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Synaptic Expression of Glutamate Receptor after Encoding of Fear Memory in the Rat Amygdala

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ABSTRACT

Fear conditioning has been ascribed to presynaptic mechanisms, particularly presynaptic facilitation of transmission at thalamo- and cortico-amygdala synapses. Here, by labeling surface receptors with biotin or using membrane fractionation approaches, we report that fear conditioning resulted in an increase in surface expression of GluR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the amygdala, whereas total GluR1 mRNA and protein levels were unchanged. The control group that received conditioned stimulus (CS) and unconditioned stimulus in an unpaired fashion did not present any increase, indicating that GluR1 increase was specific to the learning component of the task. Conditioning-induced increase in surface expression of GluR1 depended on the activation of *N*-methyl-D-aspartate receptors and protein kinases and required the synthesis of new proteins. CS-alone trials applied 24 h before training attenuated fear-potentiated startle and prevented conditioning-induced increase in surface expression of GluR1. Increase in GluR1 was also observed in the amygdala slices after delivery of tetanic stimulation that elicited long-term potentiation of synaptic transmission. Proteasome inhibitor increased surface expression of GluR1 in a time- and dose-dependent manner. Furthermore, pretraining administration of proteasome inhibitor into the amygdala facilitated the fear-potentiated startle. These results suggest that long-term memory formation is correlated with the change in synaptic expression of GluR1, and trafficking of GluR1 to the synaptic sites contributes at least in part to the expression of fear memory.

Fear conditioning, an animal model of emotional learning and post-traumatic stress disorder, is initiated by a cue (conditioned stimulus, CS) that is previously paired with an aversive stimulus (unconditioned stimulus, US) such as footshock. Neuronal changes mediating the association between the CS and US occur in the lateral (LA) and basolateral (BLA) amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Pare, 2003). It is generally recognized that consolidation of long-term memory in vertebrate and invertebrate brains requires transcription and translation of new proteins (Dudai, 1996; McGaugh, 2000). Newly synthesized proteins are believed to deposit at the synapses that encode the persistent changes in synaptic strength. Despite the signal cascades that have been identified to be responsible for the consolidation of fear memory, little is known about the downstream effectors leading to the expression of memory. Previous study has showed that conditioned fear is associated with a reduction in paired-pulse facilitation and an enhancement in the probability of transmitter release in the thalamo-amygdala pathway (McKernan and Shinnick-Gallagher, 1997). Quantal analysis of unitary synaptic responses in cortico-amygdala pathway after induction of long-term potentiation (LTP) revealed a marked decrease in the fraction of failures, with no change in potency of CV of the excitatory postsynaptic current (EPSC) (Tsvetkov et al., 2002; Humeau et al., 2003). Thus, LTP expression in the amygdala and fear conditioning in animals have a significant presynaptic component.

On the other hand, the dependence of LTP and fear memory on the *N*-methyl-D-aspartate (NMDA) receptors (Miserendino et al., 1990; Huang and Kandel, 1998; Lee and Kim, 1998; Bauer et al., 2002) and the reduction of NMDA recep-

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ABBREVIATIONS: US, unconditioned stimulus; BLA, basolateral nucleus of amygdala; CS, conditioned stimulus; D-APV, D-2-amino-5-phosphonovalerate; ER, endoplasmic reticulum; LA, lateral nucleus of amygdala; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NMDA, *N*-methyl-D-aspartate; UPR, unfolded protein response; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; TS, tetanic stimulation; ACSF, artificial cerebrospinal fluid; ITI, intertrial interval; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction; DMSO, dimethyl sulfoxide; LFS, low-frequency stimulation; PI-3, phosphatidylinositol-3; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; fEPSP, field excitatory postsynaptic potential.

tors in amygdala from fear-conditioned animals (Zinebi et al., 2003) raised the possibility that a postsynaptic component may be involved. Furthermore, studies in the hippocampus and barrel cortex revealed that activation of NMDA receptors induced LTP-like phenomenon and caused an insertion of AMPA receptors into synapses (Shi et al., 1999; Heynen et al., 2000; Takahashi et al., 2003). It is known that protein kinase A phosphorylation of Ser845 in GluR1 increases the peak open probability (Banke et al., 2000) and accompanies the surface reinsertion of GluR1 (Ehlers, 2000). The observation that coupling of GluR1 and protein kinase A by A-kinase anchoring proteins (AKAPs) through synapse-associated protein 97 kDa in the lateral amygdala is required for fear memory formation (Morita et al., 2002) indicates that surface expression of the AMPA receptor could be important for memory consolidation.

The purpose of this study was to determine whether the glutamate receptor protein level was altered after fear conditioning. We measured the expression of cell-surface GluR1 in the amygdala using biotinylation assay. We also probed the levels of GluR1 in synaptic regions using synaptoneurosome preparation. Our data provide the evidence showing that GluR1 is a potential downstream effector that contributes at least in part to the expression of fear memory. Furthermore, the increase in surface expression but not in the levels of mRNA or total GluR1 protein in the LA and BLA suggests that fear conditioning may involve a translocation of intracellular receptors into synaptic membranes.

Materials and Methods

Surgery. Sprague-Dawley rats (4–8 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and subsequently were mounted on a stereotaxic apparatus. Two cannulas made of 22-gauge stainless steel tubing (C313G; Plastic Products, Roanoke, VA) were implanted bilaterally into the LA or BLA amygdala [anteroposterior, −2.8 mm; mediolateral, ±4.5 mm; dorsoventral, −7.0 mm] (Paxinos and Watson, 1986). A 28-gauge dummy cannula was inserted into each cannula to prevent clogging. Three jewelry screws were implanted over the skull, serving as anchors, and the whole assembly was affixed on the skull with dental cement. The rats were monitored and handled daily and were given 7 days to recover. D-2-Amino-5-phosphonovalerate (D-APV), wortmannin, U0126, anisomycin (Sigma-Aldrich, St. Louis, MO), and MG-132 (Tocris Cookson Inc., Bristol, UK) were administered bilaterally to the amygdala in a volume of 1 μl at a rate of 0.1 μl/min.

Slice Preparation. Rats were decapitated, and their brains were rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (ACSF) solution. Thereafter, the brain was hemisected and glued to the chuck of a Vibroslice tissue slicer. Transverse slices of 400 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 the drug incubation experiment. ACSF solution had 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. Amygdala slices were incubated with MG-132 for various periods, and then surface GluR1 was determined.

Fear Conditioning. Rats were trained and tested in a stabilimeter device. A piezoelectric device mounted below the stabilimeter detects and transduces the motion of the cylinder produced by the whole-body startle response of the rat (San Diego Instrument, San Diego, CA). The whole set-up was enclosed in a ventilated, sound-attenuating cabinet (length, 38 cm; width, 38 cm; and height, 55 cm). The acoustic startle stimulus was a 50-ms white noise at the 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 0.6-mA footshock with a duration of 0.5 s.

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

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

Training. Rats were placed in the startle boxes and received 10 light-footshock pairings with an ITI of 2 min. Unpaired controls received the same number of light and footshock presentation but in a pseudorandom fashion, in which the US could occur at any time except at 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 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). The percentage of fear-potentiated startle was computed as follows: [(startle amplitude on CS-noise minus noise-alone trials)/(noise-alone trials)] × 100.

