Behavioral/Systems/Cognitive

$GABA_A$ Receptor Endocytosis in the Basolateral Amygdala Is Critical to the Reinstatement of Fear Memory Measured by Fear-Potentiated Startle

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Reinstatement represents a phenomenon that may be used to model the effects of retraumatization observed in patients with posttraumatic stress disorder (PTSD). In this study, we found intraperitoneal injection of the β-adrenergic receptor antagonist propranolol (10 mg/kg) 1 h before reinstatement training attenuated reinstatement of fear memory in rats. Conversely, reinstatement was facilitated by intra-amygdalar administration of β-adrenergic receptor agonist isoproterenol (Iso; 2 μg per side) 30 min before reinstatement training. The frequency and amplitude of the miniature IPSC (mIPSC) and the surface expression of the β3 and γ2 subunits of the $GABA_A$ receptor ($GABA_AR$) were significantly lower in reinstated than in extinction rats, whereas the AMPA/NMDA ratio and the surface expression of GluR1 and GluR2 in the amygdala did not differ between groups. In amygdala slices, Iso-induced decrease in the surface β3 subunit of $GABA_A$ receptor was blocked by a Tat-conjugated dynamin function-blocking peptide (Tat-P4) pretreatment (10 μM for 30 min). By contrast, Tat-scramble peptide had no effect. Intravenous injection (3 μmol/kg) or intra-amygdalar infusion (30 pmol per side) of Tat-P4 interfered with reinstatement. Reinstatement increased the association between protein phosphatase 2A (PP2A) and the β3 subunit of the $GABA_AR$, which was abolished by PP1/PP2A inhibitors okadaic acid and calyculin A. These results suggest the involvement of β-adrenergic receptor activation and $GABA_A$ receptor endocytosis in the amygdala for the reinstatement in fear memory.

Introduction

Reinstatement represents a phenomenon in patients with posttraumatic stress disorder (PTSD) in whom reexposure of the trauma evokes inappropriate fear responses despite successful reduction of fear through exposure psychotherapy (Rescorla and Heth, 1975; Laurent and Westbrook, 2009). Reinstatement together with extinction (Ext) suggests that the conditioned response acquired when a subject is exposed previously to a conditioned stimulus (CS) that was associated with an unconditioned stimulus (US) is a dynamic process. For example, if a conditioned animal was exposed to the CS without pairing with a US, previously acquired responses would be extinguished (Pavlov, 1927; Rescorla, 2001; Myers and Davis, 2002). As the extinguished response could be reinstated after experience of one or more US-only presentations, it is suggested that extinction is an active learning that inhibits the expression of the original association rather than erasing it (Pavlov, 1927; Bouton and Bolles 1979; Sotres-Bayon et al., 2004; Quirk et al., 2006; Myers and Davis, 2007). In sharp contrast with extensive studies on the neuronal circuitry and neurochemical mechanisms leading to memory extinction, few studies have focused on memory reinstatement. The effective therapeutic method by which to block fear reinstatement remains elusive.

In the lateral amygdala nucleus (LA) as well as in the cortex and hippocampus, fast synaptic inhibition is mainly mediated by type A GABA receptors ($GABA_ARs$). $GABA_ARs$ are Cl^- permeable heteropentameric ligand-gated ion channels that can be assembled from seven classes of homologous subunits with the most common subtypes in the brain being composed of α, β, and γ_2 subunits (Whiting et al., 1999). Recently, we found that fear conditioning decreased the frequency and amplitude of mIPSC and the surface protein levels of the β2 and γ2 subunits of the $GABA_A$ receptor. Extinction training, on the other hand, reversed the decreased frequency and amplitude of mIPSC and the surface protein levels of gephyrin and the β2 subunit of the $GABA_A$ receptor (Lin et al., 2009). In addition, inhibition of $GABA_A$ receptor insertion in the amygdala significantly impaired extinction. In the present study, we tested the hypothesis that restoration of fear response is associated with endocytosis of the $GABA_A$ receptor, and that disruption of $GABA_A$ receptor endocytosis will block the reinstatement of fear response.

The amygdala receives a dense norepinephrine (NE) innervation originating primarily in the locus ceruleus (LC) (Asan 1998). Stress elicits activation of LC neurons (Rasmussen et al., 1986; Abercrombie and Jacobs, 1987; Grant et al., 1988; Aston-Jones et al., 1991), which mediates the stress-induced release of NE in the amygdala (Galvez et al., 1996; Hatfield et al., 1999; Buffalari and

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Grace, 2007). A previous study using single-unit recordings showed that the firing rate of many amygdala neurons gradually increased after a footshock, peaking at 30–50 min after shock and then subsiding to baseline levels 2 h later. During this period of increased activity, the discharges of simultaneously recorded amygdala cells were more synchronized than before the shock (Abercrombie and Jacobs, 1987; Aston-Jones et al., 1991). Indeed, arousal-evoked NE release strengthens the acquisition of aversive memories via activation of β-adrenergic receptors, and blocking of β-adrenergic receptors results in the loss of this enhancement (McGaugh, 2000; Sara, 2009). In the present study, we investigated whether the β-adrenergic receptor was activated when the extinguished response was reinstated by exposing the animals to unconditional stimuli (footshocks). Rats were trained with 10 light–shock pairings on day 1, and memory retention was assessed 24 h later. One hour after the test, rats received 30 light (CS)-alone trials (extinction training), and fear-potentiated startle was measured 24 h later. The next day, the rats were administered 10 US-alone trials (reinstatement training), and retention of memory was tested 24 h later. We found previously unrecognized roles of β-adrenergic receptor and $GABA_A$ receptor endocytosis in the reinstatement of fear memory.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Cheng-Kung University. Animals were housed in cages of four rats each in a temperature-controlled (24°C) animal colony, and pelleted rat chow and water were available *ad libitum*. The animals were maintained in a 12 h light/dark cycle with lights on at 7:00 A.M. All behavioral procedures took place during the animal light cycle.

Male Sprague Dawley rats (175–200 g), anesthetized with sodium pentobarbital (50 mg/kg, i.p.), were mounted on a stereotaxic apparatus and a cannula made of 22 gauge stainless-steel tubing was implanted into the 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.

Drugs

The amino acid sequence for the dynamin inhibitory peptide (P4) is QVPSRPNRAP, and for the Scrambled control peptide (S) it is QPPASNPRVR. Tat-conjugated peptide designed to block the binding of dynamin to amphiphysin and thus preventing endocytosis (Gout et al., 1993; Lissin et al., 1998) was used. Tat-P4 or Tat- S (30 pmol per side for amygdalar injection or 3 μmol/kg, i.v.; Kelowna International Scientific) was dissolved in 0.9% NaCl. Isoproterenol (Iso; 2 μg per side for amygdalar injection; Sigma-Aldrich) was dissolved in 0.9% NaCl. Propanolol (10 mg/kg, i.p., or 5 μg per side for amygdalar injection; Tocris Bioscience) was dissolved in 0.9% NaCl. Okadaic acid (10 ng per side for amygdalar injection; Sigma-Aldrich) was dissolved in 10% DMSO. Calyculin A (5 pmol per side for amygdalar injection; Tocris Bioscience) was dissolved in 50% DMSO. Tat-P4, Tat-S, isoproterenol, okadaic acid, or calyculin A was infused into the amygdala bilaterally 30 min before reinstatement training. 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. Tat-P4, Tat-S, or propranolol was administered for system injection 60 min before reinstatement training. Rats were given intra-amygdalar injections of propranolol 30 min before Iso injection. Propranolol (10 mg/kg, i.p.) was administered 1 h before reinstatement training at a dose that was sufficient to block reconsolidation of spatial and fear memory in rats (Przybyslawski et al., 1999; Debiec and Ledoux, 2004).

Behavioral apparatus and procedures

Rats were trained and tested in a stabilimeter device. Behavioral experiments of fear conditioning and extinction training were performed in standard operant chamber (San Diego Instrument). The acoustic startle stimulus was a 50 ms 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 stabilimeter. The US was a 0.65 mA footshock with duration of 0.5 s. 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 d, the rats were handled in the same chamber before fear conditioning for preexposure. During preexposure, baseline startle was measured on each of 2 d by presenting 30 startle stimuli at a 10 s interstimulus interval (ISI). Rats with equivalent baseline mean startle amplitudes were then divided into separated matched groups. On the day of fear conditioning, each animal was brought to the room, allowed to habituate, and placed in the chamber as before. The CS–US pairing began after a 3 min acclimation period in the chamber.

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

Test. Twenty-four 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 (ISI, 30 s).

Extinction training. One hour after test 1, rats were returned to the stabilimeter and administered three 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 ISI of 1 min.

Reinstatement. Animals were returned to the testing chamber 24 h after test 2 and administered 10 footshocks. The animals then underwent a test for memory reinstatement 24 h after the footshocks (test 3).

