Synaptic expression of glutamate receptor after encoding of fear memory in the rat amygdala

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Abbreviation: AKAPs, A-kinase anchoring proteins; BLA, basolateral nucleus of amygdala;
CS, conditioned stimulus; CV, coefficient of variation; D-APV, D-2-amino-5-phosphonovalerate; EPSC, excitatory postsynaptic current; ER, endoplasmic reticulum; LA, lateral nucleus of amygdala; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; UPR, unfolded protein response; US, unconditioned stimulus; VDCC, voltage-dependent calcium channel.
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 reported that fear conditioning resulted in an increase in surface expression of GluR1 subunit of AMPA receptors in the amygdala whereas total GluR1 mRNA and protein levels were unchanged. The control group that received conditioned stimulus (CS) and unconditioned stimulus (US) 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 NMDA receptors and protein kinases, and required synthesis of new proteins. CS-alone trials applied at 24 hr 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 (TS) that elicited long-term potentiation (LTP) of synaptic transmission. Proteasome inhibitor increased surface expression of GluR1 in a time- and dose-dependent manner. Furthermore, pre-training administration of proteasome inhibitor into the amygdala facilitated 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.
Introduction

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 thought 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 showed that conditioned fear was 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 coefficient of variation (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 receptors in amygdala from fear-conditioned animals (Zinebi et al., 2003) raised the possibility that 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 PKA phosphorylation of S845 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 PKA by A-kinase anchoring proteins (AKAPs) through synapse-associated protein 97 kDa (SAP97) in the lateral amygdala is required for fear memory formation (Morita et al., 2002) indicates that surface expression of AMPA receptor could be important for memory consolidation.

The purpose of this study was to determine whether 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 provides the evidence showing that GluR1 is a potential downstream effector which contributes at least in part to the expression of fear memory. Furthermore, the increase in surface expression, but not 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 week old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and subsequently were mounted on a stereotaxic apparatus. Two cannula made of 22 gauge stainless steel tubing (C313G, Plastic Products) were implanted bilaterally into the lateral (LA) or basolateral (BLA) amygdala [anteroposterior (AP), –2.8 mm; mediolateral (ML), ±4.5 mm; dorsoventral (DV), –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-APV, wortmannin, U0126, anisomycin (Sigma, St. Louis, Missouri) and MG-132 (Tocris Cookson Ltd. Northpoint, 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 rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (ACSF) solution. Subsequently, 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 hr before drugs incubation experiment. ACSF solution had the following composition (in mM): NaCl 117, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2, and glucose 11. The ACSF was bubbled continuously with 95% O₂/5% CO₂ 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 stabilimeter detects and transduces the motion of the cylinder produced by the whole body startle response of the rat (San Diego Instrument, San Diego). 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 three consecutive days, rats were placed in the startle test boxes for 10 min and returned to their home cages.

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

**Training:** Rats were placed in the startle boxes and received 10 light-footshock pairings with an ITI of 2 min. Unpaired controls received the same number of light and footshock presentation, but in a pseudorandom fashion in which the US could occur at anytime except at the 3.2 sec following 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 sec after onset of the 3.7 sec light (light-noise trials). The two trial types were presented in a balanced mixed order (ITI, 30 sec). The percentage of fear-potentiated startle was computed as follow: \[
\frac{\text{(startle amplitude on CS-noise minus noise-alone trials)}}{\text{(noise-alone trials)}} \times 100.
\]

**Western blot analysis**

Rats were sacrificed with 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 0.5mg/ml Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, Illinois) for 1 hour on ice. Slices were rinsed in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) to quench the biotin reaction and then sonicated briefly in homogenizing buffer (1% Triton X-100, 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 (PMSF), 20 µg/ml leupeptin and 4 µg/ml aprotinin). Following sonication, the samples were centrifuged at 12000 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. 250 µg protein of the supernatant was incubated with 50 µl of 50% Neutravidin agarose (Pierce Chemical Co.) for 16 hours at 4 °C, washed 4 times with homogenizing buffer, and bound protein were re-suspended in 4 µl of SDS sample buffer and boiled. Each sample were resolved in 8.5% SDS-polyacrylamide gels, blotted electrophoretically to Immobilon, and blocked overnight in TBS buffer containing 3% bovine serum albumin. For detection of GluR1, Cadherin or Actin, blots were incubated with antibody for GluR1 (1:4000, Santa Cruz
Biotech., Santa Cruz, California), Cadherin (1:2500, Sigma, St. Louis, Missouri) or Actin (1:4000, Santa Cruz Biotech., Santa Cruz, California). 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 softward BIO-ID (Vilber Lourmat, France). In most experiments, GluR1 levels in paired or unpaired animals were expressed as a percentage of those in naïve controls.

