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Memory recall and modifications by activating neurons with elevated CREB

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Memory is supported by a specific ensemble of neurons distributed in the brain that form a unique memory trace. We previously showed that neurons in the lateral amygdala expressing elevated levels of cAMP response-element binding protein are preferentially recruited into fear memory traces and are necessary for the expression of those memories. However, it is unknown whether artificially activating just these selected neurons in the absence of behavioral cues is sufficient to recall that fear memory. Using an ectopic rat vanilloid receptor TRPV1 and capsaicin system, we found that activating this specific ensemble of neurons was sufficient to recall established fear memory. Furthermore, this neuronal activation induced a reconsolidation-like reorganization process, or strengthening of the fear memory. Thus, our findings establish a direct link between the activation of specific ensemble of neurons in the lateral amygdala and the recall of fear memory and its subsequent modifications.

It is thought that memory is represented by a sparsely distributed, specific collection of neurons in the brain that forms a unique memory trace[1]. Accordingly, only a portion of eligible neurons are recruited into a specific memory. For instance, imaging of activity-dependent induction of the immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein, also known as *Arg3.1*) revealed that a novel environment produced activation of a sparsely distributed neuronal ensemble in the hippocampus[2]. During fear conditioning, about 70% of neurons in the lateral amygdala, a critical brain site for fear memory acquisition and storage[3], are thought to receive sensory inputs, but only about one-quarter exhibit learning-related synaptic plasticity[4,5]. A study of the amygdala circuitry that combined the genetic tagging of *c-fos*–active neurons and immediate-early gene imaging found that neurons in the basolateral amygdala that are activated during fear conditioning are reactivated during fear memory recall, but only small portions of neurons activated during training are reactivated[6]. Previously, we found that neurons expressing elevated levels of cAMP response-element binding protein (CREB neurons) in lateral amygdala at the time of fear learning are preferentially selected for inclusion into fear memory trace and are essential for the later expression of that memory[7–9]. As such, these neurons may represent key components in the fear memory trace. What would happen if this specific ensemble of neurons is stimulated? It is possible that activating these selected neurons may lead to the recall of fear memory. However, whether artificially activating these selected neurons in the absence of behavioral cues is sufficient to recall this fear memory remains unknown.

Current views on the dynamic nature of the memory trace[10] suggest that recall is not just a simple neural process of memory expression, but can induce the processes of modifying recalled memory. It is well established that behavioral recall of a fear memory can induce a reconsolidation process[11,12]. In addition, it has been thought that subpopulations of neurons that are active during behavior training may be internally reactivated later in the brain and that this activity replay or reactivations of a memory trace may contribute to strengthening of a previously acquired memory[13–18]. However, it has never been directly tested whether activating particular sets of neurons in the brain is sufficient to induce modifications of established fear memory such as reconsolidation-like reorganization or strengthening of memory. To address these questions, we artificially stimulated a subset of neurons expressing elevated levels of CREB by drug-mediated control of neuronal activation approach and investigated whether this manipulation was sufficient to induce behavioral recall of fear memory and subsequent modifications of it.

RESULTS

Temporally specific activation of CREB neurons

To selectively activate lateral amygdala neurons with elevated CREB, we took advantage of rat transient receptor potential vanilloid receptor 1 (TRPV1)-mediated neuronal activation[19,20]. Ectopic expression of TRPV1 in mouse brains *in vivo* activates a genetically defined group of neurons to drive reliable behavioral responses[21,22]. Low concentrations of the selective TRPV1 ligand capsaicin induce cationic currents that evoke robust depolarization and neuronal firing in neurons ectopically expressing TRPV1 (refs. 19,21,22). By coexpressing a CREB-GFP fusion protein with TRPV1, we specifically targeted CREB-injected lateral amygdala neurons for subsequent selective activation (**Fig. 1a**). To achieve this coexpression, we used neurotropic herpes simplex virus (HSV)-mediated gene transfer. We generated an HSV-CREB-TRPV1 construct (hereafter CREB-TR) in which CREB and TRPV1 expression are independently driven by two separate promoters in the same viral vector. We also included control constructs, GFP-TR (no CREB), CREB alone (no TRPV1) and GFP alone (no CREB and