Experiment 1: reinstatement of fear response

On day 1, rats were trained with 10 light–shock pairings, and memory retention was assessed 24 h later (day 2, test 1). One hour after test 1, the rats received 30 light (CS)-alone trials, and fear-potentiated startle was measured 24 h later (day 3, test 2). In the present study, we defined the experimental procedures of the CS-alone trials as extinction training. The decrement in the conditioned response measured 24 h after extinction training was defined as extinction retention (Myers and Davis, 2002). Similarly, we defined the experimental procedures of the US-alone trials as reinstatement training. The restoration of fear-potentiated startle measured 24 h after reinstatement training was taken to be the reinstatement retention. On day 4, the rats were given 10 US-alone trials, and retention of memory was tested 24 h later (day 5, test 3).

Experiment 2: effect of reinstatement training on the excitatory and inhibitory transmission

All rats were killed at day 5 (1 h after test 3), and whole-cell patch-clamp recordings were made from the soma of visually identified pyramidal-like neurons located in the LA of the amygdala slices. AMPA receptor (AMPAR)-mediated EPSC was evoked when the neurons were voltage clamped at −70 mV, whereas NMDA receptor (NMDAR)-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). To isolate $GABA_A$ receptor-mediated IPSC, glutamatergic synaptic transmission was blocked by the NMDA receptor antagonist D-AP-5 (50 μM) and the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) at a holding potential of −70 mV. The stimulating electrode was placed in the LA to directly stimulate interneurons.

Whole-cell patch-clamp recordings

Rats were decapitated, and the brains were hemisected and cut transversely. Transverse slices (400 μm thick) were cut, and the appropriate slices were placed in a beaker of oxygenated artificial CSF (ACSF) at room temperature for at least 1 h before recording. The ACSF solution was of the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2

$MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose. The ACSF was bubbled continuously with 95% O_2/5% CO_2 and had a pH of 7.4.

Whole-cell patch-clamp recordings from LA projection neurons were performed at ~32°C in a superfusing chamber. Neurons were visualized with infrared video microscope using a 40× water-immersion objective on an upright microscope. Patch electrodes were pulled from a thick-wall glass capillary (1.17 mm inner diameter, 1.5 mm outer diameter) to a tip resistance of 3–5 MΩ. The internal solution contained the following (in mM): 140 K or Cs-gluconate, 10 KCl or CsCl, 1 EGTA, 10 phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP, and 10 HEPES. The final pH of the internal solution was adjusted to 7.3 by adding 1 M KOH or CsOH; the final osmolarity was adjusted to 280 mOsm by adding sucrose. To record neuronal properties and action potentials, we used a K-based internal solution, whereas for recording mIPSCs, Cs-based internal solution was used. Records were low-pass filtered at 2.5–20 kHz and digitized at 5–50 kHz. The signal was monitored and recorded using an Axopatch 200B amplifier. The stimulation electrode (SNE-100; Kopf Instruments) was positioned at the internal capsule to elicit monosynaptic EPSCs at LA neurons (Fourcaudot et al., 2008; Smith et al., 2000). Electrical stimuli (150 μs in duration) were delivered at a frequency of 0.05 Hz. $GABA_A$ receptor-mediated mIPSCs were isolated in the presence of CNQX (10 μM) and D-2-amino-5-phosphonovalerate (D-APV; 50 μM). The access resistance was monitored continuously throughout recordings; neurons were discarded if the access resistance exceeded 25 MΩ.

Surface biotinylation and Western blot analysis of the β3 and γ2 subunits of the $GABA_A$ receptor

Surface biotinylation and Western blot analysis were performed as described previously (Mao et al., 2006). Surface GluR1 and GluR2 subunits of the AMPA receptor and surface β3 and γ2 subunits of the $GABA_A$ receptor were detected by a biotinylation assay followed by Western blot analysis using an anti-GluR1(1:2000; Alomone Labs) and an anti-GluR2 (1:2000; Millipore) of AMPA receptor, and an anti-β3 subunit (1:1000; Millipore Bioscience Research Reagents) and anti-γ2 subunit of the $GABA_A$ receptor (1:1000; Millipore Bioscience Research Reagents), respectively, followed by an 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.

Experiment 3: effects of β-adrenergic receptor agonist and antagonist on the reinstatement of fear memory

Rats were conditioned and then extinction trained. Rats received saline or propranolol (10 mg/kg, i.p.) 1 h before reinstatement training at a dose that is sufficient to block reconsolidation of spatial and fear memory in rats (Przybyslawski et al., 1999; Debiec and Ledoux, 2004). To determine whether β-adrenergic receptor activation facilitated reinstatement, we used an experimental protocol identical to that described previously except that there were 5 instead of 10 US-alone trials to avoid the ceiling effect. β-Adrenergic receptor agonist isoproterenol (2 μg per side) was administered by intra-amygdalar injection 30 min before reinstatement training.

Experiment 4: effects of a dynamin function-blocking peptide (Tat-P4) on the surface expression of $GABA_A$ and glutamate receptors

In the initial experiment, we verified the effect of Tat-P4 on the Iso-induced decrease in surface $GABA_A$ receptors in the *in vitro* amygdala. Amygdala slices were incubated with ACSF, Tat-P4(10 μM), or Tat-scramble peptide (10 μM) for 30 min, and Iso was then added and incubated with the slices for 10 min. In the next experiment, Tat-P4 (30 pmol per side), scramble peptide (30 pmol per side), or saline was infused into the amygdala 60 min before reinstatement training. Retention of memory was tested 24 h later. Finally, to keep the integrity of the amygdala for the whole-cell recordings, saline, Tat-P4 (3 μmol/kg), or Tat-scramble peptide (3 μmol/kg) was administered intravenously 60 min before reinstatement training.

Experiment 5: effect of reinstatement training on the association of $GABA_AR$ with AP2

LA and BLA extracts from Ext and reinstated rats were immunoprecipitated with an antibody against the β subunit of AP2 and then immunoblotted with an antibody against the $GABA_AR$ β3 subunit. To determine whether reinstatement required PP2A activity, rats were administered an intra-amygdalar injection of PP1/PP2A inhibitor okadaic acid (10 ng per side) or vehicle (10% DMSO) 30 min before reinstatement training.

Immunoprecipitation

The LA and BLA subregions of the amygdala were dissected out and synaptoneurosome was prepared as described previously (Mao et al., 2006). Synaptoneurosome lysate was immunoprecipitated with anti-AP2 α subunit (2 μg; Novus Biologicals) antibodies, anti-β3 subunit (2 μg; IMGENEX) antibodies, or IgG overnight. The antibody-bound complexes were incubated with protein G agarose (Sigma) for 2 h at 4°C. The agarose beads were pelleted by centrifugation and washed three times using lysis buffer, and the immune complexes were eluted. Immunoprecipitated proteins were separated on 10% polyacrylamide gels by SDS-PAGE and then electrophoretically transferred onto nitrocellulose membrane. They were then blotted with anti-β3 subunit of the $GABA_A$ receptor and anti-PP2Ac (1:1000; Epitomics) 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.

Elevated plus maze

The elevated plus maze was made of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm) with a plus-shape platform. The apparatus was elevated 50 cm above the ground and had a 0.5 cm edge on the open arms. Rats were placed in the center of the maze facing the closed arm. During a 5 min test period, behavioral parameters were recorded and analyzed by a behavioral tracking system (Noldus Information Technology).

Data analysis

All data are presented as the mean ± SEM. Differences among multiple groups were evaluated either by two-way ANOVA or one-way ANOVA with Newman–Keuls *post hoc* test. In cases where comparisons between only two groups were of interest, unpaired *t* tests were performed. For all analyses, a p value of <0.05 indicated a statistically significant difference.

Results

US-alone trials elicit reinstatement of fear response

Rats in the paired group received 10 light–shock pairings, whereas the unpaired rats received 10 lights and 10 shocks in a pseudorandom manner. Paired ($n = 7$) and unpaired ($n = 6$) rats subsequently received extinction and reinstatement trainings as described in Materials and Methods. Figure 1 shows that paired rats exhibited significant fear-potentiated startle, which was not seen in the unpaired rats. As a result of extinction training, the difference between light and noise and noise alone in test 2 was significantly lower than in test 1 in paired rats ($t_{(12)} = 4.399$; $p < 0.001$). Finally in the paired rats, fear-potentiated startle in test 3 was significantly higher than that in test 2 ($t_{(12)} = 4.048$; $p < 0.01$), demonstrating the reinstatement of fear memory after presentations of US-alone trials.

