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GluA1 phosphorylation at serine 831 in the lateral amygdala is required for fear renewal

Sukwon Lee[1,6], Beomjong Song[1,6], Jeongyeon Kim[2,6], Kyungjoon Park[3,4], Ingie Hong[1], Bobae An[1], Sangho Song[1], Jiwon Lee[1], Sungmo Park[1], Jihye Kim[1], Dongeun Park[1], C Justin Lee[2], Kyungjin Kim[1], Ki Soon Shin[3,4], Richard W Tsien[5] & Sukwoo Choi[1]

Fear renewal, a widely pursued model of post-traumatic stress disorder and phobias, refers to the context-specific relapse of conditioned fear after extinction. However, its molecular mechanisms are largely unknown. We found that renewal-inducing stimuli, generally believed to be insufficient to induce synaptic plasticity, enhanced excitatory synaptic strength, activity of synaptic GluA2-lacking AMPA receptors and Ser831 phosphorylation of synaptic surface GluA1 in the lateral nucleus of the amygdala (LAn) of fear-extinguished rats. Consistently, the induction threshold for LAn synaptic potentiation was considerably lowered after extinction, and renewal occluded this low-threshold potentiation. The low-threshold potentiation (a potential cellular substrate for renewal), but not long-term potentiation, was attenuated by dialysis into LAn neurons of a GluA1-derived peptide that competes with Ser831-phosphorylated GluA1. Microinjections of the same peptide into the LAn attenuated fear renewal, but not fear learning. Our findings suggest that GluA1 phosphorylation constitutes a promising target for clinical treatment of aberrant fear-related disorders.

Fear extinction, an animal model for exposure therapy, has provided important insight for treatment of aberrant fear-related disorders[1–4]. However, one intrinsic property of extinction has limited the effectiveness of this well-established therapy: extinguished fear easily relapses even after extensive extinction (or exposure therapy)[5,6]. Together with other forms of fear relapse such as reinstatement and spontaneous recovery, fear renewal is thought to be one of the best models for studying recurrent relapses in patients with aberrant fear-related disorders[1]. To date, fear renewal has been studied mostly at the level of systems and behavior[7–10]. Elucidation of the precise molecular and cellular changes underlying fear renewal is believed to offer a promising target for pharmacological treatment of aberrant fear-related disorders. However, recent efforts to treat these emotional disorders have mainly focused on thwarting fear relapse without precise knowledge of the molecular mechanisms involved in fear relapse, including renewal[11–14].

Auditory fear conditioning has been used as a prevailing animal model for studying fear-related behaviors including fear renewal. The pairing of a tone (conditioned stimulus, CSt) and a shock (unconditioned stimulus) produces conditioned fear that may persist across the lifetime[15]. Thereafter, the presentation of the tone CSt alone elicits conditioned fear responses. However, repetitive tone presentation to the conditioned subject without a shock extinguishes the conditioned fear responses (fear extinction). Notably, the extinguished fear remains extinguished only when the subject is tone tested in the context in which the extinction was performed, whereas the extinguished fear is rapidly relapsed or renewed when the subject is tone tested in the contexts other than where the extinction was performed (fear renewal)[1]. Thus, both the tone CSt and proper context are required for fear renewal.

A large body of evidence suggests that fear renewal is a network phenomenon[8,16] (**Supplementary Fig. 1**). It has been proposed that the LAn is the pivotal component of the regulation of the neural network of fear renewal[8]. The LAn receives both CSt- and context-based excitatory inputs from the auditory thalamus and cortex, and the hippocampus, respectively[17]. This connectivity is also consistent with previous findings showing context- and hippocampus-dependent enhancement of CSt-evoked unit activity following renewal[18,19]. Furthermore, the LAn has been proposed to enhance central amygdala activity via the basal amygdala[20,21] and amygdala intercalated neurons[22], both of which are critical for renewal[9,16]. In summary, the LAn receives two critical inputs required for renewal (that is, CSt and context) and sends its outputs to the nuclei that are important for fear renewal. Thus, acute plastic changes at LAn synapses in response to renewal-inducing stimuli may be crucial in fear renewal. To date, this possibility has not been tested.

One mechanism that may account for the rapid renewal of extinguished fear is the phosphorylation of synaptic AMPA-type glutamate receptors (AMPARs), particularly GluA1 subunits, which enhances either channel conductance[23,24] or the lateral diffusion of AMPARs

[1]School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, Korea. [2]Center for Neural Science and Center for Functional Connectomics, Korea Institute of Science and Technology, Seoul, Korea. [3]Department of Biology, Kyunghee University, Seoul, Korea. [4]Department of Life and Nanopharmaceutical Sciences, Kyunghee University, Seoul, Korea. [5]Department of Physiology and Neuroscience, Neuroscience Institute, New York University Langone Medical Center, New York, New York, USA. [6]These authors contributed equally to this work. Correspondence should be addressed to S.C. (sukwoo12@snu.ac.kr), K.S.S. (kisoon_shin@khu.ac.kr) or K.K. (kyungjin@snu.ac.kr).

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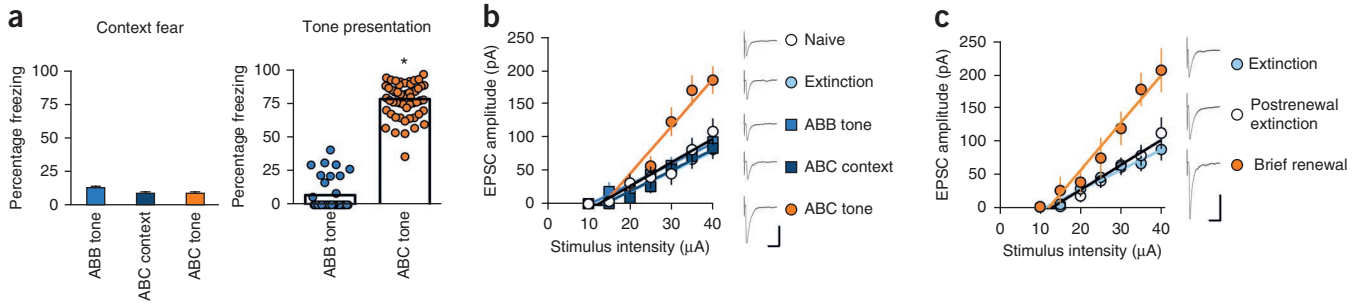

Figure 1 Renewal-inducing stimuli produce a context-specific enhancement of synaptic efficacy at T-LAn synapses. (**a**) Pooled behavioral results. Left, levels of freezing during context exposure in ABB-tone group ($n = 39$ rats), ABC-context group ($n = 54$ rats) and ABC-tone group ($n = 43$ rats) on day 7 ($F_{2,133} = 1.905$, $P = 0.1529$, one-way ANOVA). Right, levels of freezing during tone presentation in ABB-tone group ($n = 39$ rats) and ABC-tone group ($n = 54$ rats) on day 7 ($t_{91} = 27.42$, $P < 0.05$, unpaired t test). $^*P < 0.05$. (**b**) Input-output curves for EPSCs in naive group, extinction group, ABB-tone group, ABC-context group and ABC-tone group ($F_{4,45} = 7.701$, $P < 0.0001$; naive, 3.57 ± 0.63 pA μA^{-1}, $n = 8$ cells (8 rats); extinction group, 3.05 ± 0.56 pA μA^{-1}, $n = 9$ cells (9 rats); ABB-tone group, 3.10 ± 0.64 pA μA^{-1}, $n = 8$ cells (8 rats); ABC-context group, 3.13 ± 0.44 pA μA^{-1}, $n = 7$ cells (7 rats); ABC-tone group, 7.15 ± 0.84 pA μA^{-1}, $n = 14$ (13 rats); $P < 0.01$ for all the four pairs, one-way ANOVA with Newman-Keuls post-test). Series resistances of whole-cell recordings were not different between the groups (naive, 17.62 ± 0.82 MΩ; extinction group, 16.93 ± 0.63 MΩ; ABB-tone group, 16.64 ± 0.78 MΩ; ABC-context group, 17.57 ± 0.70 MΩ; ABC-tone group, 17.39 ± 0.44 MΩ). For the behavioral procedure, see **Supplementary Figure 2a**. (**c**) Input-output curves for EPSCs in extinction group, postrenewal-extinction group and brief renewal group ($F_{2,21} = 8.297$, $P = 0.0026$; extinction group, 3.02 ± 0.57 pA μA^{-1}, $n = 8$ cells (8 rats); brief renewal group, 7.17 ± 1.02 pA μA^{-1}, $n = 7$ cells (7 rats); postrenewal-extinction group, 3.77 ± 0.68 pA μA^{-1}, $n = 7$ cells (7 rats); $P < 0.01$ for all the two pairs, one-way ANOVA with Newman-Keuls post-test). For the behavioral procedure, see **Supplementary Figure 2b**. The representative traces are the averages of four responses evoked by input stimulations of 35 μA. Error bars represent s.e.m. Scale bars in **b** and **c** represent 20 ms and 100 pA.

from extrasynaptic sites to synaptic sites[25,26]. Among the phosphorylation sites of GluA1, Ser831 and Ser845 have been implicated in both synaptic plasticity and learning[27–30]. In particular, Ser831 phosphorylation is positively correlated with long-term potentiation (LTP), a cellular model for learning and memory[27]. However, disruption of Ser831 phosphorylation alone fails to reveal any clear deficits in synaptic plasticity and learning[31] (but see ref. 30). Thus, a role for Ser831 phosphorylation in synaptic plasticity and learning has not been fully characterized.

Here we extensively characterized LAn excitatory synapses following fear renewal in brain slices prepared from behavior-trained rats (**Supplementary Fig. 2**). We found that Ser831 phosphorylation of GluA1 in fear renewal mediates a low-threshold potentiation at LAn synapses primed with previous extinction of conditioned fear.