Western Blot Analysis. Rats were killed by decapitation, and the LA and BLA minislices were made, placed on ice, and washed two times with ice-cold ACSF. Slices were then incubated with ACSF containing 0.5 mg/ml Sulfo-NHS- LC-Biotin (Pierce Chemical, Rockford, IL) for 1 h on ice. Slices were rinsed in Tris-buffered saline buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) to quench the biotin reaction and then sonicated briefly in homogenizing buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 4 μg/ml aprotinin). After sonication, the samples were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was obtained. Protein concentration in the soluble fraction was then measured using a Bradford assay with bovine serum albumin as the standard. A sample consisting of 250 μg of protein of the supernatant was incubated with 50 μl of 50% Neutravidin agarose (Pierce Chemical) for 16 h at 4°C, washed four times with homogenizing buffer, and bound protein was resuspended in 4 μl of SDS sample buffer and boiled. Each sample was resolved in 8.5% SDS-polyacrylamide gels, blotted electrophoretically to Immobilon, and blocked overnight in Tris-buffered saline buffer containing 3% bovine serum albumin. For detection of GluR1, Cadherin, or Actin, blots were incubated with antibody for GluR1 (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), Cadherin (1:2500, Sigma-Aldrich), or Actin (1:4000, Santa Cruz Biotechnology). An enhanced chemiluminescence kit was used for detection. Western blots were developed in the linear range used for densitometry. The density of the immunoblots was determined by an image analysis system installed with a software BIO-ID (Vilber Lourmat, Marne-la-Vallée, France). In most experiments, GluR1 levels in paired or unpaired animals were expressed as a percentage of those in naive controls.

RT-PCR Analysis. Tissues of amygdala were mixed with 1 ml of TRI reagent (Molecular Research Center Inc., Cincinnati, OH) to extract cellular mRNA. Single-strand cDNA was synthesized from the cellular mRNA by adding 1 μl of StrataScript reverse transcriptase (200 U/μl; Stratagene, La Jolla, CA), 10 μl of buffer (250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$), 5 μl of dithiothreitol (0.1 M), 1 μl of oligo(dT), and 18 μl of mixed dNTPs (2.5 mM). The mixture was incubated for 50 min at 42°C. The reaction was terminated by heating the mixture to 70°C for 10 min and then icing. Amplification was performed on a thermal cycler (PC800; Astec, Fukuoka, Japan) using DNA polymerase (5 U/μl; Yeastern Biotech, Taipei, Taiwan). One of the primer sets was added to give a final volume of 20 μl. Reactions were run for the optimal cycles under the following conditions: denaturation at 95°C for 1 min, annealing at

60°C for 1 min, and extension at 72°C for 1 min and 30 s (repeated for a total of 30 cycles). The primers for the detection of AMPA receptor subunit GluR1 cDNA were 5′-AGGTTTGCTTTGTCACAA-3′ and 5′-CTTCTCCAGGTC CTGAAA-3′. The primers for the detection of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA were 5′-TGACAACTTTGGCATCGTGGAAGG-3′ and 5′-CAAC GGATACATTGGGGGTAGGAAC-3′. Control amplifications were done without cDNA. Amplification from primers, GluR1, or GAPDH produces fragments of 456 or 233 base pairs in length, respectively. After polymerase chain reaction amplification, an 8.5-μl aliquot of reaction product was analyzed by electrophoresis on ethidium bromide-stained agarose gel (1.8%). Images of the DNA gels after RT-PCR analyses were digitally captured by an image analysis system installed with BIO-ID software (Vilber Lourmat).

Histology. At the end of the experiments, animals received an overdose of pentobarbital (100 mg/kg), and the brains were removed from the skull and fixed in buffered 4% paraformaldehyde, pH 7.4, for 48 h. Brains were sectioned with a sliding MicroSlicer (DTK-1000; Ted Pella, Redding, CA), and sections (40 μm thickness) were stained for Nissl bodies.

Synaptoneurosome Preparation. The LA and BLA subregions of the amygdala were dissected out and homogenized in 70 μl of ice-cold lysis buffer in an Eppendorf tube. The buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose, and 10 μg/ml adenosine deaminase, with pH adjusted to 7.4 by bubbling with 95% O_2 + 5% CO_2. Proteinase inhibitors (0.0l mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.l mg/ml aprotinin, and 5 mM benzamide) were included in the buffer to minimize proteolysis. The homogenate was diluted with 350 μl of additional ice-cold lysis buffer. This mixture was loaded into a 1-ml tuberculin syringe attached to a 13-mm diameter syringe filter holder (Millipore Corporation, Billerica, MA). The diluted filtrate was forced over three layers of nylon (Tetko, 100 μm pore size), prewetted with 150 μl of lysis buffer, and collected in a 1.5-ml Eppendorf tube. The nylon prefiltered mixture was loaded into another 1-ml tuberculin syringe and forced through a prewetted 5 μm nitrocellulose filter (Millipore). The homogenate was kept ice-cold at all times to minimize proteolysis. The filtered particulate was then spun at 1000*g* for l5 min at 4°C. The supernatant was removed, and the pellet (synaptoneurosome) was resuspended in 80 μl of lysis buffer for Western blot analysis.

Data Analysis. Data were analyzed with analysis of variance. A single-factor analysis of variance and post hoc comparisons were used to analyze the time course of surface expression of GluR1 after fear conditioning. Unpaired *t* test was used to analyze the difference in startle reflex between drug-treated and vehicle-control groups. All values in the text and figure legends are mean ± S.E.M.

Results

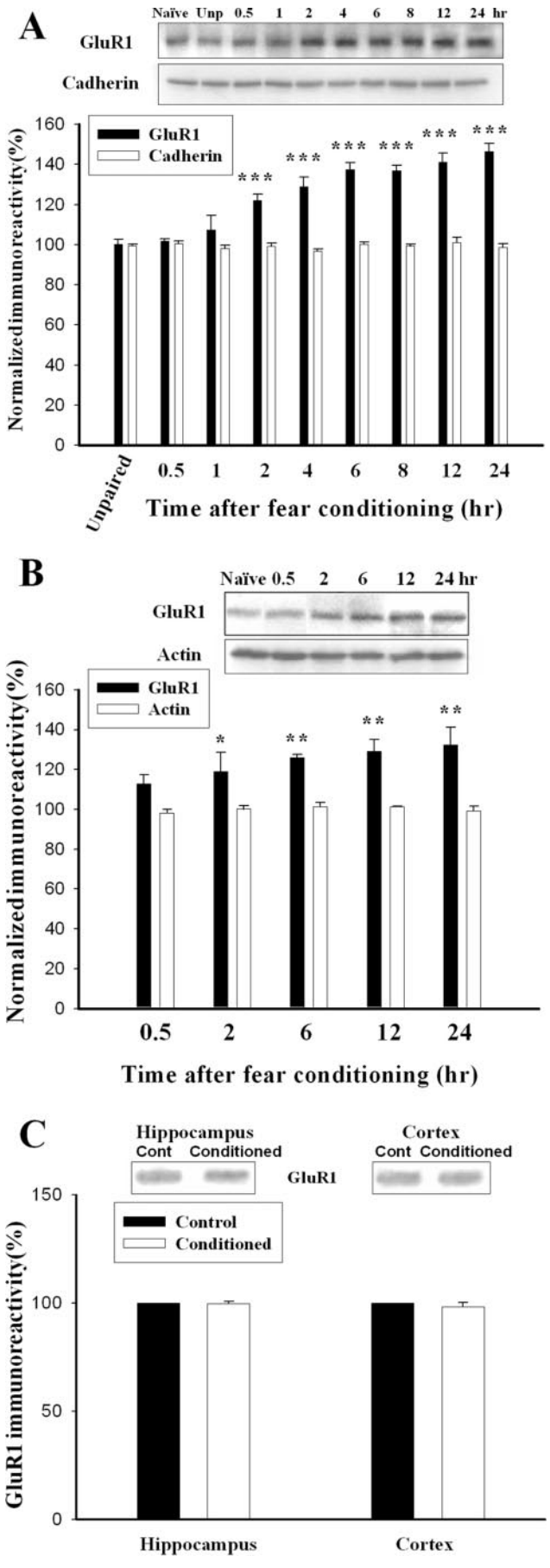
Fear Conditioning Increases Surface Expression of GluR1 in the Amygdala. At various time points after training, the tissues of LA and BLA were dissected out, and surface receptors were labeled with biotin. Biotinylated receptors were precipitated, and surface GluR1 was determined by quantitative immunoblotting. Figure 1A shows that surface expression of GluR1 was significantly increased after fear conditioning compared with naive control. The increase was detected at 2 h after training and was sustained for at least 24 h ($F_{9,20} = 35.45$, $p < 0.0001$, $n = 3$ rats in each time point). Newman-Keuls post hoc comparison revealed the differences between naive control and 2-, 4-, 6-, 8-, 12-, and 24-h time points ($p < 0.001$). No significant difference was seen between naive control and 0.5- and 1-h time points ($p > 0.05$).

