$50% freezing during fear conditioning (average of conditioning trials 2–7) were excluded from the subsequent phases of the study. Rats that satisfied the freezing criterion ($>$50% freezing) during conditioning were assigned to either an experimental or control group, matched for freezing during fear conditioning. Three different experiments were conducted in rats that met this criterion. As rats were subjected to behavioral procedures four at a time, special care was taken to test two experimental and two control rats in each batch of four. Freezing was recorded continuously during the extinction training and test sessions. Consistent with the fear conditioning procedure, throughout extinction sessions (training and test) mean intertrial interval was 4 min (2–6 min range).

Measurement of freezing behavior. Freezing was used to measure the conditional emotional fear response, and was defined as the cessation of all movement with the exception of respiration-related movement and non-awake or rest body posture (McAllister and McAllister, 1971; Fanselow, 1994). Freezing was videotaped and later scored offline with a digital stopwatch by recording the total time spent freezing during every 30-s tone CS. Freezing was scored blind with respect to the treatment group. In addition, online assessment of freezing was obtained using activity/inactivity data collected from the overhead infrared activity monitor (see Apparatus and stimuli above). These data were converted to freezing values using a custom MATLAB® (MathWorks Inc.) code, where freezing was defined as continuous inactivity lasting at least 2 s. These values were then transformed to freezing

percentage and used exclusively to match groups after conditioning.

Drugs

Two NMDAR antagonists were used: ifenprodil tartrate salt and CPP (Sigma-Aldrich Co.). Ifenprodil, a non-competitive, selective NR2B-containing NMDA receptor antagonist (Williams, 2001), was dissolved in distilled water for systemic studies, and for intra-amygdala infusions was dissolved in artificial cerebrospinal fluid, which depending on the ifenprodil dose (1 or 5 μg), respectively, contained 2 or 10% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich Co.; Yaksh *et al*, 1991), adjusted to pH = 7.4 using hydrochloric acid. CPP, a competitive, non subunit-selective NMDA antagonist, was dissolved in physiological saline (0.9%) and only used in systemic studies. A new sealed vial of drug was used each time, and all solutions were prepared the same day they were used.

Systemic injections. Extinction training took place either 2 or 24 h after fear conditioning (see Experimental design below). Before extinction training, rats were given intraperitoneal (i.p.) injections of ifenprodil or CPP. Ifenprodil (5 mg/ml/kg) or distilled water was injected 15 min before extinction training. CPP (10 mg/ml/kg) or saline was injected 60 min before extinction training (as by Santini *et al*, 2001).

Intra-amygdala injections. Cannulae were surgically implanted bilaterally to locally infuse ifenprodil or its vehicle into the amygdala. Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.; Ketaject®) and xylazine (6.0 mg/kg i.p.; Xyla-Ject®), and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Supplemental doses of the mix were given as needed to maintain a deep level of anesthesia. Body temperature was maintained with a heated gel pad. The skull was exposed and small holes were drilled. Using a stereotaxic apparatus, stainless-steel guide cannulae (22 gauge; Plastics One Inc., Roanoke, VA) fitted with infusion cannulae (28 gauge) that extended 1.5 mm beyond the base of the guide, were positioned bilaterally above the lateral amygdala (5.8 mm anterior, 5.2 mm lateral, and 2.2 mm dorsal from the interaural line; Paxinos and Watson, 1998). The guide cannulae were secured to the skull using surgical screws and acrylic dental cement. Infusion cannulae were replaced with dummy cannulae, cut to extend 0.5 mm beyond the guide cannulae, to prevent clogging. Antibiotic ointment was applied to prevent infections. After surgery, rats were administered buprenorphine hydrochloride (2.0 mg/kg, i.p.; Buprenex®) and atipamezole (1.0 mg/kg, i.p.; Antisedan®) for analgesia and reversal of the anesthetic.

After 7 days recovery from surgery, rats were subjected to habituation, fear conditioning, and then to extinction training a day later (see above). Fifteen to forty-five minutes before extinction training, rats received bilateral intra-amygdala infusions (0.25 μl/side) of either vehicle or ifenprodil (1.0 or 5.0 μg/side). Solutions were infused in freely moving rats at a rate of 0.15 μl/min through infusion cannulae attached to a 1.0 μl Hamilton syringe via polyethylene tubing (PE-10, Harvard Apparatus Inc.). Cannulae were left in place for an additional 60 s after the infusion to allow for diffusion of the solution away from the cannula tip, after which the dummy cannulae were replaced and the rat was returned to its home cage and brought back to the colony room.

Histology

To verify the intra-amygdala placement of the injection cannula tips, rats were anesthetized following completion of the behavioral procedures with an overdose of chloral hydrate (25%, 1 ml/100 g) and transcardially perfused with 10% buffered formalin. Brains were removed and stored in 10% buffered formalin with 30% sucrose. Subsequently, brains were blocked, and cut in 40 μm sections through the amygdala using a cryotome. After standard histological Nissl-staining, sections were examined on a light microscope for injector tip localization into the amygdala. Only data from rats that had the bilateral placements within the lateral amygdala were included in the study—decisions to include or exclude animals were made without knowledge of the experimental results.

Experimental Design

Four studies were performed to assess the contribution of NR2B-containing NMDARs to fear extinction. The first three studies involved systemic injections and the last study involved intra-amygdala microinfusions.

Study 1: systemic injections of ifenprodil 2 h after fear conditioning. Two hours after conditioning rats were given an i.p. injection of ifenprodil ($n = 12$) or vehicle ($n = 12$). Fifteen minutes later, they were exposed to 20 extinction training trials (CS-alone presentations). On day 2, rats received five additional extinction test trials (CS-alone presentations).

