

# Glutamate NMDA Receptors within the Amygdala Participate in the Modulatory Effect of Glucocorticoids on Extinction of Conditioned Fear in Rats

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Recent results show that brain glucocorticoids are involved in the dysregulation of fear memory extinction in post-traumatic stress disorder patients. The present study was aimed to elucidate the possible mechanism of glucocorticoids on the conditioned fear extinction. To achieve these goals, male SD rats, fear-potentiated startle paradigm, and Western blot were used. We found that (1) systemic administration of the synthetic glucocorticoid agonist dexamethasone (DEX) facilitated extinction of conditioned fear in a dose-dependent manner (0.05, 0.1, 0.5, or 1.0 mg/kg, i.p.); (2) systemic administration of the glutamate NMDA receptor antagonist ( $\pm$ )-HA966 (6.0 mg/kg, i.p.) and intra-amygdala infusion of the NMDA receptor antagonists MK801 (0.5 ng/side, bilaterally) or D,L-2-amino-5-phosphonovaleric acid (AP5, 2.0 ng/side, bilaterally) blocked the DEX facilitation effect; (3) the corticosteroid synthesis inhibitor metyrapone (25 mg/kg, s.c.) blocked extinction and this was prevented by co-administration of NMDA receptor agonist D-cycloserine (DCS, 5.0 mg/kg, i.p.); (4) co-administration of DEX and DCS in subthreshold doses provided a synergistic facilitation effect on extinction (0.2 and 5 mg/kg, respectively). Control experiments indicated that co-administration of DEX and DCS did not alter the expression of conditioned fear and the effect was not due to lasting damage to the amygdala. These results suggest that glutamate NMDA receptors within the amygdala participate in the modulatory effect of glucocorticoids on extinction.

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## INTRODUCTION

Corticosteroids play an important role in fear and anxiety. They can modulate the neurochemical changes in specific neural pathways and alter the behavioral outcomes. Glucocorticoids also play an essential role in the formation, consolidation, and retrieval of fear memory. Corticosterone levels after conditioning are correlated with fear conditioning levels (Cordero *et al*, 1998; Cordero and Sandi, 1998). Adrenalectomy can reduce the unconditioned freezing behavior of the newborn mice (Takahashi, 1994a,b). Systemic administration of the glucocorticoid synthesis inhibitor metyrapone attenuated the long-term expression of contextual fear conditioning in a dose-dependent manner (Cordero *et al*, 2002). Roozendaal and McGaugh indicated that glucocorticoids could act directly in the

amygdala to influence memory consolidation (Roozendaal and McGaugh, 1997; Roozendaal *et al*, 2002; Roozendaal, 2003). It is remarkable that glucocorticoids also participate in the extinction of conditioned fear. Subcutaneous or intracerebroventricle administration of corticosterone normalizes the extinction of avoidance behavior in adrenalectomized rats (Bohus *et al*, 1982). Recently, Barrett and Gonzalez-Lima (2004) also reported that administration of corticosterone inhibitor metyrapone impairs extinction of conditioned freezing.

Inability to extinguish intense fear memories is a significant clinical problem in several psychiatric disorders distinguished by a dysregulation of fear, such as specific phobia, panic disorder, and post-traumatic stress disorder (PTSD) (Morgan *et al*, 1995; Fyer, 1998; Gorman *et al*, 2000; Myers and Davis, 2002). Treatments of these disorders often rely on progressive extinction of fear memories (Zarate and Agras, 1994; Dadds *et al*, 1997; Foa, 2000). However, there is little direct evidence for any molecular dysfunction that may predispose individuals to such disorders. Yet there is abundant evidence that these patients have some pre-existing biological diatheses. For example, there is abundant evidence suggesting a pre-existing difference between those who do and do not develop PTSD, despite experiencing the

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same trauma. Recent clinical studies showed that PTSD patients often appear to have reduced cortisol levels (Yehuda *et al*, 2004). Aerni *et al* (2004) found that daily administration of cortisol reduced symptoms of traumatic memory in PTSD patients. Schelling *et al* (2004) showed that prolonged high-dose glucocorticoid treatment after trauma reduced the risk of humans developing PTSD. In addition, Perlman *et al* (2004) reported that reduced glucocorticoid receptor mRNA levels in the amygdala of patients with major mental illness. Recently, we showed that either systemic or intra-amygdala administration of glucocorticoid agonist dexamethasone (DEX) facilitated extinction of conditioned fear (Yang *et al*, 2006). These results suggest that brain glucocorticoids may be involved in the dysregulation of fear memory extinction in PTSD patients. Evidence also indicated that administration of the partial NMDA receptor agonist D-cycloserine (DCS) facilitated extinction of conditioned fear (Walker *et al*, 2002; Ledgerwood *et al*, 2003, 2004; Ressler *et al*, 2004; Yang and Lu, 2005) and numerous signaling cascades are involved in the DCS effect on extinction that include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI-3 kinase) and calcineurin (Yang and Lu, 2005). The amygdaloid NMDA receptors may be also involved in the facilitatory effect of glucocorticoids on the extinction. We hypothesize that glucocorticoid activation in the basolateral nucleus of the amygdala (BLA) would facilitate extinction of fear-potentiated startle in the rat via interactions with glutamatergic NMDA receptors. We also suggest that DCS might be an especially useful adjunct to exposure-based psychotherapy in this group of patients.

A clarification of the neural mechanisms of glucocorticoids in the extinction process may be useful in the future treatment of psychiatric disorders that involved dysregulated fear responses. The present study was aimed to elucidate the possible mechanisms of glucocorticoids on the extinction of conditioned fear by using the fear-potentiated startle paradigm.

## MATERIALS AND METHODS

### Animals

Adult male Sprague–Dawley rats (obtained from the Animal Center of National Taiwan University) weighing between 250 and 350 g were used. Animals were housed in cages of four rats each in a temperature (24°C)—controlled animal colony, pelleted rat chow and water were available *ad libitum*. They were maintained on a 12:12 light–dark cycle with lights on at 0700 h. All behavioral procedures took place during the animal light cycle. All procedures were conducted in accordance with the ‘Guideline for Care and Use of Laboratory Animals’ and set forth by the Institutional Animal Care and Use Committee (IACUC) at the National Taiwan Normal University. All efforts were made to minimize the animal numbers, which are required to produce meaningful experimental data.

