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Augmentation of Fear Extinction by Infusion of Glycine Transporter Blockers into the Amygdala[S]

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ABSTRACT

It is known that fear extinction is blocked by the *N*-methyl D-aspartate (NMDA) receptor antagonist. In this study, we investigate whether extinction could be facilitated by the enhancement of NMDA response, achieved by the blocking of glycine transporters. In amygdala slices, NMDA at a concentration that normally does not have a long-term effect was found to reduce the cellular levels of postsynaptic density protein 95 and synapse-associated protein 97, in addition to the surface expression of GluR1/2, in the presence of a glycine transporter blocker, *N*[3-(4-fluorophenil)-3-(4′-phenilphenoxy)] propylsarcosine (NFPS). In in vivo experiments, extinction training applied 24 h after conditioning reduced startle potentiation without influencing the conditioning-induced increase in the surface expression of GluR1/2. However, NFPS augmented extinction and reversed the conditioning-induced increase in GluR1/2 when infused bilaterally into the amygdala before extinction training. The effects of NFPS were therefore blocked by the NMDA antagonist. In parallel, NFPS treatment in conjunction with extinction reversed the conditioning-induced α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/NMDA ratio. In behavioral tests, Tat-GluR2$_{3Y}$, a synthetic peptide that has been shown to block AMPA receptor endocytosis, inhibited only the additional reduction caused by NFPS treatment, rather than returning the fear potentiation levels to those of fear-conditioned animals that did not undergo extinction. These results suggest that NFPS in combination with extinction training reverses GluR1/2 surface expression and thus augments the extinction of conditioned fear.

In the human brain, the functional magnetic resonance imaging signal in the amygdala decreases over time during repeated presentations of emotional facial expressions (Wright et al., 2001). Analogous to animal studies, this process may represent a form of extinction in which the response to a feared cue decreases as the cue is repeatedly presented without any adverse consequences (Taylor et al., 2003; Hermans et al., 2006). This exposure-based psychotherapy is a standard treatment for a number of anxiety disorders (Yehuda, 2002). However, in some patients, the successful reduction of fear through exposure therapy is followed by a return of fear, because the aversive or fear memory was stronger and extinction training did not erase the original memory (Quirk et al., 2000; Myers and Davis, 2002; Maren and Quirk, 2004; Barad, 2006).

Demonstration of the blocking of fear extinction by the NMDA receptor antagonist (Falls et al., 1992; Lee and Kim, 1998; Lin et al., 2003) led to the hypothesis that pharmacological manipulation of the NMDA receptor may influence extinction. Later, it was shown that D-cycloserine (DCS), a partial agonist at the glycine-binding site of the NMDA receptor, facilitated extinction of conditioned fear in rats (Walker et al., 2002; Ledgerwood et al., 2003). In human studies, patients who received DCS had significantly better outcomes than the placebo group in enhancing fear reduction in cases of social phobia and fear of heights (Ressler et al., 2004; Davis et al., 2006). These results suggest that agents

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ABBREVIATIONS: NMDA, *N*-methyl D-aspartate; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; BLA, basolateral nucleus of the amygdala; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; CS, conditioned stimulus; D-APV, D-2-amino-5-phosphonovalerate; DCS, D-cycloserine; DMSO, dimethyl sulfoxide; fEPSP, field excitatory postsynaptic potential; GlyT1, glycine transporter type 1; LA, lateral nucleus of the amygdala; LTD, long-term depression; NFPS, *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine; PSD-95, postsynaptic density protein 95; SAP97, synapse-associated protein 97; US, unconditioned stimulus; ITI, intertrial time interval; EPSC, excitatory postsynaptic current; PKA, protein kinase A; CGP52432, 3-[[(3,4-dichlorophenyl)methyl]amino]propyl(diethoxymethyl)phosphinic acid; HA-966, 3-amino-1-hydroxypyrrolid-2-one.

augmenting NMDA responses may be useful in the treatment of anxiety disorders.

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; Thomson et al., 1989). The 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, glycine may normally exist at nonsaturating concentrations within the synaptic regions because of the strategic placement of the high-affinity glycine transporter type 1 (GlyT1) around the synapses. Thus, NMDA responses could be enhanced after blocking GlyT1 (Bergeron et al., 1998). The purpose of this study was therefore to determine whether the pharmacological blocking of GlyT1 facilitates extinction. We also aimed to delineate the mechanism behind the action of GlyT1 blockers.

Methods and Materials

Animals. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Cheng-Kung University (Tainan, Taiwan). Animals were housed four to a cage in a temperature (24°C)-controlled animal colony under a 12:12 light/dark cycle, with lights on at 7:00 AM, and pelleted rat chow and water were available ad libitum. All behavioral procedures took place during the light cycle.

Surgery. Rats (6–8 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and subsequently mounted on stereotaxic apparatus. Two cannulae made of 22-gauge stainless steel tubing (C313G; Plastic Products, Roanoke, VA) were implanted bilaterally into the lateral (LA) or basolateral (BLA) amygdala (anteroposterior, −2.8 mm; mediolateral, ±4.5 mm; dorsoventral, −7.0 mm), and 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 from the procedure. NFPS (5 μg/side dissolved in 50% DMSO for intra-amygdalar injection; 1 or 10 mg/kg dissolved in 25% 2-hydroxypropyl-β-cyclodextrin/75% saline for intraperitoneal injection) was purchased from Tocris Bioscience (Northpoint, UK). Sarcosine (dissolved in saline) was obtained from Sigma (St. Louis, MO). Drugs were administered bilaterally to the amygdala in a volume of 0.8 μl at a rate of 0.1 μl/min. The infusion cannulae were left in place for 2 min before being withdrawn.

Behavioral Apparatus and Procedures. Rats were trained and tested in a stabilimeter device. The behavioral experiments of fear conditioning and extinction training were performed in a standard operant chamber (San Diego Instruments, San Diego, CA). The acoustic startle stimulus was 50 ms of white noise at an intensity of 95 dB; the visual CS was a 3.7-s light produced by an 8-W fluorescent bulb attached to the back of the stabilimeter, and the US was a 0.6-mA foot-shock of a 0.5-s duration. Rats were placed in the training/testing chamber for 10 min and returned to their home cages on 3 consecutive days to habituate them to the test chamber and to minimize the effect of contextual conditioning. On the following 2 days, the rats were handled in the same chamber before fear conditioning for pre-exposure, during which the baseline startle was measured on each of the 2 days by administering 10 startle stimuli with a 2-min interstimulus interval. Rats with equivalent baseline mean startle amplitudes were then divided into separated matched groups. On the day of fear conditioning, the animal was taken to the room, allowed to habituate, and placed in the chamber as before. The CS-US pairing began after a 3-min acclimatization period in the chamber.

Experiment 1: Effect of NFPS on the NMDA Receptor-Mediated Synaptic Response. Male Sprague-Dawley rats (150–200 g) were decapitated, and their brains were 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 (Camden Instruments, Sileby, UK). Transverse slices of a 450-μm thickness were cut, and the appropriate slices were placed in a beaker of oxygenated ACSF at room temperature for at least 1 h before recording. The ACSF solution was of the following composition: 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 11 mM glucose. The ACSF was bubbled continuously with 95% O_2/5% CO_2 and had a 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 to 3 ml/min. Extracellular field potentials were created by electrical stimulation of the external capsule, which contained fibers from the auditory cortex to the lateral amygdala, with a concentric bipolar stimulating electrode. Electrical stimuli (150 μs in duration) were delivered at a frequency of 0.05 Hz, and baseline field potentials were adjusted to ~30 to 40% of the maximal responses. Bicuculline (10 μM) and CGP52432 (10 μM) were present in the perfusion solution to block $GABA_A$ and $GABA_B$ receptors, respectively. 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).