Study 2: systemic injections of ifenprodil and CPP 24 h after fear conditioning. To compare the effects of ifenprodil and CPP, rats were injected with ifenprodil ($n = 11$), CPP ($n = 6$), or one of the corresponding vehicles (water, $n = 12$; saline, $n = 6$), respectively, 15 or 60 min before extinction training. Extinction training (20 CS-alone presentations) took place 24 h after conditioning (ie on day 2), in contrast to the 2-h delay used in the previous experiment. Rats received five additional extinction test trials (CS-alone presentations), drug-free, on the following day (day 3). There were no significant differences between the two vehicle groups (water or saline; $p = 0.67$), and so data from both groups were collapsed ($n = 18$). To assess the requirement for extinction training in the effects of ifenprodil, a separate group of rats received i.p. injections of ifenprodil ($n = 5$) or vehicle ($n = 4$) 24 h after conditioning (day 2), but were not given extinction training. Instead, they were put back in their home cages and returned to the colony room. The next day (day 3), they were exposed to five extinction trials to test for retention of fear memory, drug free.

Study 3: systemic injections of ifenprodil and CPP on spontaneous locomotor activity. To test whether systemic injections of ifenprodil or CPP produce nonspecific, acute behavioral impairments, separate groups of rats were given i.p. ifenprodil ($n=8$), CPP ($n=10$), or vehicle (water, $n=9$; saline, $n=9$), as above, before measuring spontaneous locomotor activity, for 90 min, in a neutral environment. Each rat was placed in a chamber equipped with an overhead infrared activity monitor (described above), which continuously recorded movement. There were no significant differences between the two vehicle groups (water or saline; $p=0.70$), and so data from both groups were collapsed ($n=18$).

Study 4: intra-amygdala infusions of ifenprodil 24 h after fear conditioning. In the final study, we evaluated the effects of local infusion of two doses of ifenprodil into the lateral amygdala. First, we tested the effect of an intra-amygdala ifenprodil dose that has been shown to be sufficient to block the acquisition of fear conditioning (ifenprodil low: 1.0 μg/side) (Rodrigues *et al*, 2001; Blair *et al*, 2005). In a separate group of animals, we assessed the effect of a higher ifenprodil dose infusion into the lateral amygdala (ifenprodil high: 5.0 μg/side). Twenty-four hours after conditioning (day 1), and 15–45 min after bilateral intra-amygdala infusion of ifenprodil low or high doses ($n=9$ and 8, respectively) or their respective vehicle ($n=6$ and 8), rats were exposed to 20 extinction training trials (day 2). The next day (day 3), as in the previous experiments, rats received five extinction test trials, drug free. There were no significant differences between the two vehicle groups ($p=0.84$), and so data from both groups were collapsed ($n=14$).

Data Analysis

Behavioral data from each experiment (percent freezing or activity scores) were analyzed using an analysis of variance (ANOVA), with drug group as a between-subjects factor, and session and/or trial as repeated measures factors. CS-presentation trials were combined into bins of four for extinction training sessions from all experiments, to equate levels for direct statistical comparison across extinction training and testing sessions. Bonferroni's *a priori* test was used for planned mean comparisons. Significant ANOVA results were followed up using Tukey's HSD *post hoc* mean comparisons. Statistica 7 (StatSoft Inc.) was used for the analyses. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

We assessed whether systemic and intra-amygdala administration of the NR2B subunit-selective antagonist ifenprodil affects the acquisition and subsequent retrieval of fear extinction. In the first experiment, we used a 2 h delay between fear conditioning and extinction training (as by Santini *et al*, 2001), and gave systemic ifenprodil 15 min before extinction. In the second experiment, we used the same procedure except that we used a 24 h delay between fear conditioning and extinction in order to rule out an effect on fear memory consolidation. Also, we compared the effects of ifenprodil with CPP, a NMDAR antagonist that is not strongly selective for a particular receptor subunit. The third study assessed whether spontaneous locomotor activity is affected by either ifenprodil or CPP, as drug-induced alterations of activity, if they exist with either drug, could interfere with the ability to measure and interpret freezing. Finally, we examined the effects of two ifenprodil doses infused locally into the lateral amygdala before extinction training.

Ifenprodil, 2 h after Fear Acquisition, Impairs the Acquisition of Fear Extinction

Rats underwent fear conditioning and were then matched for freezing scores during fear conditioning and divided into two groups that received either ifenprodil or vehicle. The matching procedure ensured that freezing was not different between ifenprodil and vehicle groups before extinction. The rats were then injected with the NR2B antagonist ifenprodil (5 mg/kg, i.p.) 15 min before extinction training, which began 2 h after fear conditioning. The next day, they received a drug-free extinction test. The results (by trial) are shown in Figure 1a.

Rats injected with ifenprodil or vehicle before extinction training exhibited similar CS-elicited freezing during the first trial of extinction training ($t_{(22)}=-1.15$; $p=0.26$). This indicated that the fear memory was acquired and expressed to the same extent in both groups, confirming previous findings, which show that ifenprodil does not disrupt the expression of fear conditioning (Rodrigues *et al*, 2001; Blair *et al*, 2005).

As extinction training progressed, vehicle-treated rats showed a gradual reduction in freezing, reaching negligible freezing levels by the end of extinction training. However, ifenprodil-treated rats showed sustained freezing across multiple extinction training trials, suggesting that ifenprodil impaired the acquisition of fear extinction.