### Surgery

All surgeries were carried out under sodium pentobarbital (50 mg/kg, *i.p.*) anesthesia. Once anesthetized, rats were

placed in a Kopf stereotaxic instrument (David Kopf instruments, Tujunga, CA, USA). The skull was exposed and 22-gauge guide cannulas (model C313G, Plastic Products, Roanoke, VA) were implanted bilaterally into the BLA (AP, -2.8; DV, -9.0, ML,  $\pm$ 5.0 from bregma, Paxinos and Watson, 1997). Size 0 insect pins (Carolina Biological Supply, Burlington, NC) were inserted into each cannula to prevent clogging. These extended approximately 2 mm past the end of the guide cannula. Screws were anchored to the skull and the assembly was cemented in place using dental cement (Plastic Products, Roanoke, VA). Rats received an antibiotic (penicillin) once every day for the first 3 days after the surgery to reduce the risk of infection.

### Behavioral Procedures

Extinction of the conditioned fear was measured using the well-established fear potentiated startle paradigm (Cassella and Davis, 1986; Lu *et al*, 2001; Walker *et al*, 2002; Yang and Lu, 2005; Yang *et al*, 2006). Rats were trained and tested in a startle chamber (San Diego Instruments, San Diego, CA, USA), in which cage movement results in the displacement of an accelerometer. Startle amplitude was defined as peak accelerometer voltage within 200 ms after startle stimulus onset.

The behavioral procedures common to all experiments consisted of an acclimation phase, a baseline startle test phase, a fear-conditioning phase, a pre-extinction test, extinction training, and a postextinction test.

**Acclimation.** On each of three consecutive days, rats were placed into the test chambers for 10 min and then returned to their home cages.

**Baseline startle test.** On each of the next two consecutive days, animals were placed in the test chambers and presented with 30 95-dB startle stimuli at a 30-s inter-stimulus interval (ISI). Animals whose baseline startle was <1% of the measurable level were not included in later analyses.

**Fear conditioning.** Twenty-four hours later, rats were returned to the test chambers and 5 min later given the first of 10 light-footshock pairings. The shock (unconditioned stimulus) was delivered during the last 0.5 s of the 3.7 s lights (conditioned stimulus). The average inter-trial interval was 4 min (range = 3–5 min).

**Pre-extinction test.** Twenty-four hours after fear conditioning, rats were returned to the test chambers and 5 min later presented with 30 startle-eliciting noise bursts (95 dB—30 s ISI). These initial startle stimuli were used to habituate the startle response to a stable baseline prior to the light-noise test trials that followed. Thirty seconds later a total of 20 startle-eliciting noise bursts was presented, 10 in darkness (noise alone) and 10 3.2 s after onset of the 3.7 s light (light-noise) in a balanced, irregular order at a 30-s ISI. Percent fear potentiated startle was computed as ((startle amplitude on light-noise minus noise-alone trials)/noise-alone trials)  $\times$  100. Based on these data, the rats were divided into equal size groups that had comparable mean levels of

percent fear potentiated startle. Animals with less than 50% fear potentiated startles during the pre-extinction test were not used.

**Extinction training.** Extinction training (cue exposure) is defined as the repetitive exposure to the conditioned stimulus cue (light) in the absence of the unconditioned stimulus. Twenty-four hours after the pre-extinction test, rats were returned to the test chamber and 5 min later presented with 30, 3.7 s light exposures at a 30-s ISI. Context control groups (context exposure) remained in the test cages for the same amount of time but did not receive light presentations.

**Postextinction test.** Twenty-four hours after the last extinction training period, rats were returned to the test chamber and 5 min later presented with 30 95-dB leader stimuli for a habituated startle baseline. This was followed by a total of 60 startle-eliciting noise bursts, 30 in darkness (noise alone), and 30 presented 3.2 s after the onset of the 3.7 s light (light-noise) in a balanced, irregular order at a 30-s ISI. Results were evaluated as with the pre-extinction test.

### Drug Treatment

DEX (Sigma, St Louis, MO, USA) and DCS (Sigma, St Louis, MO, USA) were freshly dissolved in saline. DEX (0.2 or 1.0 mg/kg) and DCS (5.0 mg or 15.0 mg/kg) were injected intraperitoneally 15 min prior to extinction training with a 26-gauge injection needle that was connected to a 1 ml syringe. ( $\pm$ )-HA966 (HA, Sigma) was freshly dissolved in saline and HA (6.0 mg/kg) was injected intraperitoneally 30 min before extinction training. D,L-2-amino-5-phosphonovaleric acid (AP5, sigma) was dissolved in artificial cerebrospinal fluid and AP5 (0.2 ng/side, bilaterally) was infused into the basolateral nucleus of amygdala (BLA) 10 min prior to saline or DEX (1.0 mg/kg). Injections were made through 28-gauge injection cannula (model C313I, Plastic Products, Roanoke, VA, USA) that was connected to a Hamilton micro-syringe via polyethylene tubing. The infusion speed was 0.25  $\mu$ l/min. Metirapone (25.0 mg/kg s.c.) was given 90 min prior to a single session of extinction training.

### Histological Assessment

Upon completion of the experiment, animals were overdosed with sodium pentobarbital and perfused intracardially with PBS followed by 10% formaldehyde. Coronal sections (40  $\mu$ m) were cut through the area of interest, stained with cresyl violet, and examined under light microscope for cannula placement.

### Statistics

Behavioral results from FPS were statistically analyzed by an analysis of variance (ANOVA) to detect overall differences. Whenever ANOVA was significant, Fisher's least significant difference *post hoc* test was employed to determine specific differences between groups. Between-group comparisons were also using two-tailed *t*-tests for independent samples. The criterion for significance for all comparisons was  $p < 0.05$ .