Experiment 2: Effects of GlyT1 Blockers on Extinction and Reinstatement. On day 1, rats were placed in the startle boxes and received 10 light–foot-shock pairings with an ITI of 2 min. On day 2, the rats were returned to the startle box and received 3 sessions of 10 presentations of the 3.7-s light in the absence of either shock or the startle-elicited noise burst (extinction training). Each session was separated by 10 min, with an ITI of 1 min. The GlyT1 blocker NFPS (5 μg/side) was infused into the amygdala 30 min before extinction training. Twenty-four hours later (day 3), rats were returned to the startle chamber for testing. Thirty initial startle stimuli were presented in darkness at a 10-s interstimulus interval (leader stimuli), followed by 30 startle-eliciting noise bursts presented alone in darkness (noise-alone trial) and 30 noise bursts presented 3.2 s after the onset of the 3.7-s light (light-noise trials). The two trial types were presented in a balanced mixed order (ITI, 30 s). Startle amplitude was averaged over all of the light-noise or noise-alone stimuli within the test session, and the percentage of fear-potentiated startle was computed as follows: [(startle amplitude on CS-noise − noise-alone trials)/(noise-alone trials)] × 100. Paired and unpaired rats were administered 10 light-shock pairings in a paired or pseudorandom manner, and retention of memory was tested 48 h later with no extinction training administered. A separate group of rats that received NFPS infusion but no extinction training served as No-CS NFPS controls. To examine whether NFPS-treated rats exhibited reinstatement of fear, the rats were given US-alone trials (10 unpaired foot shocks) 1 h after the test, and retention of memory was tested 24 h later.

Experiment 3: Effect of NFPS on the Surface Expression of GluR1 and GluR2. For the in vitro experiment, amygdala slices were treated with 20 μM NMDA, 10 μM NMDA, 10 μM NMDA plus 1 μM NFPS, or 10 μM NMDA plus 1 mM sarcosine for 3 min. One hour later, LA and BLA tissues were dissected out, placed on ice, and washed twice with ice-cold ACSF. Control slices were incubated in ACSF for 1 h. 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 ACSF and then sonicated briefly in homogenizing buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 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, pH 7.5).

After sonication, the samples were centrifuged at 18,200*g* for 30 min at 4°C and the supernatant was obtained. The 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) 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, and then biotinylated protein was resolved in 8.5% SDS-polyacrylamide gel, blotted electrophoretically on polyvinylidene difluoride membrane, and blocked overnight in TBS buffer containing 5% nonfatty milk. Surface GluR1 and GluR2 receptors and pan-cadherin (surface protein control) were detected by a biotinylation assay, followed by Western blot analysis using GluR1 (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), GluR2 (1:5000; Chemicon, Temecula, CA), PSD-95 (1:5000; Upstate, Billerica, MA), SAP97 (1:5000; Stressgen, Ann Arbor, MI), or pan-cadherin (1:2500; Sigma) antibodies, followed by horseradish peroxidase-conjugated secondary antibody for 1 h. An enhanced chemiluminescence kit was used for detection, and Western blots were developed in the linear range used for densitometry. GluR1 and GluR2 levels in the NMDA- and NFPS-treated slices were expressed as a percentage of those in the control slices from rats that did not undergo drug treatment. Likewise, the GluR1 and GluR2 levels in the conditioned animals were expressed as a percentage of those in the naïve controls that did not receive light-shock pairings.

In Vivo Experiment. On day 1, the rats were conditioned; 24 h later, they were treated with NFPS (5 μg/side) before extinction training. LA and BLA tissues were dissected out 24 h after extinction training, and the surface receptors were labeled with biotin.

Experiment 4: Effect of NFPS on the AMPA/NMDA Ratio. Whole-cell patch-clamp recordings were made from the LA neurons. EPSCs were evoked at 0.03 Hz by extracellular stimulation of fibers emerging from the internal capsule, which originate in the medial geniculate nucleus of the thalamus and project monosynaptically to the LA using a bipolar electrode. Patch electrodes were pulled from a thick-walled glass capillary (0.75 mm internal diameter, 1.5 mm outer diameter) to a tip resistance of 3 to 5 MΩ. The composition of the internal solution was 115 mM cesium gluconate, 5 mM NaCl, 1 mM EGTA, 0.3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na-ATP, 0.4 mM Na-GTP, and 10 mM HEPES. The final pH of the internal solution was adjusted to 7.3 by adding 1 M KOH; the final osmolarity was adjusted to 280 mOsm by adding sucrose. Recordings were low-pass-filtered at 2.5 to 20 kHz and digitized at 5 to 50 kHz. The signal was monitored and recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Online analysis and control of experimental acquisition was accomplished via a 586 (Intel)-based personal computer clone and a Digidata 1200 computer interface (Molecular Devices). AMPA receptor-mediated EPSC was evoked when the neurons were voltage-clamped at −70 mV, whereas NMDA receptor-mediated EPSC was determined as the current amplitude at 50 ms after the peak EPSC amplitude at a holding potential of +40 mV (Du et al., 2008).

Data Analysis. All values in the text are the mean ± S.E.M. Differences between the groups were evaluated by one-way ANOVA followed by Newman-Keuls post hoc tests. The paired *t* test was used to analyze the differences in fEPSP before and after drug treatment, and the unpaired *t* test was used to analyze the differences in startle reflex between the drug-treated and vehicle control groups. The level of significance was $P < 0.05$.

Results

Experiment 1: Effect of NFPS on the NMDA Receptor-Mediated Synaptic Response. NFPS is a potent and selective antagonist of GlyT1 (Bergeron et al., 1998; Aubrey and Vandenberg, 2001). The effect of NFPS (1 μM) on fEPSP as a function of time is illustrated in Fig. 1A. After the evoked responses had been stable for 20 to 30 min, NFPS was applied to the amygdala slices by bathing, which caused a reversible increase in the slope of fEPSP. On average, the magnitude of increase measured at 10 min after application was 85.2 ± 11.2% ($P < 0.01$). To determine the involvement of NMDA receptors in the action of NFPS, slices were pretreated with NMDA antagonist D-APV (50 μM) before the application of NFPS. Figure 1B shows that the effect of NFPS was abolished in the presence of D-APV (100.8 ± 2.3% of control). We further examined the effect of NFPS on NMDA receptor-mediated fEPSP ($fEPSP_{NMDA}$), isolated pharmacologically using Mg^{2+}-free solution in the presence of AMPA receptor antagonist CNQX (10 μM). Ten minutes after the