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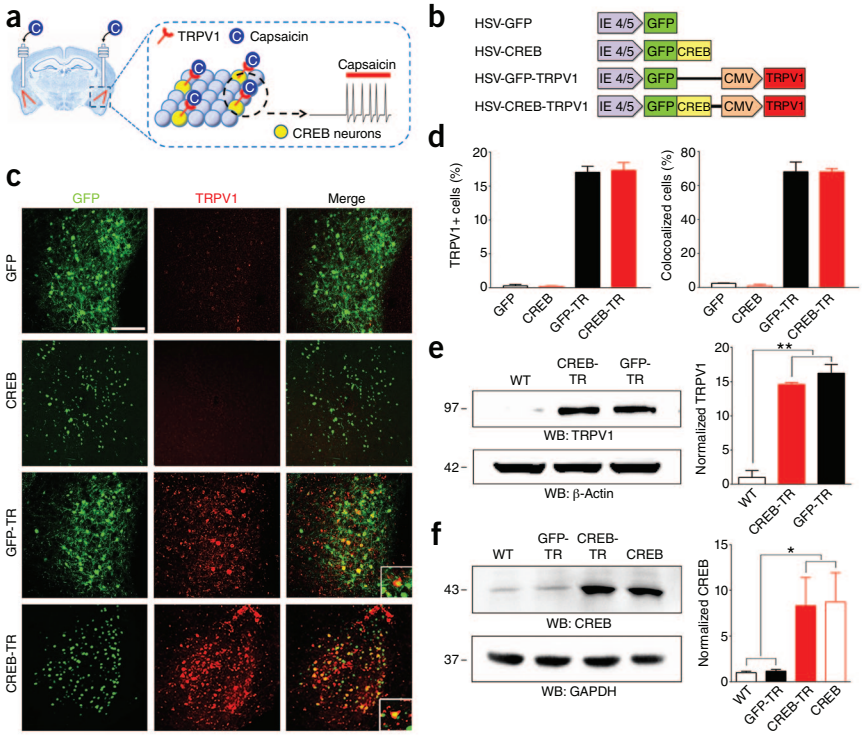

Figure 1 CREB and TRPV1 was successfully coexpressed in a subset of lateral amygdala neurons by HSV-mediated gene transfer. (**a**) Schematic experimental design for selective activation of lateral amygdala CREB neurons. TRPV1 was ectopically coexpressed with CREB in a subset of lateral amygdala neurons (yellow). Local capsaicin specifically targeted and activated this specific ensemble of CREB neurons. (**b**) The HSV vector constructs that we used. (**c**) Representative confocal microscopic images showing the successful expression (green) of HSV constructs in the lateral amygdala. All HSV constructs infected a similar percentage of neurons in lateral amygdala (**Supplementary Fig. 1**). The expression of TRPV1 (red) was detected by immunohistochemical staining using specific antibody to rat TRPV1. The yellow signal in the merged images shows colocalized expression of either CREB or GFP with TRPV1. Inset, magnified image showing colocalization pattern of either CREB or GFP with TRPV1 expression. Scale bar represents 200 μm. (**d**) Quantification of percentage numbers of total TRPV1$^+$ neurons (left) and percentage ratio of coexpressing neurons to GFP$^+$ neurons (right). Cell-counting analysis showed strong expression of ectopic TRPV1, but little expression of endogenous TRPV1 (left). TRPV1 was specifically coexpressed with GFP or CREB in most, but not all, GFP-TR– and CREB-TR–infected neurons, respectively (right). (**e**) Quantification of TRPV1 protein expression in lateral amygdala by western blots. Representative blot images (left) and histogram (right) are shown. For the comparison, the level of TRPV1 protein expression was normalized to control β-actin ($n = 2$ for all groups). Full immunoblots are shown in **Supplementary Figure 8**. (**f**) Quantification of CREB protein expression in lateral amygdala by western blots. Representative blot images (left) and histogram (right) are shown. The level of CREB protein expression was normalized to control GAPDH ($n = 4$ for all groups). Full immunoblots are shown in **Supplementary Figure 8**. $^*P < 0.05$, $^{**}P < 0.01$. Data in **d**–**f** are expressed as mean ± s.e.m.