In the drug-free extinction test conducted 24 h after extinction training, vehicle-treated rats exhibited low levels of freezing during the test trials, indicating successful retrieval of extinction learning. In contrast, the ifenprodil-treated rats exhibited relatively high levels of freezing that were comparable to the amount of freezing observed at the start of extinction training on the previous day, indicating that little or no extinction occurred.

The results were statistically evaluated using a three-way ANOVA with drug group (vehicle, ifenprodil), as a between-subjects factor and extinction session (training, testing) and trials as within-subjects factors (ANOVA: significant main effects of drug ($F_{(1,22)}=29.97$, $p<0.001$) and trial ($F_{(4,88)}=99.45$, $p<0.001$); and significant session × trial ($F_{(4,88)}=2.83$, $p=0.03$) and drug × session × trial ($F_{(4,88)}=2.68$, $p=0.04$) interactions). *Post hoc* comparisons between drug groups showed that ifenprodil-treated rats exhibited significantly higher freezing both during extinction training ($p=0.005$) and extinction testing ($p=0.004$). Despite the impaired extinction in ifenprodil-treated groups, *post hoc* comparisons showed that by the end of the session freezing was decreased to some extent in both drug groups ($p<0.001$) (Figure 1b). Thus, ifenprodil, given

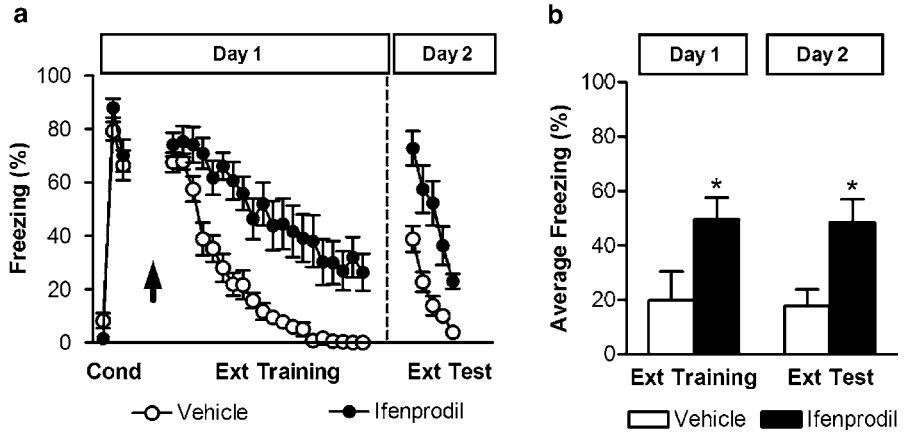

Figure 1 Ifenprodil, 2 h after fear conditioning, impairs the acquisition of fear extinction. (a) Percent freezing during conditioning (first trial and averages of 2–4 and 5–7 CS-footshock trials on day 1), trial-by-trial extinction training (20 CS-alone trials on day 1), and extinction test (5 CS-alone trials on day 2). Vehicle (white circles) or ifenprodil (black circles) injections were administered 2 h after fear acquisition, before extinction training (arrow). (b) Average percent freezing across all extinction training and extinction test trials for vehicle-treated (white bars) and ifenprodil-treated (black bars) rats. In this and all subsequent graphs, all data presented as means ± SEM. Cond: conditioning; Ext: extinction. *$p < 0.005$ relative to vehicle in the same session.

2 h after fear acquisition and just before extinction training, significantly impaired the acquisition and retention of fear extinction learning.

Ifenprodil, but not CPP, Given 24 h after Fear Acquisition, Impairs the Acquisition of Fear Extinction

In the previous experiment, only a short-time interval (2 h) separated fear conditioning and fear extinction, which leaves open the possibility that the pre-extinction ifenprodil injection may have altered fear memory consolidation rather than fear extinction learning. To address this, we repeated the procedures of the previous experiment, but used a 24 h delay between fear conditioning and extinction training to allow a full day for fear memory consolidation. In addition, we compared the effects of ifenprodil with CPP (a competitive NMDA antagonist that does not selectively affect a particular subunit). This comparison was made because previous studies (Santini *et al*, 2001; Suzuki *et al*, 2004) found no effect on the initial acquisition of extinction learning by CPP (ie CPP-treated rats showed a normal decrement in freezing during extinction training), which contrasts with the effects of ifenprodil seen in the previous experiment.

Rats underwent fear conditioning as above, and were then matched for freezing scores during fear conditioning and divided into groups that received either drug (ifenprodil or CPP) or vehicle (water or saline solution, respectively). The matching procedure ensured that freezing was not different between the drug and vehicle groups before extinction. Twenty-four hours after fear conditioning, the rats were injected with ifenprodil (5 mg/kg, i.p.) or vehicle (water) 15 min before extinction training, or CPP (10 mg/kg, i.p.) or vehicle (saline) 60 min before extinction training. The next day, the retention of extinction training was tested. As the results from the two vehicle groups were not statistically different ($p = 0.67$), the data were collapsed in the subsequent analysis. The results (by trial) are shown in Figure 2a.

Rats injected with ifenprodil, CPP or vehicle before extinction training exhibited similar CS-elicited freezing during the first trial of extinction training ($F_{(2,32)} = 2.06$; $p = 0.14$), indicating that the fear memory was acquired and expressed to the same extent in all groups. Over the course of the 20 extinction training trials, vehicle treated rats showed a gradual reduction in freezing levels, with virtually no freezing by the end of extinction training.