Effect of reinstatement training on the excitatory transmission

Five groups (naive, unpaired, paired, extinction, and reinstated) of rats received behavioral training as described in Materials and Methods. Ext rats received 10 light–shock pairings followed 24 h later by 30 CS-alone trials. Reinstated rats had the same experimental protocol as the Ext rats except that they received an additional 10 US-alone trials. As shown in Figure 2*A*, the difference between light and noise and noise alone in the Ext rats was significantly lower than in the paired and reinstated rats ($F_{(2,57)} = 6.402$; $n = 20$ in each group; $p < 0.001$). All rats were killed at day 5 (1 h after test 3), and whole-cell patch-clamp recordings were made from the soma of visually identified pyramidal-like neu-

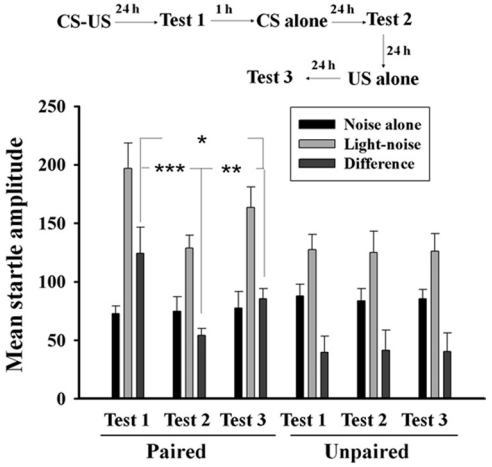

Figure 1. US-alone trials elicit reinstatement of fear response. Plot of noise alone, light and noise, and the difference of the two values in the unpaired and paired rats. Rats in the paired group received 10 light–shock pairings, whereas the unpaired rats received 10 lights and 10 shocks in a pseudorandom manner. Paired ($n = 7$) and unpaired ($n = 6$) rats subsequently received extinction and reinstatement trainings as described in Materials and Methods. Memory retention was assessed 24 h later (day 2, test 1). One hour after test 1, the rats received 30 extinction trials, and fear-potentiated startle was measured 24 h after extinction training (day 3, test 2). On day 4, the rats were given 10 US-alone trials, and retention of memory was tested 24 h later (day 5, test 3). *$p < 0.05$ versus test 1; **$p < 0.01$; ***$p < 0.001$ versus test 2.

rons located in the LA of the amygdala slices. Neurons were identified as projection neurons based on their morphology. In potassium gluconate-containing electrodes, these neurons were also identified by their intrinsic electrophysiological properties, in particular spike frequency adaptation in response to depolarizing current injection. Neither the resting membrane potential (V_m) nor the input resistance (R_{in}) of recorded LA neurons significantly differed across groups (data not shown). We measured the relative contribution of AMPARs and NMDARs to the EPSCs because this could minimize the effect of slice-to-slice variability (Ungless et al., 2001; Bellone and Luscher, 2006). As shown in Figure 2*B*, the AMPA/NMDA ratios were 1.78 ± 0.19 ($n = 7$) and 1.61 ± 0.28 ($n = 7$) in naive and unpaired rats, respectively. The ratio was significantly higher in the paired (3.79 ± 0.62; $n = 7$), Ext (4.09 ± 0.31; $n = 7$), and reinstated (3.92 ± 0.35; $n = 7$) rats ($F_{(4,30)} = 10.04$; $p < 0.001$). There were no differences in the AMPA/NMDA ratio among paired, Ext, and reinstated ($F_{(2,18)} = 0.10$; $p > 0.5$) rats, suggesting that extinction and reinstatement trainings did not affect the conditioning-induced increase in the AMPA/NMDA ratio. Furthermore, there were no differences in the reversal potentials of EPSCs among naive, unpaired, paired, Ext, and reinstated rats (Fig. 2*C*).

Consistent with the electrophysiological data, the levels of GluR1 and GluR2 expression were significantly higher in the paired, Ext, and reinstated rats than in the naive and unpaired rats ($p < 0.001$). In addition, there was no difference among the paired, Ext, and reinstated ($F_{(2,12)} = 0.04$; $p > 0.5$) rats (Fig. 2*D*,*E*). These results suggest that conditioning-induced increase in excitatory transmission in the LA was unaltered after reinstatement training.

To control the specificity of the surface binding, we blotted with antibody against actin, which is a cytoplasmic protein. However, we still could detect a trace of actin in the surface fraction, albeit at a much lower level compared to that of the total fraction (Fig. 2*F*). Indeed, actin is highly enriched at the PSD, where it anchors receptors by interacting with a cohort of scaffolding proteins (Kuriu et al., 2006; Cingolani and Goda, 2008).

Reduction of inhibitory transmission after reinstatement training

Five groups (naive, unpaired, paired, extinction, and reinstated) of rats were included in this experiment (Fig. 3*A*). All rats were killed at day 5 (1 h after test 3), and $GABA_A$ receptor-mediated IPSCs were recorded. The stimulating electrode was placed in the LA to directly stimulate interneurons. To isolate $GABA_A$ receptor-mediated IPSCs, glutamatergic synaptic transmission was blocked by the NMDA receptor antagonist D-AP-5 (50 μM) and the AMPA receptor antagonist CNQX (10 μM) at a holding potential of −70 mV. The input–output curve of the evoked IPSCs was obtained by recording 10 responses and averaging the recordings for each stimulation intensity tested. The intensity of stimulation was gradually increased from the threshold stimulus, determined in each individual experiment, to produce IPSCs of increasing amplitude. ANOVA of the input–output curves revealed that LA neurons from the extinction rats (11 neurons from five rats) had a significantly higher IPSC amplitude determined at a 4× threshold than those of the other groups ($F_{(4,45)} = 44.78$; $p < 0.001$). On the other hand, the IPSC amplitude in the reinstated rats (10 neurons from five rats) was significantly lower than that of the naive (10 neurons from five rats; $p < 0.01$) and unpaired rats (10 neurons from five rats; $p < 0.05$) (Fig. 3*B*). Importantly, the IPSC amplitude in the reinstated rats did not differ from that in the conditioned rats (nine neurons from six rats; $p > 0.05$).

We also analyzed the frequency and amplitude of mIPSCs from naive and trained rats. One-way ANOVA for the mIPSC frequency showed a significant group effect ($F_{(4,95)} = 22.92$; $p < 0.001$; $n = 20$ from 7 rats in each group), and Newman–Keuls *post hoc* tests revealed that the paired (2.34 ± 0.14 Hz; $n = 20$) and reinstated (2.18 ± 0.15 Hz; $n = 20$) groups differed from the Ext groups (5.04 ± 0.24 Hz; $n = 20$; $p < 0.001$) (Fig. 3*C*,*D*). Similarly, the amplitude of mIPSCs was lower in slices from the paired (27.3 ± 1.5 pA; $n = 20$) and reinstated animals (25.9 ± 1.4 pA; $n = 20$) than those from the extinction rats (53.3 ± 3.6 pA; $n = 20$; $F_{(4,95)} = 28.10$; $p < 0.001$). Moreover, reinstatement training appeared to reverse the extinction-induced increase in the frequency and amplitude of mIPSCs, bringing mIPSCs in the reinstated group to a level indistinguishable from that of the paired group ($p > 0.5$).

Five groups (naive, unpaired, paired, extinction, and reinstated) of rats were killed at day 5 (1 h after test 3), and the surface expression of the β3 subunit of the $GABA_A$ receptor was measured (Fig. 4*A*). The results revealed a significant effect of reinstatement ($F_{(2,12)} = 16.18$; $p < 0.001$). Newman–Keuls *post hoc* tests revealed that the paired and reinstated groups differed from the Ext groups ($p < 0.001$) (Fig. 4*B*). Similarly, the γ2 subunit level was lower in slices from the paired and reinstated animals than those from the Ext rats ($F_{(2,12)} = 11.84$; $p < 0.01$) (Fig. 4*C*). Together, these results suggest that reinstated rats displayed a lower frequency and lower amplitude of mIPSCs and reduced surface protein levels of the β3 and γ2 subunits of the $GABA_A$ receptor relative to the Ext rats.