no TRPV1) (**Fig. 1b**). When stereotaxically injected into the lateral amygdala, these viral vector constructs infected a similar percentage (15–20%) of the total lateral amygdala neuron population ($F_{3,38} = 0.49$, $P = 0.69$, one-way ANOVA; **Supplementary Fig. 1**). A colocalization analysis revealed that CREB and GFP were specifically coexpressed with TRPV1 in CREB-TR– and GFP-TR–infected neurons, respectively (**Fig. 1c**,**d**). To validate the TRPV1-capsaicin system, we first examined the expression level of endogenous TRPV1 protein in the lateral amygdala by immunohistochemical staining and western blot analysis using an antibody to TRPV1. Consistent with previous finding[23], TRPV1 was virtually undetectable without ectopic expression (**Fig. 1c**–**e**). However, we detected robust expression of TRPV1 protein in the lateral amygdala of CREB-TR– and GFP-TR–injected mice ($F_{2,3} = 76.37$, $P < 0.01$, one-way ANOVA; Bonferroni *post hoc* confirmed statistical significance, $P < 0.05$; **Fig. 1e**), indicating successful expression of ectopic TRPV1 by HSV-mediated gene transfer. To confirm that CREB was overexpressed by the injection of CREB-TR or CREB viral vectors, we also probed CREB protein expression using western blot analysis. CREB expression was robustly increased in the lateral amygdala of CREB-TR– and CREB-injected mice compared with control mice (wild-type and GFP-TR–injected mice; $F_{3,12} = 3.732$, $P < 0.05$, one-way ANOVA; **Fig. 1f**).

Next, to confirm capsaicin-mediated neuronal activation, we performed *in vivo* single-unit recordings, measuring changes in spike frequency in lateral amygdala neurons following local application of capsaicin.

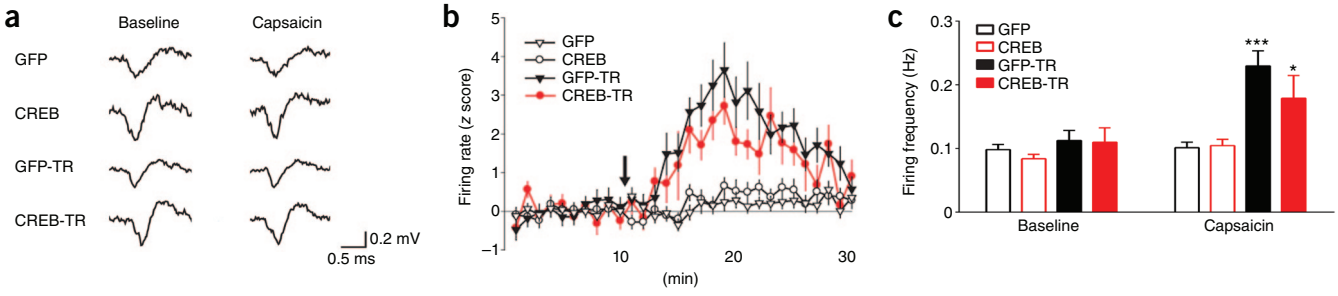

Figure 2 Capsaicin increased the firing rate in the putative ectopic TRPV1-expressing neurons in the lateral amygdala. (**a**) Sample traces of single-unit waveforms before and after capsaicin administration. (**b**) Single-unit firing rate standard score. Single units showing robust increases in firing rates were isolated only in GFP-TR ($n = 16$ of 34) and CREB-TR ($n = 17$ of 39) mice following injection of 1 μM capsaicin, whereas control groups showed no significant changes ($n = 45$, GFP group; $n = 29$, CREB group). Arrow indicates start of 5-min injection. (**c**) Single-unit firing rates before and after capsaicin injection. Baseline spike firing was determined by recording single-unit firing rates for 10 min before drug administration (left). Baseline single unit firing rates were compared with the firing rates recorded 5–15 min after capsaicin injection (right). $^*P < 0.05$, $^{***}P < 0.001$. Data in **b**,**c** are expressed as mean ± s.e.m.