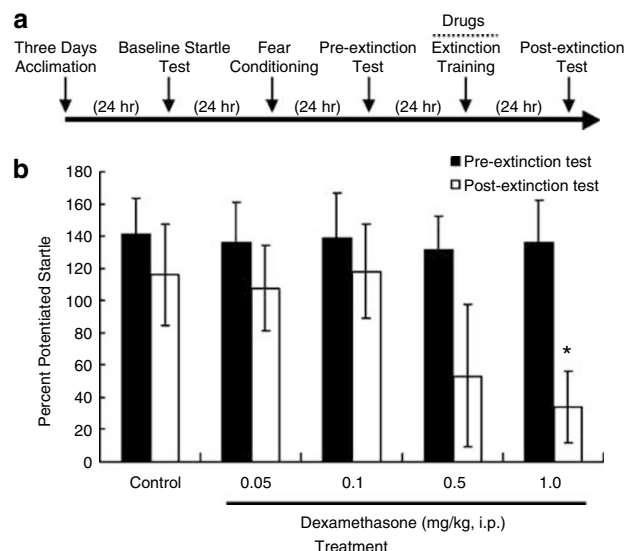
## RESULTS

### Experiment One: Systemic Administration of DEX Facilitated Extinction of Conditioned Fear in a Dose-Dependent Manner

This experiment assessed the effect on fear-potentiated startle of different doses of DEX. Initially, 35 rats were used. Five were excluded for showing  $< 50\%$  fear-potentiated startle during the pre-extinction test. The final 30 rats were assigned into five groups of six animals based on their level of fear-potentiated startle in the pre-extinction test. Twenty-four hours after the pre-extinction test, each rat received 1 day of extinction training with different doses of DEX (0.05, 0.1, 0.5, and 1.0 mg/kg, i.p.) or saline (Figure 1a). The particular dose of DEX used was based on the studies of Roozendaal (2003) and Yang *et al* (2006). Saline or DEX were injected 15 min prior to the extinction training. Figure 1b shows that DEX facilitated extinction of conditioned fear in a dose-dependent manner. ANOVA indicated a significant dose effect ( $F_{(4,25)} = 3.41$ ) with a significant linear trend ( $F_{(1,25)} = 6.23$ ). *Post hoc* comparison revealed that the doses of 1.0 mg/kg significantly decreased extinction as compared with saline control animals ( $ps < 0.05$ ). Because 1.0 mg/kg DEX produced the maximal enhancing effect, we used this dose in our subsequent experiments.

### Experiment Two: Systemic Administration of DEX Accelerated Extinction of Conditioned Fear

This experiment assessed the facilitatory effect of DEX on different amounts of extinction training. Initially, 37 rats were used. Seven were excluded for showing  $< 50\%$  fear-potentiated startle during the pre-extinction test. The final

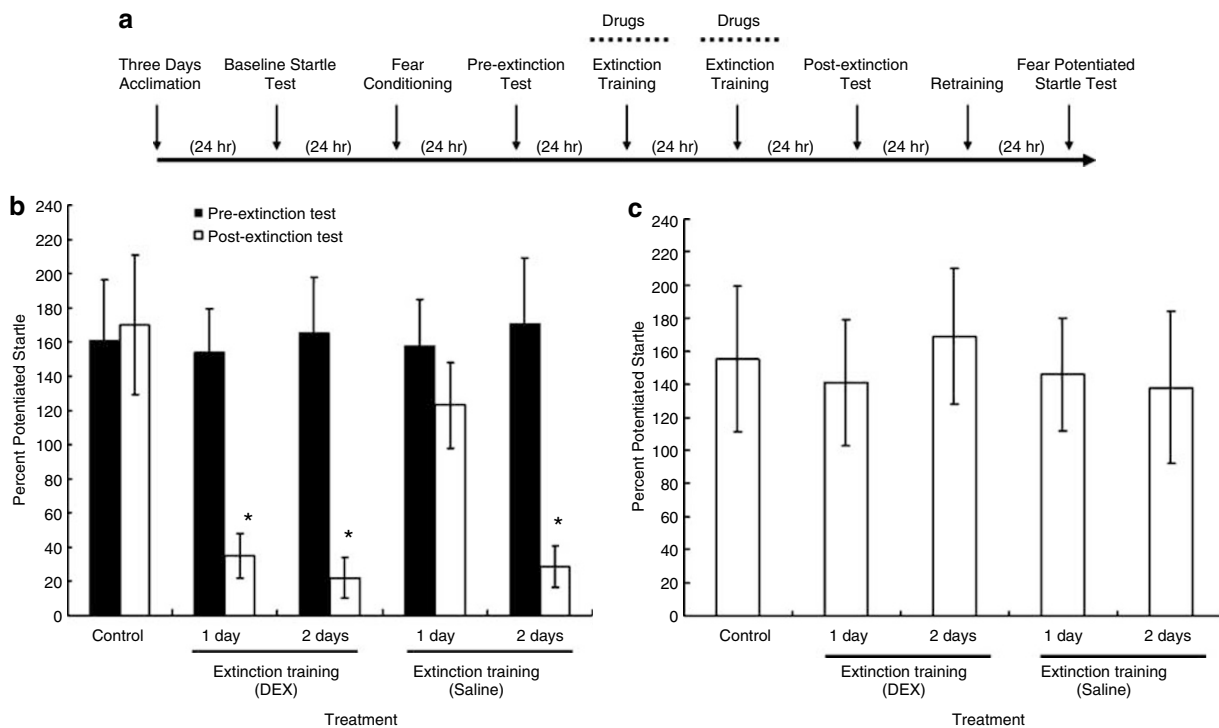


**Figure 1** Systemic administration of DEX facilitated extinction of conditioned fear in a dose-dependent manner. (a) Timeline of the behavioral procedures for experiment 1. (b) Percent fear-potentiated startle measured 24 h before (pre-extinction test) and 24 h after extinction training (postextinction test). Rats in each group were treated with either DEX (0.05, 0.1, 0.5, and 1.0 mg/kg) or saline prior to a single session of extinction training. They were tested for fear-potentiated startle response in the absence of drugs 24 h later (fear-potentiated startle test) (values are mean  $\pm$  SEM, \* $p < 0.05$  vs control group).