Synaptoneurosomes resemble isolated glutamatergic synapses located on the dendritic spines, with resealed pre- and postsynaptic compartments (Hollingsworth et al., 1985; Hampson et al., 1992; Heynen et al., 2000). We examined whether regulation of glutamate receptor availability after fear conditioning at the synaptoneurosomes occurred as that measured with biotin labeling. As shown in Fig. 1B, a similar time course of GluR1 expression was seen in synaptoneurosomes. The increase was detected 2 h after training and was sustained for at least 24 h ($F_{5,12} = 7.59$, $p < 0.01$, $n = 3$ rats in each time point). Post hoc comparison revealed the differences between naive control and 2- ($p < 0.05$), 6-, 12-, and 24-h ($p < 0.01$) time points. No significant difference was seen between naive control and the 0.5-h time point ($p > 0.05$). These results indicate that encoding of fear memory is associated with an increase in surface and synaptic expression of GluR1 in the amygdala.

To ensure that the increase in the level of GluR1 after training was specific for the association between the cue and footshock, we measured GluR1 in a group of unpaired rats. There was no difference in GluR1 immunoreactivity between naive and unpaired rats ($p > 0.1$; Fig. 1A). We determined whether the increase in glutamate receptor expression associated with fear conditioning occurred specifically within the amygdala or was a general phenomenon across the entire brain by quantifying GluR1 protein levels in the hippocampus and cortex. As illustrated in Fig. 1C, training with fear-potentiated startle paradigm did not alter the levels of GluR1 in the hippocampus and cortex. Twenty-four hours after training, GluR1 levels were 99.7 ± 1.1 ($n = 8$ rats) of unpaired control in the hippocampus and 98.4 ± 1.8 ($n = 8$ rats) in the cortex, respectively. These results indicate that the increase was restricted to a brain area related to the fear-potentiated startle.

One possibility for the increase in surface GluR1 level is an increment in total GluR1 protein expression. We therefore performed RT-PCR to measure GluR1 mRNA in tissue homogenates from LA and BLA. Figure 2A shows that the levels of GluR1 mRNA across the entire time points examined were not changed after fear conditioning. We also performed Western blot analysis of GluR1 in tissue homogenates from LA and BLA. Although there was a trend toward a small increase, the difference did not reach a significant level ($F_{6,28} = 1.71$, $p = 0.16$, $n = 5$ rats in each time point) (Fig. 2B).

Conditioning-Induced Increase in Surface GluR1 Depends on NMDA Receptor and Kinases Activation. We tested the hypothesis that fear conditioning was associated with an increase in surface expression of GluR1 by using pharmacological tools to block the receptors and their downstream signal cascades that were required for fear learning. It is known that acquisition of fear memory depends on NMDA receptor activation (Miserendino et al., 1990). In our hands, fear training resulted in 175.3 ± 3.9% ($n = 6$) of potentiation. Bilateral administration of NMDA receptor antagonist D-APV (25 nmol dissolved in 2 μl of artificial cerebrospinal fluid, 1 μl per side) to the amygdala 30 min before training blocked fear-potentiated startle (25.3 ± 3.9%, $n = 5$ rats, $p < 0.01$) (Fig. 3A). Figure 3B shows that pretreatment with D-APV also blocked the conditioning-induced increase in surface expression of GluR1 (95.3 ± 3.9% of control, $n = 4$



rats, $p < 0.05$), whereas vehicle control had no effect ($n = 4$ rats).

Consolidation of fear memory required the activation of PI-3 kinase, its downstream target Akt, mitogen-activated protein kinase (MAPK), and new protein synthesis in the amygdala (Schafe and LeDoux, 2000; Schafe et al., 2000; Lin et al., 2001). Consistent with these notions, pretraining administration of PI-3 kinase inhibitor wortmannin (2.5 μg/μl in 50% DMSO, 1 μl per side), MAPK kinase (MEK) inhibitor U0126 (2 μg dissolved in 2 μl of 50% DMSO, 1 μl per side), or anisomycin (62.5 μg dissolved in 2 μl of 50% DMSO, 1 μl per side) significantly attenuated conditioning-induced increase in surface GluR1 expression (94.4 ± 4.4%, $n = 7$ rats; 108.8 ± 10.4%, $n = 7$ rats; 104.5 ± 6.2%, $n = 6$ rats of unpaired control, respectively; $p < 0.01$ versus paired) (Fig. 3C). Block of conditioning-induced increase in GluR1 by D-APV and wortmannin was also observed in synaptoneurosomes (Fig. 3D). To examine the possibility that vehicle (50% DMSO) might damage the amygdala neurons, we performed histological analysis. Placement of the guide cannulas into the LA damaged some caudate putamen tissues. The damage was limited to the area immediately surrounding the cannulas. The pattern of results from vehicle controls (50% DMSO) did not differ from that of untreated animals, suggesting that mere placement of the guide cannulas in the LA had no significant effect on behavior. Figure 4 shows that there was no evidence of increased gliosis or cell loss in 50% DMSO-treated rats.

Pre-Exposure of CS Reverses Conditioning-Induced Increase in Surface GluR1. Next, we tested the hypothesis using behavioral manipulations. Pre-exposure to the CS can impede the development and/or expression of the CR after subsequent pairing with CS and US, a phenomenon referred to as latent inhibition (Lubow and Moore, 1959; Weiner, 2003). We examined whether fear training-induced increase in GluR1 was affected when the animal was pre-exposed to the CS. Rats were pre-exposed to 3 sessions of 10 presentations of the 3.7-s light 24 h before receiving 10 light-shock pairings. Non–pre-exposed rats were placed in the chamber for the same duration without exposing to the light. Memory retention was assessed 24 h after training. As expected, pre-exposure of CS blocked conditioning-induced startle potentiation such that there was no difference in startle reflex between pre-exposed and unpaired control rats ($t_8 = 0.43$, $p = 0.68$) (Fig. 5A). Furthermore, as illustrated in Fig. 5, B and C, conditioning-induced increase in GluR1 was not seen in pre-exposure animals measured with biotin label or in synaptoneurosomes. As a control, CS-alone trials did not

Fig. 1. Fear training increases surface expression of GluR1. A, time course of surface expression of GluR1 induced by fear conditioning. The figure shows the representative blots and mean ± S.E. of GluR1 immunoreactivities from rats decapitated at various time points after training. The tissues of LA and BLA were dissected out, and surface receptors were labeled with biotin. Biotinylated surface proteins were precipitated by immobilized avidin and analyzed by Western blot analysis with anti-GluR1 or anti-pan-cadherin (surface protein control) antibodies. ***, $p < 0.001$ versus naive control. B, increase in the expression of GluR1 in synaptoneurosomes after fear conditioning. To confirm equal protein loading, parallel blots were probed with a polyclonal goat anti-actin antibody. *, $p < 0.05$; **, $p < 0.01$ versus naive control. C, surface GluR1, measured 24 h after training, was expressed selectively in the amygdala but not hippocampus or cortex. GluR1 protein levels in paired were expressed as a percentage of that in unpaired control.