RESULTS

Enhanced T-LAn synaptic efficacy with renewal

The thalamic input synapses onto the LAn (T-LAn synapses) are one of the major LAn excitatory input synapses, and a change in the function of these input synapses is known to have a marked effect on LAn neuronal activity and fear expression[17]. Thus, we sought to detect any change in the function of these input synapses following renewal. We adopted an *ex vivo* approach in which synaptic functions were assessed in acute brain slices prepared from behavior-trained rats (**Supplementary Fig. 2**). Three tone-shock pairings (or unpairings) were presented on the first day of our experiments (day 1) and repeated on the next day (day 2). During extinction training (days 4–6), rats were repeatedly presented with a tone CSt without a foot shock. For renewal (day 7), fear-extinguished rats were placed in a different chamber (context C) from both the conditioning (context A) and extinction (context B) contexts, and were presented with a tone CSt without a foot shock (ABC-tone group). Different controls (naive group, extinction group, ABB-tone group and ABC-context group) were used to ensure that the effects seen with the renewal protocol required both the tone CSt and the renewal context (context C) (**Supplementary Fig. 2a**). In the extinction group, conditioned rats were fear-extinguished as in the ABC-tone group, but were not tone tested on day 7. In the ABB-tone group, conditioned rats were fear-extinguished as in the ABC-tone group, but the fear-extinguished rats were then tone tested in the extinction context (context B) instead of the renewal context (context C) on day 7. The ABC-context group involved the same protocol as that used for the ABC-tone group, except that the fear-extinguished rats were exposed to the renewal context (context C) without tone testing on day 7. All slices were prepared 6 d after initial conditioning (day 7). Brain slices were prepared immediately after a tone test (ABB-tone group and ABC-tone group) or context exposure (ABC-context group). To avoid possible bias, we performed our electrophysiological and behavioral experiments in an interleaved and blind fashion.

Synaptic efficacy at T-LAn synapses was assessed using whole-cell voltage-clamp recordings in the slices prepared from naive or behaviorally trained rats. The renewal protocol, which successfully induced a strong relapse of extinguished fear, produced a substantial potentiation at T-LAn synapses as compared with the four controls (**Fig. 1**), and there was no significant difference among the four controls ($P > 0.05$ for all designated pairs, Newman-Keuls post-test). We also compared series resistances of whole-cell recordings between the five groups and did not detect any significant differences ($F_{4,45} = 0.3905$, $P = 0.8142$). These results indicate that altered recording conditions do not account for the observed results. Taken together, the enhancement of T-LAn synaptic efficacy appears to be strongly associated with fear renewal.

Given that renewed fear is known to be extinguished rapidly, it is possible that the synaptic change that we observed results from the extinction of renewed fear. To test this, we compared T-LAn synaptic efficacy between three groups (extinction, brief renewal and postrenewal-extinction groups). We used the extinction group as a control in which baseline T-LAn synaptic efficacy was estimated. In the brief renewal group, slices were prepared immediately after a very brief renewal protocol (5 s of tone presentation in the context C); that is, extinction of renewed fear, if any, should be minimal as a result of the brief tone presentation. In contrast, slices

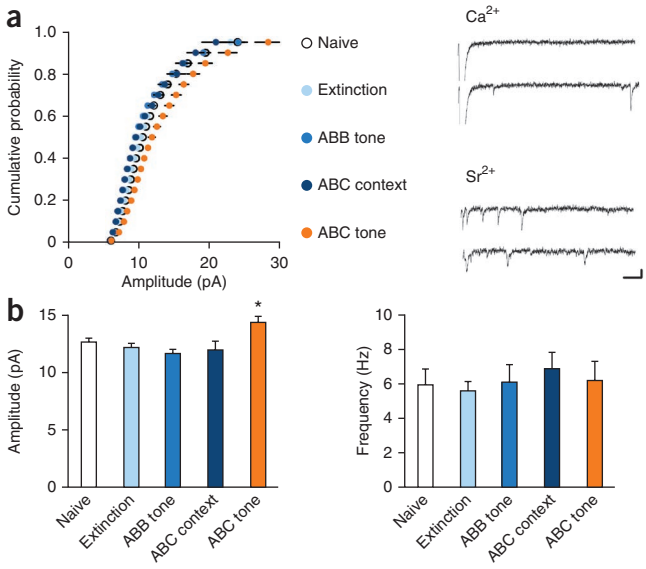

Figure 2 Renewal-inducing stimuli enhance the amplitude of AMPAR-mediated mEPSCs at T-LAn synapses in a context-specific manner. (**a**) Left, cumulative amplitude distributions of evoked mEPSCs in the presence of Sr^{2+} (naive, $n = 8$ cells (8 rats); extinction group, $n = 7$ cells (7 rats); ABB-tone group, $n = 11$ cells (11 rats); ABC-context group, $n = 4$ cells (4 rats); ABC-tone group, $n = 11$ cells (11 rats); 300 events per cell, $P < 0.01$ for the ABC-tone group versus other control groups, Kolmogorov-Smirnov test). Right, sample traces of evoked EPSCs in the presence of Ca^{2+} or Sr^{2+}. Scale bars represent 50 ms and 10 pA. Note that the representative traces were further processed with a digital Gaussian filter for better display. (**b**) Left, mean amplitude of mEPSCs evoked in the presence of Sr^{2+} ($F_{4,40} = 6.037$, $P = 0.0008$; naive, 12.66 ± 0.36 pA; extinction, 12.12 ± 0.42 pA; ABB tone, 11.67 ± 0.35 pA; ABC context, 11.95 ± 0.75 pA; ABC tone, 14.37 ± 0.57 pA; $^*P < 0.05$, one-way ANOVA with Newman-Keuls post-test). Right, mean frequency of mEPSCs evoked in the presence of Sr^{2+} (naive, 6.04 ± 0.92 Hz; extinction, 5.68 ± 0.55 Hz; ABB tone, 6.19 ± 1.03 Hz; ABC context, 6.97 ± 0.96 Hz; ABC tone, 6.29 ± 1.11 Hz; $F_{4,40} = 0.1257$, $P = 0.9722$, one-way ANOVA). Error bars represent s.e.m.

were prepared immediately after complete extinction of renewed fear in the postrenewal-extinction group (**Supplementary Fig. 2b**). The brief renewal protocol produced a substantial potentiation as compared with the extinction group and the postrenewal-extinction group (**Fig. 1c**). There was no significant difference between the extinction group and the postrenewal-extinction group ($P > 0.05$, Newman-Keuls post-test; **Fig. 1c**), indicating that extinction of renewed fear does not alter LAn synaptic strength. These findings suggest that the acute enhancement in LAn synaptic efficacy is not a result of extinction of renewed fear. Furthermore, the acute synaptic change induced by the very brief tone CSt indicates that this synaptic potentiation may be rapid enough to support fast expression of conditioned fear during renewal.

To determine whether the observed enhancement of T-LAn synaptic efficacy is a result of a presynaptic or postsynaptic change, we examined miniature AMPAR-mediated excitatory postsynaptic currents (mEPSCs) that were sampled from evoked T-LAn synapses in five groups: naive, extinction, ABB tone, ABC context and ABC tone (**Fig. 2**). Although no substantial difference in the frequency of asynchronous mEPSCs was observed in the five groups (**Fig. 2b**), the mean amplitude distribution of mEPSCs was substantially shifted to the right in the ABC-tone group relative to other controls (**Fig. 2a**), and there was no significant difference among the four controls ($P > 0.05$ for all designated pairs, Newman-Keuls post-test). These results indicate that a postsynaptic function (that is, AMPAR function and/or number) may be enhanced following renewal.

We next examined the rectification index of AMPAR-mediated EPSCs, which correlates positively with enhanced activity of GluA2-lacking AMPARs[32]. We compared rectification of EPSCs in five groups using a spermine-containing intracellular solution (**Fig. 3a**). EPSCs at LAn synapses showed a substantially higher rectification index in the ABC-tone group relative to the other four controls, and there was no significant difference among the four controls ($P > 0.05$ for all designated pairs, Newman-Keuls post-test). As an independent measure of enhanced activity of GluA2-lacking AMPARs, we examined whether AMPAR-mediated EPSCs become more susceptible to blockade by 1-naphthyl acetyl spermine (NASPM, a selective blocker for GluA2-lacking AMPARs) following renewal. The application of 50 μM NASPM inhibited AMPAR-mediated EPSCs to a substantially greater degree in the ABC-tone group compared with the four controls (**Fig. 3b–g**). Taken together,

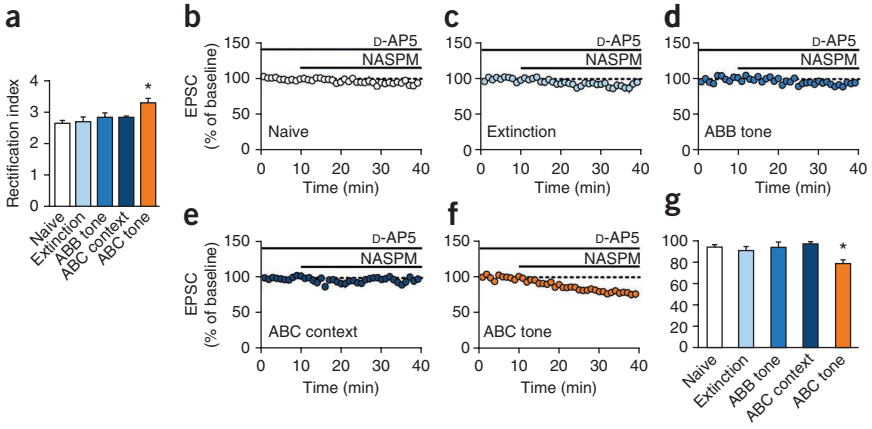

Figure 3 Renewal-inducing stimuli enhance GluA2-lacking AMPAR activity at T-LAn synapses in a context-specific manner. (**a**) The rectification index of synaptic AMPA currents was increased in the ABC-tone group relative to the four control groups ($F_{4,31} = 5.292$, $P = 0.0028$; naive, 2.64 ± 0.09, $n = 8$ cells (7 rats); extinction group, 2.69 ± 0.15, $n = 5$ cells (5 rats); ABB-tone group, 2.83 ± 0.13, $n = 6$ cells (5 rats); ABC-context group, 2.83 ± 0.04, $n = 5$ cells (4 rats); ABC-tone group, 3.30 ± 0.15, $n = 8$ cells (6 rats); $^*P < 0.05$ for all the four pairs, one-way ANOVA with Newman-Keuls post-test). The reversal potentials did not change significantly among the five groups ($F_{4,31} = 0.6574$, $P = 0.6268$; naive, 15.0 ± 0.0 mV; extinction group, 15.0 ± 0.0 mV; ABB-tone group, 14.67 ± 0.21 mV; ABC-context group, 15.0 ± 0.0 mV; ABC-tone group, 14.38 ± 0.63 mV). (**b–f**) NASPM inhibited AMPAR-mediated EPSCs in the ABC-tone group to a substantially greater degree than the other four controls (naive, $n = 5$ cells (5 rats); extinction, $n = 7$ cells (6 rats); ABB tone = 5 cells (5 rats); ABC context = 5 cells (5 rats); ABC tone = 7 cells (7 rats)). D-AP5 (50 μM) was included in the recording solution. (**g**) Results summary ($F_{4,28} = 4.573$, $P = 0.0069$; $^*P < 0.05$ ABC tone versus all other groups, one-way ANOVA with Newman-Keuls post-test). Error bars represent s.e.m.