Consistent with the previous experiment, ifenprodil-treated rats showed sustained freezing across multiple extinction training trials. CPP-treated rats, by contrast, showed a decline in freezing similar to the controls. In the drug-free test on the following day, vehicle-treated rats continued to exhibit low levels of CS-elicited freezing (indicating successful retrieval of extinction learning), but both ifenprodil and CPP-treated rats exhibited high CS-elicited freezing levels. Thus, consistent with the previous experiment, ifenprodil appeared to disrupt the acquisition of extinction. Further, consistent with previous reports (Santini *et al*, 2001; Suzuki *et al*, 2004), CPP appeared to affect only the retrieval of extinction, with no apparent effect on the rate of extinction learning during acquisition.

The results were statistically evaluated using a three-way ANOVA with drug group (vehicle, ifenprodil, CPP) as a between-subjects factor, and extinction session (training, testing) and trials as within-subjects factors (ANOVA: significant main effects of drug ($F_{(2,32)} = 25.03$, $p < 0.001$) and trial ($F_{(4,128)} = 88.90$, $p < 0.001$); and significant session × drug ($F_{(2,32)} = 9.14$, $p < 0.001$), and drug × session × trial ($F_{(8,128)} = 2.85$, $p = 0.006$) interactions). *Post hoc* comparisons between drug groups showed that ifenprodil-treated rats exhibited significantly higher freezing than both vehicle- ($p < 0.001$) and CPP-treated rats ($p = 0.002$) during extinction training. Despite the impaired extinction in ifenprodil-treated groups, *post hoc* comparisons showed that by the end of the session freezing was decreased to some extent in all drug groups ($p < 0.001$). In contrast with the different effects of ifenprodil and CPP during extinction training, both ifenprodil- and CPP-treated rats showed

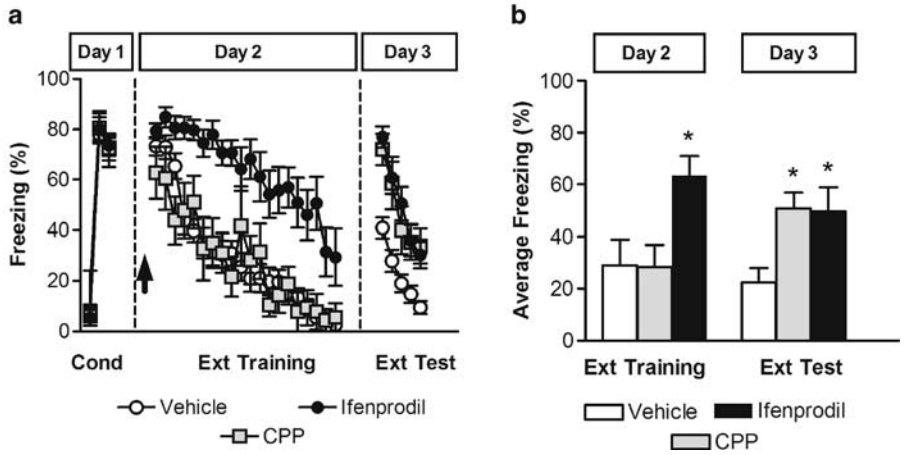

Figure 2 Ifenprodil, but not CPP, given 24 h after fear conditioning, impairs the acquisition of fear extinction. (a) Percent freezing during conditioning (first trial and averages of 2–4 and 5–7 CS-footshock trials on day 1), trial by trial extinction training (20 CS-alone trials on day 2), and extinction test (five CS-alone trials on day 3). Vehicle (white circles), CPP (gray squares), or ifenprodil (black circles) injections were administered 24 h after fear acquisition (arrow). (b) Averaged percent freezing across all extinction training and extinction test trials for vehicle-treated (white bars), CPP-treated (gray bars), and ifenprodil-treated (black bars) rats. *$p<0.01$ relative to vehicle in the same session.

similar levels of freezing during the extinction test ($p=0.999$), at levels that were significantly higher than vehicle-treated rats ($p=0.001$ and 0.009, ifenprodil and CPP, respectively) (Figure 2b). Thus, ifenprodil, but not CPP, significantly impaired the acquisition of fear extinction, whereas both ifenprodil and CPP impaired its later retention.

In addition, in a separate group of rats, we tested for possible effects of ifenprodil that may have occurred during extinction training that are not related to the CS-alone presentations. Rats that exhibited the same level of freezing during fear conditioning were divided into two groups that received either ifenprodil or vehicle 24 h after conditioning. In contrast to the previous experiments, these rats were not given extinction training. Instead, they were returned back to the colony room after the injection. The next day, as with the other groups, they were given a drug-free test session. No significant difference was observed between non-extinguished vehicle and ifenprodil groups ($p=0.79$; data not shown). Thus, the effects of ifenprodil on extinction depend on the CS-alone presentations in the presence of the drug and are not attributable to a nonspecific effect of the drug that carries over to the test session the next day.

In summary, results from the two experiments above indicate that ifenprodil and CPP have differential effects on fear extinction learning. Ifenprodil impairs extinction acquisition, regardless of the time (2 or 24 h) allowed for fear consolidation, but CPP only impairs extinction retention without producing apparent effects during the initial learning of extinction. In the next experiment, we attempt to resolve this discrepancy between the effects of ifenprodil and CPP.