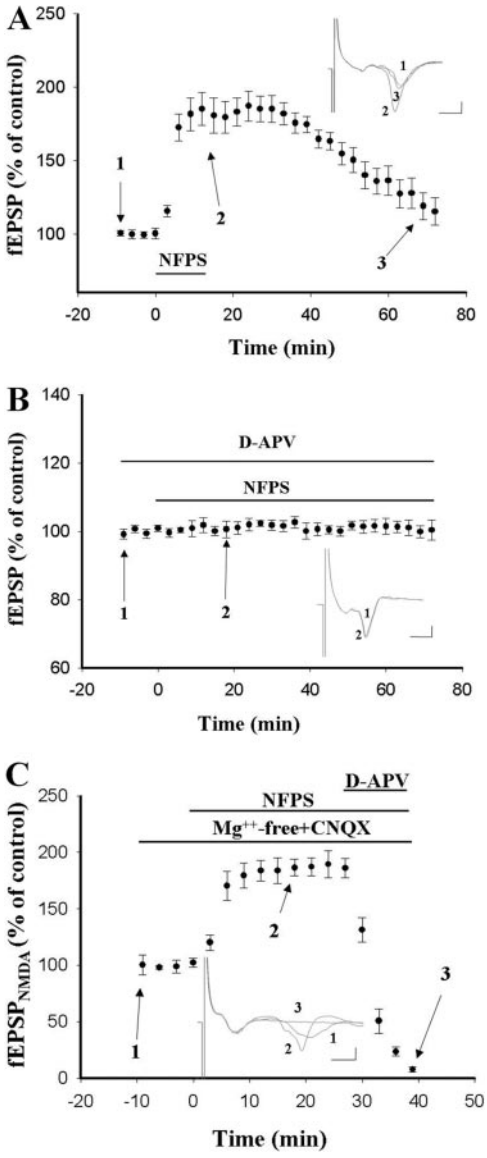

Fig. 1. Blocking of GlyT1 selectively enhances the NMDA-mediated synaptic response. A, the graph represents the mean ± S.E. slope of fEPSPs plotted against time. Application of NFPS (1 μM, $n = 8$) reversibly increased the slope of fEPSP. B, the effect of NFPS was blocked in the presence of D-APV (50 μM, $n = 6$). C, NMDA receptor-mediated fEPSP ($fEPSP_{NMDA}$) was isolated pharmacologically by using Mg^{2+}-free solution in the presence of CNQX (10 μM). Application of NFPS ($n = 6$) increased the amplitude of $fEPSP_{NMDA}$. At the end of the experiment, D-APV (50 μM) was applied to ensure that $fEPSP_{NMDA}$ was mediated by the NMDA receptors. Calibration, 1 mV, 20 ms.

application of NFPS, the $fEPSP_{NMDA}$ amplitude was increased by 83.7 ± 9.0%. At the end of the experiment, D-APV was applied to ensure that $fEPSP_{NMDA}$ was indeed mediated by the NMDA receptors (Fig. 1C). Taken together, these results indicate that NFPS selectively enhanced the NMDA receptor-mediated synaptic response.

Experiment 2: Effect of NFPS on Fear Extinction. Rats were conditioned with 10 light-shock pairings and were randomly assigned to 4 groups: Paired, Extinction, Extinction + NFPS, and Extinction + vehicle. In the Paired group, the rats were tested for fear-potentiated startle 48 h later (day 3) without receiving extinction training or any drug infusion. The Extinction group was given light-alone trials (extinction training) on day 2 without drug infusion, whereas the Extinction + NFPS and Extinction + vehicle rats were infused with NFPS (5 μg/side) or an equal amount of 50% DMSO (0.8 μl) 30 min before extinction training. Memory retention was assessed 24 h after extinction training. As expected, extinction training decreased the fear-potentiated startle from 188.5 ± 9.9% to 74.5 ± 6.6% ($P < 0.001$, unpaired t test). Furthermore, as can be seen from Fig. 2, fear-potentiated startle was significantly lower in the NFPS-treated rats compared with the vehicle-treated control and Extinction groups [$F_{(2,15)} = 8.31$, $P < 0.01$]. These results indicate that NFPS facilitated extinction. In a separate group of conditioned rats, HA-966 (6 mg/kg), an antagonist for the glycine-binding site of the NMDA receptor, was injected intraperitoneally followed by NFPS 30 min before light-alone trials, and memory retention was assessed 24 h after extinction training. As shown in Fig. 2, HA-966 blocked the effect of NFPS. The startle potentiation in rats treated with HA-966 plus NFPS was significantly higher than in those treated with NFPS alone and was comparable with those receiving extinction training only or extinction plus vehicle ($P>0.5$).

The effect of NFPS might reflect a reduction in fear response or, nonspecifically, a disruption in the startle reflex independent of fear reduction. To distinguish between these possibilities, rats were treated with NFPS without extinction training, whereupon we found that the startle potentiation in these rats (NFPS w/o Ext) was comparable with those that had not been subjected to extinction training (Paired w/o Ext) ($t_{(10)} = 0.75$, $P = 0.47$), suggesting that NFPS-augmented extinction and did not exert a general effect on the startle reflex. Furthermore, when NMDA receptor antagonist D-APV (12.5 nmol/side) was coapplied with NFPS, the effects of NFPS plus extinction training were completely abolished, indicating the requirement of NMDA receptor activation. A diagram showing the infusion cannulae tip locations is included as Supplemental Fig. 1.

We next determined whether the NFPS-treated rats exhibited reinstatement. On day 1, rats were trained with 10 light-shock pairings and 24 h later received intra-amygdala infusion of NFPS (5 μg) or vehicle 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 memory retention was tested 24 h later (day 4, test 2). Figure 3 shows that in the NFPS-treated rats, the startle potentiation in test 2 was significantly higher than in test 1 ($P < 0.01$) but was significantly less than in test 2 of the vehicle-treated rats ($P < 0.01$). These results indicate that the NFPS-treated rats exhibited reinstatement after the US presentations.

Experiment 3: Effect of NFPS on the Surface Expression of GluR1 and GluR2. Previous studies of the hippocampus have shown that NMDA-induced long-term depression (LTD) is accompanied by a persistent reduction in the surface expression of GluR1 (Lee et al., 1998; Man et al., 2007). We tested whether this was the case in the amygdala slices by using cell surface biotinylation techniques, as described under *Materials and Methods*. Cell extracts were

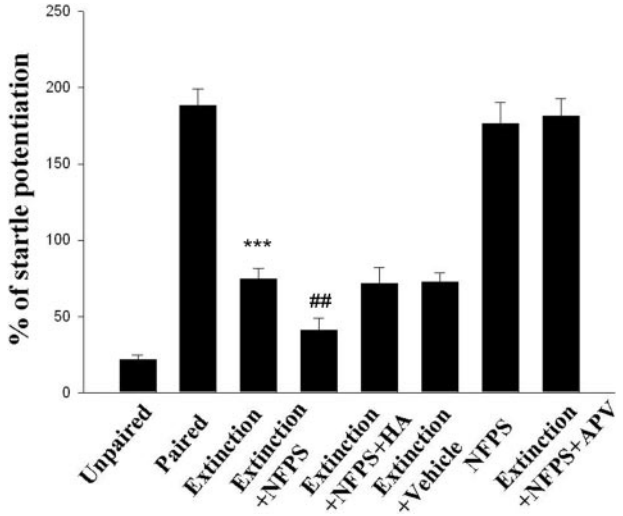

Fig. 2. Augmentation of extinction by NFPS. Rats were conditioned and 24 h later were administered light-alone trials. The percentage of fear-potentiated startle was measured 24 h after light-alone trials. NFPS (5 μg/side, 0.8 μl) or vehicle (50% DMSO, 0.8 μl) was infused bilaterally into the amygdala 30 min before the light-alone trials. The effect of NFPS plus extinction training was completely abolished when NMDA receptor antagonist D-APV (12.5 nmol/side) was coapplied with NFPS ($n = 6$). ***, $P < 0.001$ versus Paired; ##, $P < 0.01$ versus Extinction + vehicle.