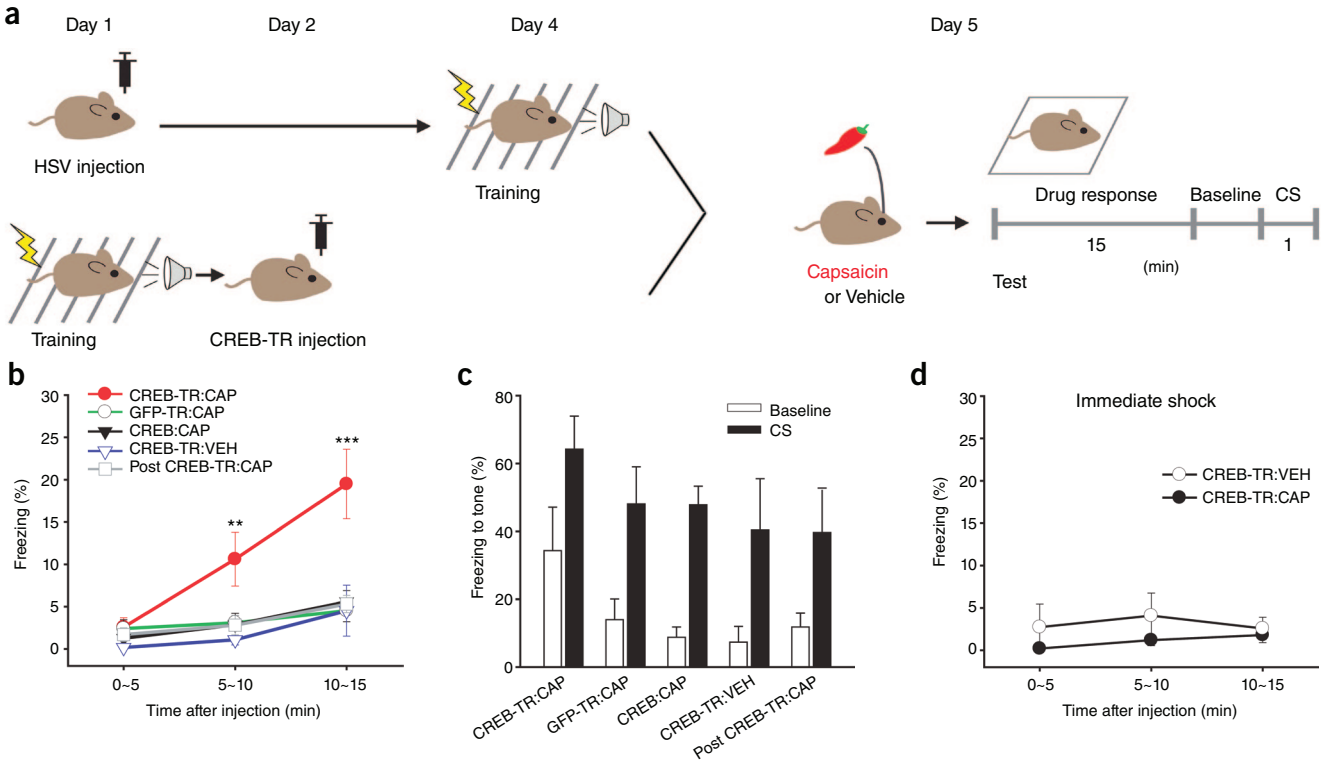

Figure 3 Fear memory was recalled by activating lateral amygdala neurons expressing elevated level of CREB. (**a**) Experimental procedure. Top, CREB-TR:capsaicin (CAP), GFP-TR:CAP, CREB:CAP, CREB-TR:vehicle (VEH) groups. Bottom, post CREB-TR:CAP group. (**b**) The percentage of freezing induced by drug administration (5-min bins). Only the CREB-TR:CAP group showed significant freezing by capsaicin at 5–10 min and 10–15 min (n = 7–8 mice per group). (**c**) Conditioned stimulus (CS)-induced freezing measured 20 min after drug infusion. Tone was presented for 1 min. The freezing level measured during 2 min right before conditioned stimulus presentation was used as baseline freezing. (**d**) Drug-induced freezing in mice trained with the immediate shock protocol. All mice were injected with CREB-TR vector (n = 4, VEH; n = 4, CAP). During training, shock was delivered immediately after mice entered the training chamber. $^{**}P < 0.01$, $^{***}P < 0.001$. Data in **b**–**d** are expressed as mean ± s.e.m.