CPP, but not Ifenprodil, Impairs Spontaneous Locomotor Activity

CPP is known to produce general behavioral changes at high doses (such as 10 mg/kg), possibly by inducing ataxia (Jerram *et al*, 1996), altering locomotor behavior (Starr and Starr, 1994), or by reducing muscle tone (Lehmann *et al*, 1987; Turski *et al*, 1987). Whether ifenprodil has such effects is not known. As altered spontaneous activity could indicate drug-induced behavioral changes that are not specific to conditioned fear, we tested the effects of these two drugs on locomotor activity to evaluate whether ifenprodil and/or CPP induce behavioral changes that could interfere with the interpretation of observed effects on freezing during the extinction training session. Rats were given systemic injections of ifenprodil or CPP, and placed in the test chamber for 90 min (the approximate duration of the extinction training session). Ifenprodil or vehicle (water) was administered 15 min before the test, whereas CPP or vehicle (saline) was injected 1 h before, as in previous behavioral procedures.

Figure 3a shows the time course (5-min bins) for locomotor activity in the three groups. There were no significant differences between the two vehicle groups (water or saline; $p=0.70$), and so data from both groups were collapsed. All groups showed similar levels of activity during the first 5 min. As the session progressed, vehicle and ifenprodil groups continued to show similar activity levels. However, the CPP group showed considerably less locomotor activity than the other two groups. This effect was evident starting at 10 min, and continued practically throughout the 90-min session. The results were statistically evaluated using a two-way ANOVA with drug group (vehicle, ifenprodil, CPP) as a between-subjects factor, and time (5-min bins) as a within-subjects factor (ANOVA: significant main effects of drug ($F_{(2,32)}=7.33$, $p=0.002$) and time ($F_{(17,561)}=48.11$, $p<0.001$); and a significant drug × time interaction ($F_{(34,561)}=9.14$, $p<0.001$)). *Post hoc* comparisons between drug groups showed that mean activity levels were similar for ifenprodil- and vehicle-treated rats ($p=0.92$), but that activity levels for CPP-treated rats were significantly lower than rats treated with vehicle ($p=0.0021$) or ifenprodil ($p=0.0025$). Thus, CPP, but not ifenprodil, impaired spontaneous activity during the 90 min session (Figure 3b). This finding is consistent with

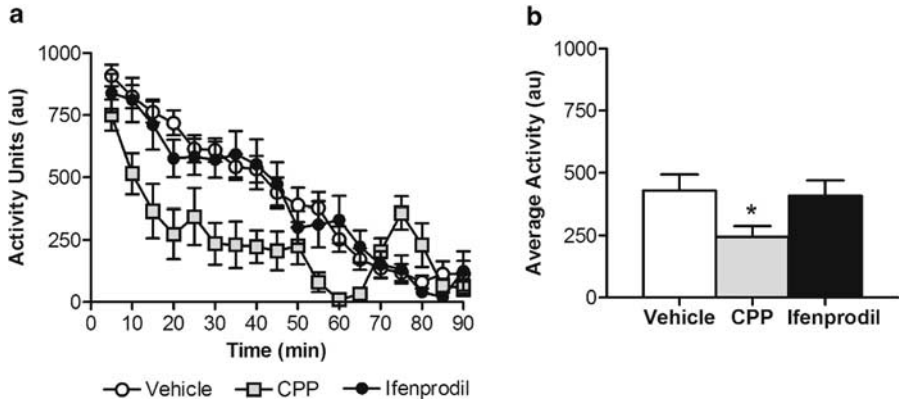

Figure 3 CPP, but not ifenprodil, impairs spontaneous locomotor activity. (a) Spontaneous locomotor activity during 90 min expressed in arbitrary units (au). Vehicle (white circles), CPP (gray squares), or ifenprodil (black circles) systemic injections were administered before locomotor activity test. (b) Average spontaneous locomotor activity across 90 min for vehicle-treated (white bars), CPP-treated (gray bars), and ifenprodil-treated (black bars) rats. *$p < 0.005$ relative to vehicle in the same session.

previous results showing that CPP can increase ataxia (Jerram *et al*, 1996) and decrease muscle tone (Lehmann *et al*, 1987; Turski *et al*, 1987; Starr and Starr, 1994) at similar doses. We discuss the implications and an alternative explanation of this finding below.

Ifenprodil in the Lateral Amygdala Impairs the Acquisition of Fear Extinction

Previous studies have implicated NMDA receptors in the lateral and basal amygdala in fear extinction. Although NMDARs that contain the NR2B subunit are prevalent in amygdala neurons (Loftis and Janowsky, 2003; Lopez de Armentia and Sah, 2003; Sah and Lopez De Armentia, 2003; Szinyei *et al*, 2003; Rodrigues *et al*, 2004), their role in fear extinction is not known. In addition, previous studies testing the effects of amygdala NMDAR blockade were not able to discriminate between effects on the acquisition or retention of fear extinction (see Introduction), leaving open the question of whether NMDARs in the amygdala are indeed involved in extinction learning. In this experiment, we therefore assessed whether blockade of NR2B-containing NMDARs in the lateral amygdala affects the acquisition of extinction learning. We repeated the procedures, described above, where we injected ifenprodil 24 h after conditioning, but in this experiment, two different doses of ifenprodil were infused directly into the lateral amygdala in two separate groups of rats.

Rats were fear conditioned 1 week after surgical implantation of cannula guides, and assigned to drug groups (ifenprodil low or high and vehicle low or high) immediately after conditioning, matched for CS-elicited freezing during conditioning. The next day (day 2), rats received intra-amygdala infusions of vehicle or ifenprodil (0, 1.0, or 5.0 μg/0.25 μl/side), 15–45 min before extinction training. On day 3, all rats received a drug-free extinction test. Following the study, rats were euthanized, transcardially perfused, and brains removed for histological analysis. Only rats with bilateral cannula placements within the lateral amygdala were included in the analysis (shown in Figure 4a). As the results from the two vehicle groups (vehicle low and high) were not statistically different ($p = 0.84$), the data were collapsed in the subsequent analysis. The results (by trial) are shown in Figure 4b.