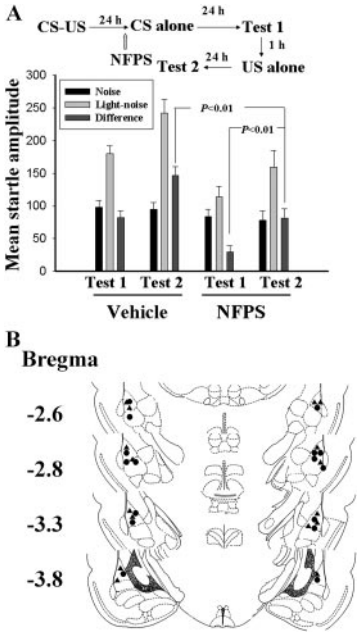

Fig. 3. Partial reinstatement after US alone presentations in rats treated with NFPS plus extinction training. A, rats were administered 10 light-shock pairings and 24 h later were given intra-amygdala infusion of NFPS (5 μg, $n = 8$) or vehicle ($n = 8$) 30 min before extinction training. Memory retention was tested 24 h later (day 3, test 1). One hour later, the rats were administered 10 US-alone trials, and retention of memory was tested 24 h later (day 4, Test 2). B, the cannulae tip placements in the rats infused with NFPS (●) or vehicle before extinction (▲).

prepared, and biotinylated surface proteins were isolated by Neutravidin precipitation 1 h after the application of NMDA (20 μM, 3 min). Control slices were incubated in ACSF solution for 1 h. Figure 4A shows that NMDA (20 μM) application caused a reduction in the GluR1 level (65.5 $\pm$ 3.4% of control, $P < 0.001$ versus control), whereas a lower concentration of NMDA (10 μM) had no effect (95.0 $\pm$ 3.4%). Only in the presence of NFPS (1 μM) did 10 μM NMDA induce a significant loss of surface GluR1 (69.2 $\pm$ 4.2%, $P < 0.001$ versus NMDA 10 μM). *N*-Methylglycine (sarcosine) is an endogenous antagonist of glycine transporter-1 (McBain et al., 1989). We tested whether sarcosine produced an effect similar to that of NFPS. As illustrated in Fig. 4B, in the presence of sarcosine (1 mM), 10 μM NMDA induced a significant loss of surface GluR1 (69.0 $\pm$ 4.2%, $P < 0.001$ versus NMDA 10 μM). We also assessed the level of surface GluR2. As shown in Fig. 4C, NMDA (20 μM) application caused a reduction in the GluR2 level (65.4 $\pm$ 4.7%, $P < 0.001$ versus control), whereas a lower concentration of NMDA (10 μM) had no effect (101.8 $\pm$ 5.8%). In the presence of NFPS (1 μM), 10 μM NMDA induced a significant loss of surface GluR2 (61.1 $\pm$ 4.8%, $P < 0.05$ versus NMDA 10 μM). NPFS alone did not affect the surface GluR2 level (96.3 $\pm$ 6.2%).

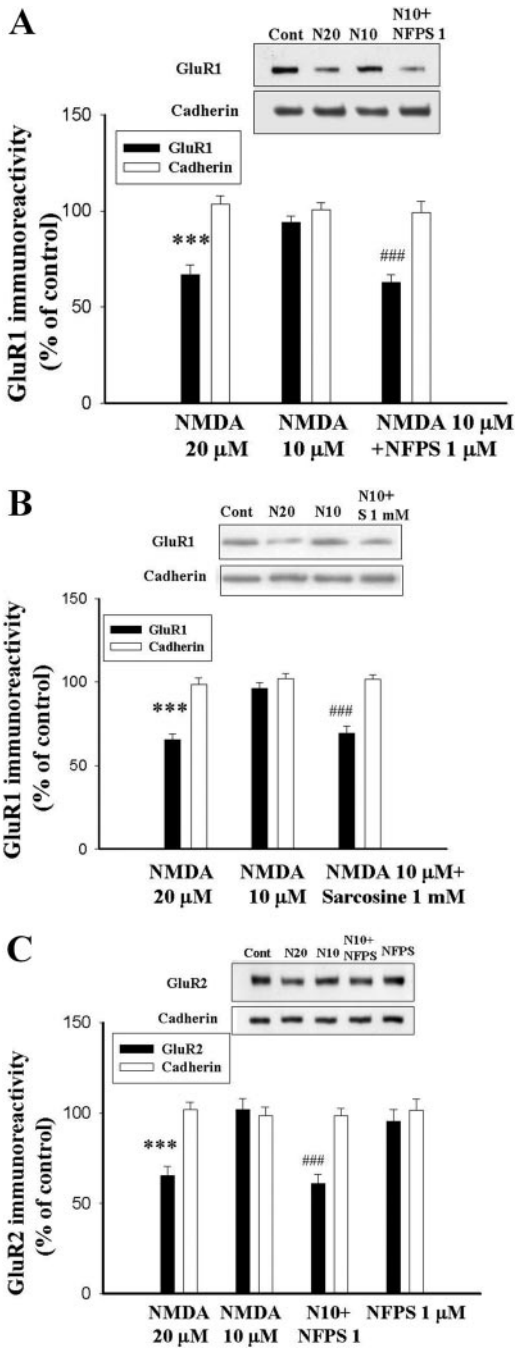

Fig. 4. GlyT1 blockers facilitate NMDA-induced reduction in the surface levels of GluR1 and GluR2. Amygdala slices were treated with 20 μM NMDA, 10 μM NMDA, 10 μM NMDA plus 1 μM NFPS (A), or 10 μM NMDA plus 1 mM sarcosine (B) for 3 min. One hour later, LA and BLA tissues were dissected out and the surface GluR1 (A and B) ($n = 7$ per group) and GluR2 (C) ($n = 8$ per group) levels were determined using biotin labeling. Control slices were incubated in ACSF for 1 h. GluR1 and GluR2 levels in the NMDA- and NFPS-treated slices were expressed as a percentage of those in the control slices. ***, $P < 0.001$ versus Control; ###, $P < 0.001$ versus 10 μM NMDA.

We examined whether NFPS affected GluR1 expression after extinction training. Rats were conditioned with 10 light-shock pairings and were randomly assigned to 4 groups. LA and BLA tissues were dissected out 24 h after extinction training, and the level of GluR1 was determined. Extinction training did not significantly influence the conditioning-induced increase in GluR1 ($p = 0.10$); however, the level was significantly lower in the NFPS-treated rats compared with the vehicle-treated control and Extinction groups [$F_{(2,15)} = 6.8, P < 0.01$] (Fig. 5A). Treatment with HA-966 (6 mg/kg i.p.) 30 min before the administration of NFPS prevented the effect of NFPS, such that the level of GluR1 in the HA-966 plus NFPS-treated rats was not significantly different from that of rats that received extinction training only ($P > 0.1$). Furthermore, NFPS-treated rats that did not undergo extinction training had a startle potentiation equivalent to that of the Paired or Extinction rats ($P>0.5$), suggesting that NFPS by itself did not influence extinction. We also assessed the level of surface GluR2. As shown in Fig. 5B, conditioning increased the surface expression of GluR2 to 141.2 $\pm$ 8.5% that of the naïve controls. Light-alone trials 24 h after training did not significantly influence the increase in GluR2 expression (141.5 $\pm$ 7.9%, $P > 0.5$); however, infusion of NFPS (5 μg/side) into the amygdala abolished the conditioning-induced increase in GluR2 (102.3 $\pm$ 2.3%, $P < 0.05$ versus Extinction). Administration of NFPS without extinction training had no effect on the increase in GluR2 expression (136.3 $\pm$ 8.6%). Treatment with D-APV prevented the effect of NFPS, such that the level of GluR2 in the D-APV plus NFPS-treated was not significantly different from that of rats that received extinction training only (139.2 $\pm$ 7.6%, $P > 0.5$).

