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Augmentation of Fear Extinction by D-Cycloserine is Blocked by Proteasome Inhibitors

Sheng-Chun Mao[1,2], Hui-Ching Lin[1,2] and Po-Wu Gean*[,1,2]

[1]Institute of Basic Medical Sciences, National Cheng-Kung University, Tainan, Taiwan; [2]Department of Pharmacology, Center for Gene Regulation and Signal Transduction Research, National Cheng-Kung University, Tainan, Taiwan

D-Cycloserine (DCS) has been shown to facilitate extinction of conditioned fear in rats and to improve fear reduction of social phobia and fear of heights in human studies. Here, we investigate the mechanism of DCS effect by measuring internalized GluR1 and GluR2 using cell-surface biotinylation techniques. DCS selectively increased NMDA receptor-mediated synaptic response without affecting AMPA receptor-mediated synaptic response. Low-frequency stimulation (LFS) when applied in the presence of DCS induced GluR1 and GluR2 internalization in the amygdala slices. Proteasome inhibitors block DCS facilitation of LFS-induced depotentiation and a reduction in surface levels of GluR1 and GluR2. Furthermore, DCS in combination with LFS reduced cellular levels of PSD-95 and synapse-associated protein 97 (SAP97), which were also blocked by proteasome inhibitors. In the *in vivo* experiments, DCS-induced reduction of fear-potentiated startle and reversal of conditioning-induced increase in surface expression of GluR1 were blocked by proteasome inhibitors. DCS-treated rats fail to exhibit reinstatement after US-alone presentations. These results suggest that DCS facilitates receptor internalization in the presence of extinction training, resulting in augmented reduction of startle potentiation.
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INTRODUCTION

Re-exposure of cue that was associated with the trauma to patients with post-traumatic stress disorders (PTSD) evokes inappropriate fear responses that can cause serious consequences on the lives of sufferers (Pitman *et al*, 2001; Bremner, 2003). Similar to animal experiment of extinction, previously acquired fear responses gradually declined if PTSD patients were exposed to the trauma-associated conditioned stimuli (CS) in the absence of aversive reinforcement (Taylor *et al*, 2003; Hermans *et al*, 2006). This exposure-based psychotherapy is a treatment of choice for a number of anxiety disorders (Yehuda, 2002). However, in some patients, successful reduction of fear through exposure therapy was often followed by a return of fear owing to persistence of the original memory (Quirk *et al*, 2000; Herry and Garcia, 2002; Myers and Davis, 2002; Maren and Quirk, 2004; Barad, 2006).

D-Cycloserine (DCS) is a partial agonist at the glycine-binding site of the NMDA receptor. Pioneer studies by Davis and co-workers have shown that DCS facilitated extinction of conditioned fear in rats (Walker *et al*, 2002). In clinical trials, patients who received DCS in conjunction with virtual reality exposure had significantly better outcome than the placebo group in their overall acrophobia symptoms (Ressler *et al*, 2004; Davis *et al*, 2006). DCS in combination with exposure therapy was also effective for the treatment of social anxiety disorder (Hofmann *et al*, 2006). Activation of glycine site by DCS enhanced the function of NMDA receptor. Therefore, the facilitation of extinction by DCS could result from its enhancement of NMDA receptor activation and subsequently the consolidation of extinction memory (Davis *et al*, 2006). The idea received support from the observation that phosphoinositide-3 kinase, mitogen-activated protein kinase, and protein synthesis were involved in the DCS effect (Yang and Lu, 2005), since in certain cases consolidation of fear conditioning and consolidation of extinction shared similar signal cascades (Lin *et al*, 2003).

In hippocampal neurons, activation of NMDA receptors induced AMPA receptor internalization and led to long-term depression (LTD) of synaptic transmission (Shi *et al*, 1999; Lin *et al*, 2000; Beattie *et al*, 2000). Therefore, facilitation of extinction and enhancement of treatment responses by DCS could be due to its promotion of receptor endocytosis. Different intracellular signals have been suggested for agonist-induced internalization of AMPA receptors. However, receptor internalization is blocked by proteasome inhibitors, indicating at least a part of shared

*Correspondence: Dr P-Wu Gean, Department of Pharmacology, National Cheng-Kung University, No. 1 Ta-Shieh Road, Tainan 701, Taiwan, Tel: +886 6 2353535 ext. 5507, Fax: +886 6 2749296, E-mail: powu@mail.ncku.edu.tw
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mechanism (Colledge *et al*, 2003). Consistent with previous reports (Walker *et al*, 2002; Ledgerwood *et al*, 2003), we found that DCS infused bilaterally into the amygdala before extinction training augmented reduction of startle potentiation. Unexpectedly, conditioning-induced increase in GluR1 was reversed by DCS in combination with extinction training (Mao *et al*, 2006). By measuring GluR1 internalization using cell-surface biotinylation techniques, the purpose of this study was to elucidate the possible mechanism of DCS and to determine whether the ubiquitin-mediated proteasome activity was required for this process.

MATERIALS AND METHODS

Surgery

Rats (6- to 8-week old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and subsequently were mounted on a stereotaxic apparatus. Two cannulas made of 22-gauge stainless steel tubing (C313G; Plastic Products) were implanted bilaterally into the lateral amygdala (LA) or basolateral amygdala (BLA) (anteroposterior, −2.8 mm; mediolateral, ± 4.5 mm; dorsoventral, −7.0 mm). A 28-gauge dummy cannula was inserted into each cannula to prevent clogging. The rats were monitored and handled daily and were given 7 days to recover. DCS (10 μg/side dissolved in saline) and lactacystin (10 μg/side dissolved in saline) were obtained from Sigma (St Louis, MO), whereas MG-132 (9.5 μg/side dissolved in 50% DMSO) was purchased from Tocris Cookson Ltd (Northpoint, UK). Drug was administered bilaterally to the amygdala in a volume of 1 μl at a rate of 0.1 μl/min. The infusion cannulas were left in place for 2 min before being withdrawn. Antibodies used in this study were PSD-95 (mouse monoclonal K28/43, 1 : 5000; Upstate), synapse-associated protein 97 (SAP97) (mouse monoclonal RPI 197.4, 1 : 5000; Stressgen), ubiquitin (mouse monoclonal P4D1, 1 : 5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), normal mouse IgG (Santa Cruz Biotechnology Inc.).

Fear Conditioning

Rats were trained and tested in a stabilimeter device as described previously (Mao *et al*, 2006).

Acclimation. On 3 consecutive days, rats were placed in the startle test boxes for 10 min and returned to their home cages.

Matching. On 2 consecutive days, rats were placed in the startle box and 3 min later presented with 10 startle stimuli at 2 min intertrial interval (ITI). On the basis of their mean startle amplitudes in the second of these two sessions, rats were matched into groups with similar response levels.

Training. Rats were placed in the startle boxes and received 10 light–footshock pairings with an ITI of 2 min. Unpaired controls received the same number of light–footshock presentation, but in a pseudorandom manner in which the US could occur at anytime except at the 3.2 s following the CS.

Extinction training. Twenty-four hours following the training, rats were returned to the stabilimeter and given 3 sessions of 10 presentations of the 3.7 s light in the absence of either shock or the startle-elicited noise burst (light-alone trials). Each session was separated by 10 min with an ITI of 1 min.

Test. Forty-eight hours after training, rats were tested for fear-potentiated startle. This involved 10 startle-eliciting noise bursts presented alone (noise-alone trial) and 10 noise bursts presented 3.2 s after onset of the 3.7 s light (light-noise trials). The two trial types were presented in a balanced mixed order (ITI, 30 s). The percentage of fear-potentiated startle was computed as follows: [(startle amplitude on CS-noise)−(noise-alone trials)/(noise-alone trials)] × 100.