Cue-conditioned fear is a context-dependent process. To control for possible effects of conditioning to the context, we used distinct contexts for conditioning and extinction training ($n = 6$). Acquisition (conditioning) took place in context A, whereas extinction and reinstatement trainings took place in context B. Figure 5*A* shows that the fear-potentiated startle of test 3 was significantly higher than that of test 2 ($t_{(10)} = 3.506$; $p < 0.01$), demonstrating the reinstatement of fear memory after presentations of US-alone trials. Furthermore, reinstatement training also

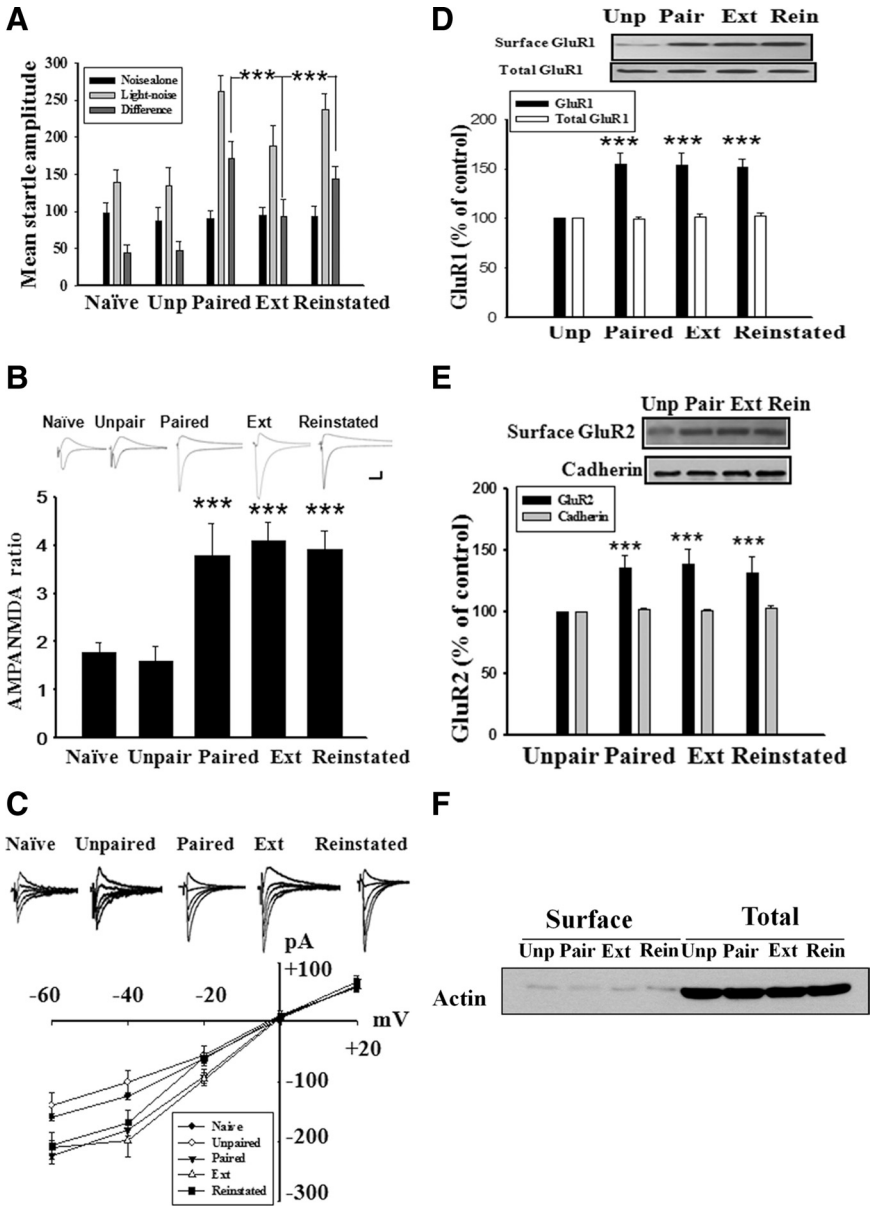

Figure 2. Conditioning-induced increase in excitatory transmission in the LA is unaltered after reinstatement. ***A***, Plot of noise alone, light and noise, and the difference of the two values in the naive, unpaired, paired, Ext, and reinstated (Rein) rats. ***B***, Plot of AMPA/NMDA ratio in naive, unpaired, paired, Ext, and reinstated rats ($n = 7$ from 7 rats in each group). ***$p < 0.001$ versus unpaired and naive rats. Calibration: 50 ms, 100 pA. ***C***, Plot of AMPA receptor-mediated EPSCs at −60, −40, −20, 0, and +20 mV in naive ($n = 7$, from 4 rats), unpaired ($n = 7$, from 4 rats), paired ($n = 7$, from 4 rats), Ext ($n = 7$, from 5 rats), and reinstated ($n = 7$, from 5 rats) rats. Calibration: 100 ms, 200 pA. ***D***, ***E***, LA and BLA tissues were dissected out from the naive, unpaired, paired, Ext, and reinstated rats, and the surface GluR1 (***D***) and GluR2 (***E***) levels were determined using cell surface biotinylation techniques. ***$p < 0.001$ versus unpaired. ***F***, Surface and total levels of actin were determined using antibody against actin.

reduced the surface expression of the β3 subunit of the GABA$_A$ receptor ($F_{(2,6)} = 29.5$; $n = 3$ in each group; $p < 0.001$) without affecting the surface expression of GluR1 ($F_{(2,6)} = 0.112$; $n = 3$ in each group; $p > 0.5$) (Fig. 5*B*,*C*), a result similar to that of using the same chamber for conditioning and extinction training.

Footshock-induced reinstatement of fear memory is mediated by the activation of β-adrenergic receptors in the amygdala

We examined whether footshock-induced reinstatement of fear memory was mediated by the activation of β-adrenergic receptors in the amygdala. Indeed, the previous study by Morris et al. (2005) showed that the β-adrenergic receptor blocker propranolol inhibited reinstatement of contextual fear memory. Rats received saline or propranolol (10 mg/kg, i.p.) 1 h before reinstatement training. The fear-potentiated startle measured 24 h later revealed that the startle potentiation in the saline-treated group was significantly higher than that in the propranolol-treated group ($p < 0.01$). In addition, there was no difference between the Ext and propranolol groups ($p > 0.1$), suggesting that propranolol blocked reinstatement (Fig. 6*A*). We examined whether fear responses could be recovered spontaneously 7 d after propranolol treatment and found that fear responses recovered to the level of the saline-treated rats.

In the next experiment, we examined whether β-adrenergic receptor activation facilitated reinstatement. The experimental protocol was identical to that described previously except that there were 5 instead of 10 US-alone trials to avoid the ceiling effect. β-Adrenergic receptor agonist isoproterenol (2 μg per side) was administered by intra-amygdalar injection 30 min before the US-alone trials. As shown in Figure 6*B*, Iso significantly increased fear potentiation ($p < 0.05$ vs saline), and the effect was blocked by propranolol (5 μg per side) administered 30 min before Iso. These results suggest that footshocks induce NE release in the amygdala, and the subsequent activation of β-adrenergic receptors leads to reinstatement of fear memory.

Promotion of GABA$_A$ receptor endocytosis by activation of β-adrenergic receptors

As reinstatement results from reduced inhibitory transmission and is mediated by β-adrenergic receptors, we determined whether activation of β-adrenergic receptor promoted GABA$_A$ receptor endocytosis. Amygdala slices were pretreated with either ACSF or propranolol (10 μM) for 10 min. Then, Iso (10 μM) was added for another 10 min and all drugs were washed out. Two-way ANOVA revealed a significant group effect (Iso vs ACSF, $p < 0.01$) but no significant effect of drug treatment (propranolol vs. ACSF, $p = 0.0589$) and no effect of interaction ($p = 0.0613$). Iso application caused a reduction in the β3 subunit of the GABA$_A$ receptor to 75.1 ± 6.7% ($n = 6$) that of the control. Pretreatment of slices with propranolol abolished the effect of Iso (95.9 ± 7.6%; $n = 6$; $p < 0.05$ vs saline). Propranolol alone without Iso application had no effect (100.1 ± 2.8%; $n = 6$) (Fig. 6*C*).

We compared the levels of the β3 subunit of the GABA$_A$ receptor in rats that received 10 US-alone trials, 5 US-alone trials plus Iso pretreatment, 5 US-alone trials plus saline, and 5 US-

alone trials plus Iso and propranolol. As shown in Figure 6*D*, the level of the $\beta 3$ subunit of the $GABA_A$ receptor in 10 US-alone trials in rats was equivalent to that in rats subject to 5 US-alone trials plus Iso pretreatment, but was significantly lower than the level in the rats subject to 5 US-alone trials plus Iso and propranolol and 5 US-alone trials plus saline pretreatment ($F_{(3,20)} = 7.09$; $n = 6$ in each group; $p < 0.001$).

A dynamin function-blocking peptide (P4 peptide) has been shown to inhibit $GABA_A$ receptor endocytosis, resulting in increased synaptic $GABA_A$ receptors in the cortical and nucleus accumbens neurons (Kittler et al., 2000). To test the effect of P4 on $GABA_A$ receptor endocytosis, we examined its effect on Iso-induced reduction of surface $GABA_A$ receptors. The peptide was N-terminally fused to the transduction domain of the Tat protein from the human immunodeficiency virus type 1 (Tat-P4) to permit its intracellular delivery. In the initial experiment, we examined whether Tat-P4 affected the Iso-induced decrease in surface $GABA_A$ receptors. Amygdala slices were incubated with ACSF, Tat-P4 (10 μM), or Tat-scramble peptide (10 μM) for 30 min. Iso was then added and incubated with the slices for 10 min. Two-way ANOVA revealed a significant group effect (Iso vs ACSF, $p < 0.01$) but no significant effect of drug treatment (Tat-P4 vs ACSF, $p=0.0549$) and no effect of interaction ($p = 0.101$). Iso application (10 μM, 10 min) caused a decrease in surface $GABA_A$ receptors to 71.8 ± 9.0% ($n = 5$) that of the control. Pretreatment of Iso with Tat-P4 blocked the effect of Iso (101.9 ± 9.2%; $n = 5$; $p < 0.05$ vs Iso plus ACSF). In contrast, Tat-scramble peptide had no effect (71.8 ± 8.7% of control; $n = 5$) (Fig. 7*A*). It was noted that application of Tat-P4 or Tat-scramble peptide alone without adding Iso did not influence the surface levels of $GABA_A$ receptor. These results demonstrate that Tat-P4 is able to block the Iso-induced reduction of surface $GABA_A$ receptors in the *in vitro* amygdala slices.