PSD-95 is a scaffolding protein of the postsynaptic density; it interacts with GluR indirectly through stargazin (Chen et al., 2000) and regulates GluR trafficking (El-Husseini Ael et al., 2002; Schnell et al., 2002). We therefore examined whether PSD-95 expression was altered after treatment with NFPS. Figure 6A shows that NMDA (20 μM) application caused a reduction in the PSD-95 level (69.4 $\pm$ 5.7%, $P <$ 0.001 versus control), whereas a lower concentration of NMDA (10 μM) had no effect (98.3 $\pm$ 3.7%). In the presence of NFPS (1 μM), 10 μM NMDA induced a significant loss of PSD-95 (67.0 $\pm$ 7.4%, $P < 0.01$ versus 10 μM NMDA). NPFS alone did not affect the PSD-95 level (100.0 $\pm$ 5.7%). The insertion and removal of AMPA receptors are regulated by PSD-95 and postsynaptic density 95/disc-large/zona occludens domain-containing protein SAP97, which binds to

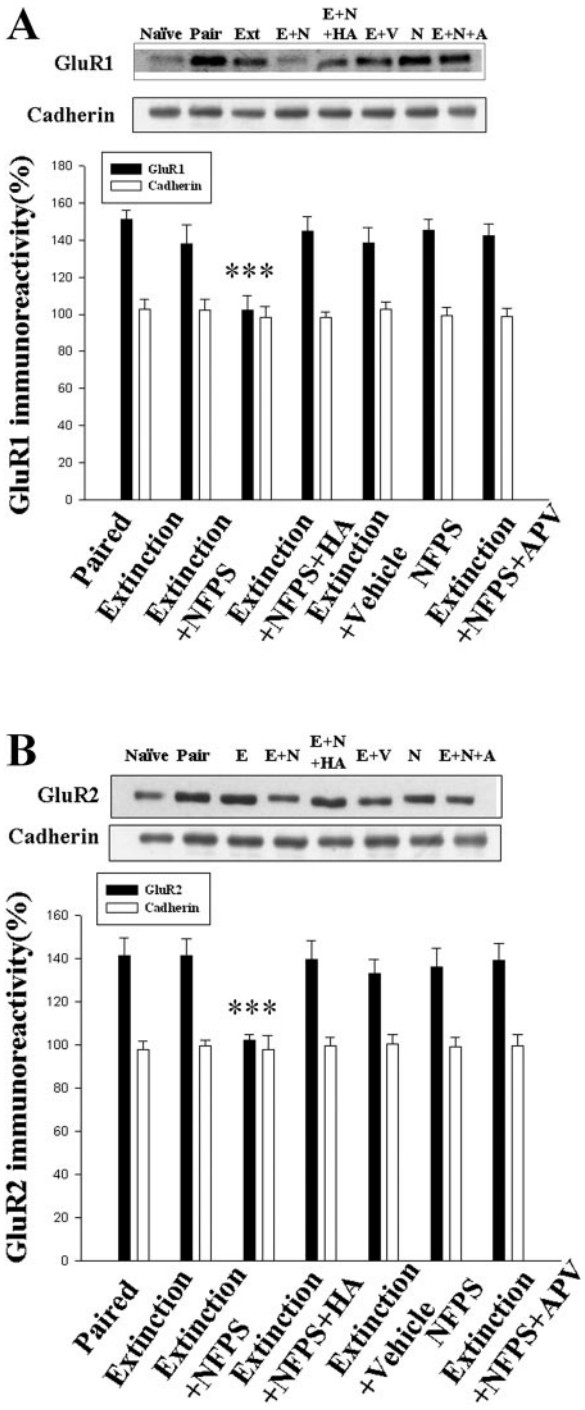

Fig. 5. Extinction training plus NFPS reverses the conditioning-induced increase in the surface expression of GluR1 and GluR2. Rats were conditioned and randomly assigned to three groups: Extinction, Extinction + vehicle, and Extinction + NFPS on day 1. On day 2, the rats in the Extinction group were given light-alone trials (extinction training), whereas the Extinction + NFPS rats were infused with NFPS (5 μg/side) bilaterally into the amygdala 30 min before extinction training. Twenty-four hours after extinction training, LA and BLA tissues were dissected out, and the surface GluR1 (A) ($n = 6$ per group) and GluR2 (B) ($n = 6$ per group) levels were determined using biotin labeling. A separate group of rats was infused with HA-966 (6 mg/kg i.p.) 30 min before NFPS; 30 min later, light-alone trials were administered. Cotreatment of NFPS with light-alone trials blocked the conditioning-induced increases in the surface expression of GluR1 and GluR2. The GluR1 and GluR2 levels in the conditioned animals were expressed as a percentage of those of the naïve controls that did not undergo light-shock pairings. ***, $P < 0.001$ versus Extinction.

GluR1 and traffics GluR1 into the spine (Rumbaugh et al., 2003; Schlüter et al., 2006). In the LA, the coupling of A-kinase-anchoring proteins and protein kinase A (PKA) to GluR1 through SAP97 is essential for memory formation (Moita et al., 2002). We therefore examined whether SAP97 expression was involved in the extinction. Figure 6B shows that NMDA (20 μM) application caused a reduction in the SAP97 level (68.9 $\pm$ 5.9%, $P < 0.001$ versus control), whereas a lower concentration of NMDA (10 μM) had no effect (98.5 $\pm$ 6.2%). In the presence of NFPS (1 μM), 10 μM NMDA induced a significant loss of SAP97 (68.0 $\pm$ 6.0%, $P < 0.01$ versus NMDA 10 μM). NPFS alone did not affect the SAP97 level (95.4 $\pm$ 6.0%).