Reinstatement. Animals were conditioned following by extinction training and test, and then returned to testing chamber 1 h after testing and presented with 10 footshocks. Animals underwent a test for memory reinstatement 24 h after footshock.

Slice Preparation and Electrophysiological Recordings

Male Sprague–Dawley rats (150–200 g) were decapitated, and their brains rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (ACSF) solution. Subsequently, the brain was hemisected and cut transversely posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting section was glued to the chuck of a Vibroslice tissue slicer. Transverse slices of 450 μm thickness were cut and the appropriate slices placed in a beaker of oxygenated ACSF at room temperature for at least 1 h before recording. ACSF solution had the following composition (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, and glucose 11. The ACSF was bubbled continuously with 95% O_2–5% CO_2 and had the pH of 7.4.

A single slice was transferred to the recording chamber, in which it was held submerged between two nylon nets and maintained at 32 ± 1°C. The chamber consisted of a circular well of a low volume (1–2 ml) and was perfused constantly at a rate of 2–3 ml/min. Extracellular field potentials were made by electrical stimulation of the external capsule (EC), which contained fibers from the auditory cortex to the LA amygdala, with a concentric bipolar stimulating electrode. Electrical stimuli (150 μs in duration) were delivered at a frequency of 0.05 Hz. Baseline field potentials were adjusted to ∼30–40% of the maximal responses. Long-term potentiation (LTP) was elicited by five trains of tetanic stimulation (TS) (100 Hz, 1 s at 1 min interval) at the same stimulation intensity used for baseline. Low-frequency stimulation (LFS) was elicited by 5 Hz stimulation for 3 min delivered at 60 min following the onset of TS. Bicuculline (10 μM) and CGP52432 (10 μM) were present in the perfusion solution.

Surface Biotinylation and Western Blot Analysis of Surface GluR1

Brain slices containing only LA and BLA were placed on ice and washed twice with ice-cold ACSF. The slices were then incubated with ACSF containing 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) for 1 h on ice.

Next, the slices were rinsed in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) to quench the biotin reaction and then sonicated briefly in homogenizing buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 4 μg/ml aprotinin). After sonication, the samples were centrifuged at 14 000 r.p.m. for 30 min at 4°C and the supernatant was obtained. Protein concentration in the soluble fraction was then measured using a Bradford assay, with bovine serum albumin as the standard. Biotinylated protein (400 μg) from the supernatant was precipitated with 50 μl of 50% Neutravidin agarose (Pierce Chemical Co.) for 16 h at 4°C and washed four times with homogenizing buffer. Bound protein was resuspended in 4 μl of SDS sample buffer and boiled. Biotinylated protein was resolved in 8.5% SDS-polyacrylamide gels, blotted electrophoretically to PVDF membrane, and blocked overnight in TBS buffer containing 5% non-fatty milk. Surface GluR1 receptors and pan-cadherin (surface protein control) were detected by a biotinylation assay, followed by western blot analysis that used a GluR1 (1 : 4000; Santa Cruz Biotechnology Inc.), GluR2 (1 : 5000; Chemicon, Temecula, CA), pan-cadherin antibody (1 : 2500; Sigma), or actin (1 : 4000; Santa Cruz Biotechnology Inc.) antibody, followed by HRP-conjugated secondary antibody for 1 h. An enhanced chemiluminescence kit was used for detection. Western blots were developed in the linear range used for densitometry. GluR1 and GluR2 levels in the TS slices were expressed as a percentage of those in control slices without receiving TS. Similarly, GluR1 and GluR2 levels in the conditioned animals were expressed as a percentage of those in naive controls without receiving light–shock pairings.

Internalization Assay

The internalizations of GluR1 and GluR2 were analyzed using cell-surface biotinylation techniques (Cao *et al*, 1998; Liang and Huganir, 2001) modified for slice preparation. Amygdala slices were biotinylated with the reversible membrane-impermeable derivative of biotin 1 mg/ml Sulfo-NHS-S-S-Biotin (Pierce Chemical Co.) for 20 min at 4°C. The slices were then incubated at 37°C in ACSF with NMDA or any other treatment to allow internalization of biotinylated cell-surface proteins. The slices were then cooled to 4°C for 1 h and the remaining biotinylated proteins on the cell surface were stripped by treating the slices with 50 mM DTT. The internalized biotinylated proteins were protected from stripping and subsequent analysis of biotinylated proteins provided an assay for protein internalization.

Data Analysis

All values represent mean ± SEM. Differences among the groups were evaluated with ANOVA followed by the Newman–Keuls *post hoc* tests. The level of significance was $p < 0.05$.

RESULTS

In Vitro Studies

We first examined whether DCS affected basal synaptic transmission and enhanced NMDA receptor-mediated synaptic response in our experimental condition. Rats were killed and amygdala slices were prepared. Field excitatory postsynaptic potentials (fEPSPs) in the LA were elicited by stimulating EC. The effect of DCS (10 μM) on fEPSP as a function of time is illustrated in Figure 1a. After the evoked responses were stable for 20–30 min, DCS was bath-applied to the amygdala slices that did not affect the slope of fEPSP. NMDA receptor-mediated fEPSP ($fEPSP_{NMDA}$) was isolated pharmacologically by using Mg^{2+}-free solution in the presence of AMPA receptor antagonist CNQX (10 μM). Application of DCS increased the slope of $fEPSP_{NMDA}$ by 79.3 ± 11.1% ($n = 6$) (Figure 1b). At end of the experiment, D-APV was applied to ensure that $fEPSP_{NMDA}$ was indeed mediated by the NMDA receptors (data not shown). It has been shown that DCS decreased AMPA receptor-mediated synaptic response in the hippocampal CA1 region. Furthermore, in the presence of D-APV (50 μM), DCS did not affect the AMPA receptor-mediated synaptic response (Figure 1c).

We examined whether DCS enhanced receptor internalization using cell-surface biotinylation techniques, as described in Materials and Methods. Slices were randomly assigned to naive, TS, LFS, and LFS + DCS groups. In the TS group, amygdala slices were given five sets of TS at an interstimulus interval of 1 min to the EC. In the LFS group, LFS was delivered at 60 min following the onset of TS. DCS was applied 5 min before and during the LFS in the DCS + LFS group. Two hours following the onset of TS, a relatively small portion of the LA and BLA was dissected out from each slice. To measure internalized receptors, 8–12 slices from two rats were pooled together for biochemical analysis. ANOVA of TS, LFS, and LFS + DCS groups showed a main effect for group ($F_{(2, 15)} = 10.72$, $n = 6$ in each group, $p < 0.01$), and Newman–Keuls *post hoc* tests revealed that the levels of internalized GluR1 were not significantly different between TS and TS + LFS ($p > 0.05$). However, DCS treatment increased the level of internalized GluR1 to 150.0 ± 9.2% of naive controls ($p < 0.01$ *vs* TS + LFS) (Figure 2a). Similar experimental procedures were employed to probe the level of surface GluR2. As shown in Figure 2b, DCS treatment also increased the level of internalized GluR2 to 159.7 ± 15.1% of naive controls ($n = 6$, $p < 0.01$ *vs* TS + LFS).