30 rats were assigned into five groups of six animals based on their level of fear-potentiated startle in the pre-extinction test. Twenty-four hours after the pre-extinction test, each rat received 1 or 2 consecutive days of extinction training with DEX (1.0 mg/kg, i.p.) or saline (Figure 2a). The particular dose of DEX used was based on the result of experiment 1. Saline or DEX were injected 15 min prior to the extinction training. An additional control group was tested 2 days after the pre-extinction training without intervening exposures to visual CS. Figure 2b shows that DEX accelerated extinction of conditioned fear. A two-way ANOVA for differences in treatment (DEX vs saline) and days (one or two extinction sessions) between-subjects indicated a significant treatment effect ( $F_{(1,20)} = 10.41$ ) and a significant treatment  $\times$  days interaction ( $F_{(2,20)} = 8.86$ ). Thus, the reduction of fear-potentiated startle after 1 day of extinction training was greater in the group that received DEX than in the group that received saline (Figure 2b). *Post hoc* analysis showed significant decrease in fear-potentiated startle in DEX treated rats compared with saline treated rats and control animals ( $ps < 0.05$ ). Previous studies have shown that lesions of the BLA block the expression of fear-potentiated startle (Campeau and Davis, 1995). To test for toxicity, all animals of experiment 1 were retrained and tested 24 h later. Animals previously injected with DEX or saline showed significant fear-potentiated startle (Figure 2c). Thus, the facilitatory effect of DEX observed during the post-extinction test 1 appeared to result from the acute drug effect rather than from a more permanent, perhaps toxic, action of DEX.

### Experiment Three: DEX Facilitation Effect on Extinction was Blocked by Glutamate NMDA Receptor Antagonists

Recent studies indicate that glutamate NMDA receptors in the amygdala play a critical role in the extinction of conditioned fear (Walker *et al*, 2002; Ledgerwood *et al*, 2003, 2004; Yang and Lu, 2005). This experiment assessed the role of amygdaloid NMDA receptors in the DEX effect, two glutamate NMDA receptor antagonists ( $\pm$ )-HA966 and MK801 were used. Initially, 55 rats were used. Animals of showing less than 50% fear-potentiated startle during the pre-extinction test were excluded. In addition, animals with misplacement of cannula were also excluded. There were seven animals excluded in this experiment. The final 48 rats were assigned to six groups based on their level of fear-potentiated startle in the pre-extinction test and received various combinations of drug treatments as shown in Figure 3a. Group one to group three animals received saline + saline, saline + DEX and ( $\pm$ )-HA966 + DEX, respectively. Group four to group six animals received ACSF + saline, ACSF + DEX, and MK801 + DEX respectively. ( $\pm$ )-HA966 (6.0 mg/kg, i.p.) and MK-801 (0.5 ng/side, bilaterally) was given 10 and 5 min prior to DEX injection, respectively. Thirty minutes later, animals were then receive a single session of extinction training. Twenty-four hours later, animals were tested for fear-potentiated startle in the absence of drug. Results demonstrated that either systemic injection of ( $\pm$ )-HA966 or intra-amygdala infusion of MK801 completely blocked the DEX enhancement effect on extinction ( $t_{(14)} = 2.86$  and  $t_{(12)} = 2.68$ , respectively)



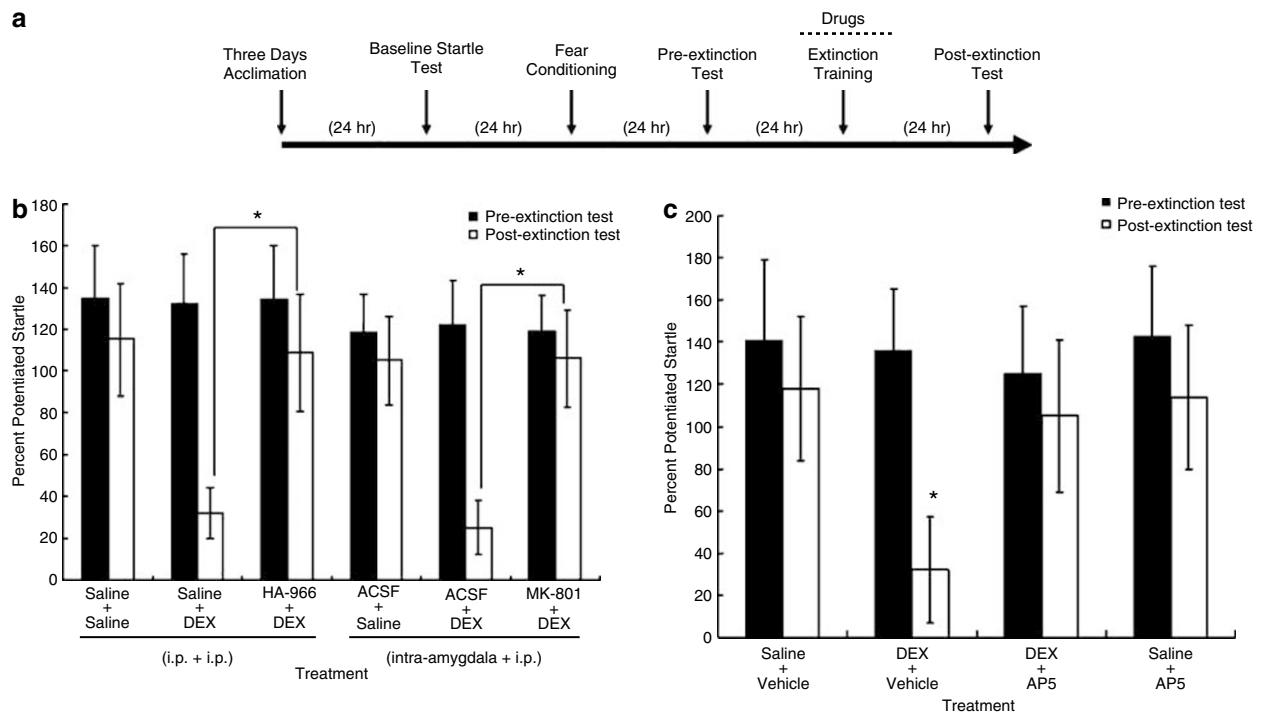
**Figure 2** Systemic administration of DEX accelerated extinction of conditioned fear. (a) Timeline of the behavioral procedures for experiment 2. (b) Percent fear-potentiated startle measured 24 h before (pre-extinction test) and 24 h after extinction training (postextinction test). Rats in each group were treated with either DEX (1.0 mg/kg) or saline prior to a single session of extinction training. (c) To test for toxicity, after 24 h all animals of experiment 1 were retrained. They were tested for fear-potentiated startle response in the absence of drugs 24 h later (fear-potentiated startle test) (values are mean  $\pm$  SEM, \* $p < 0.05$  vs control group).