A recording electrode attached to the injection pipette containing capsaicin solution was lowered into HSV-injected lateral amygdala areas. After a 10-min baseline recording, 1 μM capsaicin was locally injected through the injection pipette into lateral amygdala (**Fig. 2a**,**b**). We isolated single-unit responses that showed a significant capsaicin-induced increase in the firing rates in CREB-TR– and GFP-TR–injected mice, but found no significant firing changes in control mice (group × drug interaction, $F_{3,103} = 40.76$, $P < 0.0001$, two-way repeated-measures ANOVA). Bonferroni *post hoc* test revealed that the firing frequencies of CREB-TR and GFP-TR were significantly higher than those of control groups ($F_{3,104} = 11.095$, $P < 0.001$, one-way ANOVA; $P < 0.05$ for CREB-TR, $P < 0.001$ for GFP-TR; **Fig. 2**). Notably, capsaicin elicited similar increases in the firing rates of CREB-TR– and GFP-TR–injected mice. Typically, the spike-firing rate began increasing within 5 min of injection, peaked at 4–5 min post-injection and then returned to baseline approximately 15 min after drug infusion (**Fig. 2b**). There was no difference in baseline firing rates among different experimental groups ($F_{3,104} = 1.010$, $P = 0.39$, one-way ANOVA; **Fig. 2c**), suggesting that CREB and TRPV1 expression had no effect on baseline firing rates in lateral amygdala neurons. These results validate that, under the conditions used, the TRPV1-capsaicin system allows temporally specific activation of infected neurons.

Activating CREB neurons induced fear memory recall

Using this system, we first investigated whether capsaicin-induced activation of neurons overexpressing CREB can induce behavioral recall of a fear memory. We trained mice for auditory fear conditioning 3 d after bilateral injection of viral vectors and cannula implantation. The next day, we monitored freezing behavior for 15 min after bilateral local infusion of 1 μM capsaicin into the lateral amygdala. Mice were placed in a novel testing chamber immediately after drug administration (**Fig. 3a**). Following capsaicin infusion, CREB-TR–injected mice, but not GFP-TR mice, showed a significantly increased level of freezing (group × time interaction, $F_{8,66} = 4.284$, $P = 0.0003$; significant effect of group, $F_{4,33} = 6.942$, $P = 0.0004$; significant effect of time, $F_{2,66} = 24.79$, $P < 0.0001$; two-way repeated-measures ANOVA). Freezing responses in CREB-TR mice were first evident within 5–10 min ($F_{4,33} = 4.68$, $P < 0.01$, one-way ANOVA) of capsaicin administration and further increased, lasting to 10–15 min ($F_{4,33} = 6.94$, $P < 0.001$, one-way ANOVA) (**Fig. 3b**). Capsaicin did not induce freezing in GFP-TR mice, suggesting that simply activating a subset of random lateral amygdala neurons (approximately 15–20% of total lateral amygdala cells) does not induce freezing. To further confirm the specificity of this behavior, we performed control experiments. We found no substantial freezing in CREB mice after capsaicin infusion or in CREB-TR mice after vehicle application (**Fig. 3b**). Simply activating neurons overexpressing CREB may evoke freezing behavior unrelated to memory. We tested this possibility by injecting CREB-TR virus 1 d after auditory fear conditioning (post CREB-TR). Thus, CREB-injected neurons are not associated with the memory in this condition. We found no freezing by capsaicin in this condition, thereby excluding the possibility mentioned above.

To ensure that the mice learned auditory fear conditioning normally, we measured freezing induced by re-exposure to the conditioned stimulus (tone) that originally predicted the shock during training. We presented an auditory conditioned stimulus to the mice after the drug-induced freezing test, ~17 min after drug infusion. We measured freezing for 2 min before conditioned stimulus presentation to determine

baseline freezing for the subsequent conditioned stimulus–induced freezing. Following the 2-min baseline period, we presented the auditory conditioned stimulus to mice for 1 min to measure conditioned stimulus–induced freezing. Notably, mice from all of the experimental groups exhibited significant freezing in response to the tone, with no significant differences among groups (group × test interaction, $F_{4,33} = 0.1886$, $P = 0.9427$; significant effect of test, $F_{1,33} = 65.58$, $P < 0.0001$; two-way repeated-measures ANOVA; **Fig. 3c**). One-way ANOVA revealed no significant differences in baseline freezing ($F_{4,33} = 2.344$, $P = 0.075$) and conditioned stimulus–induced freezing ($F_{4,33} = 0.7914$, $P = 0.5392$) among groups. Thus, auditory fear memory was formed and expressed normally in these mice.