**RT-PCR analysis**

Tissues of Amygdala were mixed with 1 ml of TRI reagent (MRC Inc, USA) 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, USA), 10 µl of buffer (250 mM Tris-HCl, 375 mM KCl, and 15 mM MgCl₂), 5 µl of DTT (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). 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 sec (repeated for a total of 30 cycles). The primers for the detection of AMPA receptor subunit GluR1 cDNA were 5’-AGGTTTGCTTTGTCACAA-3’ and 5’-CTTCTCCAGGTCCTGAAA-3’. The primers for the detection of GAPDH cDNA were
5'-TGACAACTTTGGCATCGTGGAAGG-3’ and 5’-CAACGGATACATTGGGGGTAGGAAC-3’. Control amplifications were done without cDNA. Amplification from primers, GluR1 or GAPDH produces fragments of 456 or 233 bp in length respectively. After PCR amplification, 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 a software BIO-ID (Vilber Lourmat, France).

**Histology**

At the end of experiments, animals received an overdose of pentobarbital (100mg/kg) and the brains were removed from the skull and fixed in buffered 4% paraformaldehyde (PH 7.4) for 48 hrs. Brains were sectioned with a sliding MicroSlicer (DKT-1000, Ted Pella Inc., 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, 4.7mM KCl, 1.18 mM MgSO₄, 2.5 mM CaCl₂, 1.18mM KH₂PO₄, 24.9 mM NaHCO₃, 10mM dextrose, 10µg/ml adenosine deaminase, pH adjusted to 7.4 by bubbling with 95% O₂+5% CO₂. Proteinase inhibitors (0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.1 mg/ml aprotinin, 5mM 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 Millipore syringe filter holder. The diluted
filtrate was forced over three layers of nylon (Tetko, 100 µm pore size) pre-wetted with 150 µl of lysis buffer, and collected in a 1.5 ml Eppendorf tube. The nylon pre-filtered mixture was loaded into another 1 ml tuberculin syringe and forced through a pre-wetted 5 µm Millipore nitrocellulose filter. The homogenate was kept ice cold at all times to minimize proteolysis. The filtered particulate was then spun at 1000 x g for 15 min at 4°C. The supernatant was removed, and the pellet (synaptoneurosome) was re-suspended in 80 µl of lysis buffer for Western blot analysis.

Data analysis

Data were analyzed with ANOVA. A single-factor ANOVA 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 difference in startle reflex between drug-treated and vehicle control groups. All values in the text and figure legends are mean±SEM.

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 as compared to naïve control. The increase was detected at 2 hr after training and sustained for at least 24 hours ($F_{9,20}=35.45$, $p<0.0001$, 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 hr time points ($p<0.001$). No significant difference was seen between naïve control and 0.5 and 1 hr time points ($p>0.05$).