Rats infused with vehicle, low or high ifenprodil doses into the lateral amygdala before extinction training exhibited similar CS-elicited freezing during the first trial of extinction training ($F_{(2,28)} = 0.84$; $p = 0.44$), indicating that the fear memory was acquired and expressed to the same extent in all groups. Over the course of the 20 extinction training trials, vehicle-treated rats showed a gradual reduction in freezing levels, with virtually no freezing by the end of extinction training. Similar to systemic injections, rats that received intra-amygdala ifenprodil, either low or high dose, showed higher freezing to the CS across multiple extinction training trials relative to vehicle controls, indicating that both ifenprodil doses impaired the acquisition of fear extinction. Both ifenprodil group doses also showed higher levels of freezing than controls during the drug-free extinction test the next day.

The results were statistically evaluated using a three-way ANOVA with drug group (vehicle, ifenprodil low and ifenprodil high doses) as a between-subjects factor, and extinction session (training, testing) and trials as within-subjects factors (ANOVA: significant main effects of drug ($F_{(2,28)} = 6.96$, $p < 0.0035$) and trial ($F_{(4,112)} = 78.96$, $p < 0.001$). The drug × session × trial interaction approached significance ($F_{(8,112)} = 1.79$, $p = 0.087$)). *Post hoc* comparisons between drug groups showed that both ifenprodil low-treated and ifenprodil high-treated rats exhibited significantly higher freezing than vehicle-treated rats ($p = 0.045$ and 0.017, respectively) during extinction training. Despite the impaired extinction in ifenprodil-treated groups, *post hoc* comparisons showed that by the end of the session freezing was decreased to some extent in all drug groups ($p < 0.001$). Consistent with the ifenprodil-induced impairment on extinction training, both ifenprodil low- and high-treated rats showed significantly higher freezing than vehicle-treated rats ($p = 0.047$ and 0.019) during the extinction test (Figure 4c). These results suggest that NR2B-containing

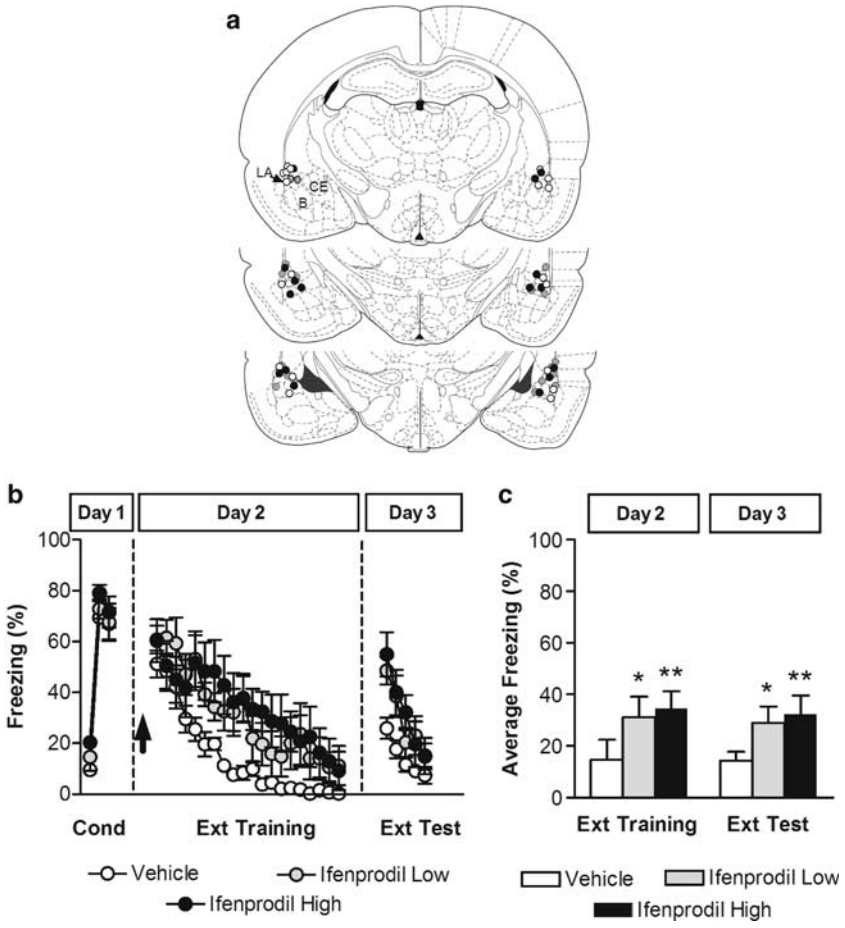

Figure 4 Ifenprodil in the lateral amygdala impairs the acquisition of fear extinction. (a) Coronal drawings show the localization of injector tips (top to bottom relative to interaural line: + 6.44, + 5.86, and + 5.40 mm; adapted from Paxinos and Watson, 1998) from rats infused with vehicle (white circles), ifenprodil low (1 μg; gray circles), or ifenprodil high doses (5 μg; black circles). LA: lateral amygdala; B: basal amygdala; CE: central amygdala. (b) Percent freezing during conditioning (first trial and averages of 2–4 and 5–7 CS-footshock trials on day 1), trial-by-trial extinction training (20 CS-alone trials on day 2), and extinction test (5 CS-alone trials on day 3). Vehicle (white circles), ifenprodil low (gray circles), or ifenprodil high (black circles) injections were administered 24 h after fear acquisition (arrow). (c) Averaged percent freezing across all extinction training and extinction retention trials for vehicle-treated (white bars), and ifenprodil-treated low (gray bars) and high (black bars) rats. $^{*}p<0.05$ and $^{**}p<0.02$ relative to vehicle in the same session.