(Figure 3b). It has been shown that administration of MK801 and ( $\pm$ )-HA966 prior to extinction training blocked extinction (Baker and Azorlosa, 1996; Walker *et al*, 2002; Yang and Lu, 2005). Thus, it is difficult to conclude that the effect of ( $\pm$ )-HA-966 and MK801 on DEX's facilitatory effect is caused by a disruption of facilitation as opposed to a direct blockade of extinction itself. To answer this question, a subthreshold dose of AP5 and additional 38 rats were used. Six animals were excluded. The final 32 rats with intra-amygdala cannulation were then assigned to four groups and received various combinations of drug treatments as shown in Figure 3c. Group one to group four animals received vehicle + saline, vehicle + DEX (1.0 mg/kg), AP5 (2.0 ng/side) + DEX (1.0 mg/kg), AP5 (2.0 ng/side) + saline, respectively. The particular doses of AP5 and DEX we used here were followed the studies of Falls *et al* (1992) and Yang *et al* (2006). AP5 was given 10 min prior to DEX injection. Fifteen minutes after DEX injection, animals were then receive a single session of extinction training. ANOVA indicated a significant interaction of DEX and AP5 factors ( $F_{(2,28)} = 11.02$ ). The *post hoc t*-tests results demonstrated that infusion of 2.0 ng/side AP5 into the BLA completely blocked the DEX enhancement effect on extinction ( $t_{(14)} = 3.66$ ;  $p < 0.05$ ). It has been shown that infusion of 2.0 ng/side AP5 into the BLA did not affect extinction (Figure 3c). In conclusion, these results strengthened our hypothesis and indicate that the DEX facilitatory effect on

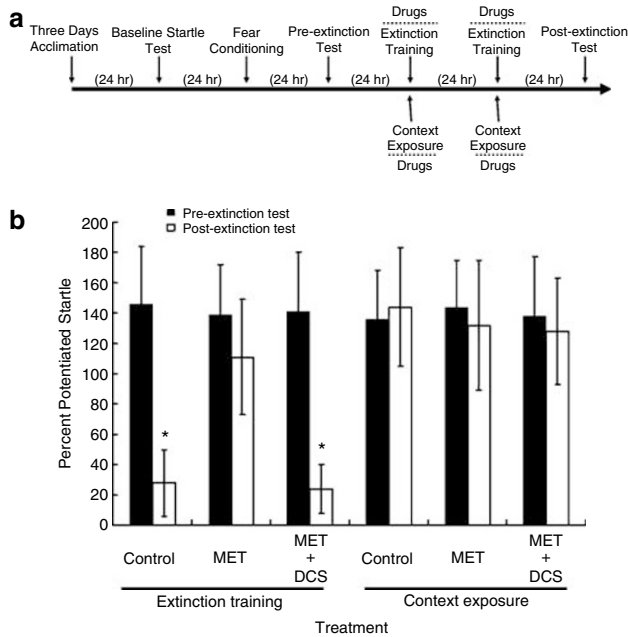
extinction is most likely mediated by amygdaloid glutamate NMDA receptors.

#### Experiment Four: Blockage of Extinction by the Corticosteroid Synthesis Inhibitor Metyrapone was Prevented by D-Cylloserine

In this experiment we examined whether the corticosteroid synthesis inhibitor metyrapone injected systematically would interfere with extinction and whether this effect could be prevented by co-administration of DCS, a partial NMDA receptor agonist. The particular doses of metyrapone and DCS were based on the studies of Yang *et al* (2006) and Walker *et al* (2002). Initially, 38 rats were used. Six were excluded for showing less than 50% fear-potentiated startle during the pre-extinction test. The final 32 rats received fear conditioning, extinction training, and testing for fear-potentiated startle as described previously. Group one, group two, and group three animals received vehicle + saline, metyrapone + saline, and metyrapone + DCS, respectively. Metyrapone (25 mg/kg, s.c.) and DCS (15 mg/kg, i.p.) were given 90 and 15 min prior to a single session of extinction training for a total of 2 days extinction training, respectively (Figure 4a). Twenty-four hours after the second extinction training, animals were tested for fear-potentiated startle in the absence of drug. Animals of group four to six received similar drug treatments as described above and were placed in the test chamber without



**Figure 3** DEX facilitation effect on extinction was blocked by glutamate NMDA receptor antagonists. (a) Timeline of the behavioral procedures for experiment 3. (b) Percent fear-potentiated startle measured 24 h before and 24 h after extinction training. To test the effect of NMDA receptor antagonist ( $\pm$ )-HA966 and MK801 on extinction. Animals were randomly assigned to six different groups and underwent either saline + saline, saline + DEX, or ( $\pm$ )-HA966 + DEX, vehicle + saline, vehicle + DEX, or MK801 + DEX, 15 min prior to a single session of extinction training. Twenty-four hours later, animals were tested for fear-potentiated startle in the absence of drug (values are mean  $\pm$  SEM,  $*p < 0.05$ ). (c) To test the effect of subthreshold dose of NMDA receptor antagonist AP5 on extinction. Animals were randomly assigned to four different groups and underwent either vehicle + saline, vehicle + DEX (1.0 mg/kg), AP5 (2.0 ng/side) + DEX (1.0 mg/kg), AP5 (2.0 ng/side) + saline, respectively. AP5 was given 10 min prior to DEX injection (values are mean  $\pm$  SEM,  $*p < 0.05$  vs control group).