Previously, we have shown that delivery of five sets of TS to the EC produced a robust enhancement of synaptic responses in the LA neurons that persisted for more than 2 h. LFS applied at 60 min after TS failed to induce depotentiation. However, when DCS was applied 5 min before and during LFS, fEPSP declined to the baseline level at 2 h after TS (Mao *et al*, 2006). In cultured hippocampal neurons, NMDA-induced receptor internalization was blocked by proteasome inhibitors, indicating the regulation of receptor trafficking by ubiquitin–proteasome system (UPS) (Patrick *et al*, 2003; Colledge *et al*, 2003). We tested whether proteasome inhibitors affected DCS-induced facilitation of depotentiation. The results revealed that when DCS was coapplied with MG-132 (10 μM) or lactacystin (5 μM) 5 min before and during the LFS, fEPSP remained 190.6 ± 10.8% ($n = 6$) and 198.0 ± 8.7% ($n = 6$) of baseline at 2 h after TS, respectively, which were significantly higher than that of DCS + LFS slices ($p < 0.001$) (Figure 3). Thus, DCS facilitated depotentiation, and the effect was blocked by proteasome inhibitors.

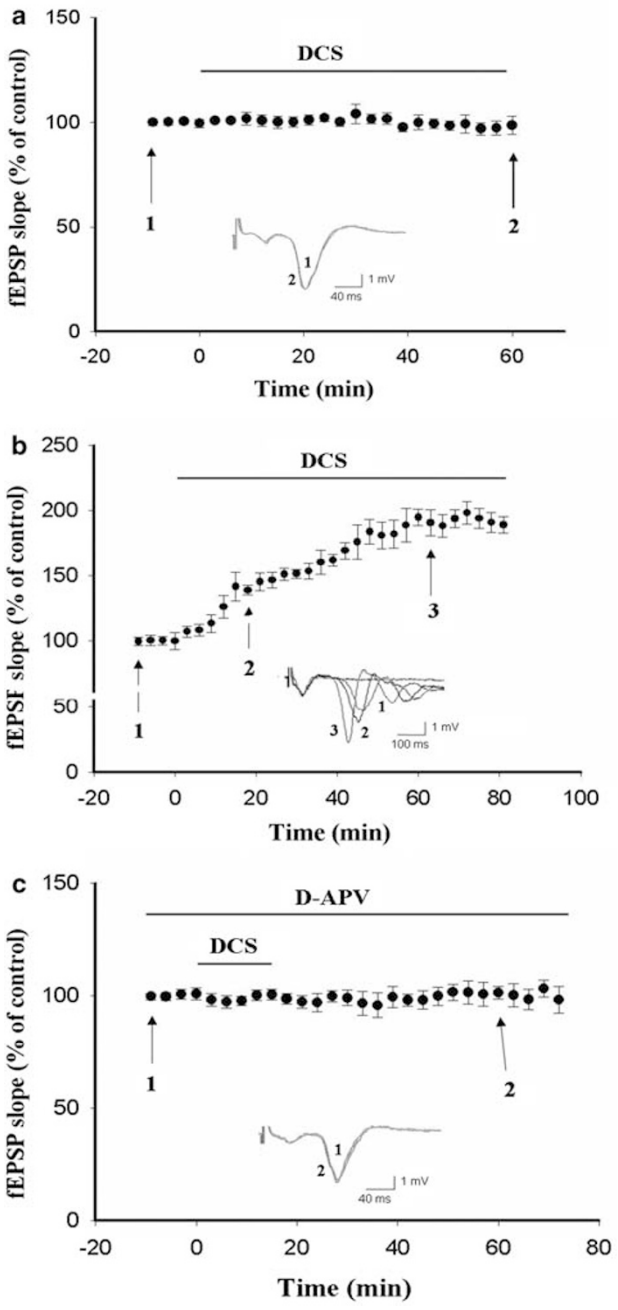

Figure 1 DCS enhances NMDA-mediated synaptic responses. (a) The graph represents the mean ± SE slope of fEPSPs plotted against time. Application of DCS (10 μM) failed to affect the slope of fEPSP. (b) The NMDA receptor-mediated fEPSP ($fEPSP_{NMDA}$) was isolated pharmacologically by using Mg^{2+}-free solution in the presence of CNQX (10 μM). Application of DCS increased the slope of $fEPSP_{NMDA}$. (c) DCS failed (10 μM) to affect fEPSP in the presence of D-APV (50 μM).

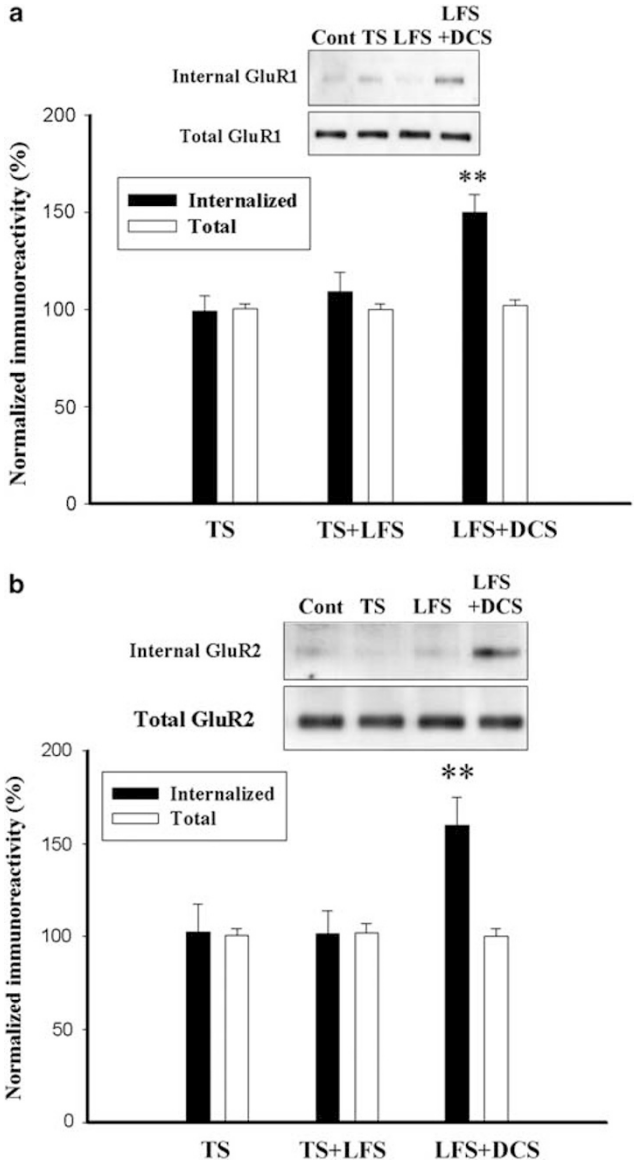

Figure 2 DCS enables LFS-induced internalization of GluR1 and GluR2. Amygdala slices were biotinylated with the reversible membrane-impermeable derivative of biotin Sulfo-NHS-S-S-Biotin for 20 min. Slices were given five sets of TS at an interstimulus interval of 1 min to the EC in the TS group. In LFS group, LFS was delivered at 60 min following the onset of TS. DCS (10 μM) was applied 5 min before and during the LFS in the DCS + LFS group. Two hours following the onset of TS, the slices were cooled to 4°C and the biotinylated proteins on the cell surface were stripped by treating the slices with 50 mM DTT. A relatively small portion of the LA and BLA was dissected out from each slice. Eight to twelve slices from two rats were pooled together for biochemical analysis. GluR1 (a) and GluR2 (b) levels in the TS slices were expressed as a percentage of those in control slices without receiving TS. $^{**}p<0.01$ *vs* TS + LFS.