NMDARs in the amygdala contribute to the acquisition of extinction learning, and further support the notion that NMDA-mediated plasticity in the lateral amygdala is necessary for the initial acquisition of fear extinction.

DISCUSSION

Synaptic plasticity is considered to be essential for the acquisition and storage of new memories and NMDARs contribute to experience-dependent synaptic plasticity (Martin *et al*, 2000). Given that extinction involves learning, and that NMDARs participate in both learning and synaptic plasticity in a variety of situations, it is important to ask whether NMDARs contribute to extinction learning. In this study, we examined the contribution of NMDAR to extinction learning, focusing particularly on NMDARs that contain the NR2B subunit. Our results indicate that, similar to the acquisition of fear conditioning (Rodrigues *et al*, 2001; Blair *et al*, 2005), the acquisition of fear extinction requires the activation of NR2B-containing NMDARs, and that the lateral amygdala is an essential brain region underlying this mechanism.

NMDAR subunit composition confers distinct functional roles to this receptor (Cull-Candy and Leszkiewicz, 2004), with evidence suggesting that the NR2B subunit may be particularly important for NMDAR contributions to synaptic plasticity underlying learning and memory (Wong *et al*, 2002; Yoshimura *et al*, 2003; Liu *et al*, 2004; Kojima *et al*, 2005; Zhao *et al*, 2005), apparently more so than NR2A subunits (Barria and Malinow, 2005). For example, overexpression of the NR2B subunit in mice results in enhanced learning and memory in several behavioral tasks, including fear conditioning and fear extinction (Tang *et al*, 1999). Further, blockade of NR2B-containing NMDARs with ifenprodil impairs synaptic plasticity in the amygdala without impairing routine neural transmission (Li *et al*, 1995; Weisskopf and LeDoux, 1999; Bauer *et al*, 2002), and ifenprodil also impairs fear acquisition without affecting its expression (Rodrigues *et al*, 2001; Blair *et al*, 2005). Our findings extend the role of NR2B-containing NMDARs in the acquisition of new learning to the acquisition of fear extinction.

Systemic Blockade of NR2B in Fear Extinction Learning

Until now, evidence in support of a role for NMDARs in fear extinction have come from experiments using systemic (Baker and Azorlosa, 1996; Santini *et al*, 2001; Suzuki *et al*, 2004) or intra-amygdala (Falls *et al*, 1992; Lee and Kim, 1998; Lin *et al*, 2003) injections of NMDAR antagonists that do not discriminate among NMDA subunit compositions. In addition to the fact that these studies do not distinguish between subunits, other issues complicate the interpretation of the results. First, the antagonist APV, which was used in some of the studies, disrupts synaptic transmission in the amygdala (Li *et al*, 1995; Maren, 1996; Weisskopf and LeDoux, 1999), and in some cases also interferes with fear expression (Maren *et al*, 1996; Lee and Kim, 1998; Lee *et al*, 2001). This does not occur in the CA1 region of hippocampus, where APV disrupts synaptic plasticity without affecting synaptic transmission (Bliss and Collingridge, 1993), but is problematic in the amygdala. Second, several of the past studies used the fear-potentiated startle paradigm, which does not allow for fear responses to be measured during fear extinction training, and thus precludes the possibility of dissociating acquisition and retention of the fear extinction memory (see Introduction). Our study was not hindered by either of these complications. First, ifenprodil, by selectively blocking NR2B subunits, allowed us to explore the importance of NR2B-containing NMDA receptors, initially with systemic injections that would affect all potential brain regions involved, and then with local infusions into the lateral amygdala. Second, our behavioral paradigm allowed us to measure conditioned responses (freezing responses) elicited by each tone CS, both during the acquisition of extinction learning and in a later drug-free retention test.

Santini *et al* (2001), using freezing as a measure of fear, also tested the effects of NMDA receptor blockade by giving systemic injections of CPP (another non-subunit selective NMDA antagonist) before extinction training, and found no apparent impairment during the extinction training session, but did find an effect on fear extinction in a later retention test. They concluded that NMDA receptors are involved in the consolidation of the long-term memory of extinction, but not in the acquisition of extinction. This CPP effect on extinction was later replicated in mice by a different group (Suzuki *et al*, 2004). However, our results indicate that NMDAR, particularly the ones containing the NR2B subunit, are required for the acquisition, rather than *only* for the consolidation of the extinction memory.

Two possible explanations might explain why ifenprodil, but not CPP, revealed a role of NMDARs in the initial acquisition of extinction. First, it is possible that the NR2B subunit of the NMDAR plays a special role in extinction learning that is not detectable by CPP because of its slightly higher affinity ($\sim$10-fold) for NR2A than for NR2B subunits, compared with the strong selective affinity ($\sim$400-fold) of ifenprodil for NR2B (Lozovaya *et al*, 2004). A second possible explanation is that CPP may have masked the role of NMDARs in extinction by producing acute behavioral effects, such as a disruption of overall behavioral responsivity (Starr and Starr, 1994), which interfered with behavioral measurements during the extinction training session. Consistent with this, we found in a separate experiment that CPP (10 mg/kg) also impaired spontaneous activity, whereas ifenprodil (5 mg/kg) did not. The demonstrated muscle-relaxant properties of CPP (Lehmann *et al*, 1987; Turski *et al*, 1987; Jerram *et al*, 1996) are consistent with a possible CPP-induced disruption that could impair the performance of both freezing, which is an effortful behavioral response, and spontaneous activity. Indeed, Santini *et al* (2001) and later Goosens and Maren (2004) found that systemic CPP decreased the expression of conditioned freezing. Moreover Goosens and Maren (2004), observed that systemic CPP reduced conditional lateral amygdala spike firing, suggesting that systemic CPP (similar to APV) altered synaptic transmission within amygdala pathways involved in fear expression. Thus, ifenprodil revealed an effect of NMDAR blockade on the acquisition of fear extinction, possibly by avoiding the nonspecific pharmacological/behavioral effects caused by CPP.