**Figure 4** Blockage effect of the corticosteroid synthesis inhibitor metyrapone on extinction was removed by DCS. (a) Timeline of the behavioral procedures for experiment 4. (b) Percent fear-potentiated startle measured 24 h before and 24 h after extinction training or context exposure (post-extinction test). Rats in each group underwent either systematic administration of vehicle (control), metyrapone alone (MET) or metyrapone + DCS (MET + DCS) 15 min prior to a single session of extinction training (with presenting of the CS) or context exposure only (without presenting of the CS). Twenty-four hours later, animals were tested for fear-potentiated startle in the absence of drug. Values are mean  $\pm$  SEM, \* $p$  < 0.05 comparing to the control group.

extinction training (context exposure only). Results showed that administration of metyrapone blocked extinction of conditioned fear significantly ( $t_{(14)} = 2.83$ ;  $p < 0.05$ ). This effect was prevented by co-administration of DCS (15 mg/kg, i.p.). ANOVA indicated a significant treatment (metyrapone alone vs metyrapone + DCS)  $\times$  training (extinction training vs context exposure) interaction ( $F_{(1,24)} = 6.52$ ) (Figure 4b). *Post hoc* comparison showed the fear-potentiated startle was significantly lower in the rats that received co-administration of metyrapone and DCS before extinction training compared with the rats that received metyrapone and saline when both groups had received extinction training ( $ps < 0.05$ ). Furthermore, the blockade of extinction by metyrapone and the reversal of this effect by DCS required exposure to the fear stimulus because neither of these effects was seen in animals that were only exposed to the context (Figure 4b). These results indicated that the facilitatory effect of DCS on the extinction was not the result of impaired expression of conditioned fear or accelerated forgetting.

#### Experiment Five: Administration of DEX and DCS in Sub-Threshold Doses Provided Synergistic Facilitation of Extinction

Experiments 2–4 showed that activation of amygdaloid NMDA receptors are required for the facilitatory effect on extinction of DEX. In this experiment, we examined if the co-administration of sub-threshold doses of DEX and DCS

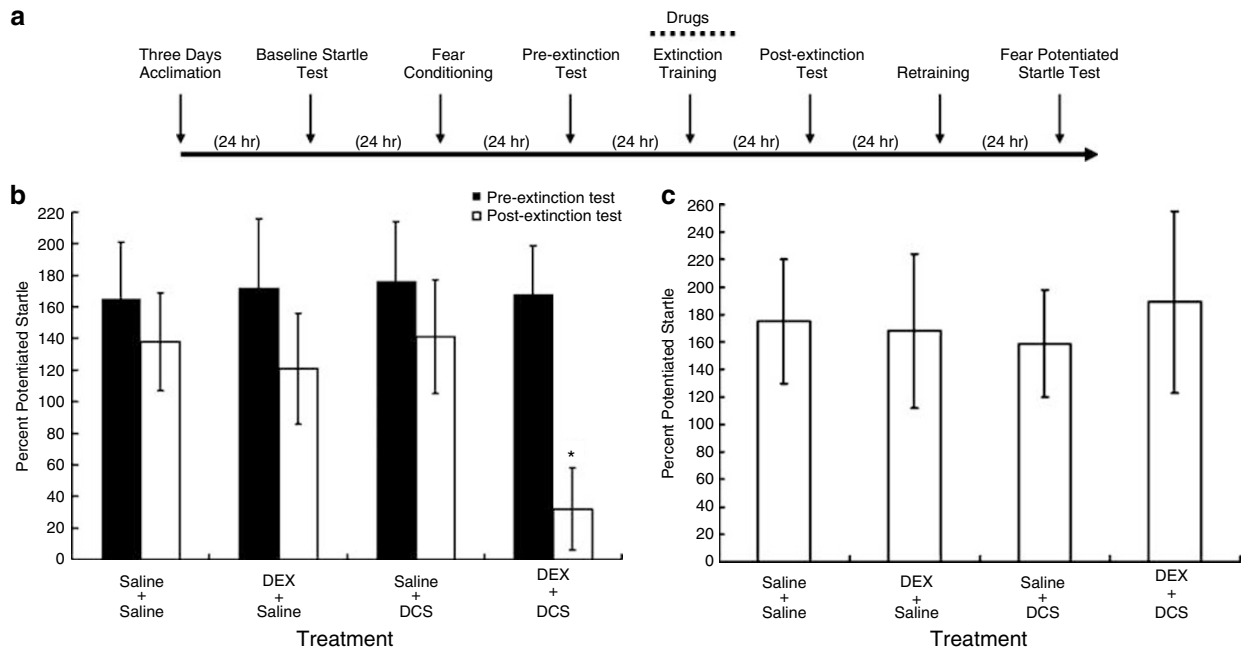
would provide a synergistic effect and accelerate extinction. Initially, 30 rats were used. Six were excluded for showing less than 50% fear-potentiated startle during the pre-extinction test. The final 24 rats received fear conditioning, extinction training, and testing for fear-potentiated startle as described previously. They were then randomly assigned to four groups based on their level of fear-potentiated startle in the preextinction test and received various combinations of drug treatments. Group one to group four animals received saline + saline, saline + DEX (0.2 mg/kg), saline + DCS (5.0 mg/kg), DEX + DCS, respectively (Figure 5a). Two-way ANOVA indicated a significant interaction of DEX and DCS factors ( $F_{(2,20)} = 9.04$ ). *Post hoc* comparison revealed that animals treated with DEX and DCS in a subthreshold doses significantly accelerated extinction as compared with DEX treated alone animals ( $ps < 0.05$ ). Neither DEX nor DCS treated alone in a subthreshold dose had any effect on the extinction (Figure 5b). To exclude the possible toxic effect of DEX + DCS on BLA, all animals of experiment 5 were retrained and tested 24 h later. Under these conditions, animals previously injected with DEX + DCS showed significant fear-potentiated startle (Figure 5c). Thus, the synergistic effect of DEX + DCS observed during the post-extinction test appeared to result from the acute drug effect rather than from a more permanent, perhaps toxic, action of the combined treatment of DEX and DCS.