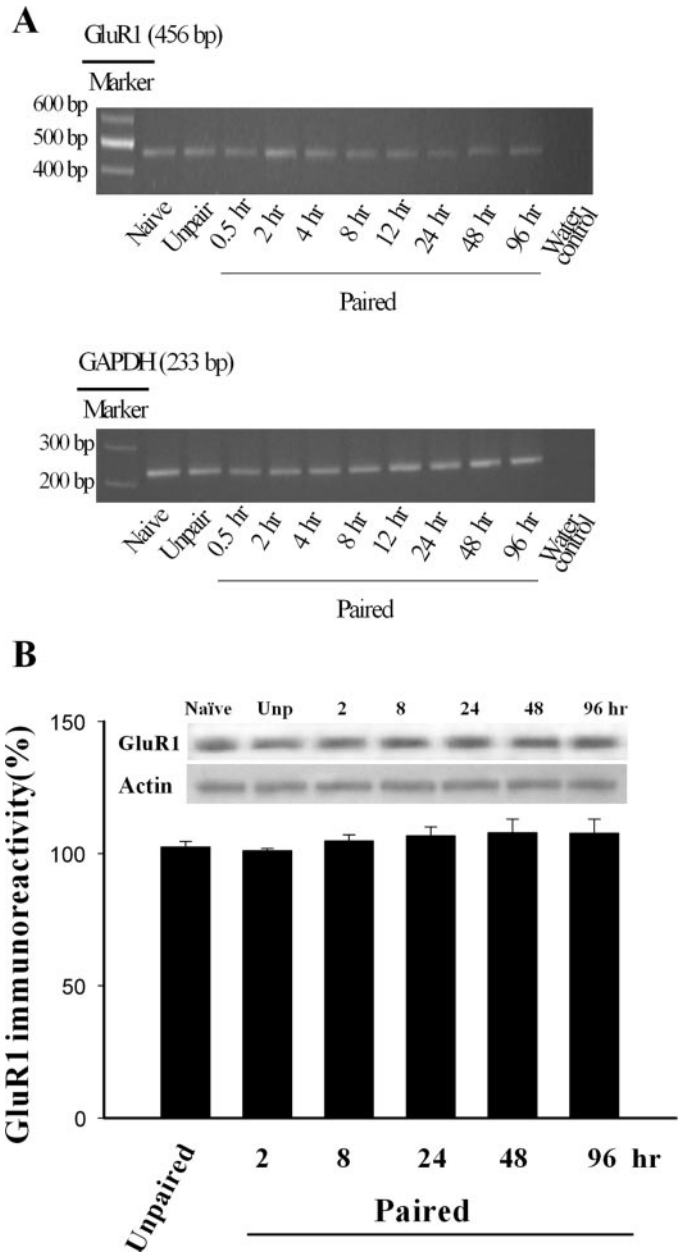


Fig. 2. Fear conditioning does not affect the total amount of GluR1 mRNA and protein levels. A, total RNA was isolated from the LA and BLA, and mRNA expression for GluR1 was analyzed with RT-PCR. mRNA expression for GAPDH was used as an internal control. B, time course of total GluR1 expression level in amygdala tissue homogenates induced by fear conditioning. GluR1 protein levels in paired and unpaired were expressed as a percentage of that in naive control.

affect surface expression of GluR1 (102.0 ± 1.8% of naive control, $n = 6$ rats; Fig. 5D).

LTP in the LA Is Accompanied by an Increase in the Surface Expression of GluR1. We further determined whether synaptic plasticity in the amygdala, namely LTP in the LA, was associated with an increase in the level of GluR1. A stimulating electrode was placed in the external capsule 200 μm lateral to the recording electrode. After the establishment of a stable baseline recording, three sets of tetanic stimulation (TS) were delivered to the EC at an interstimulus interval of 1.5 min. This stimulating protocol produced a robust enhancement of synaptic responses. The slopes of field excitatory postsynaptic potential (fEPSP) were 213.4 ± 18.2 and 200.7 ± 15.2 of pretetanus level at 1 and 2 h after the TS, respectively (Fig. 6A). Six hours after the onset of TS, a relatively small portion of the LA was dissected from each slice. Four to six slices from each animal were pooled together for biochemical analysis. Biotinylated receptors were precipitated, and surface GluR1 was determined by quantitative immunoblotting. Figure 6B shows that GluR1 level was significantly elevated in the LA compared with control slices (135.5 ± 8.5%, $n = 6$ rats). LTP of synaptic responses could be reversed by low-frequency stimulation (LFS) of afferent fibers, a phenomenon termed "depotentiation" (Staubli and Chun, 1996). Figure 6A shows that when delivered 10 min after TS, reliable depotentiation could be induced by 3 min of 5-Hz stimulation. The slope of fEPSP 2 h after TS was 104.9 ± 11.4 of the pretetanus level ($n = 6$, $p < 0.001$ versus control). We determined whether the increase in GluR1 expression was affected by subsequent LFS. As illustrated in Fig. 6B, TS-induced increase in surface expression of GluR1 was completely blocked in LFS group (98.4 ± 6.2%, $n = 6$ rats, $p < 0.005$ versus TS group). These results suggest that synaptic plasticity in the cortico-amygdala pathway correlate with surface expression of GluR1.

Proteasome Inhibitor Promotes Surface Expression of GluR1 and Facilitates Fear Conditioning. Although fear-potentiated startle could be blocked by the inhibition of protein synthesis, the total levels of GluR1 mRNA and protein were unaltered after fear conditioning. Thus, it is likely that new proteins synthesized in response to learning-associated activity promote trafficking of AMPA receptors to the synaptic regions. It was shown recently that LTP in the hippocampus required stargazin phosphorylation that promotes AMPA receptor trafficking via up-regulation of endoplasmic reticulum (ER) chaperones as part of unfolded protein response (UPR). Proteasome inhibitors increased Ig binding protein levels and greatly increased surface expression of GluR1 (Tomita et al., 2005; Vandenberghe et al., 2005). We therefore examined whether the administration of proteasome inhibitors increased the surface expression of GluR1 and facilitated fear conditioning. Amygdala slices were treated with MG-132 (10 μM) for various periods, and then biotinylated receptors were precipitated and surface GluR1 was determined by quantitative immunoblotting. Figure 7A shows that surface expression of GluR1 was significantly increased after MG-132 treatment compared with naive control ($F_{4,15} = 26.31$, $p < 0.001$). The increase was detected at 1 h and peaked at 4 h of incubation. The dose-dependent effect of MG-132 is shown in the Fig. 7B.

We examined whether MG-132 affected fear-potentiated startle. Rats were conditioned with 1 or 10 light-shock pairings, and MG-132 (9.5 μg dissolved in 1 μl of 50% DMSO, 1 μl per side) or vehicle was given bilaterally into the amygdala 30 min before training. Retention of memory was assessed 24 h later. As illustrated in Fig. 8, startle amplitude in MG-132-treated rats was significantly higher than those of vehicle control rats both in 1 ($t_8 = 2.41$, $p < 0.05$) and 10 pairings ($t_{11} = 2.24$, $p < 0.05$).