Overall, our results with systemic ifenprodil indicate that NMDARs, and specifically NMDARs containing the NR2B subunit, play a key role in the acquisition of extinction. Thus, the effects on the acquisition of extinction, rather than its consolidation, may be a prerequisite to effects observed on subsequent tests for the extinction memory. This view is consistent with the traditional idea that NMDARs are involved in learning rather than consolidation (Martin *et al*, 2000). However, several studies have also reported effects of NMDA blockade on consolidation (Kentros *et al*, 1998; Shimizu *et al*, 2000). Further experiments involving post-extinction treatments with ifenprodil are needed to investigate the possibility that NMDA receptors might also be involved in the consolidation of extinction learning.

Lateral Amygdala Blockade of NR2B in Fear Extinction Learning

Notably, our results indicate that, as with initial fear acquisition, the lateral nucleus is a key amygdala region for NMDA-dependent plasticity processes underlying extinction acquisition. Infusions of NMDAR antagonists targeted for the lateral nucleus (this study) or the basal nucleus (most previous studies) of the amygdala likely affect both regions, as well as the intercalated cell masses and the central nucleus (Paré *et al*, 2004). Although we cannot rule out an effect on regions surrounding the lateral amygdala in this study, given that lesions of the basal amygdala do not affect extinction learning (Sotres-Bayon *et al*, 2004; Anglada-Figueroa and Quirk, 2005), it seems likely that the lateral amygdala was the key subregion affected by previous studies. In addition, we used an infusion volume that was one-half of that used in most previous studies, and so our results more specifically implicate the lateral amygdala. Thus, we propose that NMDA-dependent synaptic plasticity in the lateral amygdala is a likely mechanism underlying extinction learning. However, as NMDARs are critically involved in several different forms of synaptic plasticity, including long-term potentiation, long-term depression, and depotentiation, further experiments will be necessary to specify what form of plasticity is required for extinction learning.

Decreases in freezing did occur to some extent during the extinction training session in groups infused with

ifenprodil, both with systemic and lateral amygdala injections, suggesting that some extinction learning took place. Given that the extinction training sessions lasted ~90 min, the decrease in freezing, and thus the acquisition of extinction, could be attributable to a gradual reduction of ifenprodil bioavailability as the session progressed. Alternatively, activation of other ion channel systems, such as voltage-gated calcium channels (Cain *et al*, 2002), or NMDA receptors that involve subunits other than NR2B (eg NR2A), may contribute to extinction learning.

Lateral Amygdala in the Acquisition of Fear Extinction

Although evidence suggests a role for the mPFC in extinction consolidation (reviewed by Quirk *et al*, 2006), the brain circuitry required for the acquisition of extinction is not yet clear. The amygdala has received much attention in this respect. However, because most of the studies that involve the amygdala in fear extinction did not discriminate between acquisition and consolidation of extinction, its precise role in fear extinction has remained unclear until now. Here, we used a task that allows for fear assessment during extinction training, and we used a more selective drug that has fewer side effects, and so we were able to demonstrate that amygdala NMDARs are required for the acquisition of fear extinction. Notably, a recent study found that amygdala kinase signaling pathway is also involved in the acquisition of extinction (Herry *et al*, 2006). Together with our findings, these results suggest that increased intracellular calcium in the amygdala, mediated at least in part through NMDARs, activates kinase signaling required for the initial neural plasticity that underlies the acquisition of extinction.

The effects on extinction following both doses of intra-amygdala ifenprodil were relatively weaker than those following systemic injections. This may indicate that other brain regions, in addition to the amygdala, are involved in fear extinction learning. Thus, our data are consistent with a neural model for fear extinction that involves a distributed network, where the lateral amygdala plays a central role. Other likely candidate sites are the medial prefrontal cortex (Morgan *et al*, 1993; Quirk *et al*, 2000; Milad and Quirk, 2002; Santini *et al*, 2004), the periaqueductal gray matter (McNally *et al*, 2004), and the hippocampus (Corcoran *et al*, 2005).

Current models for fear extinction propose interactions between the amygdala, medial prefrontal cortex, and hippocampus (Maren and Quirk, 2004; Sotres-Bayon *et al*, 2004, 2006). Understanding the phase of learning and memory (acquisition, consolidation, retention, retrieval) during which each structure participates in extinction will provide a deeper understanding of the mechanisms involved and may lead to better ways to treat fear and anxiety disorders. For example, recent studies in rodents showing that systemic and intra-amygdala enhancement of NMDAR function using *d*-cycloserine facilitates extinction (Walker *et al*, 2002; Ledgerwood *et al*, 2003; Yang and Lu, 2005) have led to clinical studies showing that the same drug facilitates exposure therapy (itself a form of extinction) in human patients with anxiety disorders (Ressler *et al*, 2004; Hofmann *et al*, 2006). Our results clearly indicate that amygdala NMDARs, especially those containing the NR2B subunit, are required for the neural changes underlying the acquisition of fear extinction, and should help in conceptualizing further advances in the clinical use of NMDAR manipulations.

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