#### Experiment Six: Effect of Pretest DEX and DCS Co-Administration on Fear-Potentiated Startle

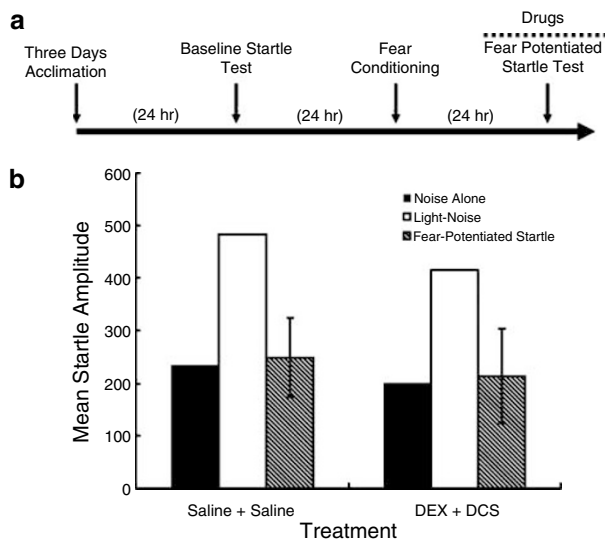
Administration of low doses of glucocorticoids can increase startle reflexes in humans (Buchanan *et al*, 2001). Larger doses can decrease startle reflexes to emotional stimuli (Buchanan and Lovallo, 2001). Here, we tested whether the co-administration of DEX and DCS might have had a secondary effect on fear itself or to CS processing. For example, if DEX + DCS enhanced CS-elicited fear, this might facilitate extinction by increasing the discrepancy between what the CS predicted and what actually occurred. To evaluate these possibilities, 16 rats received acclimation, baseline startle test, and fear conditioning. Initially, 20 rats were used, but four of them were excluded. Animals were injected with either saline or DEX (0.2 mg/kg) + DCS (15 mg/kg) (Figure 6a). At 15 min after infusion, rats were tested for fear-potentiated startle. As shown in Figure 6b, the co-administration of DEX and DCS did not significantly influence fear-potentiated startle when administered before testing ( $F_{(1,14)} = 0.692$ ). Thus, it is unlikely that these drugs influenced extinction by increasing fear.

#### DISCUSSION

The present study demonstrated that systemic administration of synthetic glucocorticoid agonist DEX facilitated extinction of conditioned fear in a dose-dependent manner (experiment 1), which is consistent with previous studies that brain glucocorticoids modulated extinction of conditioned fear (Bohus *et al*, 1982; Barrett and Gonzalez-Lima, 2004; Yang *et al*, 2006). The enhancement effect of DEX was prevented by co-administration of NMDA receptor antagonists ( $\pm$ )-HA966, MK801, or intra-amygdala infusion of



**Figure 5** Co-administration of DEX and DCS in subthreshold dose provided synergistic facilitation effect on extinction. (a) Timeline of the behavioral procedures for experiment 4. (b) Percent fear-potentiated startle measured 24 h before and 24 h after extinction training. Animals were randomly assigned to four different groups and underwent either saline + saline, DEX (0.2 mg/kg) + saline, DCS (5.0 mg/kg) + saline, DEX + DCS, respectively. Drugs were given 15 min prior to a single session of extinction training. Twenty-four hours later, animals were tested for fear-potentiated startle in the absence of drug. (c) To test for nonspecific effect, after 24 h all animals of experiment 5 were retrained. They were tested for fear-potentiated startle response in the absence of drugs 24 h later. Values are mean  $\pm$  SEM, \* $p$  < 0.05 comparing to the control group.



**Figure 6** Effect of pretest DEX and DCS co-administration on fear-potentiated startle. (a) Timeline of the behavioral procedures for experiment 6. (b) Percent fear-potentiated startle measured 24 h before and 24 h after extinction training. Animals in each group underwent either with either saline or DEX (0.2 mg/kg) + DCS (5.0 mg/kg). At 15 min after infusion, rats were tested for fear-potentiated startle. Values are mean  $\pm$  SEM.

AP5 at a subthreshold dose (experiment 3). In addition, extinction of the conditioned fear was blocked by prior administration of corticosteroid synthesis inhibitor metyrapone 90 min before extinction training and this effect of metyrapone was prevented by systemic injection of NMDA

receptor agonist DCS (experiment 4). Co-administration of subthreshold doses of DEX and DCS provided a synergistic effect and accelerated extinction of conditioned fear. Control experiments indicated that co-administration of DEX and DCS did not alter the expression of conditioned fear and the effect was not due to lasting damage to the amygdala (experiments 2 and 5). Co-treatment of DEX and DCS did not alter the expression of conditioned fear (experiment 6). These results provided evidence that activation of amygdaloid glutamate NMDA receptors are involved in the modulatory effect of glucocorticoids on extinction.

#### Amygdaloid Glutamate NMDA Receptors Participate in the Modulatory Effect of Glucocorticoids on Extinction

In this study, we found that intra-amygdala administration of glutamate NMDA receptor antagonists blocked DEX's facilitatory effect on extinction, which was consistent with our previous result that intra-amygdala infusion of glucocorticoid agonist RU28362 facilitated extinction in a dose-dependent manner (Yang *et al*, 2006). These results suggest that BLA is a critical locus for the glucocorticoid enhancement effect on fear extinction. Numerous studies suggest that several brain structures are also involved in the extinction of conditioned fear that includes the BLA (Maren, 2001; Davis and Whalen, 2001; LeDoux, 2002), medial prefrontal cortex (mPFC) (Quirk *et al*, 2000; Grace and Rosenkranz, 2002; Barrett *et al*, 2003; Quirk and Milad, 2002) and dorsal hippocampus (Corcoran and Maren, 2001; Barrett *et al*, 2003). Recent evidence showed that extensive damage to the BLA did not interfere with the extinction of