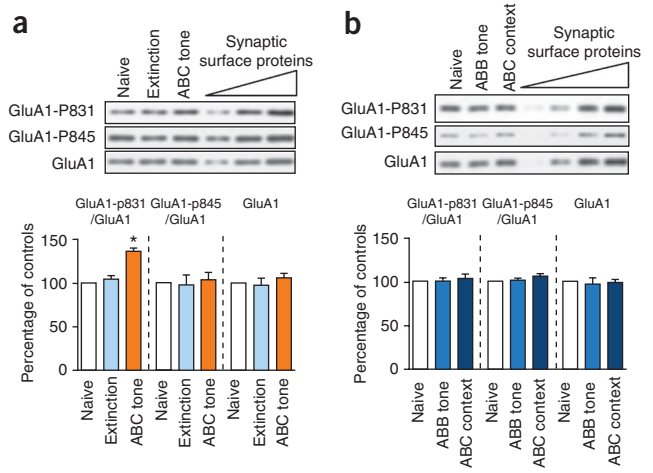

Figure 4 Renewal-inducing stimuli enhance Ser831 phosphorylation of surface GluA1 in LAn synaptosomes in a context-specific manner. (**a**) Top, representative immunoblot. Bottom, pooled result showing that Ser831 phosphorylation was enhanced in the ABC-tone group relative to the other controls (naive and extinction group). There was no significant difference across the three groups in terms of the amount of surface GluA1 from the LAn synaptosomes (naive, $n = 3$ (9 rats); extinction group, $n = 3$ (9 rats); ABC-tone group, $n = 3$ (9 rats)). (**b**) Top, representative immunoblot. Bottom, there was no significant difference across the three groups (naive, ABB tone, ABC context) in terms of the Ser831 phosphorylation level and the amount of surface GluA1 from the LAn synaptosomes (naive, $n = 3$ (9 rats); ABB-tone group, $n = 3$ (9 rats); ABC-context group, $n = 3$ (9 rats); GluA1-p831/GluA1, $F_{2,8} = 0.2664$, $P = 0.7747$, one-way ANOVA; GluA1, $F_{2,6} = 0.1273$, $P = 0.8828$, one-way ANOVA). The full blots are shown in **Supplementary Figure 10**. Error bars represent s.e.m.

these results suggest that the activity of GluA2-lacking AMPARs at LAn synapses was enhanced following renewal.

Enhanced Ser831 phosphorylation of GluA1 with renewal

We next searched for molecular changes associated with renewal. Given that the amplitude of mEPSCs changed following renewal, we predicted that there would be postsynaptic changes. It is widely held that phosphorylation of AMPARs, especially the C terminus of GluA1 subunit, is critical for enhancing synaptic AMPAR activity[26,33]. Among the known phosphorylation sites, Ser831 of GluA1 is known to be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) and protein kinase C (PKC) (but predominantly by CaMKII) after both LTP[27] and learning[34]. To examine any changes in phosphorylation of synaptic surface AMPARs following renewal, we used a biochemical surface biotinylation technique to isolate the surface proteins of LAn synaptosomes of hippocampal cultures and slices[35]. We compared GluA1 phosphorylation in five groups: naive, extinction, ABB tone, ABC context and ABC tone.

The Ser831 phosphorylation of synaptic surface GluA1 was markedly increased in the ABC-tone group relative to the naive and extinction groups ($F_{2,8} = 28.35$, $P = 0.0009$, one-way ANOVA; $P < 0.05$ for ABC tone versus naive controls or extinction group, Newman-Keuls post-test; **Fig. 4a**), without any significant changes in expression of synaptic surface GluA1 in these groups ($F_{2,8} = 0.5929$, $P = 0.5821$; **Fig. 4a**). There was no significant difference between naive controls and the extinction group ($P > 0.05$, Newman-Keuls post-test). Ser831 phosphorylation was not changed in the ABB-tone and ABC-context groups relative to naive controls ($F_{2,8} = 0.2665$, $P = 0.7747$; **Fig. 4b**). In addition, Ser845 phosphorylation was not altered in the four groups (extinction, ABB tone, ABC context and ABC tone) relative to naive controls (GluA1-p845/GluA1,$F_{2,8} = 0.1354$, $P = 0.8760$ for **Fig. 4a**; GluA1-p845/GluA1, $F_{2,8} = 1.568$, $P = 0.2832$ for **Fig. 4b**). Thus, fear renewal appears to be associated with Ser831 phosphorylation, but not with Ser845 phosphorylation. This finding contrasts with previous reports demonstrating changes in both Ser831 and Ser845 phosphorylation during hippocampal learning[29,34].

Tone CSt effects following renewal require prior extinction

Our results indicate that the acute enhancement that we observed in synaptic efficacy and GluA1 phosphorylation is associated with fear renewal. However, it is also possible that the changes observed following renewal are associated merely with a tone CSt that produces fear responses. To eliminate this possibility, we asked whether the mere presentation of a fearful tone CSt to conditioned rats alters LAn synaptic strength. Conditioned rats were subdivided into two groups. One group consisted of conditioned rats that were tone tested on day 7 (conditioning-tone group) and the other consisted of conditioned rats that were exposed to the testing context without tone testing on day 7 (conditioning-context group). The two groups of conditioned rats were exposed to the extinction context (context B), as was the extinction group, but did not receive tone CSts. Slices were prepared on the same day (day 7) from the two groups and the rats were killed immediately after the tone test or the context exposure (**Supplementary Fig. 3a**).

Presentation of a tone CSt did not produce any substantial change in the input-output relation in the conditioning-tone group as compared with the conditioning-context group (**Fig. 5a**), consistent

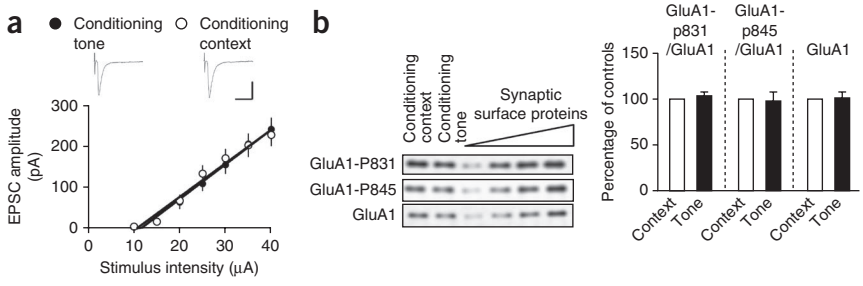

Figure 5 The presentation of a tone CSt to conditioned rats does not change both synaptic efficacy and GluA1 phosphorylation at LAn synapses. (**a**) Input-output curves for EPSCs in conditioning-context group and conditioning-tone group (conditioning tone, 8.50 ± 1.01 pA μA^{-1}, $n = 16$ cells (12 rats); conditioning context, 8.23 ± 0.94 pA μA^{-1}, $n = 21$ cells (17 rats); $t_{35} = 0.1957$, $P = 0.846$, unpaired t test). The representative traces are the averages of four responses evoked by input stimulations of 35 μA. Scale bars represent 20 ms and 100 pA. (**b**) Left, representative immunoblot. Right, pooled result showing that there was no significant difference between conditioning-tone and conditioning-context groups in terms of the level of GluA1 phosphorylation and the amount of surface GluA1 from the LAn synaptosomes (conditioning-tone group, $n = 3$ (9 rats); conditioning-context group, $n = 3$ (9 rats); $t_2 = 0.5171$, $P = 0.6566$ for GluA1-p831/GluA1, unpaired t test; $t_2 = 0.2586$, $P = 0.8201$ for GluA1-p845/GluA1, unpaired t test). The full blots are shown in **Supplementary Figure 10**. Error bars represent s.e.m. For the behavioral procedure, see **Supplementary Figure 3a**.