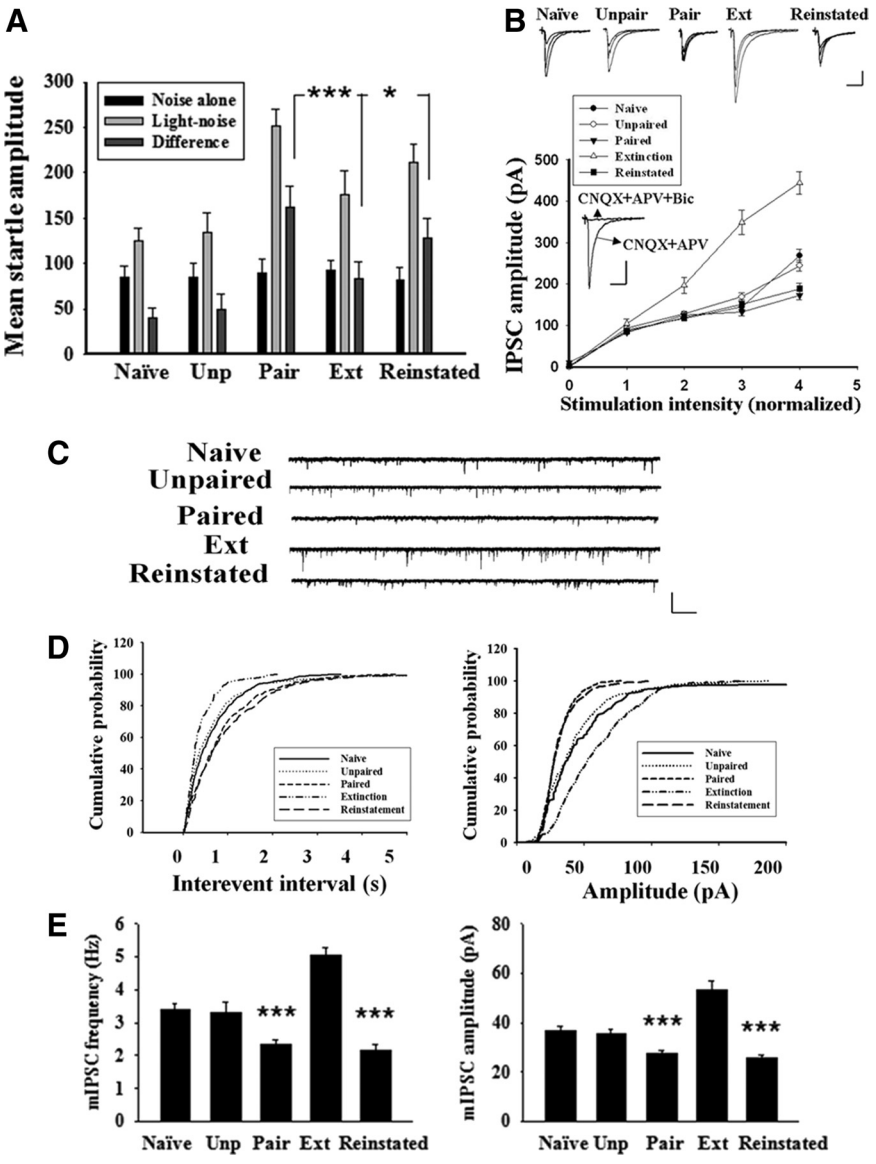

Figure 3. Decreases in the frequency and amplitude of mIPSCs of the LA neurons after reinstatement training. ***A***, Plot of noise alone, light and noise, and the difference of the two values in the naive, unpaired, paired, Ext, and reinstated rats. *$p < 0.05$; ***$p < 0.001$ versus Ext. ***B***, Input–output curves of the evoked IPSCs in naive, unpaired, paired, Ext, and reinstated rats. The intensity of stimulation was gradually increased from the threshold stimulus determined in each individual experiment to produce IPSCs of increasing amplitude. Inset shows that the evoked IPSCs recorded in the presence CNQX (10 μM) and D-APV (50 μM) were blocked by bicuculline (10 μM). ***C***, Sample traces of mIPSCs taken from slices of the naive, unpaired, paired, Ext, and reinstated rats. mIPSCs were recorded in the presence of CNQX (10 μM), D-APV (50 μM), and TTX (1 μM). ***D***, Cumulative interevent interval (left) and amplitude (right) histograms. ***E***, Summary plot of the frequency (left) and amplitude (right) of mIPSCs in naive, unpaired, paired, Ext, and reinstated rats (in each group, $n = 20$ from 7 rats). ***$p < 0.001$ versus Ext. Calibration: ***B***, inset, 50 ms, 100 pA; ***C***, 2 s, 40 pA.

We determined whether $GABA_A$ receptor endocytosis was required for the reinstatement of fear memory by application of Tat-P4. Extinction rats were infused with Tat-P4 (30 pmol per side), scramble peptide (30 pmol per side), or saline into the amygdala 30 min before reinstatement training. Retention of memory was tested 24 h later. One-way ANOVA for the difference between light–noise and noise-alone animals showed a significant effect of group ($F_{(2,15)} = 8.67$; $n = 6$ in each group; $p < 0.01$); the Tat-P4 group differed from the scramble and saline groups (Fig. 7*B*, top graph). Furthermore, rats ($n = 5$) with a cannula implanted 2 mm dorsal to the LA that were infused with Tat-P4 before US-alone trials showed the same level of startle potentiation as the saline and Tat-scramble peptide groups ($F_{(2,14)} = 0.53$; $p > 0.5$), suggesting that the effect of Tat-P4 was site specific.

To maintain the integrity of the slices, saline, Tat-P4 (3 μmol/kg), or Tat-scramble peptide (3 μmol/kg) was administered intravenously 60 min before reinstatement training. Retention of memory was tested 24 h later. One-way ANOVA for the difference between light–noise and noise-alone animals showed a significant effect of group ($F_{(2,15)} = 5.16$; $p < 0.05$); the Tat-P4 group differed from the Tat-scramble peptide and saline groups ($p < 0.05$) (Fig. 7*B*, bottom graph). Amygdala slices from each