Amygdala slices were prepared and classified into naive, TS, LFS, and LFS + DCS groups, as described above. We found that GluR1 and GluR2 levels were significantly elevated in the LA and BLA (GluR1: 148.6 ± 9.7%, $n=6$; GluR2: 163.9 ± 6.4%, $n=6$) after TS when compared to naive slices. LFS applied 60 min after TS did not significantly influence the TS-induced increases in GluR1 (GluR1: 141.2 ± 9.5%, $n=6$; GluR2: 149.0 ± 9.8%, $n=6$), but DCS treatment reversed them (GluR1: 102.4 ± 6.6%, $n=6$; GluR2: 95.0 ± 6.1%, $n=6$). Coadministration of proteasome inhibitors MG-132 (10 μM) or lactacystin (5 μM) blocked the effect of DCS such that there was no difference in GluR1 (Figure 4a) and GluR2 (Figure 4b) levels between TS + LFS and TS + LFS + DCS + MG-132 ($p>0.1$) and between TS + LFS and TS + LFS + DCS + lactacystin ($p>0.1$). LFS plus MG-132 or LFS plus lactacystin without adding DCS did not influence the GluR1 and GluR2 levels in LFS slices ($p>0.1$).

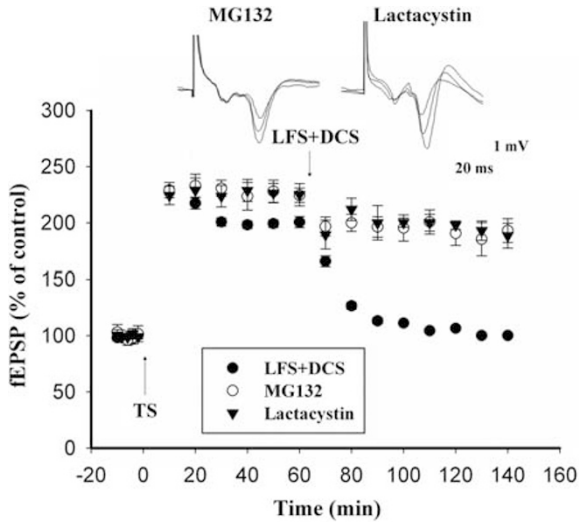

Figure 3 Proteasome inhibitors block DCS facilitation of LFS-induced depotentiation. The graph represents the mean ± SE slope of fEPSPs plotted against time. In the presence of DCS (10 μM), LFS applied 60 min after the TS induced depotentiation (data taken from Mao *et al*, 2006). Depotentiation was blocked when DCS was coapplied with MG-132 (10 μM) or lactacystin (5 μM) 5 min before and during the LFS.

We next measured the internalized GluR1 and GluR2. Figures 4c and d show that the levels of internalized GluR1 and GluR2 were not significantly different between naive and TS ($p>0.05$) and between naive and TS + LFS ($p>0.05$). However, in the DCS group, LFS increased the level of internalized GluR1 (148.3 ± 9.9% of naive, $p<0.05$ *vs* TS + LFS) (Figure 4c) and GluR2 (159.7 ± 13.3% of naive, $p<0.05$ *vs* TS + LFS) (Figure 4d). Coadministration of MG-132 (10 μM) with DCS blocked the effect of DCS (GluR1: 99.6 ± 2.0%, $n=6$; GluR2: 99.5 ± 9.0%, $n=6$). Similarly, coadministration of lactacystin (5 μM) with DCS blocked the effect of DCS (GluR1: 99.5 ± 2.5%, $n=6$; GluR2: 99.8 ± 5.3%, $n=6$). LFS plus MG-132 or LFS plus lactacystin without adding DCS did not influence the GluR1 and GluR2 levels in LFS slices ($p>0.1$).

Previous studies have shown that brief application of NMDA at a concentration of 20 μM but not at 10 μM induced AMPA receptor internalization, leading to LTD in the hippocampal neurons (Lee *et al*, 1998; Beattie *et al*, 2000). We further tested whether DCS facilitated NMDA-induced receptor internalization by using this protocol in the amygdala slices. One hour after treating slices with NMDA (20 μM, 3 min), cell extracts were prepared and biotinylated surface proteins were isolated by Neutravidin precipitation. Supplementary Figure 1A shows that NMDA (20 μM) application caused reduction in GluR1 level (65.5 ± 8.2%, $n=8$, $p<0.001$ *vs* control), whereas lower concentration of NMDA (10 μM) was without effect (99.4 ± 3.5%, $n=8$, $p=0.42$ *vs* control). Only in the presence of DCS (10 μM), 10 μM NMDA induced a significant loss of surface GluR1 (67.9 ± 7.5%, $n=8$, $p<0.05$ *vs* NMDA 10 μM). We also probed the level of surface GluR2. As shown in Supplementary Figure 1B, in the presence of DCS (10 μM), 10 μM NMDA induces a significant loss of surface GluR2 (64.8 ± 9.4%, $n=6$, $p<0.05$ *vs* NMDA 10 μM).

We measured the internalized receptors of GluR1 and GluR2. Supplementary Figures 1C and D show that NMDA (20 μM) application increased internalized GluR1 and GluR2 levels to 144.8 ± 8.2% ($n=6$) and 142.9 ± 6.2% ($n=6$) of naive controls, respectively, whereas lower concentration of NMDA (10 μM) was without effect (GluR1: 103.9 ± 7.0%, $n=6$; GluR2: 96.9 ± 6.8%, $n=6$). In the presence of DCS (10 μM), however, 10 μM NMDA induced significant increases in internalized levels of GluR1 (143.8 ± 9.7%, $n=6$, $p<0.01$ *vs* NMDA 10 μM) and GluR2 (140.2 ± 6.9%, $n=6$, $p<0.01$ *vs* NMDA 10 μM).

As GluR is primarily degraded by the lysosome (Ehlers, 2000), it is likely that GluR endocytosis is regulated by the targeted degradation of a GluR-interacting protein, leading to receptor destabilization and subsequent endocytosis. One of the proteins that regulates GluR trafficking is PSD-95 (EL-Husseini Ael *et al*, 2002; Schnell *et al*, 2002; Beique and Andrade, 2003), which interacts with GluR indirectly through stargazin (Chen *et al*, 2000). We examined whether PSD-95 expression was altered by DCS treatment. Amygdala slices were prepared and classified into naive, TS, LFS, and LFS + DCS groups. ANOVA of TS, LFS, and LFS + DCS groups showed a significant effect for group ($F_{(2,27)}=17.38$, $n=10$ in each group, $p<0.001$), and *post hoc* test revealed that PSD-95 level was significantly elevated in the TS group (170.0 ± 9.5%, $n=10$) when compared to naive slices without receiving TS. LFS applied 60 min after TS did not influence TS-induced increases in PSD-95 (144.9 ± 8.3%, $p>0.05$), but DCS treatment decreased the level of PSD-95 to 96.8 ± 9.2% of naive controls ($p<0.001$ *vs* TS + LFS) (Figure 5a). Furthermore, coadministration of MG-132 (10 μM) or lactacystin (5 μM) with DCS blocked the effect of DCS such that there was no difference in PSD-95 levels between TS + LFS and TS + LFS + DCS + MG-132 ($p>0.1$), and between TS + LFS and TS + LFS + DCS + lactacystin ($p>0.1$). MG-132 or lactacystin did not influence PSD-95 level in the TS + LFS slices.