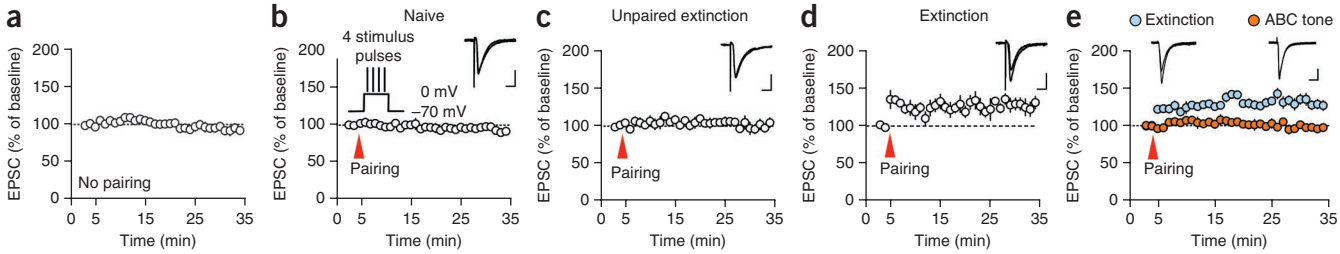

Figure 6 Induction threshold for T-LAn synaptic potentiation is lowered after extinction of conditioned fear. (**a**) Basal transmission without any treatments at T-LAn synapses ($n = 9$ cells (7 rats), $t_8 = 0.9112$, $P = 0.39$, paired t test). (**b**–**d**) Pairing of a single burst (four stimulus pulses) with a postsynaptic depolarization produced a lasting potentiation in the extinction group, but not in the unpaired-extinction or naive controls (averaged responses from 20 min after the pairing to 30 min: naive, 95.38 ± 3.86% of baseline, $n = 7$ cells (6 rats), $t_6 = 1.197$, $P = 0.2766$; unpaired extinction, 101.8 ± 5.24% of baseline, $n = 7$ cells (7 rats), $t_6 = 1.638$, $P = 0.1624$; extinction, 128.2 ± 6.83% of baseline, $n = 5$ cells (5 rats), $t_4 = 4.126$, $P = 0.0145$; paired t test). (**e**) Renewal occluded this low-threshold potentiation. Pairing of four stimulus pulses with a postsynaptic depolarization induced significantly less potentiation in the ABC-tone group than in the extinction controls (first 10 min: extinction group, 125.4 ± 1.88% of baseline, $n = 8$ cells (7 rats); ABC-tone group, 103.2 ± 4.09% of baseline, $n = 6$ cells (5 rats); $t_{12} = 5.390$, $P = 0.0002$, unpaired t test; last 10 min: extinction group, 129.7 ± 6.31% of baseline; ABC-tone group, 97.80 ± 4.53% of baseline; $t_{12} = 3.840$, $P = 0.0024$, unpaired t test). The representative paired traces are the averages of four traces before and after the indicated treatment. Error bars represent s.e.m. Scale bars represent 10 ms and 50 pA. For the behavioral procedure, see **Supplementary Figure 3b**.

with our previous result[36]. The presentation of a tone CSt also did not induce any substantial change in GluA1 phosphorylation in the conditioning-tone group as compared with the conditioning-context group (**Fig. 5b**). Thus, these findings, together with the results described above, suggest that a fearful tone CSt induces acute enhancement in both synaptic efficacy and GluA1 phosphorylation only when the fearful tone CSt is associated with renewal.

Low-threshold potentiation as a mechanism for renewal

Thus far, our results indicate that renewal is tightly associated with enhancement in LAn synaptic efficacy. However, fear renewal is triggered by much weaker stimuli than those needed for amygdala synaptic plasticity (for example, amygdala synaptic plasticity underlying fear memory formation), and it would be difficult to imagine that such weak stimuli is sufficient for the acute enhancement in LAn synaptic efficacy following renewal. One potential mechanism is that extinction of conditioned fear primes LAn synapses for subsequent renewal via a reduction of the threshold for synaptic potentiation. To determine whether the induction threshold of synaptic potentiation is altered after extinction, we assessed the threshold for synaptic potentiation of field potentials using different numbers of stimulus pulses at 100 Hz in fear-extinguished rats. We found a clear difference in the induction of an intermediate-lasting potentiation (<60 min) between the extinction group and naive controls with four individual stimulus pulses at 100 Hz, but failed to detect any difference with six or more stimulus pulses, consistent with a lowered threshold of synaptic potentiation (**Supplementary Fig. 4**).

To confirm the findings based on field-potential recordings (**Supplementary Fig. 4**), we developed a pairing protocol (four individual stimulus pulses at 100 Hz with a postsynaptic depolarization) to induce a low-threshold potentiation in whole-cell recordings. The pairing protocol was applied 5 min after the start of each whole-cell recording; the pairing protocol failed to induce low-threshold potentiation when applied >5 min after the start of the whole-cell recording, possibly as a result of a 'washout' effect (data not shown, also see ref. 37). During the first 3 min after the start of the whole-cell recording, the amplitude of the baseline responses was set to ~150 pA. Data points collected 3–5 min after the start of the recording were used as a baseline and recordings that showed a baseline drift of >10% were discarded. To test the stability of the recordings, we recorded synaptic responses for >30 min without any treatments. In this condition, the synaptic responses were stable relative to the baseline (**Fig. 6a**); we obtained similar results in the control experiments (**Fig. 6b**,**c**).

We first determined whether pairing of both presynaptic stimulation and postsynaptic depolarization is required for the induction of low-threshold potentiation. All slices were prepared on day 7 (6 d after initial conditioning or unpairing; **Supplementary Fig. 3b**). Slices from the extinction group failed to show low-threshold potentiation following the application of four stimulus pulses (100 Hz) to thalamic afferents when the postsynaptic neuron was held at −70 mV or in response to postsynaptic depolarization alone (40 ms in duration; data not shown). The pairing protocol (a 40-ms postsynaptic depolarization paired with the four stimulus pulses at 100 Hz) induced an instantaneous and lasting potentiation in the extinction group, but not in naive and unpaired-extinction controls (**Fig. 6b**–**d**). The protocol applied to the unpaired-extinction controls was the same as that applied to the extinction group except that the tones and shocks were not paired, thereby demonstrating the necessity of initial conditioning for eliciting a low-threshold potentiation. Taken together, these findings suggest that extinction of conditioned fear induces a decrease in the induction threshold for LAn synaptic potentiation.

If the renewal-inducing stimuli cause low-threshold potentiation at LAn synapses, one would predict that further potentiation would be decreased or impaired in slices prepared after renewal. To test this possibility, we determined whether renewal occludes low-threshold potentiation. For this, slices were prepared either 1 d after the last extinction session (day 7) or immediately after fear renewal (which was performed 1 d after the last extinction session; day 7). The pairing protocol produced substantially less potentiation in the ABC-tone group than in the extinction group (**Fig. 6e**). The occlusion of low-threshold potentiation by renewal suggests that fear renewal shares some mechanisms with the low-threshold potentiation.

Low-threshold potentiation requires Ser831 phosphorylation

Having observed that Ser831 phosphorylation of GluA1 following fear renewal is enhanced and that low-threshold potentiation is an underlying mechanism of fear renewal, we hypothesized that Ser831 phosphorylation could be the molecular mechanism underlying both low-threshold potentiation and fear renewal. To test this possibility, we employed short peptide fragments (11-mers) to dissect the downstream consequences of endogenous GluA1 carboxyl tail phosphorylation at the Ser831 site. The short fragments contained

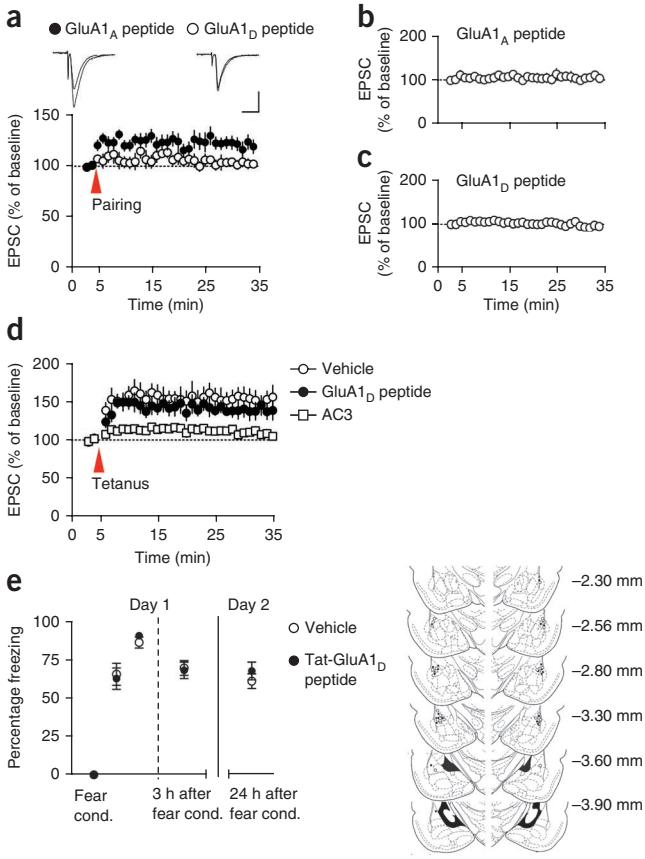

Figure 7 The GluA1$_D$ peptide inhibits low-threshold potentiation, but not LTP or fear conditioning. (**a**) Inclusion of GluA1$_D$ peptide in the internal solution inhibited the low-threshold potentiation relative to GluA1$_A$ peptide in slices prepared from fear-extinguished rats (GluA1$_A$, 122.7 ± 6.37% of baseline, n = 13 cells (12 rats); GluA1$_D$, 103.6 ± 3.79% of baseline, n = 9 cells (9 rats)). The representative traces are superimposed averages of EPSCs obtained before and 20 min after pairing. Scale bars represent 10 ms and 100 pA. (**b**,**c**) Inclusion of GluA1$_A$ peptide (**b**) or GluA1$_D$ peptide (**c**) in the internal solution did not have a significant effect on basal synaptic transmission in slices prepared from fear-extinguished rats (GluA1$_A$ peptide, 106.2 ± 6.02 of baseline, n = 4 cells (4 rats), t_3 = 1.026, P = 0.3806, paired t test; GluA1$_D$ peptide, 98.34 ± 4.69% of baseline, n = 10 cells (9 rats), t_9 = 0.3549, P = 0.7309, paired t test). (**d**) The GluA1$_D$ peptide did not affect LTP at the T-LAn synapses in slices prepared from naive rats, but this potentiation was blocked by the AC3 peptide, a well-known CaMKII inhibitor (vehicle, n = 5 cells (5 rats); GluA1$_D$ peptide group, n = 5 cells (5 rats); AC3 group, n = 5 cells (5 rats); $F_{2,12}$ = 5.945, P = 0.0161; P < 0.05, AC3 group versus vehicle or GluA1$_D$ peptide group, one-way ANOVA with Newman-Keuls post-test). (**e**) Left, microinjection of the GluA1$_D$ peptide into the LAn of naive rats did not have any significant effect on fear conditioning (naive, n = 15 rats and Tat-GluA1$_D$ peptide group, n = 13 rats; 3 h after fear conditioning, t_{26} = 0.2052, P = 0.84, unpaired t test; 24 h after fear conditioning, t_{26} = 0.8937, P = 0.38, unpaired t test). Right, schematic representation of the injector cannula tips. Histological plates illustrating the injection site in the LAn were adopted from the rat brain atlas[50]. Error bars represent s.e.m.