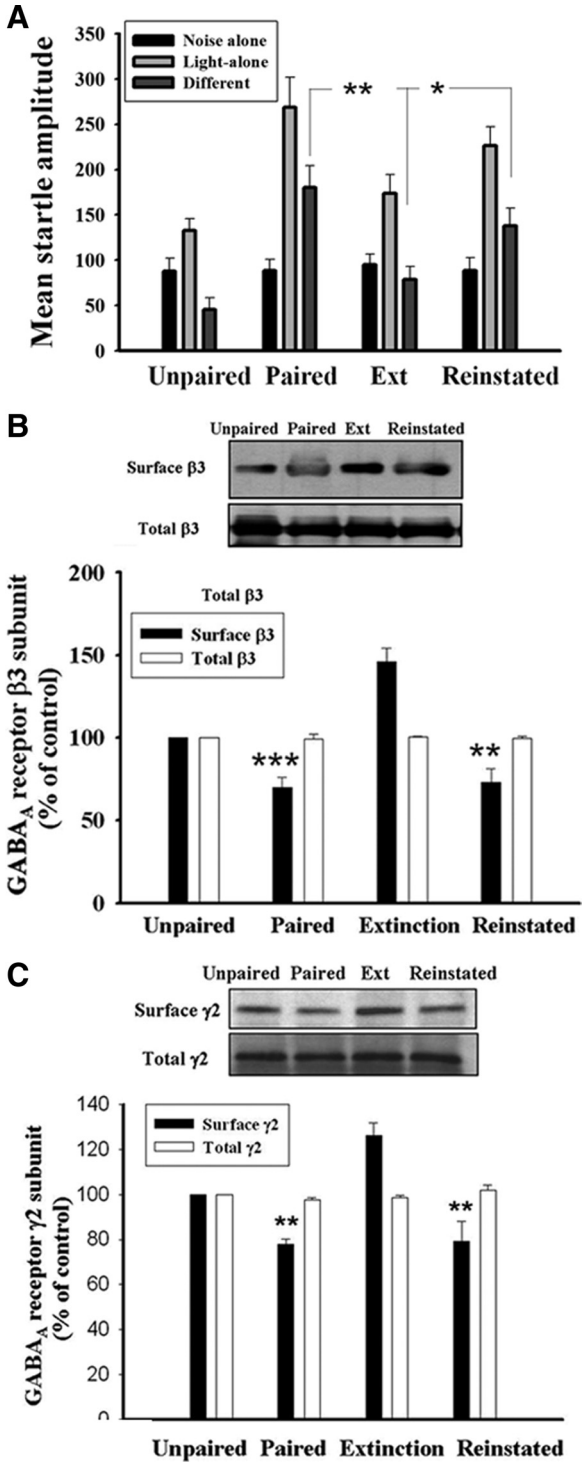

Figure 4. Decrease in $\beta 3$ and $\gamma 2$ subunits of $GABA_A$ receptor after reinstatement. ***A***, Plot of noise alone, light and noise, and the difference of the two values in the unpaired, paired, Ext, and reinstated rats. $^*p < 0.05$; $^{**}p < 0.01$ versus Ext. ***B***, ***C***, Plots of total and surface protein levels of $\beta 3$ (***B***) and $\gamma 2$ (***C***) subunits of the $GABA_A$ receptor in the unpaired, paired, Ext, and reinstated rats. $^{**}p < 0.01$; $^{***}p < 0.001$ versus Ext.

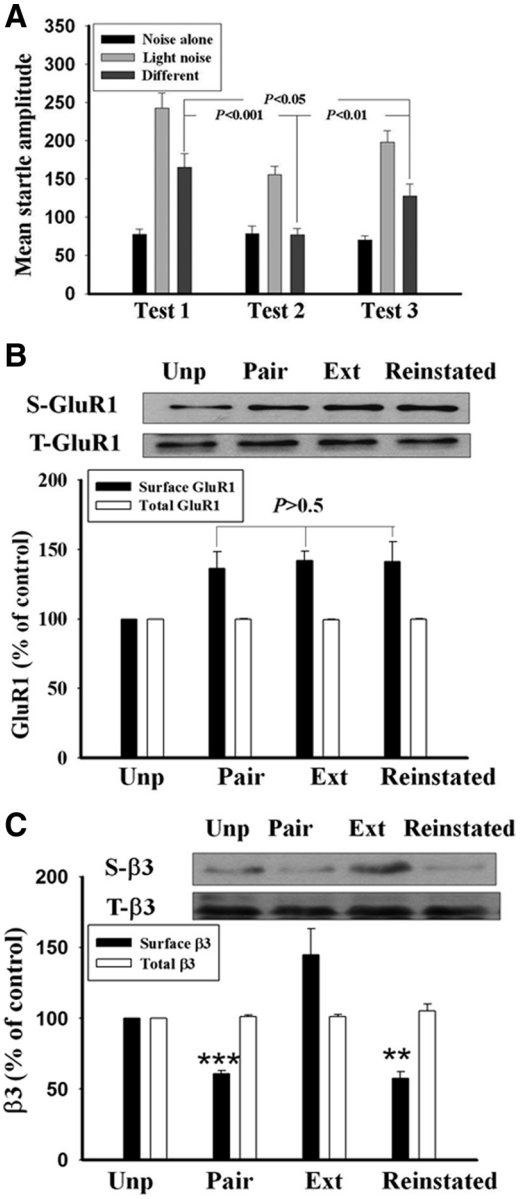

Figure 5. Extinction and reinstatement trainings in different context also reduce surface expression of the $\beta 3$ subunit of the $GABA_A$ receptor without affecting surface expression of GluR1. ***A***, Plot of noise alone, light and noise, and the difference of the two values in the reinstated rats. Acquisition (conditioning) took place in context A, whereas extinction and reinstatement trainings took place in context B. ***B***, ***C***, In Ext and reinstated rats, acquisition (conditioning) took place in context A, whereas extinction and reinstatement trainings took place in context B. LA and BLA tissues were dissected out from the unpaired (Unp), paired (Pair), Ext, and reinstated rats, and the AMPA receptor surface GluR1 and $GABA_A$ receptor surface $\beta 3$ subunit levels were determined using cell surface biotinylation techniques. $^{**}p < 0.01$; $^{***}p < 0.001$ versus Ext.

group were prepared 1 h after the test. Figure 7*C* shows that the IPSC elicited by the 4× threshold in the Tat-P4 group was significantly higher than that in the saline- and scramble peptide-treated rats ($F_{(2,15)} = 29.52$; $n = 6$ from 3 rats in each group; $p < 0.001$). In addition, the frequency and amplitude of mIPSC in the Tat-P4 group was significantly higher in the saline and scramble peptide-treated rats (frequency, $F_{(3,36)} = 16.75$, $n = 10$ from 4 rats in each group, $p < 0.001$; amplitude, $F_{(3,40)} = 8.10$, $n = 11$ from 4 rats in each group, $p < 0.001$) (Fig. 7*D*). Importantly, there was no difference between the extinction and Tat-P4-treated rats ($p > 0.05$).

The surface expression of the $\beta 3$ subunit of the $GABA_A$ receptor was significantly higher in the Tat-P4-treated rats than in the Tat-scramble peptide and saline groups ($p < 0.01$) (Fig. 7*E*). In addition, we found that Tat-P4 had no significant effect on the surface expression of GluR1 and GluR2 (Fig. 7*F*).

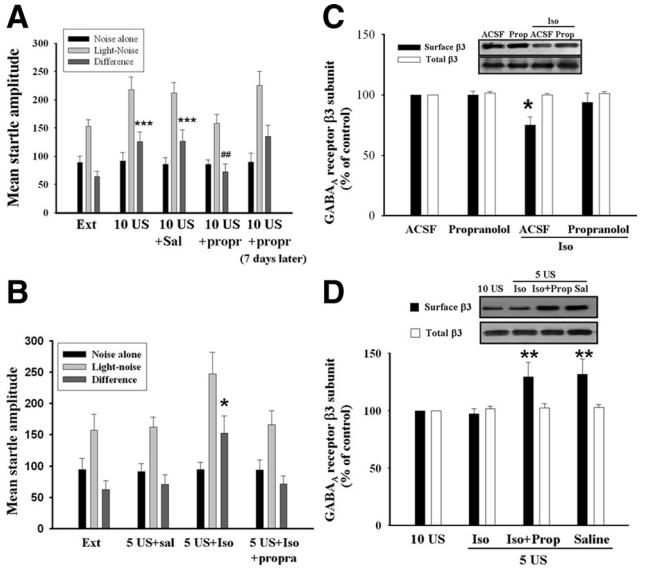

Figure 6. Inhibition of reinstatement by a β-adrenergic receptor antagonist. ***A***, Rats were injected intraperitoneally with saline (+Sal) or propranolol (+propr; 10 mg/kg) 30 min before 10 US-alone trials. Fear-potentiated startle was measured 24 h later. Startle potentiation in the saline-treated group was significantly higher in the propranolol-treated group ($p < 0.01$). ***$p < 0.001$ versus Ext; ##$p < 0.01$ versus saline and propranolol tested at 7 d. ***B***, Plot of noise alone, light and noise, and the difference of the two values in the Ext, Ext plus 5 US-alone trials plus saline (5 US+sal), Ext plus 5 US-alone trials plus Iso (5 US+Iso), and Ext plus 5 US-alone trials plus Iso and propranolol (5 US+Iso+propra) rats. The Ext+5 US-alone trials group was given 5 US-alone trials, whereas the 5 US+Iso rats were infused with Iso (2 μg per side) bilaterally into the amygdala 30 min before US-alone trials. Propranolol (5 μg per side) was given 30 min before Iso for 5 US+Iso+propra rats. *$p < 0.05$ versus 5 US+sal. ***C***, Amygdala slices were treated with 10 μM of Iso for 10 min, and propranolol (10 μM) or ACSF was applied to the slices 10 min before the addition of Iso. One hour later, the tissues of LA and BLA were dissected out, and the GABA$_A$ receptor surface β3 subunit level was determined using biotin labeling. *$p < 0.05$ versus 5 US+Iso+propra. ***D***, Ext rats were given 5 or 10 US-alone trials. Five US-alone trials were divided into three groups: rats that were given saline, Iso, or Iso plus propranolol 30 min before the US-alone trial. Twenty-four hours later, the tissues of the LA and BLA were dissected out, and the GABA$_A$ receptor surface β3 subunit level was determined using biotin labeling. **$p < 0.01$ versus 5 US+Iso.