We examined whether NFPS affected the levels of PSD-95 and SAP97 after extinction training. Rats were conditioned with 10 light-shock pairings and were randomly assigned to 4 groups: Paired, Extinction, Extinction + NFPS, and Extinction + vehicle, as described under *Materials and Methods*. LA and BLA tissues were dissected out 24 h after extinction training, and the levels of PSD-95 and SAP97 were determined. Extinction training did not significantly influence the conditioning-induced increase in PSD-95 ($P = 0.68$); however, the PSD-95 level was significantly lower in the NFPS-treated rats compared with the levels in the vehicle-treated control and Extinction groups [$F_{(2,15)} = 26.89$, $P < 0.001$] (Fig. 6C). Treatment with HA-966 (6 mg/kg i.p.) 30 min before the administration of NFPS prevented the effect of NFPS, such that the level of PSD-95 in the HA-966 plus NFPS-treated rats was not significantly different from that of rats that received extinction training only ($P > 0.5$). Furthermore, NFPS-treated rats that did not undergo extinction training had a PSD-95 level comparable with those of Paired or Extinction rats ($P > 0.5$), suggesting that NFPS by itself did not influence the PSD-95 level. We also probed the level of SAP97. As shown in Fig. 6D, conditioning increased the SAP97 expression level to 189.2 $\pm$ 14.2% that of the naïve controls. Light-alone trials 24 h after training did not significantly influence the increase in SAP97 expression (185.7 $\pm$ 12.1%, $P > 0.5$); however, infusion of NFPS (5 μg/side) into the amygdala abolished the conditioning-induced increase in SAP97 (101.9 $\pm$ 11.2%, $P < 0.001$ versus Extinction). Administration of NFPS without extinction training had no effect on the increase in the SAP97 level (197.3 $\pm$ 13.9%). Treatment with D-APV prevented the effect of NFPS, such that the level of SAP97 in the D-APV plus NFPS-treated rats was not significantly different from the levels in the rats that underwent extinction training only (208.4 $\pm$ 12.7%, $P > 0.1$).

Experiment 4: Effect of NFPS on the AMPA/NMDA Ratio. To establish whether the decrease in surface receptors by extinction plus NFPS resulted in changes in excitatory synaptic transmission, we measured the relative contributions of AMPA receptors and NMDA receptors to the EPSCs, a method that has been shown to be a sensitive assay for detecting differences in glutamatergic synaptic strength (Ungless et al., 2001). Rats were conditioned and 24 h later were injected intraperitoneally with NFPS (1 or 10 mg/kg) or vehicle 30 min before light-alone trials. The rats were tested for fear-potentiated startle 24 h after extinction training. ANOVA for startle scores showed a significant effect for the group [$F_{(3,21)} = 51.15$, $P < 0.001$], and Newman-Keuls post hoc tests showed that the high-dose NFPS group differed

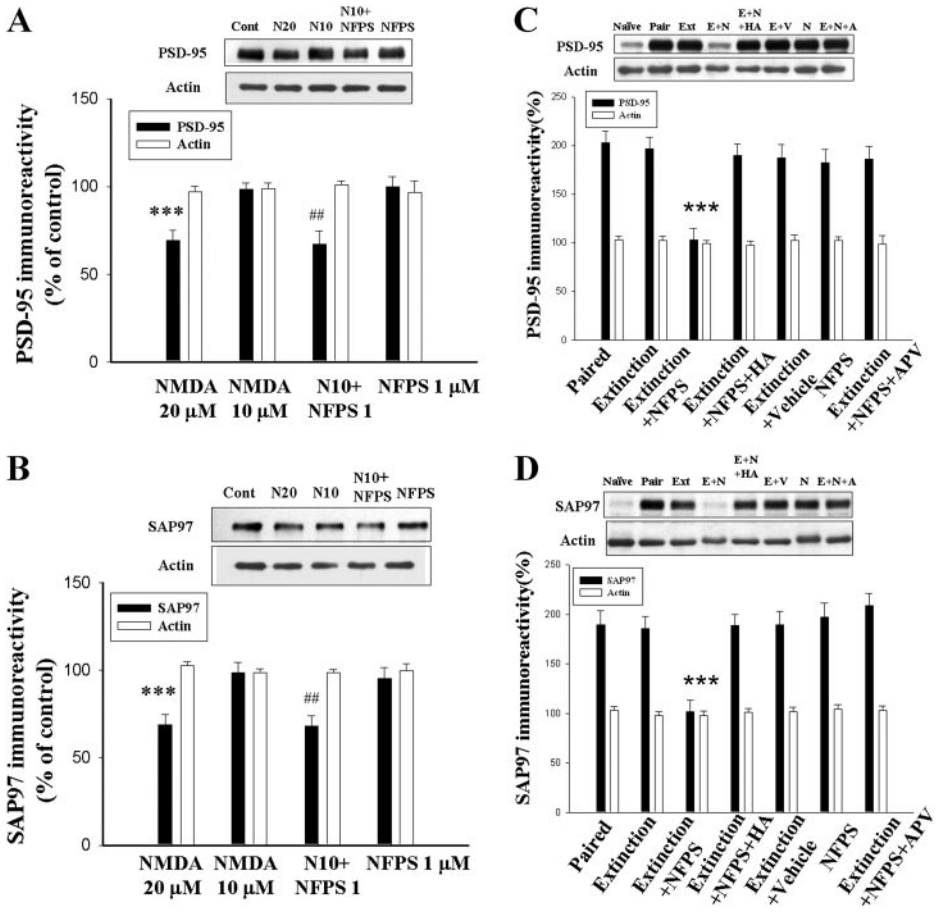

Fig. 6. NFPS facilitates NMDA-induced reduction in the cellular levels of PSD-95/SAP97 and reverses the conditioning-induced increase in PSD-95/SAP97 in conjunction with extinction training. Amygdala slices were treated with 20 μM NMDA, 10 μM NMDA, or 10 μM NMDA plus 1 μM NFPS for 3 min. One hour later, LA and BLA tissues were dissected out, and PSD-95 (A) ($n = 6$ per group) and SAP97 (B) ($n = 6$ per group) levels were determined by Western blotting analysis. Control slices were incubated in ACSF for 1 h. ***, $P < 0.001$ versus Control; ##, $P < 0.01$ versus NMDA 10 μM. C and D, rats were conditioned and randomly assigned to three groups: Extinction, Extinction + vehicle, and Extinction + NFPS on day 1. On day 2, the rats in the Extinction group were given light-alone trials (extinction training), whereas the Extinction + NFPS rats were infused with NFPS (5 μg/side) bilaterally into the amygdala 30 min before extinction training. Twenty-four hours after extinction training, LA and BLA tissues were dissected out, and PSD-95 (C) ($n = 6$ per group) and SAP97 (D) ($n = 6$ per group) levels were determined. A separate group of rats was infused with HA-966 (6 mg/kg i.p.) 30 min before NFPS; 30 min later, light-alone trials were given. Cotreatment of NFPS with light-alone trials blocked the conditioning-induced increases in PSD-95 and SAP97. PSD-95 and SAP97 levels in the conditioned animals were expressed as a percentage of those in the naïve controls that did not undergo light-shock pairings. ***, $P < 0.001$ versus Extinction.

from the vehicle group ($P < 0.05$) (Fig. 7A). This result was consistent with the facilitation of extinction by NFPS.

One hour after the test, amygdala slices were taken from rats of the four groups (Naïve, Paired, Extinction + vehicle, Extinction + NFPS). The AMPA/NMDA ratio was 1.26 ± 0.11 in slices from the naïve rats; this ratio was significantly higher in the Paired rats (3.35 ± 0.11, $P < 0.001$, unpaired t test), suggesting that fear conditioning persistently increased AMPA-mediated synaptic transmission. There was no difference in the AMPA/NMDA ratio between the Paired and vehicle-treated extinction rats (3.56 ± 0.29, $P > 0.1$), suggesting that extinction did not affect the conditioning-induced increase in the AMPA/NMDA ratio. However, in the NFPS-treated extinction rats, the AMPA/NMDA ratio (1.17 ± 0.15) was significantly lower than that of the vehicle-treated extinction rats ($P < 0.001$) (Fig. 7B). It is noteworthy that there was no difference in the AMPA/NMDA ratio between the naïve and NFPS-treated extinction rats ($P > 0.5$). These results suggest that extinction failed to affect the conditioning-induced increase in the AMPA/NMDA ratio. Only in the presence of NFPS did extinction training reverse this increase.