Discussion

Formation of fear memory is believed to involve long-term enhancement of synaptic efficacy within neural circuits in

the amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Pare, 2003). In this report, we showed that fear conditioning increased surface expression of GluR1 in the amygdala. The control group that received CS and US in an unpaired fashion did not present any increase, indicating that GluR1 increase was specific for the learning component of the task. In addition, the increase was restricted to the amygdala but not the hippocampus or cortex. However, it might be inherently difficult to completely avoid a rapid form of context conditioning that implicated both the hippocampus and amygdala when rats were placed in the startle boxes. Because only a single time point (24 h after training when GluR1 expression reached its peak in the amygdala) was tested for the hippocampus, it is possible that learning-related surface GluR1 expression may occur in this area at other time points that we have not examined. Second, conditioning-induced increase in surface expression of GluR1 depended on the activation of NMDA receptors and protein kinases and required the synthesis of new proteins. The inhibitors for NMDA receptor, protein kinases, or protein synthesis, in parallel, impaired fear memory. Third, light-alone trials given 24 h before training attenuated fear-potentiated startle and prevented conditioning-induced increase in surface expression of GluR1. Taken together, these data demonstrate a correlation between long-term synaptic plasticity and memory formation, with changes in surface expression of GluR1.

Previous studies have demonstrated that fear conditioning in rodents depended on presynaptic mechanisms. On the other hand, it has been hypothesized that acquisition of fear

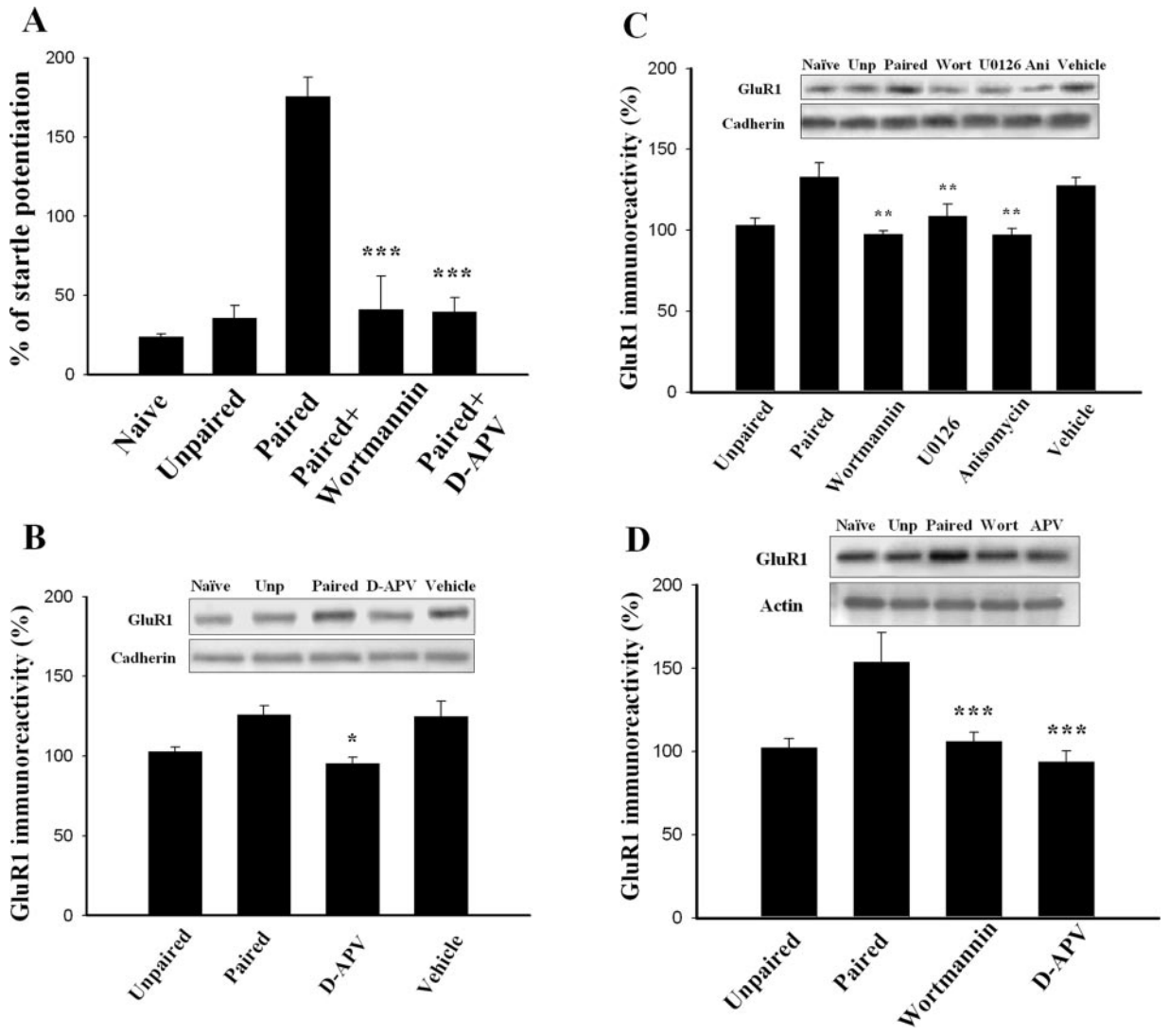


Fig. 3. Block of fear conditioning-induced increase in surface expression of GluR1 by NMDA receptor, kinases, and protein synthesis inhibitors. A, rats were infused with D-APV (25 nmol dissolved in 2 μl of ACSF, 1 μl per side) or wortmannin (2.5 μg/μl in 50% DMSO, 1 μl per side) bilaterally into the amygdala 30 min before training. Memory retention was assessed 24 h after training. Fear-potentiated startle was significantly reduced by D-APV and wortmannin treatment. ***, $p < 0.001$ versus paired. B, 24 hours after training, the tissues of LA and BLA were dissected out, and surface GluR1 was determined using biotin labeling. Pretreatment with D-APV blocked conditioning-induced increase in surface expression of GluR1. *, $p < 0.05$ versus paired. C, block of fear conditioning-induced increase in surface expression of GluR1 by PI-3 kinase, MEK, and protein synthesis inhibitors. Wortmannin (2.5 μg/μl in 50% DMSO, 1 μl per side), U0126 (2 μg dissolved in 2 μl of 50% DMSO, 1 μl per side), or anisomycin (62.5 μg dissolved in 2 μl of 50% DMSO, 1 μl per side) were infused into the amygdala 30 min before training. Twenty-four hours after training, the tissues of LA and BLA were dissected out, and surface GluR1 was determined with biotin labeling. **, $p < 0.01$ versus paired. D, rats were infused with wortmannin (2.5 μg/μl in 50% DMSO, 1 μl per side) or D-APV (25 nmol dissolved in 2 μl of ACSF, 1 μl per side) 30 min before training. Synaptoneurosomes were prepared and GluR1 in each group was determined by quantitative immunoblotting. ***, $p < 0.005$ versus paired.

memory was initiated by calcium influx through NMDA receptors or voltage-dependent calcium channels. The increase in intracellular calcium resulted in the activation of protein kinases such as PI-3 kinase and MAPK. Once activated, MAPK could translocate to the nucleus in which they activated cAMP response element-binding protein and promoted new protein synthesis (Lin et al., 2003; Maren and Quirk, 2004; Rodrigues et al., 2004). This hypothesis implicated a postsynaptic expression of fear memory. In the present study, we demonstrated that fear memory was associated with an increase in the surface expression of GluR1 protein in the synaptic regions. Consistent with behavioral tests, change in GluR1 depended on the activation of NMDA receptor, PI-3 kinase, and MAPK. Furthermore, adding light-alone trials 24 h before training (latent inhibition paradigm) effectively attenuated fear-potentiated startle and prevented conditioning-induced increase in GluR1 levels. These results suggest that GluR1 is a potential downstream effector, which contributes at least in part to the expression of fear memory.