The insertion and removal of AMPA receptors are regulated by the scaffold proteins PSD-95 and a PDZ domain-containing protein SAP97, which binds to GluR1 and traffics GluR1 into spines (Rumbaugh *et al*, 2003; Schluter *et al*, 2006). In the LA, the coupling of A-kinase anchoring proteins and protein kinase A to GluR1 through SAP97 is essential for memory formation (Moita *et al*, 2002), whereas the involvement of SAP97 in memory extinction remains to be elucidated. We examined whether SAP97 expression was altered by DCS. Amygdala slices were prepared as described previously. ANOVA showed a significant effect for group ($F_{(2,15)}=17.54$, $n=6$ in each group, $p<0.001$), and *post hoc* test revealed that SAP97 level was significantly elevated in the TS group (191.5 ± 13.6%, $p<0.001$) compared to naive slices. LFS applied 60 min after TS did not influence TS-induced increases in SAP97 (174.0 ± 11.4%, $p>0.05$), but DCS treatment decreased the level of SAP97 to 100.2 ± 9.3% of controls ($p<0.001$ *vs* TS + LFS) (Figure 5b). Furthermore, coadministration of MG-132 (10 μM) or lactacystin (5 μM) with DCS blocked the effect of DCS such that there was no difference in the SAP97 levels between TS + LFS and TS + LFS + DCS + MG-132 ($p>0.1$), and between TS + LFS and TS + LFS + DCS + lactacystin ($p>0.1$). MG-132 or lactacystin did not influence SAP97 level in the TS + LFS slices.

In Vivo Studies

Rats were conditioned with 10 light–shock pairings and were randomly assigned to three groups: paired, extinction,

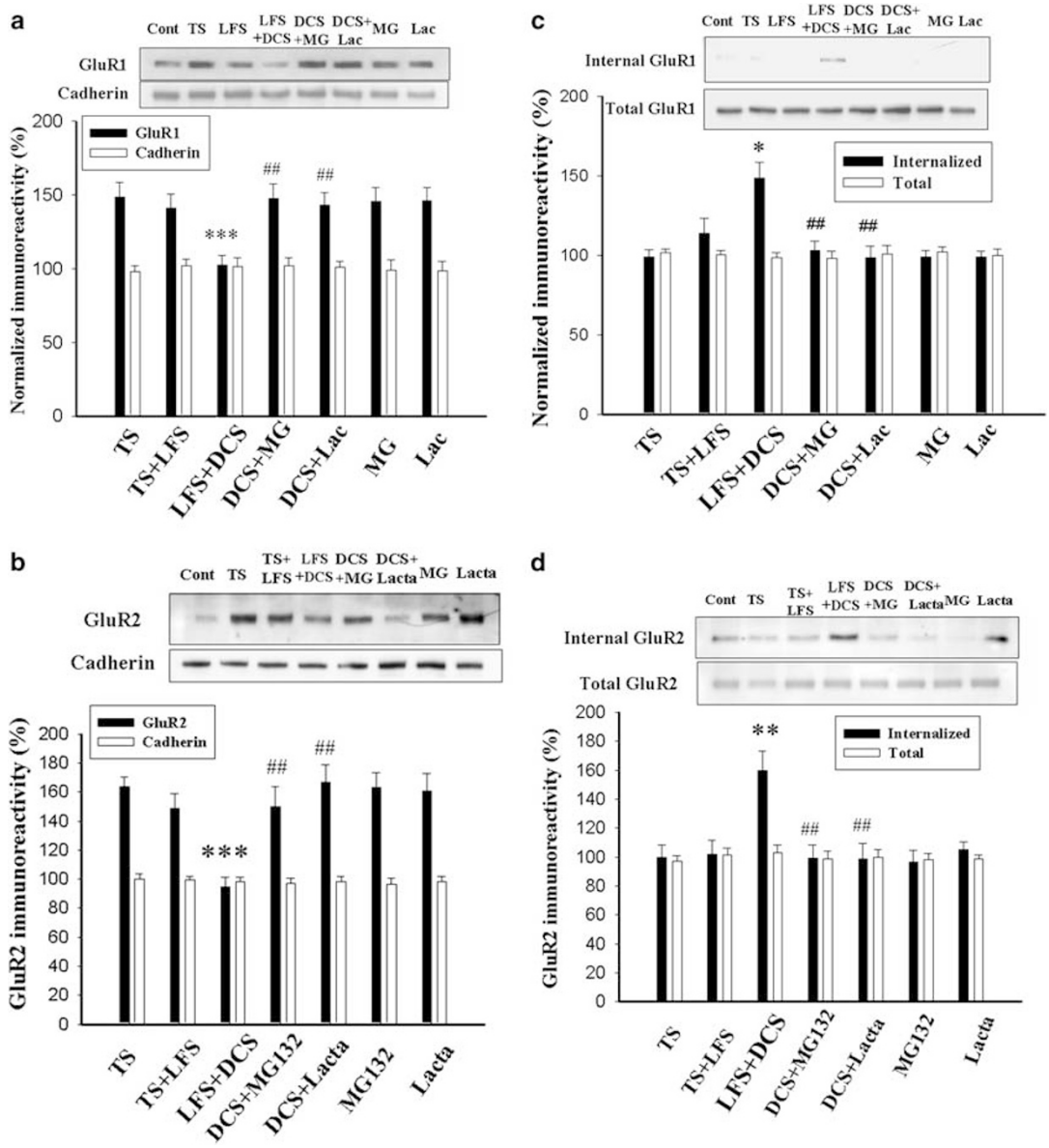

Figure 4 Proteasome inhibitors block DCS facilitation of LFS-induced reduction in surface levels of GluR1 and GluR2. Amygdala slices were randomly assigned to naive, TS, LFS, and LFS + DCS groups. Slices were given five sets of TS at an interstimulus interval of 1 min to the EC in the TS group. In LFS group, LFS was delivered at 60 min following the onset of TS. DCS (10 μM) was applied 5 min before and during the LFS in the DCS + LFS group. Separate groups of TS + LFS and DCS slices were treated with MG-132 (10 μM) or lactacystin (5 μM). Two hours following the onset of TS, a relatively small portion of the LA and BLA was dissected out from each slice. Surface GluR1 (a) and GluR2 (b) levels were determined by biotin labeling as described in Materials and Methods. $^{***}p<0.001$ *vs* TS + LFS; $^{\#\#}p<0.01$ *vs* DCS. (c and d) Proteasome inhibitors block DCS facilitation of LFS-induced increase in internalized levels of GluR1 and GluR2. Internalized GluR1 (c) and GluR2 (d) levels were determined by biotin labeling as described in Materials and Methods. $^{*}p<0.05$; $^{**}p<0.01$ *vs* TS + LFS; $^{\#\#}p<0.01$ *vs* DCS.

and extinction + DCS groups. In the paired group, the rats were tested for fear-potentiated startle 48 h later (day 3) without any drug infusion. The extinction group was given light-alone trials (extinction training) on day 2 while the extinction + DCS rats were infused with DCS (10 μg/side) bilaterally into the amygdala 30 min before extinction training. Memory retention was assessed 24 h after extinction training. In all three groups, a comparison of the startle magnitudes between 'noise-alone' trials and 'light (CS)-noise' trials showed significant fear potentiation by the light CS ($n=10$ in each group, $p<0.001$, paired *t*-test). ANOVA showed a main effect for group ($F_{(2,27)}=22.09$, $n=10$ in each group, $p<0.001$), and the magnitude of startle potentiation (difference) was significantly less in extinction group than that in paired group ($p<0.01$). In addition, the magnitude of startle potentiation was significantly less in DCS group than that in extinction group ($p<0.05$). Baseline startle levels were not significantly affected by the DCS infusion ($p>0.5$). These results were consistent with previous reports showing that DCS facilitated extinction without altering baseline acoustic startle or sensorimotor responses (Walker *et al*, 2002; Ledgerwood *et al*, 2003).