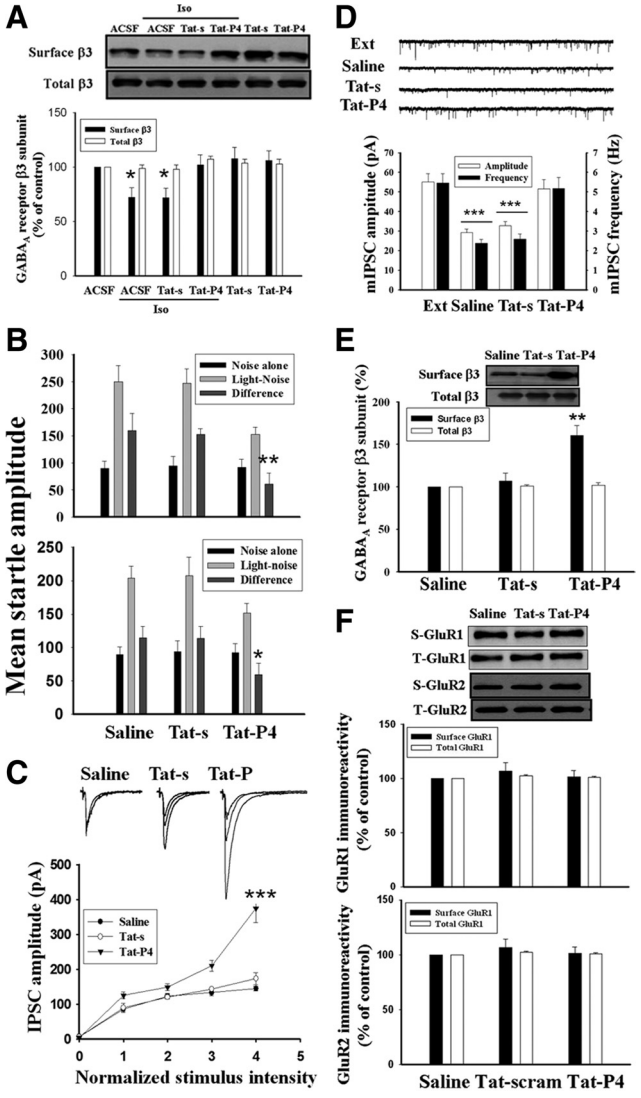

Figure 7. Disruption of GABA$_A$ receptor endocytosis with a dynamin function-blocking peptide inhibits reinstatement. ***A***, Amygdala slices were treated with 10 μM of Iso for 10 min. Tat-P4 (10 μM), Tat-scramble peptide (10 μM), or ACSF was applied to the slices 30 min before the addition of Iso. One hour later, tissues of the LA and BLA were dissected out, and the GABA$_A$ receptor surface β3 subunit level was determined using biotin labeling. *$p < 0.05$ versus Tat-scramble peptide. ***B***, Extinction rats were injected with Tat-P4 (30 pmol per side), scramble peptide (30 pmol per side), or saline into the amygdala bilaterally 30 min before reinstatement training. Retention of memory was tested 24 h later. **$p < 0.01$ versus Tat-scramble peptide (top graph). Ext rats were administered Tat-P4 (3 μmol/kg), Tat-scramble peptide (3 μmol/kg), or saline intravenously 60 min before 10 US-alone trials. Retention of memory was tested 24 h after US- alone trials. *$p < 0.05$ versus Tat-scramble peptide (bottom graph). ***C***, Amygdala slices from each group were prepared 1 h after the test. IPSCs were elicited in amygdala slices from each group. At 4× threshold stimulation intensity, the evoked IPSC in the Tat-P4 group was significantly higher than that in the saline- and scramble peptide-treated rats. ***$p < 0.001$ versus Tat-scramble peptide (bottom). ***D***, Amygdala slices from each group were prepared 1 h after the Test. mIPSCs were recorded in the presence of CNQX (10 μM), D-APV (50 μM), and TTX (1 μM) from each group. ***$p < 0.001$ versus Tat-scramble peptide. ***E***, One hour after the test, the tissues of LA and BLA were dissected out from the reinstated rats. The level of GABA$_A$ receptor surface β3 subunit was determined using biotin labeling. **$p < 0.01$ versus Tat-scramble peptide. ***F***, Ext rats were given Tat-P4 (3 μmol/kg), Tat-scramble peptide (3 μmol/kg), or saline intravenously 60 min before 10 US-alone trials. One hour after the test, the tissues of LA and BLA were dissected out from the reinstated rats. The surface GluR1 and GluR2 levels were determined using biotin labeling.

It has been shown that GluR1-containing AMPA receptors are inserted into synapses of the LA after fear conditioning, and GluR2/GluR3-containing receptors replace existing synaptic AMPA receptors continuously and may act to maintain synaptic efficacy. AMPAR expression is regulated by the direct interaction between *N*-ethylmaleimide-sensitive factor (NSF) and the GluR2 subunit because inhibition of the GluR2–NSF interaction reduced surface expression of AMPAR and impaired fear memory (Nishimune et al., 1998; Joels and Lamprecht, 2010). The adaptor complex AP2 interacts with a region on GluR2 that overlaps with the NSF-binding site (Lee et al., 2002) suggests a possible mechanism for the lack of effect of Tat-P4 on the surface expression of GluR1 and GluR2. Since most GluR2 binds NSF when rats were fear conditioned, therefore, Tat-P4 was less effective.

Since GABA receptors undergo substantial rates of constitutive endocytosis, it is expected to see the effect of dynamin-blocking peptide on basal expression of GABA$_A$ receptors. Indeed, a previous study in the hippocampal slices did observed a significant increase in β3 subunit of the GABA$_A$ receptor 30 min after the application of P4 and the effect sustained for up to 60 min (Terunuma et al., 2008). We repeated the experiment in the amygdala slices and found that after the application of Tat-P4, the β3 subunit of the GABA$_A$ receptor increased in 30 min, lasting for 2 h, and then subsided 24 h later. In our experimental protocol, Tat-P4 was given 30 min before reinstatement training, and the

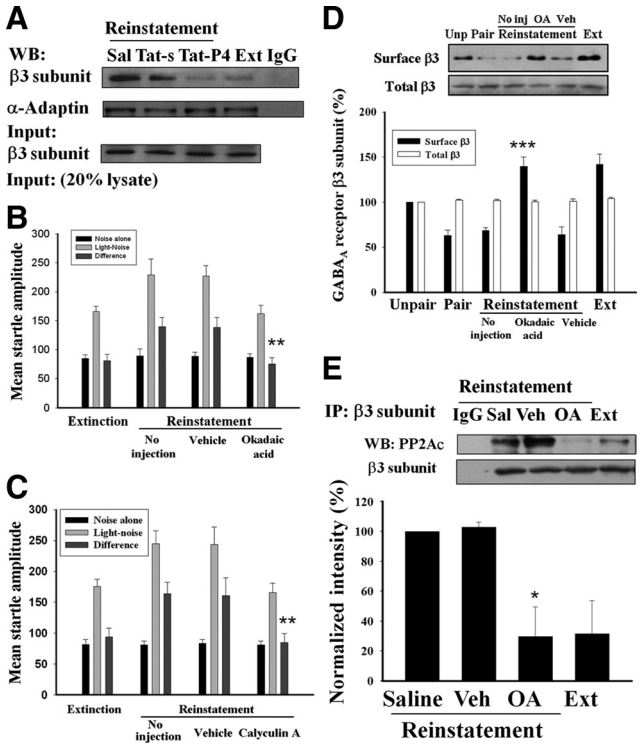

Figure 8. Reinstatement increases association of $GABA_A$Rs with AP2. ***A***, Representative Western blots for immunoprecipitation with an antibody to the α subunit of AP2 are shown, and resulting samples were sequentially immunoblotted with antibodies to the $GABA_A$R β3 subunit and AP2 α subunit in LA and BLA tissues from Ext and reinstated rats. Reinstatement-induced increase in association of $GABA_A$Rs with AP2 was blocked by Tat-P4 administration. ***B***, ***C***, Inhibition of PP1/PP2A blocks reinstatement. Rats were given an intra-amygdalar injection with okadaic acid (10 ng per side) (***B***), calyculin A (5 pmol per side) (***C***), or vehicle (10% DMSO) 30 min before 10 US-alone trials. Fear-potentiated startle was measured 24 h later. Startle potentiation in the vehicle-treated group was significantly higher than those of okadaic acid and calyculin A-treated groups. $^{**}p < 0.01$ versus vehicle. ***D***, One hour after the test, the tissues of LA and BLA were dissected out from the reinstated rats. The level of $GABA_A$ receptor surface β3 subunit was determined using biotin labeling. $^{***}p < 0.001$ versus vehicle. ***E***, Representative Western blots for immunoprecipitation with an antibody to $GABA_A$R β3 subunit are shown, and resulting samples were sequentially immunoblotted with antibodies to the PP2Ac subunit and $GABA_A$R β3 subunit in LA and BLA tissues from Ext and reinstated rats. Application of okadaic acid resulted in a significant decrease in the association of $GABA_A$Rs with PP2Ac. $^{*}p < 0.05$ versus vehicle.

behavioral test was performed 24 h later, the time when the Tat-P4-induced increase in $GABA_A$ receptors subsided (data not shown). Therefore, as shown in Figure 7*B*, Tat-P4 did not affect the basal startle amplitude.