a small part of GluA1 that included the Ser831 site, with the serine residue modified to a phosphomimetic aspartate (D) or non-phosphorylatable alanine (A). We refer to these fragments as GluA1$_D$ peptide (LIPQQD^{831}INEAI) and GluA1$_A$ peptide (LIPQQA^{831}INEAI). The rationale for these phosphomimetic peptides is that phosphorylation at the Ser831 site of endogenous GluA1 subunit is critical for binding to adaptor and auxiliary proteins and that this binding is important for downstream events leading to increased conductance or synaptic localization of the phosphorylated GluA1 subunits[24,26,38]. Thus, GluA1$_D$ peptide is expected to inhibit the downstream events by competing with the endogenous Ser831 phosphorylated GluA1 subunits, whereas the non-phosphorylatable, non-phosphomimetic peptide and non-competing GluA1$_A$ peptide act as a negative control. Thus, inhibition by the GluA1$_D$ peptide, but not by the GluA1$_A$ peptide, would suggest that Ser831 phosphorylation of endogenous GluA1 subunits is important. A phosphorylated form[39] or phosphomimetic form (see Fig. 7 of ref. 40) of peptide fragment has been used in a previous study.

We found that the GluA1$_D$ peptide specifically blocked low-threshold potentiation (**Fig. 7a**) compared with GluA1$_A$ peptide. Inclusion of the GluA1$_D$ peptide (300 μg ml^{-1}) in internal solution attenuated low-threshold potentiation relative to the GluA1$_A$ peptide (300 μg ml^{-1}) (t_{20} = 2.298, P = 0.0325, unpaired t test; **Fig. 7a**), whereas neither peptide had a substantial effect on basal transmission in slices prepared from extinguished rats (**Fig. 7b**,**c**). These findings suggest that the GluA1$_D$ peptide inhibits low-threshold potentiation via competition with the corresponding carboxyl tail sequence in Ser831-phosphorylated GluA1 subunits.

Alternatively, this inhibitory effect by GluA1$_D$ peptide could be a result of a nonselective blocking of CaMKII and PKC, as the GluA1$_D$ peptide may compete for the active site of CaMKII and PKC. To rule out this possibility, we measured the kinase activities of CaMKII and PKC in the presence of these peptides and estimated both the efficacy and potency of these peptides as competitors for CaMKII and PKC. The GluA1$_D$ peptide showed little inhibitory effects on the enzymatic activities of either CaMKII or PKC (**Supplementary Fig. 5**).

We then examined whether the GluA1$_D$ peptide could inhibit well-known CaMKII-dependent processes such as amygdala LTP and cued fear conditioning[41] at the concentration that we used. Consistent with the previous findings[41], dialysis of AC3 peptide (300 μg ml^{-1}), a general peptide inhibitor of CaMKII[42], blocked LTP at T-LAn synapses relative to vehicle controls in slices prepared from naive rats. However, dialysis of the same concentration of the GluA1$_D$ peptide failed to inhibit LTP at the same synapses (**Fig. 7d**). Similarly, bilateral microinjection of a cell-permeable form of GluA1$_D$ peptide (Tat-GluA1$_D$ peptide, 25 pmol) into the LAn of naive rats failed to inhibit the acquisition of fear memory, which requires CaMKII activity (**Fig. 7e**)[41]. These findings indicate that the GluA1$_D$ peptide did not affect the phosphorylation of the CaMKII substrates, which is in turn required for amygdala LTP and fear conditioning. Thus, the GluA1$_D$ peptide, at the concentration we used, is not likely to act as a general inhibitor of CaMKII and PKC. Taken together, our findings suggest that Ser831 phosphorylation of endogenous GluA1 subunits in LAn neurons is required for low-threshold potentiation, but not for long-term potentiation.

Fear renewal requires Ser831 phosphorylation

We next reasoned that, if Ser831 phosphorylation of GluA1 subunits is required for low-threshold potentiation, it could be the molecular basis for fear renewal. Accordingly, we asked whether the GluA1$_D$ peptide could attenuate fear renewal. We performed intracranial microinfusion of a cell-permeable form of GluA1$_D$ or GluA1$_A$ peptide (Tat-GluA1$_D$ peptide or Tat-GluA1$_A$ peptide; 25 pmol, 60 min before the tone presentation for renewal) into the LAn of fear-extinguished rats (**Supplementary Fig. 6**). Microinfusion of Tat-GluA1$_D$ peptide into the LAn attenuated fear renewal (ABC renewal) compared with the control peptide (Tat-GluA1$_A$ peptide–infused group and the vehicle-infused group; **Fig. 8**)

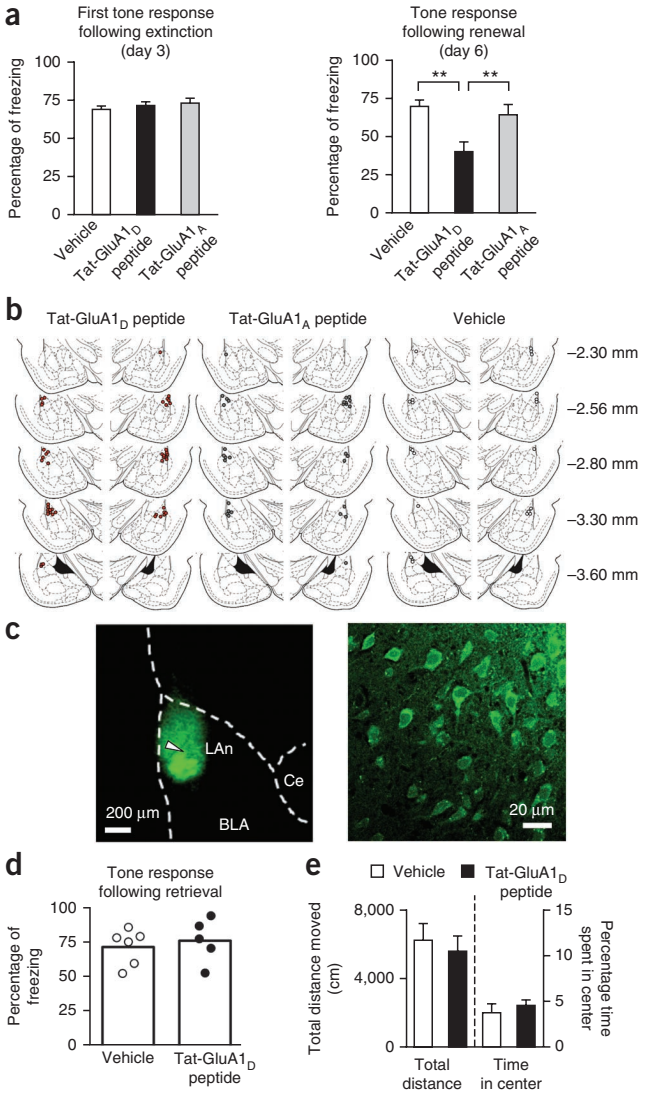

Figure 8 Microinjection of a cell–permeable form of the $GluA1_D$ peptide into the LAn attenuates fear renewal. (**a**) Left, freezing levels in response to the first tone CSt on day 3. This was the first tone testing after conditioning, and the peptides were not injected at this stage. There was no significant difference in freezing levels between the three groups ($F_{2,48} = 0.4628$, $P = 0.6324$, one-way ANOVA). Right, microinjection of Tat-$GluA1_D$ peptide into the LAn impaired fear renewal relative to the $GluA1_A$-infused and vehicle-infused groups (Tat-$GluA1_A$ peptide, 63.94 ± 6.82%, $n = 15$ rats; Tat-$GluA1_D$ peptide, 39.92 ± 6.23%, $n = 23$ rats; vehicle, 69.55 ± 4.22%, $n = 11$ rats; $F_{2,48} = 6.371$, $P = 0.0036$; $^{**}P < 0.01$ Tat-$GluA1_D$ peptide versus the other groups, one-way ANOVA with Newman-Keuls post-test). The peptides were injected 60 min before the tone presentation for renewal on day 6. For the behavioral procedure, see **Supplementary Figure 6**. (**b**) Schematic representation of injector cannula tips. Histological plates illustrating the injection site in the LAn were adopted from the rat brain atlas[50] (left, Tat-$GluA1_D$ peptide; middle, Tat-$GluA1_A$ peptide; right, vehicle). (**c**) Diffusion of the fluorescent dansyl-tat-$GluA1_D$ peptide (1.5 nmol) within 1 h after microinjection, as visualized by confocal microscopy (left). The white arrowhead indicates the end of the injector cannula. Peptide transduction into individual LAn neurons at high magnification (right). The white dashed lines indicate the boundaries for the subnuclei of the amygdala (LAn, lateral nucleus of amygdala; Ce, central nucleus of amygdala; BLA, basolateral nucleus of amygdala). (**d**) Microinjection of the Tat-$GluA1_D$ peptide into the LAn of naive rats did not have any significant effect on retrieval of conditioned fear (vehicle group, $n = 6$ rats; Tat-$GluA1_D$ peptide group, $n = 5$ rats; $t_9 = 0.5306$, $P = 0.6085$, unpaired t test). Conditioned fear memory was retrieved by tone-testing 24 h after conditioning. (**e**) Microinjection of Tat-$GluA1_D$ peptide into the LAn of fear-extinguished rats did not alter exploratory behavior in an openfield relative to vehicle controls (microinjection of the peptide or vehicle was performed 90 min before the test; 25 pmol of the peptide was microinjected 60 min before the test; Tat-$GluA1_D$, $n = 9$ rats; vehicle, $n = 7$ rats; percentage time in center, $t_{14} = 0.7262$, $P = 0.4797$, unpaired t test; distance traveled, $t_{14} = 0.4758$, $P = 0.6416$, unpaired t test). The open field test was performed using fear-extinguished rats on day 6. Schematic representation of the injector cannula tips is presented in **Supplementary Figure 7**. Error bars represent s.e.m.

and there was no significant difference between the two control groups ($P > 0.05$, Newman-Keuls post-test; **Fig. 8a**).