In a separate group of conditioned rats, proteasome inhibitor MG-132 (9.5 μg/side) or vehicle was infused into

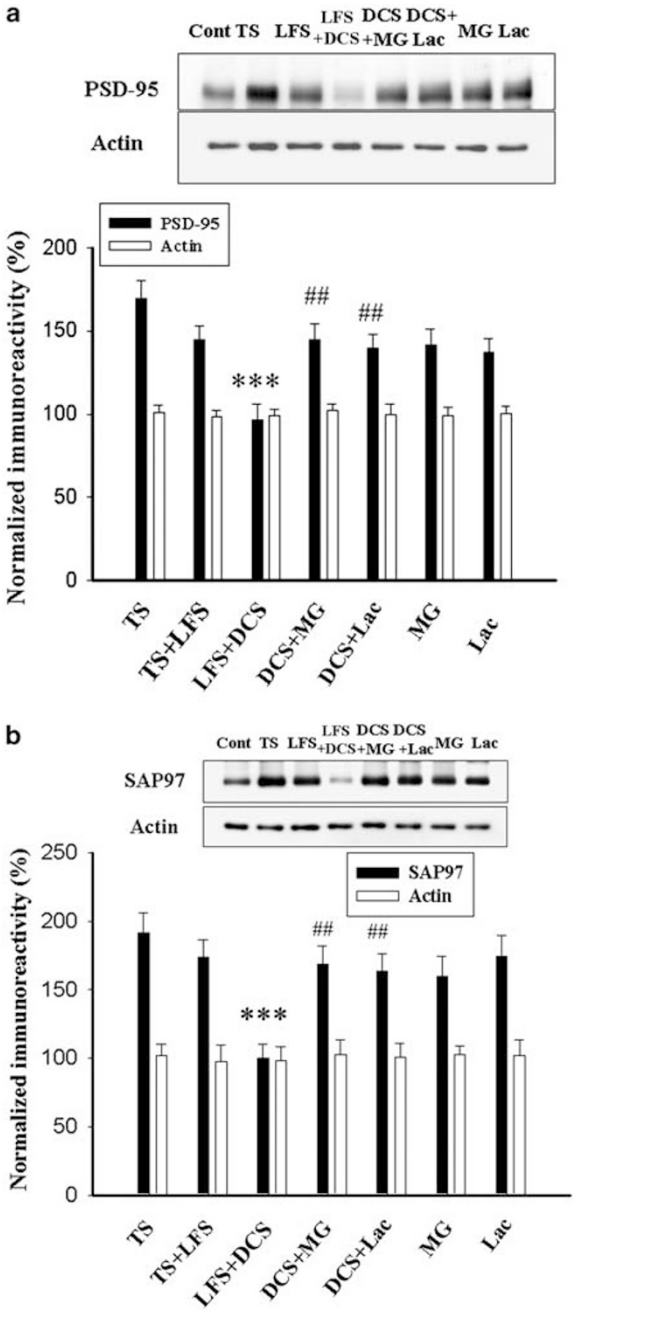

Figure 5 LFS plus DCS causes a proteasome-sensitive reduction of PSD-95 and SAP97. Amygdala slices were randomly assigned to naive, TS, LFS, and LFS + DCS groups. Eight to twelve slices from two rats were pooled together for biochemical analysis. Cells were lysed and the levels of PSD-95 (a) and SAP97 (b) were analyzed. LFS plus DCS caused a loss of PSD-95 and SAP97. Pretreating slices with MG-132 (10 μM) or lactacystin (5 μM) 5 min before and during LFS prevented the loss. $^{***}p<0.001$ *vs* TS + LFS; $^{\#\#}p<0.01$ *vs* DCS.

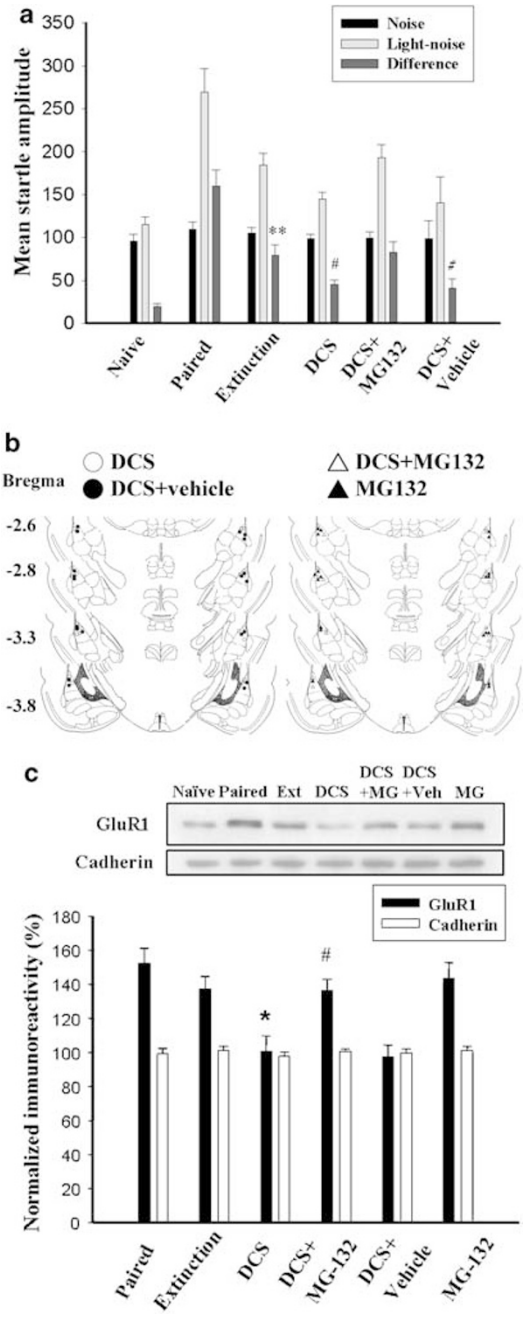

Figure 6 Proteasome inhibitor MG-132 blocks the effect of DCS on fear extinction. (a) Rats were conditioned and randomly assigned to extinction and extinction + DCS on day 1. On day 2, rats in the extinction group were given light-alone trials (extinction training) while the extinction + DCS rats were infused with DCS (10 μg/side) bilaterally into the amygdala 30 min before extinction training. Fear memory was measured 24 h after extinction training. MG-132 (9.5 μg/side) was given 30 min before DCS; 30 min later light-alone trials were given. A separate group of rats was infused with MG-132 without DCS treatment. The percentage of fear-potentiated startle was measured 24 h after the light-alone trials. $^{**}p<0.01$ *vs* paired; $^{\#}p<0.05$ *vs* extinction. (b) Cannula tip placements in rats infused with DCS (○), DCS + vehicle (●), DCS + MG-132 (△), or MG-132 (▲) in experiments shown in panel a. (c) The same conditioning and extinction training procedures as experiment shown in panel a were used on another group of rats. Twenty-four hours after extinction, LA and BLA tissues were dissected out and the surface GluR1 levels were determined using biotin labeling. GluR1 levels in the conditioned animals were expressed as a percentage of those in naive controls without receiving light–shock pairings. $^{*}p<0.05$ *vs* extinction; $^{\#}p<0.05$ *vs* DCS and DCS + vehicle.

the amygdala followed by DCS, 30 min before light-alone trials. As shown in Figure 6a, MG-132 blocked the effect of DCS such that the difference in startle amplitude between light–noise and noise-alone in extinction rats treated with DCS + MG-132 was not different from that in extinction rats ($p>0.1$). The infusion cannula tip locations are shown in Figure 6b.