Memory formation is believed to involve long-term reten-

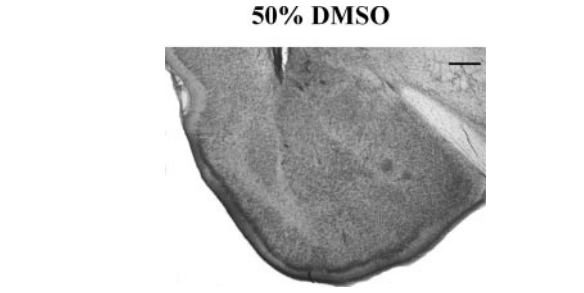


Fig. 4. Vehicle did not lesion the amygdala. Representative photomicrographs show amygdala slices from rats infused with 50% DMSO. There was no evidence of increased cell loss or gliosis in the 50% DMSO-treated animals. Scale bar, 0.5 mm.

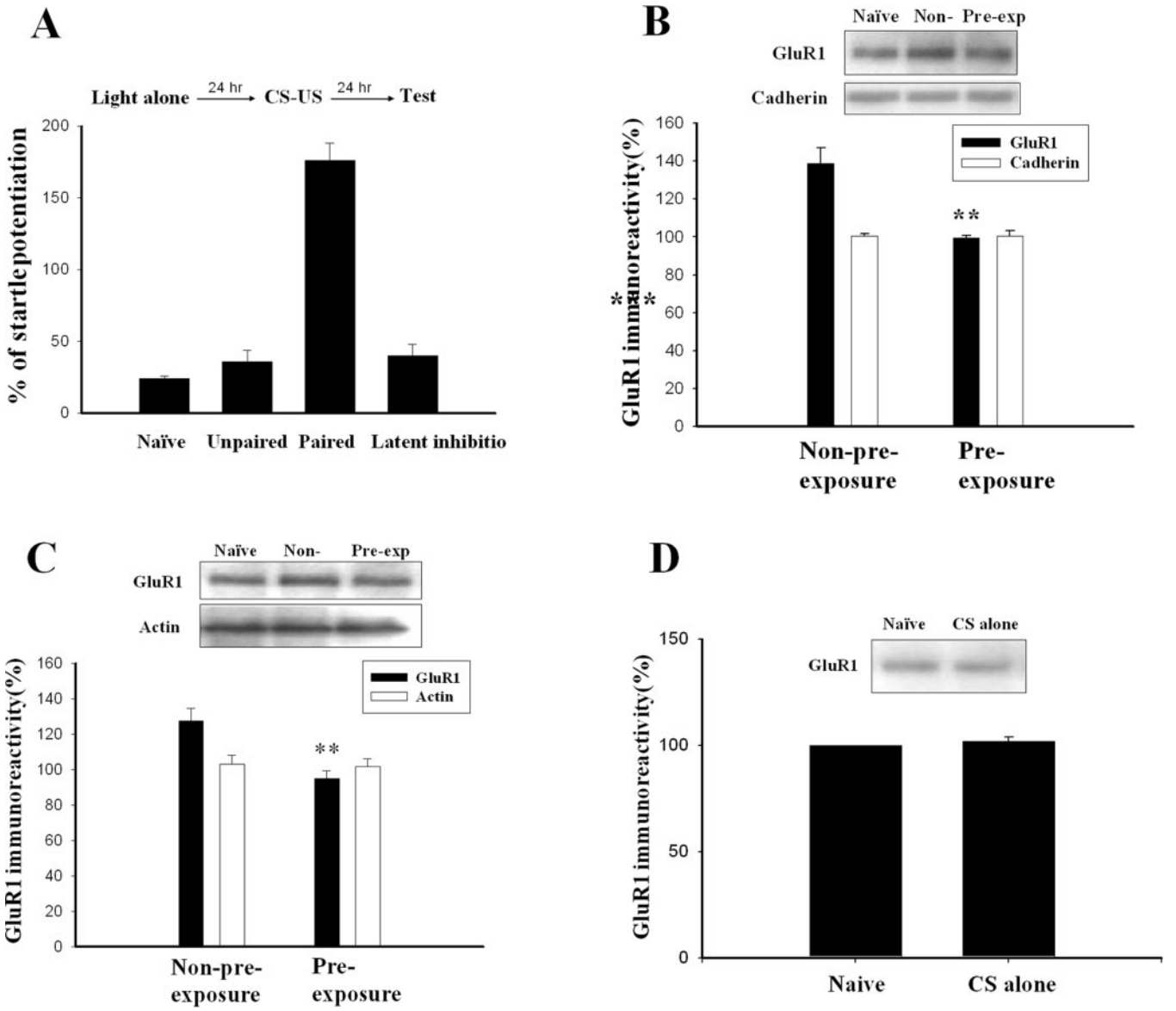


Fig. 5. Pre-exposure of CS alone prevents conditioning-induced increase in surface expression of GluR1. A, rats were given three sessions of 10 presentations of light 24 h before training. Pre-exposure to the CS significantly attenuated startle reflex. ***, $p < 0.001$ versus paired. B and C, pre-exposure to the CS prevented conditioning-induced increase in surface expression of GluR1 measured with biotin labeling (B) and in synaptoneurosomes (C). **, $p < 0.01$ versus non–pre-exposure. D, rats were given three sessions of 10 presentations of light and 24 h later, surface expression of GluR1 was measured with biotin labeling. CS-alone trials did not affect GluR1 expression in the amygdala.

tion of synaptic potentiation within relevant circuits and is stabilized by protein synthesis that targets newly synthesized proteins to the recently activated synaptic sites. We noted that the total mRNA and protein levels of GluR1 were unaltered after fear conditioning, although fear-potentiated startle and conditioning-induced surface expression of GluR1 was blocked by protein synthesis inhibitor. One explanation is that new proteins synthesized in response to learning-associated activity promote trafficking of AMPA receptors to the synaptic regions. AMPA receptor trafficking was regulated by numerous AMPA receptor binding proteins, including transmembrane protein stargazin (Chen et al., 2000; Song and Huganir, 2002). Incompletely folded or assembled proteins were often retrotranslocated into the cytosol and eliminated by ER-associated degradation. Accumulation of unfolded or unassembled proteins in the ER rapidly induced the unfolded protein response, leading to increased transcription of genes encoding ER chaperones such as Ig binding protein (Ma and Hendershot, 2001; Zhang and Kaufman, 2004). It has been shown that neurons require UPR to move the AMPA receptor-like subunit, GLR-1, out of the ER in *Caenorhabditis elegans* (Shim et al., 2004), and LTP in the hippocampus required stargazin phosphorylation that promotes AMPA receptor trafficking via up-regulation of ER chaperones as part of UPR (Vandenberghe et al., 2005). In the present study, we demonstrated that proteasome inhibitor increased surface expression of GluR1 in a time- and dose-dependent manner. Furthermore, administration of proteasome inhibitor into the amygdala-facilitated fear-potentiated startle. We speculate that fear conditioning may