To determine whether the Tat-$GluA1_D$ peptide effect is specific to renewal, we examined the effects of Tat-$GluA1_D$ peptide on retrieval of conditioned fear. Microinfusion of Tat-$GluA1_D$ peptide into the LAn of conditioned rats (60 min before the tone presentation for memory retrieval) did not alter retrieval of conditioned fear as compared with the vehicle-infused group ($P > 0.05$, unpaired t test; **Fig. 8d** and **Supplementary Fig. 7**). In addition, the effect observed with Tat-$GluA1_D$ peptide was not attributable to nonspecific increases in anxiety levels or motility. Microinjection of Tat-$GluA1_D$ peptide into the LAn of fear-extinguished rats did not change exploratory behavior in an open field in Tat-$GluA1_D$–injected groups relative to vehicle controls (**Fig. 8e** and **Supplementary Fig. 7**). Thus, these findings suggest that the Ser831 phosphorylation of GluA1 subunits in the LAn is required for fear renewal, but not for fear conditioning. Taken together, our findings suggest a unique role for Ser831 phosphorylation of GluA1 subunits in plasticity and learning.

DISCUSSION

Our findings suggest a mechanism underlying fear renewal: renewal involves acute enhancement in the efficacy of the input synapses onto the LAn. Excitatory synaptic strength, GluA2-lacking AMPAR activity and Ser831 phosphorylation of GluA1 in the LAn are all enhanced in a context-specific manner, as is fear renewal[8,16]. Notably, a threshold for LAn synaptic potentiation appeared to be reduced after extinction and renewal occluded subsequent low-threshold potentiation, suggesting that renewal shares some mechanisms with the low-threshold potentiation. The $GluA1_D$ peptide, which competes with Ser831-phosphorylated GluA1 subunits, inhibited the low-threshold potentiation, and the cell-permeable form of the peptide attenuated fear renewal when microinfused into the LAn. These findings suggest that both low-threshold potentiation and renewal require phosphorylation of GluA1 subunits at Ser831 in LAn synapses and that renewal may be triggered, at least in part, through low-threshold potentiation–like mechanisms.

Considerable progress has been made in understanding the neural network responsible for the context-specific modulation of conditioned fear after extinction[16]. Depending on the memory retrieval context (that is, extinction or renewal context), the hippocampus selectively drives one of the two distinct neural circuits, resulting in either suppression or renewal of conditioned fear[16]. When fear-extinguished subjects are tested in the extinction context, the hippocampus is believed to drive a prefrontal activation of both amygdala intercalated neurons[20] and 'extinction neurons' in the basal amygdala[9], thereby suppressing fear expression. Following renewal, prefrontal activation is reduced, whereas the hippocampus is thought to enhance activity of 'fear neurons' in the basal amygdala[9,43], leading to renewed fear. The LAn synaptic mechanisms that we observed fit nicely with this neural network of fear renewal. First, the LAn low-threshold potentiation was associative, requiring postsynaptic

depolarization coupled to CSt-based synaptic inputs. Thus, when extinguished fear is renewed, context-based excitatory inputs onto LAn neurons from the hippocampus[18,19] may provide a source for the postsynaptic depolarization, permitting the acute synaptic enhancement following tone CSt input. Next, the resulting increased output from LAn neurons may drive the activation of fear neurons[9] in the basal amygdala, as LAn neurons are known to send excitatory inputs to basal amygdala neurons[20,21]. Another possibility is that it may produce the disynaptic inactivation of amygdala intercalated neurons[22], which leads to central amygdala disinhibition, thereby producing fear relapse. In this context, LAn neurons could be regarded as integrators for CSt- and context-driven neural inputs from sensory and upstream modulatory brain regions, providing a decisive danger signal to downstream brain regions. Further studies will be required to verify the precise functional connections between the groups of neurons involved and the relative contributions of each connection to fear renewal.

Excitatory input synapses onto the LAn are known to exhibit a long-term potentiation of synaptic strength, and the enhanced synaptic efficacy at LAn input synapses is thought to be a cellular substrate underlying conditioned fear memory[44]. Although extinction of conditioned fear is also thought to be a network phenomenon, as mentioned above[3,16], additional evidence has suggested a weakening (or a depotentiation) of some of the original fear memory traces (or conditioning-induced synaptic potentiation at LAn synapses) after extinction[35,45–47]. In this context, the acute synaptic enhancement at the low-threshold synapses in the LAn following renewal could be envisioned as the reconstruction of the original memory traces. In other words, at least some of the information encoded by initial fear conditioning could be retained after extinction as a different form of synaptic plasticity (that is, a reduction in the threshold for synaptic potentiation). One prediction from the reconstruction hypothesis is that acute enhancement in LAn synaptic efficacy following renewal occurs exclusively at the depotentiated synapses (that is, only the depotentiated synapses are low-thresholded), but testing this prediction will require new techniques for identifying the depotentiated synapses.

The seeds of renewal appear to be sown in extinction of conditioned fear as a means of reducing the threshold for synaptic potentiation at LAn synapses. This type of mechanism has been defined as metaplasticity[48], in which previous learning stimuli produce a long-term change of the threshold for subsequent plasticity induction. Albeit preliminary, we tested two independent possibilities. One possibility is that extinction dephosphorylates the Ser831 sites while other conditioning-induced changes are spared, and the threshold of re-potentiation following renewal is therefore lowered, as only the Ser831 site needs to be re-phosphorylated. However, this was not the case: the conditioning-induced enhancement in Ser831 phosphorylation appeared to return to baseline even without fear extinction (**Supplementary Fig. 8**). The second possibility is that extinction produces GluA1 nitrosylation, which is known to facilitate Ser831 phosphorylation[49]. In fact, we observed enhanced GluA1 nitrosylation after extinction (**Supplementary Fig. 9**). Thus, it is possible that this extinction-induced nitrosylation of GluA1 underlies a reduction in the threshold for Ser831 phosphorylation and for LAn synaptic potentiation. Future work will be necessary to determine the molecular identity for this metaplastic process in more details. Our findings imply the presence of a previously unknown form of *in vivo* metaplastic processes (that is, an altered threshold of plasticity induction) and lay the foundation for a new and effective strategy for treating aberrant relapse of fear memory–related diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L., B.S., Jeongyeon Kim, K.K., K.S.S. and S.C. designed the experiments. S.L., B.S., Jeongyeon Kim, K.P., I.H., B.A., S.S., J.L., S.P. and Jihye Kim performed the experiments. S.L., B.S., Jeongyeon Kim, K.K., K.S.S., R.W.T. and S.C. analyzed the data. S.L., B.S., Jeongyeon Kim, D.P., C.J.L., K.K., K.S.S., R.W.T. and S.C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Bouton, M.E. Context and ambiguity in the extinction of emotional learning: implications for exposure therapy. *Behav. Res. Ther.* **26**, 137–149 (1988).
2. Myers, K.M. & Davis, M. Behavioral and neural analysis of extinction. *Neuron* **36**, 567–584 (2002).
3. Maren, S. & Quirk, G.J. Neuronal signalling of fear memory. *Nat. Rev. Neurosci.* **5**, 844–852 (2004).
4. Sotres-Bayon, F., Bush, D.E.A. & Ledoux, J.E. Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction. *Learn. Mem.* **11**, 525–535 (2004).
5. Rachman, S. The return of fear: review and prospect. *Clin. Psychol. Rev.* **9**, 147–168 (1989).
6. Hermans, D., Craske, M.G., Mineka, S. & Lovibond, P.F. Extinction in human fear conditioning. *Biol. Psychiatry* **60**, 361–368 (2006).
7. Corcoran, K.A. & Maren, S. Hippocampal inactivation disrupts contextual retrieval of fear memory after extinction. *J. Neurosci.* **21**, 1720–1726 (2001).
8. Bouton, M.E., Westbrook, R.F., Corcoran, K.A. & Maren, S. Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biol. Psychiatry* **60**, 352–360 (2006).
9. Herry, C. *et al.* Switching on and off fear by distinct neuronal circuits. *Nature* **454**, 600–606 (2008).
10. Knapska, E. & Maren, S. Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learn. Mem.* **16**, 486–493 (2009).
11. Woods, A.M. & Bouton, M.E. D-cycloserine facilitates extinction but does not eliminate renewal of the conditioned emotional response. *Behav. Neurosci.* **120**, 1159–1162 (2006).
12. Gogolla, N., Caroni, P., Lüthi, A. & Herry, C. Perineuronal nets protect fear memories from erasure. *Science* **325**, 1258–1261 (2009).
13. Kindt, M., Soeter, M. & Vervliet, B. Beyond extinction: erasing human fear responses and preventing the return of fear. *Nat. Neurosci.* **12**, 256–258 (2009).
14. Monfils, M.-H., Cowansage, K.K., Klann, E. & Ledoux, J.E. Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *Science* **324**, 951–955 (2009).
15. Gale, G.D. *et al.* Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *J. Neurosci.* **24**, 3810–3815 (2004).
16. Maren, S. Seeking a spotless mind: extinction, deconsolidation, and erasure of fear memory. *Neuron* **70**, 830–845 (2011).
17. LeDoux, J.E. Emotion circuits in the brain. *Annu. Rev. Neurosci.* **23**, 155–184 (2000).
18. Maren, S. & Hobin, J.A. Hippocampal regulation of context-dependent neuronal activity in the lateral amygdala. *Learn. Mem.* **14**, 318–324 (2007).
19. Hobin, J.A., Goosens, K.A. & Maren, S. Context-dependent neuronal activity in the lateral amygdala represents fear memories after extinction. *J. Neurosci.* **23**, 8410–8416 (2003).
20. Amano, T., Duvarci, S., Popa, D. & Paré, D. The fear circuit revisited: contributions of the basal amygdala nuclei to conditioned fear. *J. Neurosci.* **31**, 15481–15489 (2011).