Same protocol of conditioning followed by extinction training was applied to another group of animals. Twenty-four hours after extinction, the tissues of LA and BLA were dissected out and surface receptors were labeled with biotin. As shown in Figure 6c, light-alone trials 24 h after training did not significantly influence the conditioning-induced increase in surface expression of GluR1 ($p > 0.1$), a result consistent with the notion that extinction is an active learning and not an erasure of the original memory (Berman and Dudai, 2001; Myers and Davis, 2002). However, when rats were infused with DCS (10 μg/side) bilaterally into the amygdala 30 min before light-alone trials, conditioning-induced increase in GluR1 was abolished. Treatment with MG-132 prevented the effect of DCS such that the level of GluR1 in MG-132 plus DCS-treated rats was not significantly different from that in rats received extinction training only ($n = 10$, $p > 0.1$).

A structurally distinct proteasome inhibitor lactacystin (10 μg/side) was infused into the amygdala 30 min before DCS. Figure 7a shows that the magnitude of startle potentiation was significantly less in DCS group than that in extinction group ($p < 0.05$). Lactacystin blocked the effect of DCS such that the difference in startle amplitude between light–noise and noise-alone in extinction rats treated with DCS + lactacystin was not different from that in extinction rats ($n = 10$, $p > 0.5$). The infusion cannula tip locations are shown in Figure 7b. Figure 7c shows that lactacystin blocked the effects of DCS on conditioning-induced increase in GluR1 and the levels of GluR1 in lactacystin plus DCS-treated rats were not significantly different from those that received extinction training only ($p > 0.1$).

We next determined whether DCS-treated rats exhibited reinstatement. On day 1, rats were trained with 10 light–shock pairings and 24 h later they received intra-amygdala infusion of DCS (10 μg, $n = 8$) or vehicle ($n = 6$) 30 min before extinction training. Memory retention was tested 24 h later (day 3, test 1). One hour later, the rats were given 10 US-alone trials and retention of memory was tested 24 h later (day 4, test 2). A two-way ANOVA revealed a significant increase in fear-potentiated startle in the vehicle-treated group, but no change in the DCS-treated group after US-alone trials ($F_{(1,28)} = 11.06$, $p < 0.01$) (Figure 8). These results indicate that DCS-treated rats failed to exhibit reinstatement after US presentations.

In Figure 5a, the reversal of TS-induced PSD-95 by DCS + LFS is blocked by proteasome inhibitors suggests that PSD-95 may be the target for UPS. We tested this possibility by pull-downing ubiquitinated proteins and then conducted western blotting analysis for PSD-95. As shown in Supplementary Figure 2A, the PSD-95 was not detected when the ubiquitinated proteins were reprobed with an anti-PSD-95 antibody. We also conducted a reverse immunoprecipitation experiment, in which we first immunoprecipitated PSD-95 and then probed the blot with an anti-ubiquitin antibody. Again, we failed to detect ubiquitin immunoreactivity (Supplementary Figure 2C).

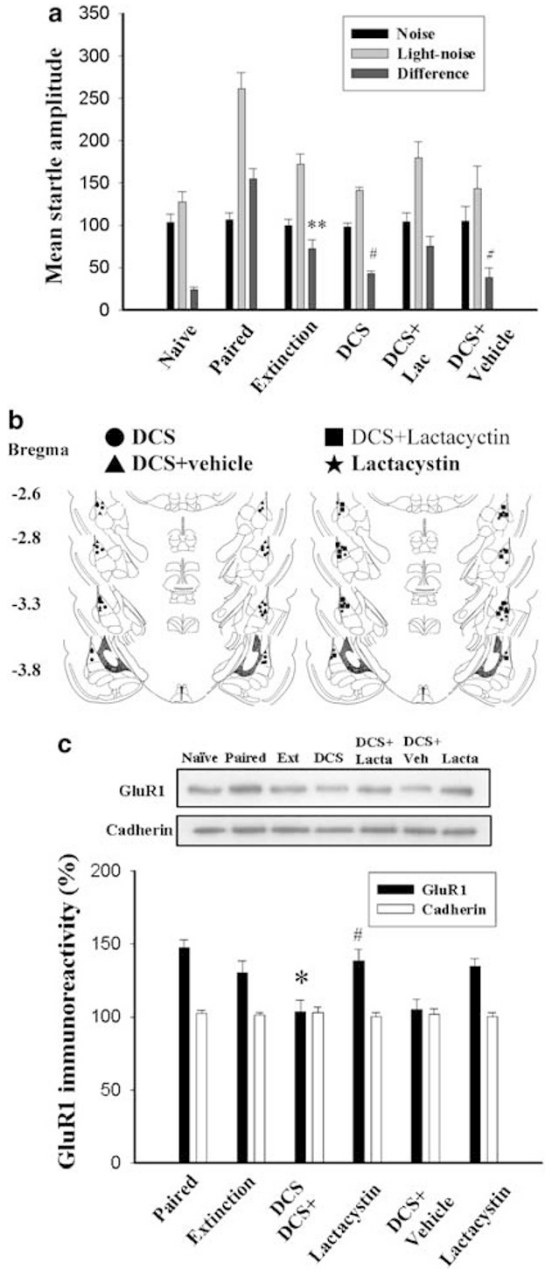

Figure 7 Block of the effect of DCS on extinction by lactacystin. (a) Rats were conditioned and 24 h later were infused with vehicle or lactacystin (10 μg/side) followed by DCS; 30 min later all the rats were given light-alone trials. A separate group of rats was infused with lactacystin without DCS treatment. The percentage of fear-potentiated startle was measured 24 h after the light-alone trials. $^{**}p < 0.01$ *vs* paired; $^{\#}p < 0.05$ *vs* extinction. (b) Cannula tip placements in rats infused with DCS (●), DCS + vehicle (▲), DCS + lactacystin (■) or lactacystin (★) in experiments shown in panel a. (c) The same conditioning and extinction training procedures as experiment shown in panel a were used on another group of rats. $^{*}p < 0.05$ *vs* extinction; $^{\#}p < 0.05$ *vs* DCS and DCS + vehicle.

DISCUSSION

Inability to extinguish or suppress invasive fear responses is a fundamental challenge to the treatment of most anxiety disorders, PTSD in particular. The elegant study by Davis and co-workers showing that DCS can facilitate extinction of fear memory in rat is being successfully translated into novel treatment for human disorders (Ressler *et al*, 2004; Barad, 2005; Anderson and Insel, 2006). There are two major types of neurons in the LA and BLA. Excitatory pyramidal neurons, which use glutamate as neurotransmitter, are responsible for synaptic transmission. Local circuit inhibitory

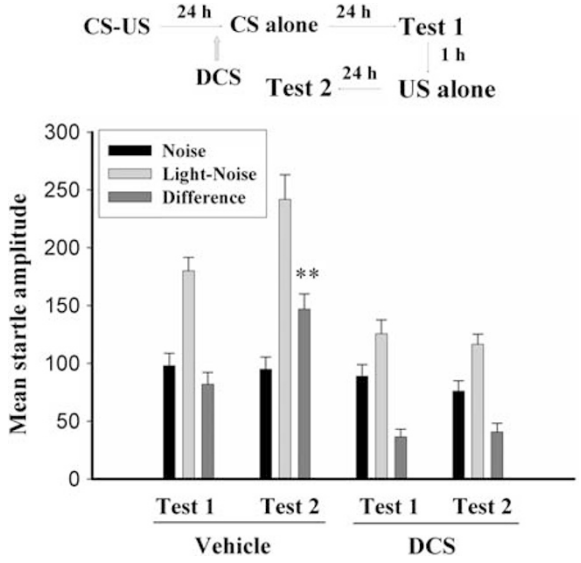

Figure 8 DCS-treated rats fail to exhibit reinstatement after US-alone presentations. Rats were given 10 light–shock pairings and 24 h later were given intra-amygdala infusion of DCS (10 μg, $n = 8$) or vehicle ($n = 8$) 30 min before extinction training. Memory retention was tested 24 h later (day 3, test 1). One hour later, the rats were given 10 US-alone trials and retention of memory was tested 24 h later (day 4, test 2). $^{**}p < 0.01$ *vs* test 1.