tone-elicited fear response indicating that structures other than BLA are essential for the extinction (Sotres-Bayon *et al*, 2004). In addition, postextinction infusion of an MAPKs inhibitor PD98059 into the mPFC impairs memory of the extinction of conditioned fear (Hugues *et al*, 2004). Furthermore, Herry and Mons (2004) demonstrated that the spontaneous recovery of the conditioned fear (resistance to extinction) in mice is associated with a prolonged expression of long-term depression of synaptic transmission in the mPFC and the failure of induction of the immediate-early gene *c-Fos* and *zif268* expression in the mPFC and the BLA. It should be noted that our results showing that activation of the amygdaloid NMDA receptors modulate the extinction of conditioned fear does not necessarily question whether other structures contribute to fear extinction. The fact that the BLA plays an important role in DEX facilitation effect on extinction does not mean that it is the only structure involved in this process. Roozendaal and McGaugh found that lesions of the amygdala do not necessarily result in memory impairment but block stress hormone effects on memory consolidation. This is because the amygdala interacts with other brain regions including the hippocampus and mPFC, to regulate stress hormone effects on memory (Roozendaal *et al*, 2002; Roozendaal, 2003). In addition, the GABAergic intercalated cells of the amygdala, which receive input from the mPFC and control amygdala input, exhibit NMDA-dependent long-term potentiation (Royer and Pare, 2002). Thus, mPFC and the amygdala may work together to control the consolidation and expression the extinction. Our findings match previous findings by indicating that the glucocorticoids acted in the amygdala to influence extinction (Yang *et al*, 2006), but BLA lesions alone did not block extinction of conditioned fear (Sotres-Bayon *et al*, 2004). Further experiments such as local infusion of glucocorticoid agonists and antagonists into the mPFC and hippocampus are needed to clarify the possible role of other structures in the DEX effect on extinction.

The results of the present study suggest that an NMDA-dependent mechanism in the amygdala underlies the facilitatory effect of DEX on extinction. A large literature indicates amygdaloid glutamate NMDA receptors play an essential role in the extinction of fear memory (Walker *et al*, 2002; Ledgerwood *et al*, 2003, 2004; Yang and Lu, 2005). In the present study, we found that either systemic or intra-amygdala administration of glutamate NMDA receptor antagonists ( $\pm$ )-HA966 and MK801 blocked the facilitatory effect of DEX on extinction significantly. We demonstrate that administration of AP5 at the dose of 2.0 ng/site did not affect extinction but blocked the facilitatory effect of DEX and the blocking effect of metyrapone on extinction was also prevented by systemic injection of DCS, which provides evidence that an NMDA-dependent mechanism is involved in the modulatory effect of glucocorticoids on extinction. Furthermore, administration of DEX and DCS in subthreshold doses led to a synergistic facilitation of extinction. These results suggest that NMDA receptors are involved in the DEX facilitatory effect on extinction. We also excluded the possibility that co-administration of DEX and DCS had a secondary effect on fear itself or to CS processing.

How does DEX lead to amygdaloid NMDA receptor activation and facilitate extinction of conditioned fear? As a

synthetic glucocorticoid agonist, DEX can activate glucocorticoid receptors. The amygdala and hippocampus, specific to the extinction of conditioned fear, contain glucocorticoid receptors (Fuxe *et al*, 1985; Van Eekelen *et al*, 1987; Korte, 2001). There is also evidence that glucocorticoids alter calcium conductance and calcium channel subunit expression in BLA (Karst *et al*, 2002). Recent findings demonstrate that corticosterone has acute effects on NMDA receptors and results in  $Ca^{2+}$  elevation in mouse hippocampal slices (Sato *et al*, 2004). Stress impairs glutamate uptake in the hippocampal CA1 region and the impairment is prevented by administration of glucocorticoid receptor antagonist RU38486 (Yang *et al*, 2005). We suggest that a similar mechanism also is involved in the facilitatory effect of DEX on extinction. It is well known that numerous signaling cascades and immediate early genes are involved in extinction which include MAPKs, PI-3 kinase (Lu *et al*, 2001; Lin *et al*, 2003; Mansuy, 2003; Yang and Lu, 2005), calcineurin, *zif268*, and *c-fos* (Quirk *et al*, 2000; Herry and Garcia, 2002; Vianna *et al*, 2001). Activation of the amygdaloid glucocorticoid receptors may impair glutamate uptake in the amygdala, which could prolong the activation of amygdaloid NMDA receptors and result in activation of MAPKs signal cascades. Our results showing that the blockade of extinction by metyrapone was prevented by systemic injection of DCS supports this hypothesis. In addition, our previous results showed that phosphorylation of MAPKs in the amygdala can be induced by DCS administration and blocked by NMDA receptor antagonist provides the direct evidence that DCS has direct effect on the amygdaloid glutamate NMDA receptors and elevates intracellular calcium concentration and resulted in MAPKs activation. Further experiments such as local infusion of specific kinase inhibitors into BLA are required to identify the related signal cascades responsible for the facilitation effect of DEX.

### Clinical Implications

There is abundant evidence suggesting a pre-existing difference between those who do and do not develop PTSD, despite experiencing the same trauma (Yehuda, 1999; Perlman *et al*, 2004; Yehuda *et al*, 2004). It is possible that the dysfunction in these disorders is related to the inability to normally extinguish fear. Recent clinical studies showed that PTSD patients often appear to have reduced cortisol levels (Yehuda *et al*, 2004) and daily cortisol administration reduced symptoms of traumatic memory in PTSD patients (Aerni *et al*, 2004). These results suggest that brain glucocorticoid is involved in the dysregulation of fear memory extinction. In addition, Perlman reported that glucocorticoid receptor mRNA level is significantly decreased in the amygdala of patients with major mental illness (Perlman *et al*, 2004) and administration of metyrapone and mifepristone blocked the normal extinction (Barrett and Gonzalez-Lima, 2004; Yang *et al*, 2006). All of these results suggest amygdaloid glucocorticoid receptor dysfunction impairs normal fear extinction. Our results are consistent with the growing body of evidences implying that the extinction process can be enhanced by pharmacological intervention (Quartermain *et al*, 1994; Roscorla, 2000; Walker *et al*, 2002; Pitman *et al*, 2002; Ledgerwood *et al*,



2003, 2004; Richardson *et al*, 2004; Yang and Lu, 2005). Our studies suggest that glucocorticoid replacement during a controlled therapy session might restore the normal extinction in PTSD patients.

## Conclusion

We showed that facilitation of conditioned fear extinction by synthetic glucocorticoid agonist DEX is mediated by amygdaloid glutamate NMDA receptors. Increased understanding of the mechanisms involved in the extinction process will likely be extremely relevant to treating psychiatric disorders involving dysregulated fear responses.

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