21. Anglada-Figueroa, D. & Quirk, G.J. Lesions of the basal amygdala block expression of conditioned fear but not extinction. *J. Neurosci.* **25**, 9680–9685 (2005).
22. Paré, D., Quirk, G.J. & Ledoux, J.E. New vistas on amygdala networks in conditioned fear. *J. Neurophysiol.* **92**, 1–9 (2004).
23. Derkach, V., Barria, A. & Soderling, T.R. Ca^{2+}/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc. Natl. Acad. Sci. USA* **96**, 3269–3274 (1999).
24. Kristensen, A.S. *et al.* Mechanism of Ca^{2+}/calmodulin-dependent kinase II regulation of AMPA receptor gating. *Nat. Neurosci.* **14**, 727–735 (2011).
25. Oh, M.C., Derkach, V.A., Guire, E.S. & Soderling, T.R. Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. *J. Biol. Chem.* **281**, 752–758 (2006).
26. Derkach, V.A., Oh, M.C., Guire, E.S. & Soderling, T.R. Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat. Rev. Neurosci.* **8**, 101–113 (2007).
27. Lee, H.K., Barbarosie, M., Kameyama, K., Bear, M.F. & Huganir, R.L. Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405**, 955–959 (2000).
28. Lee, H.-K. *et al.* Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112**, 631–643 (2003).
29. Hu, H. *et al.* Emotion enhances learning via norepinephrine regulation of AMPA-receptor trafficking. *Cell* **131**, 160–173 (2007).
30. Crombag, H.S. *et al.* A necessary role for GluR1 serine 831 phosphorylation in appetitive incentive learning. *Behav. Brain Res.* **191**, 178–183 (2008).
31. Lee, H.-K., Takamiya, K., He, K., Song, L. & Huganir, R.L. Specific roles of AMPA receptor subunit GluR1 (GluA1) phosphorylation sites in regulating synaptic plasticity in the CA1 region of hippocampus. *J. Neurophysiol.* **103**, 479–489 (2010).
32. Bowie, D. & Mayer, M.L. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* **15**, 453–462 (1995).
33. Shepherd, J.D. & Huganir, R.L. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu. Rev. Cell Dev. Biol.* **23**, 613–643 (2007).
34. Whitlock, J.R., Heynen, A.J., Shuler, M.G. & Bear, M.F. Learning induces long-term potentiation in the hippocampus. *Science* **313**, 1093–1097 (2006).
35. Kim, J. *et al.* Amygdala depotentiation and fear extinction. *Proc. Natl. Acad. Sci. USA* **104**, 20955–20960 (2007).
36. Kim, J. *et al.* Reactivation of fear memory renders consolidated amygdala synapses labile. *J. Neurosci.* **30**, 9631–9640 (2010).
37. Malinow, R. & Tsien, R.W. Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* **346**, 177–180 (1990).
38. Kessels, H.W., Kopec, C.D., Klein, M.E. & Malinow, R. Roles of stargazin and phosphorylation in the control of AMPA receptor subcellular distribution. *Nat. Neurosci.* **12**, 888–896 (2009).
39. Ahmadian, G. *et al.* Tyrosine phosphorylation of GluR2 is required for insulin-stimulated AMPA receptor endocytosis and LTD. *EMBO J.* **23**, 1040–1050 (2004).
40. Boehm, J. *et al.* Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. *Neuron* **51**, 213–225 (2006).
41. Rodrigues, S.M., Farb, C.R., Bauer, E.P., Ledoux, J.E. & Schafe, G.E. Pavlovian fear conditioning regulates Thr286 autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II at lateral amygdala synapses. *J. Neurosci.* **24**, 3281–3288 (2004).
42. Otmakhov, N., Griffith, L.C. & Lisman, J.E. Postsynaptic inhibitors of calcium/calmodulin-dependent protein kinase type II block induction but not maintenance of pairing-induced long-term potentiation. *J. Neurosci.* **17**, 5357–5365 (1997).
43. Orsini, C.A., Kim, J.H., Knapska, E. & Maren, S. Hippocampal and prefrontal projections to the basal amygdala mediate contextual regulation of fear after extinction. *J. Neurosci.* **31**, 17269–17277 (2011).
44. Maren, S. Synaptic mechanisms of associative memory in the amygdala. *Neuron* **47**, 783–786 (2005).
45. Lin, C.-H., Lee, C.-C. & Gean, P.-W. Involvement of a calcineurin cascade in amygdala depotentiation and quenching of fear memory. *Mol. Pharmacol.* **63**, 44–52 (2003).
46. Dalton, G.L., Wang, Y.T., Floresco, S.B. & Phillips, A.G. Disruption of AMPA receptor endocytosis impairs the extinction, but not acquisition of learned fear. *Neuropsychopharmacology* **33**, 2416–2426 (2008).
47. Mao, S.-C. *et al.* Inhibition of spontaneous recovery of fear by mGluR5 after prolonged extinction training. *PLoS ONE* **8**, e59580 (2013).
48. Abraham, W.C. & Bear, M.F. Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci.* **19**, 126–130 (1996).
49. Selvakumar, B. *et al.* S-nitrosylation of AMPA receptor GluA1 regulates phosphorylation, single-channel conductance, and endocytosis. *Proc. Natl. Acad. Sci. USA* **110**, 1077–1082 (2013).
50. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates* (Academic Press, 1997).

ONLINE METHODS

Behavioral procedures. All procedures were approved by the Institute of Laboratory Animal Resources of Seoul National University. Male Sprague-Dawley rats (3–5 weeks old) were maintained with free access to food and water and group housed (1–3 rats per cage) under an inverted 12/12-h light/dark cycle (lights off at 09:00 h). Behavioral training was done during the dark portion of the light/dark cycle. For fear conditioning in all experiments except those shown in **Figures 7e** and **8**, rats were placed in a conditioning chamber and left undisturbed for 2 min. A neutral tone (30 s, 2.8 kHz, 85 dB) co-terminating with an electrical foot shock (1.0 mA, 1 s) was then presented three times at an average interval of 100 s (day 1). The three tone-shock pairings were repeated the next day (day 2). At each of the days, rats were returned to their home cages 60 s after the last shock was applied. For extinction training (days 4 through 6), rats were placed in the new chamber, allowed to settle for 4 min, and then presented with 20 (day 4) and 15 shock-free tones (day 5 and 6), respectively, at an average interval of 100 s. A Plexiglas chamber distinct from conditioning chamber was used for both extinction training and tone test. For renewal (day 7), fear-extinguished rats were placed in the novel chamber for 4 min and then given the same neutral tone for 30 s, but without a foot shock. Brain slices were prepared immediately after a tone test (extinction-tone controls and renewal groups) or context exposure (context control) on day 7. Conditioned freezing was defined as immobility except for respiratory movements and was quantified by trained observers who were blind to the experimental groups. Total freezing time during a test period was normalized versus the duration of the tone presentation (30 s) or context exposure. For the experiments shown in **Figures 7e** and **8**, no fear conditioning was performed on day 1, so the rats received only the three tone-shock pairings (0.5 mA and 1 s) on day 2. In **Figure 8**, the subsequent procedures were as described above, except that the tone stimulus used for both memory testing and renewal was 60 s in duration. To test general activity and anxiety of rats, open field test was performed. Rats were placed in an open field that consisted of a square divided it into peripheral (within 20 cm from the walls) and central (40 × 40 cm) zones for 10 min. The total distance moved in the arena and the time spent in the central zone were measured using EthoVision 3.1 software (EthoVision 3.1, Noldus Information Technology).

Cannula implantation and peptide infusion. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg per kg), mounted on a stereotaxic apparatus (Storting), and 26-gauge stainless-steel cannulas (model C315G, Plastic Products) were implanted bilaterally into the LAn (anterior-posterior; − 2.35 mm, medial-lateral; ± 5.05 mm and dorsal-ventral; − 6.7 mm) using previously described techniques[35,51]. A 32-gauge dummy cannula was inserted into each cannula to prevent clogging. Two jewelry screws were implanted over the skull to serve as anchors, and the whole assembly was affixed on the skull with dental cement. Rats were given at least 1 week to recover before experiments were performed. Following completion of the experiments, the intra-LAn placement of the injection cannula tips was confirmed. Briefly, rats were anesthetized with urethane (1 g per kg of body weight, intraperitoneal) and transcardially perfused with 0.9% saline solution (wt/vol) followed by 10% buffered formalin (vol/vol). Brains were removed and post-fixed overnight. Coronal sections (80 μm thick) were cut using a vibroslicer (NVSL, World Precision Instruments), stained with cresyl violet, and examined under a light microscope. For the microinfusion of peptides, Tat-GluA1–derived peptides (Peptron) were dissolved in artificial cerebrospinal fluid (aCSF). The peptides were administrated bilaterally into the LAn via 33 gauge injector cannulas (C315I, Plastic Products) attached to a 10-μl Hamilton syringe at a rate of 0.25 μl min^{-1} (60 min before tone presentation in the renewal group or the start of the open field test). Following peptide infusion, the cannulas were left in place for an additional minute to allow the peptides to diffuse away from the cannula tip. The dummy cannulas were then replaced and the rats were returned to their home cages.

Slice preparation. Brain slices were prepared using previously described techniques[35]. In brief, Sprague-Dawley rats (4–5 weeks old) were anesthetized with isoflurane and decapitated. Whole brains were isolated and placed in an ice-cold modified aCSF solution containing 175 mM sucrose, 20 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$ and 11 mM D-(+)-glucose, and were gassed with 95% O_2/5% CO_2. Coronal slices (300 μm) including the LAn were cut using a vibroslicer (HA752, Campden Instruments) and incubated in normal aCSF containing 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$ and 11 mM D-(+)-glucose, and was continuously bubbled at 23–24 °C with 95% O_2/5% CO_2. Just before a given slice was transferred to the recording chamber, the cortex overlying the LAn was cut away with a scalpel so that the addition of picrotoxin (100 μM, Sigma-Aldrich) would block cortical epileptic burst discharges from invading the LAn.