$GluR2_{3Y}$, a synthetic peptide containing a short C-terminal sequence of GluR2 ($_{869}$YKEGYNVYG$_{877}$), which is critical for the expression of hippocampal CA1 LTD (Ahmadian et al., 2004), has been shown to block LTD, depotentiation, and AMPA receptor endocytosis in the hippocampus, nucleus accumbens, and amygdala (Ahmadian et al., 2004; Brebner et al., 2005; Kim et al., 2007). When $GluR2_{3Y}$ was fused to the cell membrane transduction domain of the HIV-1 Tat protein (Tat-$GluR2_{3Y}$), it became membrane-permeable and was able to impair the extinction of fear memory (Kim et al., 2007; Dalton et al., 2008). We performed behavioral assessment to determine whether Tat-$GluR2_{3Y}$ influenced the effect of NFPS. Rats were divided into four groups: rats that received Tat-$GluR2_{3Y}$ (15 pmol in 0.8 μl of saline per side) or Tat-$GluR2_{3A}$($_{869}$AKEGANVAG$_{877}$); and a control peptide that had no effect on AMPA receptor endocytosis followed by NFPS (10 mg/kg, i.p.) or vehicle 30 min before extinction training. Two-way ANOVA revealed no group effect [Tat-$GluR2_{3Y}$ versus Tat-$GluR2_{3A}$, $F_{(1,20)} = 3.29$, $P = 0.08$] or effects of drug treatment [NFPS versus vehicle, $F_{(1,20)} = 1.48$, $P = 0.24$] but a significant effect of interaction [$F_{(1,20)} = 4.75$, $P < 0.05$]. Post hoc comparisons revealed that the startle potentiation in the Tat-$GluR2_{3A}$-NFPS rats was significantly less than that in the Tat-$GluR2_{3Y}$-NFPS rats ($P < 0.05$). Furthermore, the startle potentiation in the Tat-$GluR2_{3Y}$-vehicle rats was comparable with that in the Tat-$GluR2_{3A}$-vehicle rats ($P = 0.81$), indicating that Tat-$GluR2_{3Y}$ had no effect in the absence of NFPS. It is noteworthy that in the Tat-$GluR2_{3Y}$ rats, the startle potentiation in the NFPS-treated rats was comparable with that in the vehicle-treated rats ($P = 0.61$), indicating that Tat-$GluR2_{3Y}$ specifically blocked the effect of NFPS on extinction (Fig. 8).

Discussion

From a classic viewpoint, extinction results from a positive cognitive enhancement of CS-no-US learning concomitant with the increased inhibitory tone coming from ventromedial

PFC projection neurons to the amygdala (Myers and Davis, 2002; Quirk et al., 2003; Maren and Quirk, 2004). Extinction can also be mediated by an increase in the inhibitory transmission within the amygdala, probably via modulation of the $GABA_A$ receptor (Chhatwal et al., 2005). In the present study, we combined behavioral, biochemical, and electrophysiological experiments to explore the effects of GlyT1 blockers on fear extinction. We showed for the first time that 1) the GlyT1 blocker NFPS selectively enhanced the NMDA receptor-mediated synaptic response in the amygdala; 2) NFPS augmented extinction without exerting a general effect on the startle reflex; 3) the NFPS-treated rats exhibited partial reinstatement after the US presentations; 4) NMDA at a concentration that is normally ineffective reduced the surface expression of GluR1 and GluR2 and the levels of PSD-95 and SAP97 in the presence of NFPS; 5) NFPS plus extinction training reversed the conditioning-induced increases in PSD-95 and SAP97, and in the surface expression of GluR1 and GluR2; 6) NFPS treatment in conjunction with extinction training reversed the conditioning-induced AMPA/NMDA ratio; and 7) $GluR2_{3Y}$, a synthetic peptide that is critical for the expression of hippocampal CA1 LTD, specifically blocked the effect of NFPS on extinction. These results suggest that NFPS augments extinction via enhancement of NMDA-mediated AMPA receptor endocytosis in the amygdala. It is noteworthy that DCS, which binds at the glycine-binding site of the NMDA receptor, produced similar facilitation of extinction, reversal of GluR1/2 expression, and less reinstating effect (Ledgerwood et al., 2004). These results suggest that agents augmenting NMDA responses may be useful in the treatment of anxiety disorders.

NFPS Potentiates NMDA Receptor-Mediated Synaptic Response. Synaptic transmission in the internal capsule-LA is mediated primarily by AMPA receptors. In this study, we showed that exogenous application of the GlyT1 inhibitor NFPS increased fEPSP and that this effect was not seen in the presence of an NMDA receptor antagonist. In addition, the pharmacologically isolated NMDA receptor-me-

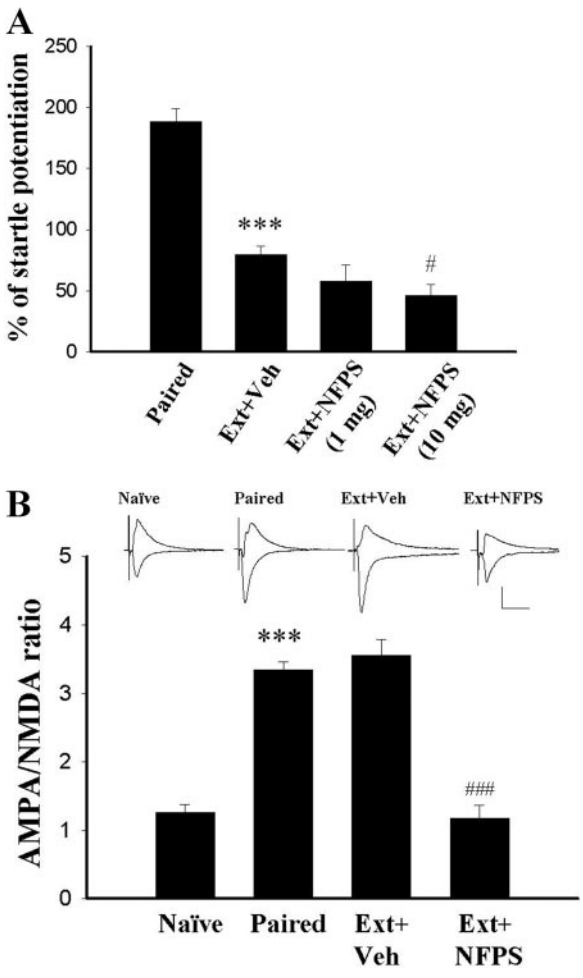

Fig. 7. Extinction training plus NFPS reverses the conditioning-induced increase in the AMPA/NMDA ratio. A, rats received 10 light-shock pairings and 24 h later were injected intraperitoneally with NFPS (1 or 10 mg/kg) or vehicle 30 min before extinction training (3 blocks of 10 presentations of light-alone trials). Retention of memory was assessed 24 h after extinction training. ***, $P < 0.001$ versus Paired; #, $P < 0.05$ versus Extinction + Vehicle. B, plot of the AMPA/NMDA ratio in slices taken from Naïve, Paired, Extinction + Vehicle, and Extinction + NFPS (10 mg/kg i.p.) animals ($n = 6$ per group). ***, $P < 0.001$ versus Naïve; ###, $P < 0.001$ versus Extinction + Vehicle scale, 50 ms, 100 pA.