$GABA_A$Rs undergo significant rates of constitutive endocytosis, which is regulated by the direct binding of specific endocytosis motifs within the intracellular domains of receptor β and γ subunits to the clathrin adaptor protein AP2. Therefore, we assessed whether reinstatement training led to enhanced association of $GABA_A$R with AP2. LA and BLA extracts from Ext and reinstated rats were immunoprecipitated with an antibody against the β subunit of AP2 and then immunoblotted with an antibody against the $GABA_A$R β3 subunit. As shown in Figure 8*A*, the association of the $GABA_A$R β3 subunit with AP2 was significantly increased in the reinstated rats compared with the Ext rats. Furthermore, the enhanced association was blocked when Tat-P4 was administered to the rats 60 min before reinstatement training.

$GABA_A$R β subunits have an AP2-binding motif, which includes the major protein kinase A (PKA) phosphorylation sites, and PKA phosphorylation of these sites reduces the affinity of the AP2 complex for $GABA_A$ receptor β subunits (Kittler et al., 2005), leading to decreased endocytosis of $GABA_A$ receptors and an enhanced $GABA_A$R function. In contrast to this observation, we found in this study that Iso reduced the surface expression of $GABA_A$Rs, suggesting the involvement of a dephosphorylating factor. Previous studies have demonstrated that PP2A is a major serine phosphatase that mediates the dephosphorylation of $GABA_A$R β subunits (Jovanovic et al., 2004; Zhang et al., 2007). We examined whether Iso may work through PP2A to enhance $GABA_A$R endocytosis. This would explain why activation of β-adrenergic receptor reduced rather than increased the surface expression of $GABA_A$Rs. To this end, we first determined whether reinstatement required PP2A activity. Rats were administered an intra-amygdalar injection of PP1/PP2A inhibitor okadaic acid (10 ng per side) or vehicle (10% DMSO) 30 min before reinstatement training. A group of rats ($n = 8$) that received conditioning and extinction trainings but did not get reinstatement training served as extinction controls. One-way ANOVA for the difference between light and noise and noise alone showed a significant effect for group ($F_{(3,28)} = 11.03$; $n = 8$ in each group; $p < 0.001$); the okadaic group differed from the vehicle ($p < 0.01$) (Fig. 8*C*). Additional support for an involvement of PP1/PP2A came from the observation that direct injection of another PP1/PP2A inhibitor, calyculin A (5 pmol per side), into the amygdala blocked reinstatement; the calyculin A group differed from the vehicle ($p < 0.01$; $F_{(3,20)} = 6.837$, $n = 6$ in each group, $p < 0.01$) (Fig. 8*C*). In addition, the reinstatement training-induced decrease in surface expression of the β3 subunit of the $GABA_A$R was inhibited by okadaic acid (Fig. 8*D*). Protein extracts from the Ext and reinstated rats were subjected to immunoprecipitation with an antibody directed to the β3 subunit of the $GABA_A$R, and the catalytic subunit of PP2A (PP2Ac) immunolabeling was performed. Rats were given an intra-amygdalar injection of PP1/PP2A inhibitor okadaic acid (10 ng per side) or vehicle (10% DMSO) 30 min before reinstatement training. In Ext rats, a low level of $GABA_A$R β3 subunit coprecipitated with PP2Ac, indicative of a relatively low rate of β3 $GABA_A$R dephosphorylation. The PP2Ac level that was associated with β3 $GABA_A$R was significantly increased in reinstated rats injected with vehicle or saline. Okadaic acid treatment abolished the association (Fig. 8*E*). These data provide additional evidence that $GABA_A$Rs are internalized via the clathrin-mediated pathway and that reinstatement training promotes receptor internalization by recruiting $GABA_A$Rs to the AP2 complex.

Discussion

It has been shown that there are two distinct classes of neurons, fear and extinction neurons, in the basolateral amygdala. During and after fear conditioning, fear neurons exhibit a selective increase in CS^+-evoked spike firing, which is abolished by subsequent extinction training. On the other hand, extinction neurons respond selectively to extinction training with an increase in CS^+-evoked spike firing (Herry et al., 2008). We integrate this observation with that proposed by Rosenkranz and Grace (2001) by suggesting that increased neural activity in IL projection neurons by extinction training activates BLA inhibitory interneurons and increases $GABA_A$R surface expression in the fear neurons, leading to feedforward inhibition of fear neurons. In the present study, it was conceived that reinstatement training (footshocks) elicits epinephrine release from adrenals. Epinephrine activates the vague nerve, which synapses on a wide range of neurons in the nucleus tractus solitarius (NTS) (Sumal et al., 1983; Hassert et al.,

2004). NTS neurons project directly to the LC, and the latter presumably mediates the stress-induced increase in NE in the amygdala (Buffalari and Grace, 2007).

NE could suppress GABAergic inhibition of projection neurons in the lateral amygdala by decreasing the excitability of local circuit interneurons (Tully et al., 2007). In this study, we made an intriguing finding, showing that application of Iso in the amygdala reduced the surface expression of the β3 subunit of the $GABA_A$ receptor. The effect was blocked by propranolol, confirming the mediation of β-adrenergic receptors. Furthermore, both Iso-induced and reinstatement-elicited reduction of the surface expression of β3 subunit of the $GABA_A$ receptor could be blocked by a dynamin function-blocking peptide, suggesting that $GABA_A$ receptor endocytosis contributes significantly to the reinstatement of fear memory.

Several lines of evidence have shown that PKA is involved in several receptors trafficking. For example, $GABA_AR$ β subunits have an AP2-binding motif that includes the major PKA phosphorylation sites (Brandon et al., 2003), and PKA phosphorylation of these sites reduces the affinity of the AP2 complex for $GABA_A$ receptor β subunits, and therefore decreases the internalization of $GABA_A$Rs (Kittler et al., 2005). In contrast with these results, we found that Iso reduced instead of increased the surface expression of $GABA_A$Rs. One possible mechanism is that activation of β-adrenergic receptors decreases the phosphorylation of $GABA_A$ receptors through the activity of PP2A. Indeed, this has been demonstrated in other systems (Jovanovic et al., 2004; Goffin et al., 2010). An active PP2A enzyme consists of a heterotrimer of the structural A subunit, a catalytic C subunit, and a regulatory B subunit (McCright et al., 1996; Ruvolo et al., 2002). Previous studies have shown that the B56δ subunit of PP2A is phosphorylated by PKA, resulting in a fivefold increase of PP2A activity, and that this mechanism is responsible for cAMP-dependent dephosphorylation of Thr-75 of DARPP-32 (Ahn et al., 2007). In addition, we found that reinstatement enhanced the $GABA_AR$ and PP2A physical association in the LA neuronal cell lysate. To determine whether the physical association was accompanied by a functional interaction, we measured surface $GABA_A$Rs under conditions in which PP2A activity was inhibited. The results showed that treatment with the PP2A inhibitor okadaic acid abolished the physical association of $GABA_A$Rs and PP2A, markedly increased the surface expression of $GABA_A$Rs, and blocked reinstatement. These results suggest that PP2A effectively regulates the physical association of $GABA_A$Rs and PP2A and $GABA_AR$ endocytosis. Collectively, our data suggest that footshocks during US-alone trials induce NE release in the amygdala and that the subsequent activation of β-adrenergic receptor stimulates PKA. PKA phosphorylates the B56δ subunit of PP2A, resulting in an increased PP2A activity and an enhanced affinity of the AP2 complex for $GABA_AR$, and therefore the internalization of $GABA_A$Rs.

LC neurons also send projections to many forebrain nuclei including the prefrontal cortex. Although, NE acting on β-adrenergic receptors enhances excitatory transmission, increases neuronal excitability, and facilitates extinction memory (Ji et al., 2008; Mueller et al., 2008), the prefrontal cortex in general plays an inhibitory role in the stress response, leading to dampening of the respond to stressful stimuli (Herman and Cullinan, 1997). Thus, it is likely that reinstatement training eliciting NE release in the IL could offset extinction training-induced activation of ITC neurons leading to reinstatement of fear memory.

Similar to animal experiments of extinction, exposure psychotherapy in a clinical setting is based on the theory that previously acquired fear responses gradually decline if PTSD patients are exposed to trauma-associated conditioned stimuli in the absence of aversive reinforcement (Taylor et al., 2003; Hermans et al., 2006). However, successful reduction of fear through exposure therapy is often followed by a return of fear when patients encounter the aversive stimuli (reinstatement). The present study suggests the involvement of β-adrenergic receptor activation and $GABA_A$ receptor endocytosis in the fear reinstatement, which may open up new avenues for the treatment of PTSD.

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