Afferent stimulation and recording conditions. We chose brain slices containing a well-isolated, sharply defined trunk (containing thalamic afferents) crossing the dorsolateral division of the LAn where the somatosensory and auditory inputs converge[52]. The sizes of the LAn and central amygdala were relatively constant in the used slices; when multiple trunks were observed, we used the closest trunk to the central nucleus of the amygdala. Unless otherwise noted, thalamic afferents were stimulated at a frequency of 0.067 Hz using a concentric bipolar electrode (MCE-100, Rhodes Medical Instruments). The stimulation electrode was placed at the midpoint of the trunk between the internal capsule and the medial boundary of the LAn. Regions and cells of interest for all recordings were located beneath the midpoint of the trunk spanning the LAn horizontally.

Whole-cell patch-clamp recordings. Whole-cell recordings were made using an Axopatch 200A amplifier or Multiclamp 700A (Molecular Devices, Sunnyvale, CA). For experiments that required a higher quality of voltage clamping (**Figs. 1**–**3a** and **5**), recordings were obtained using a cesium-based internal solution containing 100 mM cesium gluconate, 0.6 mM EGTA, 10 mM HEPES, 5 mM NaCl, 20 mM TEA, 4 mM Mg-ATP, 0.3 mM Na-GTP and 3 mM QX314 with the pH adjusted to 7.2 with CsOH and the osmolarity adjusted to around 297 mmol kg^{-1} with sucrose. In the remaining experiments, the pipettes were filled with a potassium-based internal solution containing 120 mM potassium gluconate, 0.2 mM EGTA, 10 mM HEPES, 5 mM NaCl, 1 mM $MgCl_2$, 2 mM Mg-ATP and 0.3 mM Na-GTP, with the pH adjusted to 7.2 with KOH. The cells used were classified as principal neurons on the basis of the pyramidal shape of their somata and their ability to show spike-frequency adaptation in response to current injection in potassium-filled cells and the slower decay time of spontaneous EPSCs in cesium-filled cells (see also supplementary texts in ref. 35 for additional details). We included picrotoxin (100 μM) in our recording solution to isolate excitatory synaptic transmission and block feed-forward GABAergic inputs to the principal neurons in the LAn. The pipette resistances ranged from 2.5 to 3.5 MΩ. Infrared differential interference contrast–enhanced visual guidance was used to select neurons that were 3–4 cell layers below the surface of the 300-μm-thick slices, which were held at 32 ± 1 °C. The neurons were voltage clamped at −70 mV, and the various solutions were delivered to the slices via gravity-driven superfusion at a flow rate of 1.5 ml min^{-1}. The pipette series resistance was monitored throughout each experiment, and the data were discarded if it changed by >20%. Whole-cell currents were filtered at 1 kHz, digitized at up to 20 kHz, and stored on a microcomputer (Clampex 8 software, Molecular Devices). Pairing-induced low-threshold potentiation was induced by four individual stimulus pulses at 100 Hz with postsynaptic depolarization (0 or −10 mV for 40 ms). The LTP induction protocol for **Figure 7d** consisted of a 30-Hz tetanus (100 pulses, given twice with a 20-s interval at test intensity). One or two neurons were recorded per rat (a single neuron per slice). All recordings were initiated by 4 h after slice preparation, mainly because of cell viability in the 300-μm-thick slices. For better display, running averages of four or six data points were applied in the time-lapse experiments.

Analysis of evoked mEPSCs. AMPAR-mediated, asynchronous evoked mEPSCs were collected during a 400-ms period beginning 50 ms after each stimulus of a 1.67-Hz, ten-pulse train delivered once every 30 s in a bath solution containing D-AP5 (50 μM, Tocris), 5 mM $MgCl_2$ and 3 mM Sr^{2+} (or D-AP5, 5 mM $MgCl_2$ and 3 mM Ca^{2+} for the measurement of synchronous evoked EPSCs before each Sr^{2+} experiment). Quantal events were analyzed using the Minianalysis software (Synaptosoft) with the detection parameters set at amplitude >6 pA and rise time <3 ms, and the results were visually verified. For each cell, a random stretch of 300 mEPSCs was used to construct a cumulative probability plot and calculate the mean mEPSC amplitude. Data from the cumulative EPSC histograms were statistically compared using the Kolmogorov-Smirnov test.

Estimation of rectification index. The rectification index was calculated as the ratio of the peak amplitudes ($EPSC_{hyperpolarized}/EPSC_{depolarized}$) obtained with an internal solution that contained spermine (100 µM). The reversal potential (E_{rev}) was measured in each experiment. This method is based on the fact that AMPARs lacking GluA2 subunits display inward rectification due to the voltage-dependent block of the channel pore by polyamines at positive membrane potentials[32]. The rectification index (E_{rev} –60 mV / E_{rev} + 30 mV) was compared 20 min after the start of whole-cell recordings to ensure complete diffusion of the exogenous spermine into the cell interior. D-AP5 (50 µM) was applied 5 min before the rectification index estimation, allowing us to isolate the AMPAR-mediated EPSCs at positive potentials.

Biochemical measurements of surface AMPARs on LAn synaptosomal membranes. Biotinylation experiments monitoring the expression of surface AMPARs were performed as described previously[35,53,54] with modifications. LAn areas microdissected from 400-µm-thick brain slices were pooled (three to four pieces per rat), incubated with aCSF containing 1 mg ml^{-1} sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce Chemical) for 30 min on ice, and then quenched by two successive 20-min washes in aCSF containing 100 mM glycine, followed by two washes in ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl). The microdissected LAn were then lysed in ice-cold homogenization buffer containing 10 mM Tris (pH 7.6), 320 mM sucrose, 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA and 1 mM EGTA. A 10-µg aliquot of each lysate was retained as a total protein fraction, and the remainder was centrifuged at 1,000 *g* for 10 min at 4 °C for removal of nuclei and large debris. The supernatant was further centrifuged at 10,000 *g* at 4 °C for 30 min to obtain a crude synaptosomal fraction, which was then lysed in modified RIPA buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100 (vol/vol), 0.5% sodium deoxycholate (wt/vol), 0.1% SDS (wt/vol), 1 mM phenylmethylsulfonyl fluoride, 100 µg ml^{-1} aprotinin and 100 µg ml^{-1} leupeptin. The samples were sonicated and spun down at 15,000 *g* at 4 °C for 15 min. The supernatant was mixed with 400 µl of modified RIPA buffer and 100 µl of 50% Neutravidin agarose (vol/vol, Pierce Chemical Company), and incubated for 3 h at 4 °C, for isolation of the biotinylated proteins from the crude synaptosomal complexes. The Neutravidin agarose was washed four times with modified RIPA buffer, and the bound proteins were eluted with SDS sample buffer by boiling for 5 min. The isolated biotinylated proteins were subsequently analyzed by immunoblotting with monoclonal antibody to GluA1 (1:1,000, SC-13152, Santa Cruz Biotechnology), monoclonal antibody to p-Ser-831 (1:1,000, 04-823, Millipore), polyclonal antibody to p-Ser-845 (1:1,000, ab-5849, Millipore). The immunoblot was probed with a horseradish peroxidase–conjugated secondary antibody (donkey antibody to rabbit IgG: 1:5,000, 711-035-152; donkey antibody to mouse IgG: 1:5,000, 715-035-150; Jackson ImmunoResearch) for 1 h and developed using an ECL-based immunoblotting detection system (Pierce Chemical Company). The relative optical densities of the bands were quantified using the ImageJ image analysis software (US National Institutes of Health). We confirmed equal loading of proteins based on densitometric quantification of silver-stained band profiles obtained from gels that were pre-run with small aliquots of the loaded samples. The linearity of the immunoblotting results was confirmed by analyzing the relative optical band densities of serially diluted samples (amygdala whole-cell extracts) loaded on each gel. The optical densities of the GluA1 bands in the extinction and renewal groups were normalized with respect to those of the control group in each experiment. It should be noted that the best results for the Western experiments could be obtained when all the procedures were completed within 48 h. Initially, we experienced a great difficulty with quantitation of synaptosomal surface proteins using a silver staining method, especially when we handled more than three experimental groups (**Fig. 5**). Thus, we had to further divide the five groups in two, each included naive controls for comparison; one group of naive controls, extinction group, ABC-tone group and the other group of naive controls, ABB-tone group, ABC-context group in the **Figure 5** experiments.

Statistical analysis. Sample size were determined *post hoc* on the basis of those used in our previous studies[35,36,51]. Subjects were randomly assigned to the experimental groups by a person who was not involved in the experiments. No subjects or data points were excluded for further data analyses. Data distribution was assumed to be normal but this was not formally tested. Between-group comparisons of data were made using either a two-sided unpaired *t* test or one-way ANOVA with subsequent Newman-Keuls *post hoc* comparison. A two-sided paired *t* test was used to determine whether the post-treatment responses differed significantly from the baseline responses. $P < 0.05$ was considered indicative of statistical significance. The data from each neuron and slice were treated as independent samples. In all experiments with behaviorally trained rats, the data include samples from three or more rats.

51. Kim, J. *et al.* Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction. *Biochem. Biophys. Res. Commun.* **355**, 188–193 (2007).
52. Pitkänen, A., Savander, V. & Ledoux, J.E. Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends Neurosci.* **20**, 517–523 (1997).
53. Chung, H.J., Xia, J., Scannevin, R.H., Zhang, X. & Huganir, R.L. Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain–containing proteins. *J. Neurosci.* **20**, 7258–7267 (2000).
54. Dunah, A.W. & Standaert, D.G. Dopamine D1 receptor–dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. *J. Neurosci.* **21**, 5546–5558 (2001).

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