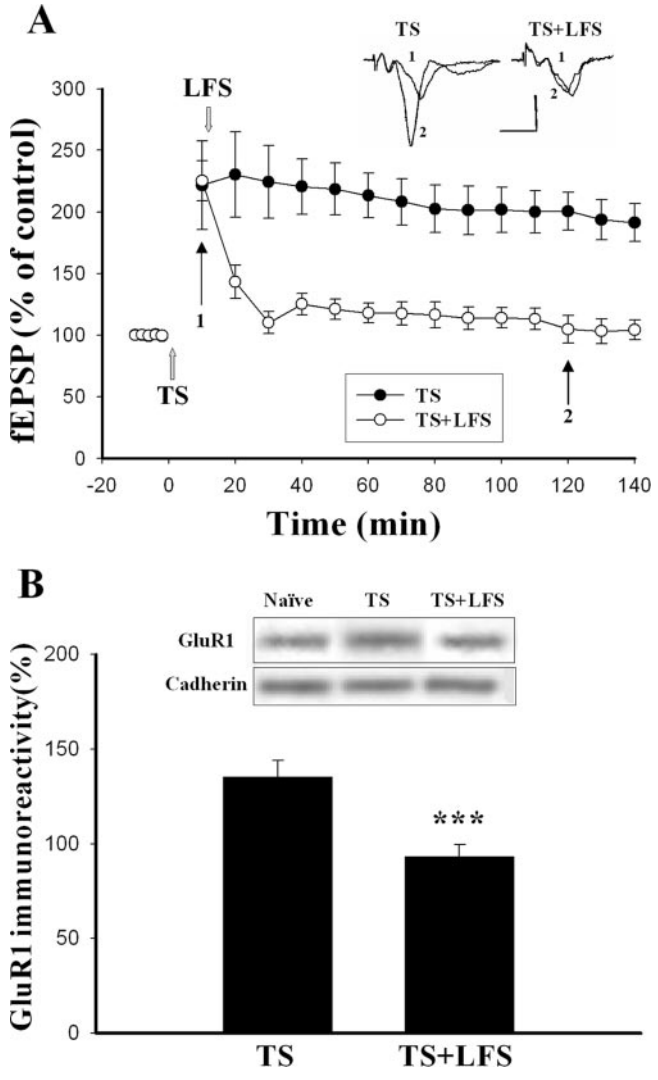


Fig. 6. Tetanic stimulation that evokes LTP increases surface expression of GluR1 in the amygdala slices. A, mean ± S.E. slope of fEPSPs plotted against time. LFS was delivered at 10 min after TS. B, surface GluR1 levels were measured with biotin labeling and expressed as a percentage of that in naive slices. ***, $p < 0.005$ versus TS.

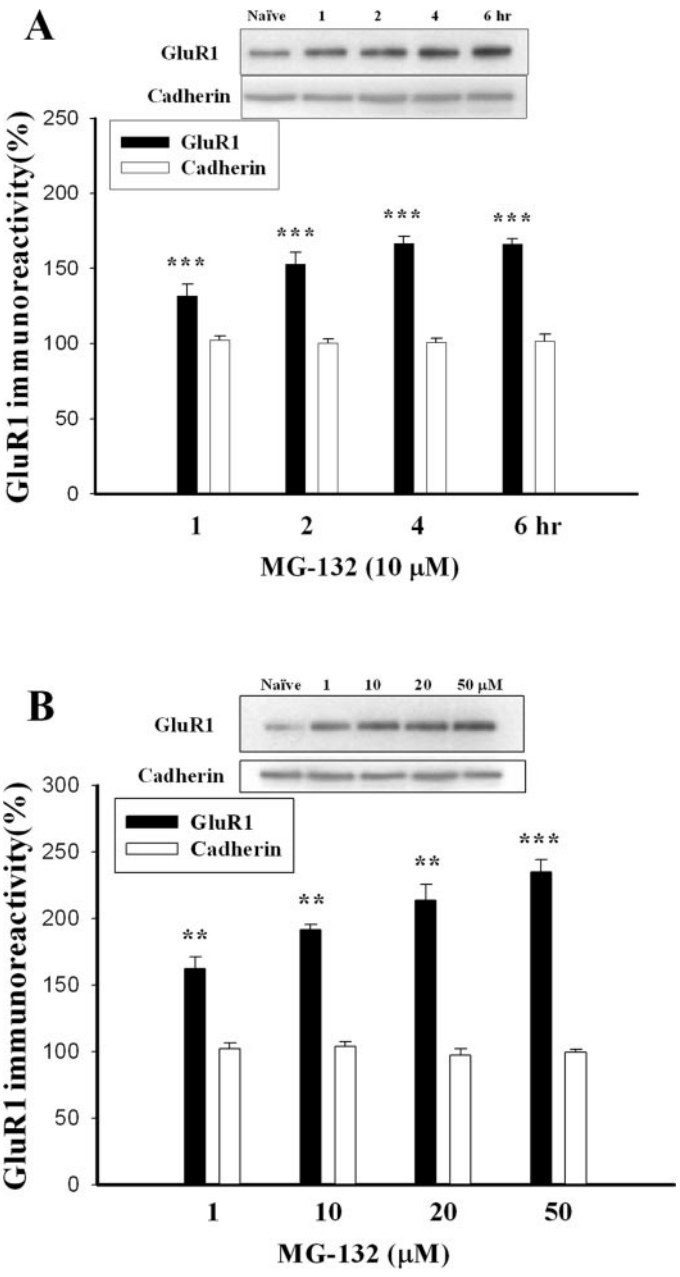


Fig. 7. Proteasome inhibitor increases GluR1 surface expression. A, amygdala slices were incubated with MG-132 (10 μM) for various periods, biotinylated receptors were precipitated, and surface GluR1 was determined by quantitative immunoblotting. GluR1 protein levels in MG-132 treatment were expressed as a percentage of that in naive control slices. ***, $p < 0.001$ versus naive slices. B, dose dependence of the effect of MG-132 (incubation of 4 h) on surface expression of GluR1. **, $p < 0.01$; ***, $p < 0.001$ versus naive slices.

induce stargazin phosphorylation and UPR and that this response promotes AMPA receptor surface trafficking.

In summary, many different mechanisms have been proposed to underlie the expression of LTP and long-term memory. Here, we made biochemical measurements of GluR1 protein levels at the synaptic sites by surface biotinylation and membrane fractionation approaches. We found that pairing the CS with US (fear training) in rats significantly increased the levels of GluR1, and the increase was abolished when animals were pre-exposed to the CS alone at 24 h before training. These results suggest that long-term memory formation is correlated with change in modification of GluR1 expression. Furthermore, our data have an important implication. The increase in surface expression of GluR1 without affecting the total GluR1 mRNA or protein levels suggests that fear conditioning may promote AMPA receptor trafficking to the synaptic sites. Indeed, proteasome inhibitor increased surface expression of GluR1 and facilitates fear conditioning. It is speculated that fear conditioning may induce stargazin phosphorylation and UPR and that this response promotes AMPA receptor surface trafficking.