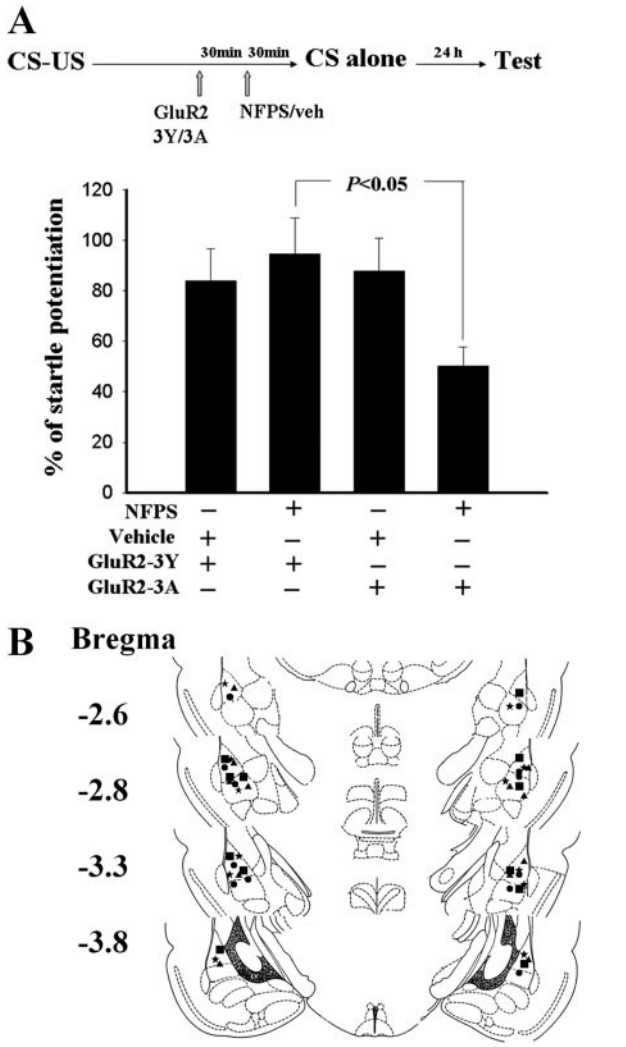

Fig. 8. Tat-$GluR2_{3Y}$ specifically blocks the effect of NFPS on extinction. A, rats were divided into four groups: those that received Tat- $GluR2_{3Y}$ (15 pmol in 0.8 μl of saline per side) or Tat-$GluR2_{3A}$, followed by NFPS (10 mg/kg, i.p.) or vehicle administered bilaterally into the amygdala 30 min before extinction training. Retention of memory was assessed 24 h after extinction training. B, the cannulae tip placements in the rats infused with Tat-$GluR2_{3Y}$ + NFPS (●), Tat-$GluR2_{3Y}$ + vehicle (▲), Tat-$GluR2_{3A}$ + NFPS (■), or Tat-$GluR2_{3A}$ + vehicle (★) 30 min before extinction training.

diated response was enhanced by NFPS. These results demonstrate that the glycine concentration within the synaptic regions of the amygdala is strongly regulated by glycine transporters and under normal conditions is not sufficient to saturate the glycine-binding site of the NMDA receptor. Thus, the blocking of GlyT1 selectively enhances the NMDA response without affecting the AMPA receptor-mediated synaptic response.

Mechanism of Augmented Extinction by NFPS. In hippocampal and cortical neurons, a brief application of NMDA induces LTD that is associated with a reduction in surface GluR1 (Lee et al., 1998; Beattie et al., 2000). Using a similar procedure, we found that NMDA at a concentration that is normally unable to elicit a long-term effect reduced the surface expression of GluR1 and GluR2 in the presence of NFPS. NFPS per se had no effect on the conditioning-induced GluR1 expression; only in conjunction with extinction training did NFPS reverse the increased GluR1 expression. These results are consistent with the idea that a threshold activation of NMDA receptors and subsequent Ca^{2+} influx must be reached to trigger endocytosis of AMPA receptors. In this way, NFPS specifically produced cue-induced extinction rather than exerting a general reduction of fear.

In behavioral tests, Tat-GluR2$_{3Y}$, a synthetic peptide that is critical for the expression of hippocampal CA1 LTD, blocked only the additional reduction caused by NFPS treatment, rather than returning the fear potentiation level to that of fear-conditioned animals that had not undergone extinction. These results suggest that extinction training-induced inhibitory learning is intact, and NFPS only adds a bit of erasure. NFPS augments extinction via the facilitation of NMDA receptor activation, which induces Ca^{2+} influx, resulting in the activation of calcineurin. Calcineurin activation leading to local reorganization of F-actin causes displacement of PKA from the synapse, which may initiate endocytosis of receptors and trigger a loss of GluR1 and GluR2 from the surface of neurons (Gomez et al., 2002; Snyder et al., 2005).

PSD-95 is a prominent postsynaptic density molecule that interacts indirectly with AMPA receptors through the transmembrane protein stargazin (Chen et al., 2000) and regulates the trafficking and localization of AMPA receptors at synapses (El-Husseini Ael et al., 2002; Schnell et al., 2002; Beique and Andrade, 2003). In the present study, we showed that the PSD-95 level was down-regulated in response to NMDA plus NFPS stimulation, which suggests that the degradation of GluR-interacting proteins leads to destabilization of the receptor and its subsequent endocytosis. In the LA, the coupling of A-kinase-anchoring proteins and PKA to GluR1 through SAP97 is essential for memory formation (Moita et al., 2002). Here, we provide evidence suggesting that SAP97 is also involved in extinction, because the level of SAP97 decreased in response to NFPS stimulation, leading to augmentation of extinction.

Two recent reports showed that extinction reversed both the enhanced synaptic efficacy observed in conditioned rats and the conditioning-induced enhancement of surface expression of AMPA receptors (Kim et al., 2007; Dalton et al., 2008), implying the unlearning of extinction. In contrast to these reports, we found that extinction training failed to influence the conditioning-induced increase in the surface expression of GluR1 and GluR2, as well as the AMPA/NMDA ratio. Only in the presence of NFPS did extinction training reverse these changes. In addition, the NFPS-treated rats exhibited partial reinstatement after US-alone presentations. The reasons for the difference are not known but could be attributable to the different tests (freezing versus fear-potentiated startle) used. The extinction protocol in the recent reports consisted of 20 tone presentations, each 20 to 30 s in duration, whereas we used 30 light presentations lasting 3.7 s. The more extensive extinction protocol used in the previous studies may have reached the threshold for the activation of NMDA receptors and subsequent Ca^{2+} influx to trigger endocytosis of AMPA receptors.

In summary, facilitation of extinction could result from a positive cognitive enhancement of CS-no-US learning or an erasure of the original CS-US association. In the present study, we found that coadministration of NFPS with extinction training facilitated extinction and reversed the conditioning-induced increase in GluR1 and GluR2, as well as the AMPA/NMDA ratio. In this context, NFPS in combination with extinction training does erase the original excitatory association. Because high-affinity glycine transporters are usually located around the synapses, glycine may normally exist at nonsaturating concentrations within the synaptic regions. Therefore, it would be beneficial to treat patients with post-traumatic stress disorder with a glycine transporter blocker in conjunction with exposure therapy.

Acknowledgments

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