Synaptoneurosomes resemble isolated glutamatergic synapses located on the dendritic spines, with resealed pre- and postsynaptic compartments (Heynen et al., 2000; Hollingsworth et al., 1985; Hampson et al., 1992). We examined whether regulation of glutamate receptor availability after fear conditioning at the synaptoneurosomes occurred as that measured with biotin labeling. As shown in figure 1B, a similar time course of GluR1 expression was seen in synaptoneurosomes. The increase was detected at 2 hr after training and sustained for at least 24 hr ($F(5,12)=7.59$, $p<0.01$, n=3 rats in each time point). Post hoc comparison revealed the differences between naïve control and 2 ($p<0.05$), 6, 12 and 24 hr ($p<0.01$) time points. No significant difference was seen between naïve control and 0.5 hr 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 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 naïve and unpaired rats ($p>0.1$) (Fig.1A). We determined whether 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 figure 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 small increase, the difference did not reach the 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 upon 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-2-amino-5-phosphonovalerate (D-APV, 25 nmol dissolved in 2 µl 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 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 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, pre-training 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 50% DMSO, 1 µl per side) or anisomycin (62.5 µg dissolved in 2 µl 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 vs. 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 conditioned stimulus (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 conditioned response (CR) following 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 animal was pre-exposed to the CS. Rats were pre-exposed to 3 sessions of 10 presentations of the 3.7 sec light 24 hr 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 at 24 hr 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 figures 5B and 5C, 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 affect surface expression of GluR1 (102.0\(\pm\)1.8% of naïve 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 \(\mu\)m lateral to the recording electrode. After 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 fEPSP were 213.4\(\pm\)18.2 and 200.7\(\pm\)15.2 of pre-tetanus level
at 1 and 2 hr after the TS (Fig.6A). Six hours following the onset of TS, a relatively small portion of the LA was dissected from each slice. Four to 6 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 when compared to 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 at 10 min after TS, reliable depotentiation could be induced by 3 min of 5 Hz stimulation. The slope of fEPSP at 2 hr after TS was 104.9±11.4 of pre-tetanus level (n=6, p<0.001 vs. control). We determined whether the increase in GluR1 expression was affected by subsequent LFS. As illustrated in figure 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 vs. 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 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. Recently, it has been shown 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 BiP protein levels and greatly increased surface expression of GluR1 (Vandenberghe et al., 2005; Tomita et al., 2005). We therefore examined whether administration of proteasome inhibitors increased 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 as compared to naïve control ($F_{(4,15)}=26.31, p<0.001$). The increase was detected at 1 hr and peaked at 4 hr of incubation. The dose-dependent effect of MG-132 is shown in the figure 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 50% DMSO, 1 µl per side) or vehicle was given bilaterally into the amygdala 30 min before training. Retention of memory was assessed 24 hr later. As illustrated in figure 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 thought 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. Since only a single time point (24 hr 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. Secondly, conditioning-induced increase in surface expression of GluR1 depended on the activation of NMDA receptors and protein kinases, and required synthesis of new proteins. The inhibitors for NMDA receptor, protein kinases or protein synthesis, in parallel, impaired fear memory. Thirdly, light-alone trials given at 24 hr 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 memory was initiated by calcium influx through NMDA receptors or voltage-dependent calcium channels (VDCCs). 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 where they activated CREB and promoted new protein synthesis (Lin et al., 2003;
Rodrigues et al., 2004; Maren and Quirk, 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 hr 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 thought to involve long-term retention 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 retro-translocated 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 (BiP) (Ma and Hendershot, 2001; Zhang and Kaufman, 2004). It has been shown that neurons require UPR to move 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 have 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 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 hr 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.
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Figure Legends

Figure 1. Fear training increases surface expression of GluR1. A, Time course of surface expression of GluR1 induced by fear conditioning. Figure shows the representative blots and Mean±SE 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 vs. naïve 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 vs. naïve control. C, Surface GluR1, measured at 24 hr 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.

Figure 2. Fear conditioning does not affect 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 glyceraldehydes-3-phosphate dehydrogenase (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 naïve control.
Figure 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 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 hr after training. Fear-potentiated startle was significantly reduced by D-APV and wortmannin treatment. ***p<0.001 vs. paired. B, Twenty-four hours after training, the tissues of LA and BLA were dissected out and surface GluR1 was determined using biotin labeling. Pre-treatment with D-APV blocked conditioning-induced increase in surface expression of GluR1. *p<0.05 vs. 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 50% DMSO, 1 µl per side) or anisomycin (62.5 µg dissolved in 2 µl 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 vs. 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 vs. paired.

Figure 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.
Figure 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 hr before training. Pre-exposure to the CS significantly attenuated startle reflex. ***p<0.001 vs. 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 snaptoneuroosomes (C). **p<0.01 vs. non-pre-exposure. D, Rats were given three sessions of 10 presentations of light and 24 hr later surface expression of GluR1 was measured with biotin labeling. CS-alone trials did not affect GluR1 expression in the amygdala.

Figure 6. Tetanic stimulation that evokes LTP increases surface expression of GluR1 in the amygdala slices. (a) Mean±SE 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 naïve slices. ***p<0.005 vs.TS.

Figure 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 vs. naïve slices. B, Dose dependence of the effect of MG-132 (incubation of 4 hr) on surface expression of GluR1. **p<0.01, ***p<0.001 vs naïve slices.
Figure 8. Proteasome inhibitor promotes surface expression of GluR1 and facilitates fear conditioning. A, Plot of percent 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 hr 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.
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