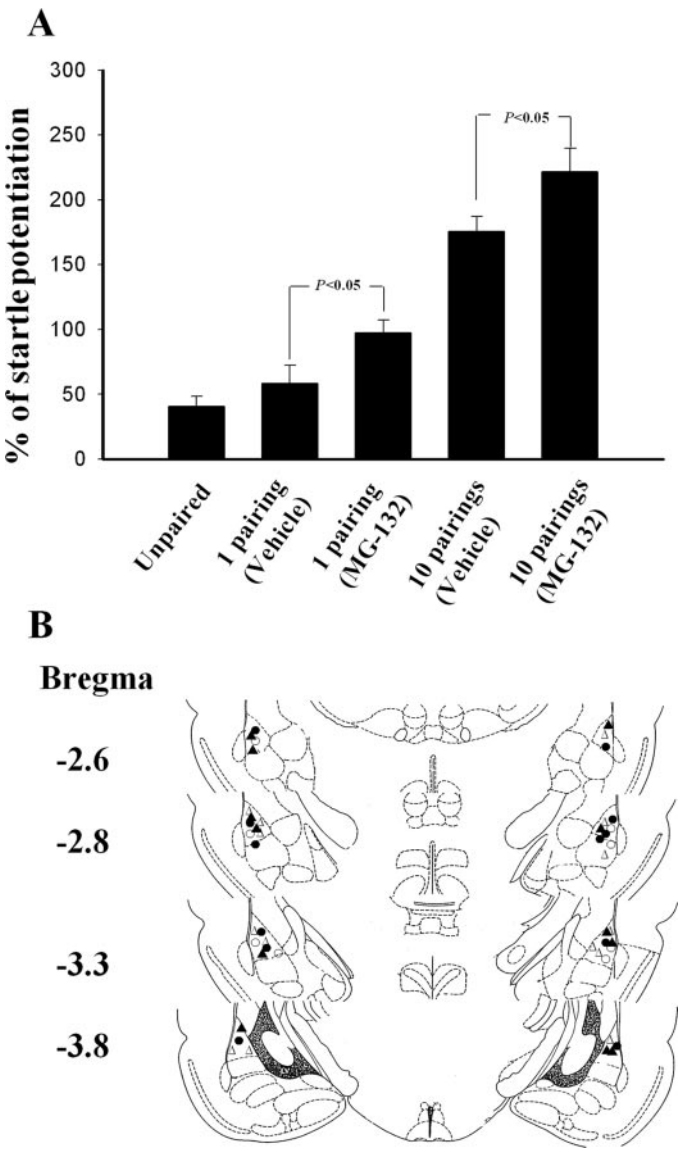


Fig. 8. Proteasome inhibitor promotes surface expression of GluR1 and facilitates fear conditioning. A, plot of percentage of startle potentiation in MG-132- or vehicle-treated animals. Rats were conditioned with 1 or 10 light-shock pairings and MG-132 (9.5 μg per side) was microinjected bilaterally into the amygdala 30 min before training. Retention of memory was assessed 24 h later. B, cannula tip placements from rats infused with vehicle (○) or MG-132 (●) in 1 pairing and with vehicle (△) or MG-132 (▲) in 10 pairings.

References

Banke TG, Bowie D, Lee HK, Huganir RL, Schousboe A, and Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *J Neurosci* **20:**89–102.

Bauer EP, Schafe GE, and LeDoux JE (2002) NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *J Neurosci* **22:**5239–5249.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, and Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature (Lond)* **408:**936–943.

Dudai Y (1996) Consolidation: fragility on the road to the engram. *Neuron* **17:**367–370.

Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* **28:**511–525.

Hampson DR, Huang XP, Oberdorfer MD, Goh JW, Auyeung A, and Wenthold RJ (1992) Localization of AMPA receptors in the hippocampus and cerebellum of the rat using an anti-receptor monoclonal antibody. *Neuroscience* **50:**11–22.

Heynen AJ, Quinlan EM, Bae DC, and Bear MF (2000) Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo. *Neuron* **28:**527–536.

Hollingsworth EB, McNeal ET, Burton JL, Williams RJ, Daly JW, and Creveling CR (1985) Biochemical characterization of a filtered synaptoneurosome preparation from guinea pig cerebral cortex: cyclic adenosine 3′,5′-monophosphate-generating systems, receptors and enzymes. *J Neurosci* **5:**2240–2253.

Huang YY and Kandel ER (1998) Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. *Neuron* **21:**169–178.

Humeau Y, Shaban H, Bissiere S, and Luthi A (2003) Presynaptic induction of heterosynaptic associative plasticity in the mammalian brain. *Nature (Lond)* **426:**841–845.

Lee H and Kim JJ (1998) Amygdalar NMDA receptors are critical for new fear learning in previously fear-conditioned rats. *J Neurosci* **18:**8444–8454.

Lin CH, Yeh HW, Lin CH, Lu KT, Leu TH, Chang WC, and Gean PW (2001) A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. *Neuron* **31:**841–851.

Lin CH, Yeh SH, and Gean PW (2003) The similarities and diversities of signal pathways leading to consolidation and extinction of fear memory. *J Neurosci* **23:**8310–8317.

Lubow RE and Moore AU (1959) Latent inhibition: the effect of non-reinforced pre-exposure to the conditional stimulus. *J Comp Physiol Psychol* **52:**415–419.

Maren S and Quirk GJ (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* **5:**844–852.

Ma Y and Hendershot LM (2001) The unfolded tale of the unfolded protein response. *Cell* **107:**827–830.

McGaugh JL (2000) Memory-a century of consolidation. *Science (Wash DC)* **287:**248–251.

McKernan MG and Shinnick-Gallagher P (1997) Fear conditioning induces a lasting potentiation of synaptic currents in vitro. *Nature (Lond)* **390:**607–610.

Miserendino MJ, Sananes CB, Melia KR, and Davis M (1990) Blocking of acquisition but not expression on conditioned fear-potentiated startle by NMDA antagonists in the amygdala. *Nature (Lond)* **345:**716–718.

Morita MA, Lamprecht R, Nader K, and LeDoux JL (2002) A-kinase anchoring proteins in amygdala are involved in auditory fear memory. *Nat Neurosci* **5:**837–838.

Pare D (2003) Role of the basolateral amygdala in memory consolidation. *Prog Neurobiol* **70:**409–420.

Paxinos G and Watson C (1986) *The Rat Brain in Stereotaxic Coordinates.* Academic Press, New York.

Rodrigues SM, Schafe GE, and LeDoux JE (2004) Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. *Neuron* **44:**75–91.

Rogan MT, Staubli UV, and LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. *Nature (Lond)* **390:**604–607.

Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, and LeDoux JE (2000) Activation of ERK/MAPK kinase in the amygdala is required for memory consolidation of Pavlovian fear conditioning. *J Neurosci* **20:**8177–8187.

Schafe GE and LeDoux JE (2000) Memory consolidation of auditory Pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J Neurosci* **20:**RC96.

Shim J, Umemura T, Nothstein E, and Rongo C (2004) The unfolded protein response regulates glutamate receptor export from the endoplasmic reticulum. *Mol Biol Cell* **15:**4818–4828.

Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, and Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science (Wash DC)* **284:**1811–1816.

Song I and Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci* **25:**578–588.

Staubli U and Chun D (1996) Factors regulating the reversibility of long-term potentiation. *J Neurosci* **16:**853–860.

Takahashi T, Svoboda K, and Malinow R (2003) Experience strengthening transmission by driving AMPA receptors into synapses. *Science (Wash DC)* **299:**1585–1588.

Tomita S, Stein V, Stocker TJ, Nicoll RA, and Bredt DS (2005) Bidirectional synaptic

plasticity regulated by phosphorylation of stargazin-like TARPs. *Neuron* **45:**269–277.
Tsvetkov E, Carlezon WA Jr, Benes FM, Kandel ER, and Bolshakov VY (2002) Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. *Neuron* **34:**289–300.
Vandenberghe W, Nicoll RA, and Bredt DS (2005) Interaction with the unfolded protein response reveals a role for stargazing in biosynthetic AMPA receptor transport. *J Neurosci* **25:**1095–1102.
Weiner I (2003) The two-headed latent inhibition model of schizophrenia: modeling positive and negative symptoms and their treatment. *Psychopharmacology (Berl)* **169:**257–297.
Zhang K and Kaufman RJ (2004) Signaling the unfolded protein response from the endoplasmic reticulum. *J Biol Chem* **279:**25935–25938.
Zinebi F, Xie J, Liu J, Russell RT, Gallagher JP, McKernan MG, and Shinnick-Gallagher P (2003) NMDA currents and receptor protein are down-regulated in the amygdala during maintenance of fear memory. *J Neurosci* **23:**10283–10291.

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