interneurons, upon their activation, release γ-aminobutyric acid (GABA) and exert powerful control on the pyramidal cells. Repetitively pairing animals a cue with an aversive stimulus increases LTP of synapses from auditory thalamus and cortex to the LA (McKernan and Shinnick-Gallagher, 1997; Rogan *et al*, 1997) and synaptic GluR1 subunit of AMPA receptors (Rumpel *et al*, 2005; Yeh *et al*, 2006). Extinction training, on the other hand, increases function of $GABA_A$ receptors in the BLA (Chhatwal *et al*, 2005) without affecting surface GluR1 level (Mao *et al*, 2006). The IL subregion of the mPFC exerts an inhibitory tone over the main output regions of the amygdala (Quirk *et al*, 2003; Likhtik *et al*, 2005), and extinction is an active learning that inhibits expression of the original association rather than erasing it (Myers and Davis, 2002; Sotres-Bayon *et al*, 2004). In the present study, we found that coadministration of DCS with extinction training facilitated extinction and reversed conditioning-induced increase in GluR1 and GluR2. Neither extinction training nor DCS alone altered surface expression of GluR1 and GluR2. These results suggest that DCS may transform the effect of light-alone trials from inhibitory learning (extinction) to erasure.

By binding to a strychnine-insensitive site on the NR1 NMDA receptor subunit, glycine acts as a necessary coagonist at the NMDA receptor (Johnson and Ascher, 1987; Thompson *et al*, 1989). Glycine concentration in the cerebrospinal fluid is estimated to be in the low micromolar range (Westergren *et al*, 1994), a concentration that is sufficient to saturate the site under physiological conditions. However, the present result suggests that glycine concentration within synaptic regions of the amygdala is not sufficient to saturate the glycine-binding site of the NMDA receptor, as DCS still can exert its potentiating effect on the NMDA responses. Consistent with this notion, because of the strategic placement of the high-affinity glycine transporter type 1 (GlyT1) around the synapses, NMDA responses could be enhanced after blocking GlyT1 (Bergeron *et al*, 1998). In addition, the facilitation of LFS-induced depotentiation by DCS indicates that normal glycine level is subsaturating in the proximity of NMDA receptors mediating depotentiation in the amygdala slices. However, there was a recent report showing that D-serine levels are near-saturating at NMDA receptors mediating the induction of LTP and depotentiation, but are subsaturating at those NMDA receptors mediating LTD (Duffy *et al*, 2007). The discrepancy between these results is not clear but could be due to the different brain areas studied.

Because of technique limitation, we were unable to label GluR1 with biotin *in vivo* to test whether the effect of DCS in the *in vitro* amygdala slices also occurred in the *in vivo* animal studies. Instead, we have shown that augmented reduction of fear-potentiated startle and reversal of GluR1 increase by DCS were blocked by proteasome inhibitors. The rationale is that NMDA-induced AMPA receptor endocytosis requires ubiquitin-mediated proteasome activity (Patrick *et al*, 2003; Colledge *et al*, 2003). We first tested this idea in the amygdala slices. Facilitation of DCS-induced GluR1 internalization was blocked by proteasome inhibitors. Next, in the *in vivo* experiments, two structurally distinct proteasome inhibitors (MG-132 and lactacystin) both produced similar effects on fear-potentiated startle and surface expression of GluR1, suggesting the mediation via inhibition of proteasome activity. These results suggest that DCS facilitates receptor internalization, resulting in augmented reduction of startle potentiation.

Many observations in animal studies, including reinstatement after US presentations (Rescorla and Heth, 1975), indicate that extinction is a new inhibitory learning, which leaves the original memory intact (Myers and Davis, 2002; Maren and Quirk, 2004). We and others showed that animals that had received DCS before extinction training exhibited less reinstatement effect (Ledgerwood *et al*, 2004). It is possible that extinction seen following DCS treatment was more robust and less susceptible to subsequent US reinstatement. Alternatively, it may suggest the possibility of additional mechanisms. One mechanism identified by the present study is the promotion of receptor internalization by DCS.

Opposite results have been reported regarding the role of UPS in the consolidation of memory. When degradation of an inhibitory protein is necessary for memory consolidation, proteasome inhibitors blocked the formation of long-term memory (Chain *et al*, 1999; Lopez-Salon *et al*, 2001; Upadhya *et al*, 2004). Conversely, when UPS functioned as an inhibitory constraint, removal of constraint by proteasome inhibitor enhanced synaptic transmission and facilitated memory formation (Zhao *et al*, 2003; Yeh *et al*, 2006). In the present study, we found that proteasome inhibitors blocked DCS-induced GluR1 internalization. In cultured hippocampal neurons, there is a negative correlation between neurotransmitter-stimulated GluR1 internalization and PSD-95 staining (Bingol and Schuman, 2004). Palmitoylation of PSD-95 is essential for clustering at the postsynaptic density (Craven and Bredt, 1998), whereas blocking of palmitoylation disperses synaptic clusters of PSD-95 and causes a selective loss of synaptic AMPA receptors (EL-Husseini Ael *et al*, 2002). Since GluR is primarily degraded by the lysosome (Ehlers, 2000), GluR is unlikely the target for proteasome degradation. GluR-interacting proteins may serve as a critical

proteasome target (Bingol and Schuman, 2004). The degradation of GluR-interacting proteins leads to destabilization of the receptor and its subsequent endocytosis (Bingol and Schuman, 2005). We showed that there was a proteasome-dependent downregulation of PSD-95 levels in response to DCS stimulation. These data point to the possibility that PSD-95 may be a target of UPS regulation. However, we were unable to detect polyubiquitinated PSD-95 in lysates prepared from the amygdala neurons raising the possibility that PSD-95 may be an indirect target of UPS regulation (Bingol and Schuman, 2004).

Cadherin has been used as a neuronal surface protein marker (Du *et al*, 2004). A recent study has shown that the rate of N-cadherin endocytosis is significantly reduced following activation of NMDA receptors (Tai *et al*, 2007). In the present study, we used monoclonal anti-pan cadherin, which reacts extensively with all known members of the cadherin family, including N-cadherin, E-cadherin, P-cadherin, V-cadherin, R-cadherin, and T-cadherin. We found that the level of pan-cadherin was unaltered after NMDA treatment, suggesting that the change of N-cadherin was diluted by the other cadherin family. Nonetheless, one should be cautious in using N-cadherin as a negative control of surface proteins.

In summary, augmentation of extinction is a potential approach to the treatment of maladaptive memory disorders such as post-traumatic stress phobias (Walker *et al*, 2002; Ressler *et al*, 2004; Richardson *et al*, 2004). Extinction training involves repeated non-reinforced re-exposure to the CS, resulting in a new memory being formed (CS−no US), and so the fear response to the CS is subsequently attenuated (Bouton and Bolles, 1979; Rescorla, 2001). The demonstration of DCS-induced GluR1 and GluR2 internalization suggests that facilitation of extinction by DCS not only could result from the enhancement of new extinction memory but also could be attributed, at least in part, to an erasure of the original fear memory.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)