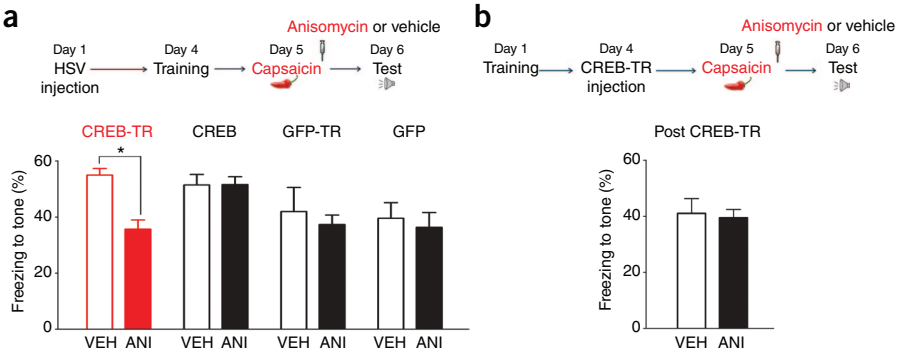

Figure 4 Fear memory was rendered labile after activating CREB neurons. (**a**) Experimental procedure and histogram showing the percentage of freezing in response to conditioned stimulus presentation during testing ($n = 7$, CREB-TR/VEH; $n = 9$, CREB-TR/anisomycin (ANI); $n = 9$, CREB/VEH; $n = 9$, CREB/ANI; $n = 7$, GFP-TR/VEH; $n = 11$, GFP-TR/ANI; $n = 8$, GFP/VEH; $n = 9$, GFP/ANI). Mice were trained for auditory fear conditioning and treated with either anisomycin or vehicle as a control immediately after capsaicin application. (**b**) For the post CREB-TR group, CREB-TR vector was injected 1 d after training as before. Drug was applied 3 d after viral vector injection ($n = 9$, VEH; $n = 10$, ANI). $^*P < 0.05$. Data in **a**,**b** are expressed as mean ± s.e.m.

Specificity of memory recall by activation of CREB neurons

The lack of capsaicin-induced freezing in the GFP-TR group (**Fig. 3b**) suggests that the fear memory recall induced by capsaicin was specific to activation of CREB neurons. To further confirm this, we examined whether freezing induced by capsaicin was influenced by memory strength. We further analyzed GFP-TR group data by sorting these mice into two subgroups: those with strong memory (>50% freezing) and those with weak memory (<50% freezing). We found no significant differences between the two subgroups ($P = 0.9181$, Student's t test; **Supplementary Fig. 3a**). This result suggests that simply activating a subset of lateral amygdala neurons does not elicit freezing even when fear memory is strong.

Freezing may occur by stimulation of any set of neurons in animals trained with elevated CREB expression. To test this possibility, we stimulated neurons infected with GFP-TR with CREB overexpression in different population of neurons in the lateral amygdala network. We injected two different viruses into the same mouse: HSV-GFP-TR, followed 2 d later by HSV-CREB. These vectors were injected at slightly different sites in the lateral amygdala. We trained the mice for auditory fear conditioning 3 d after viral injection and tested them 24 h later. During testing, we infused either capsaicin or vehicle as a control and measured freezing. Like mice in the GFP-TR alone condition, mice in this condition showed no significant freezing to capsaicin compared with the vehicle control group (drug × time interaction, $F_{2,14} = 1.378$, $P = 0.2842$, two-way repeated-measures ANOVA; **Supplementary Fig. 3b**). Taken together, these results indicate that freezing induced by capsaicin is specific to the activation of neurons with increased CREB expression and are not a result of the stimulation of any set of neurons in mice conditioned with CREB overexpression in lateral amygdala, highlighting the specificity of drug-induced memory recall.

Freezing induced by capsaicin could result from a non-associative process. For example, the shock could preferentially and more strongly activate lateral amygdala neurons overexpressing CREB during training and produce non-associative plasticity in the fear circuitry. To test this possibility, we employed an immediate shock training procedure[24], a non-associative condition in which the shock is not or is very weakly associated with a context. If the freezing was the result of a non-associative process, then capsaicin should also induce freezing in this condition. However, capsaicin did not induce freezing compared with vehicle control in this condition (drug × time interaction, $F_{2,12} = 0.3436$, $P = 0.7160$, two-way repeated-measures ANOVA; **Fig. 3d**). Thus, freezing induced by drug was not a result of a non-associative process, supporting the specificity of memory recall by capsaicin.

In our experimental condition, background contextual fear conditioning was also produced with tone fear conditioning. Thus, fear memory associated with a context might also be recalled by drug. To test this possibility, we used an unpaired training. In this condition, a tone presentation is unpaired with a shock, but a context fear memory is normally formed. During testing, capsaicin did not induce statistically significant freezing compared with vehicle control (drug × time interaction, $F_{2,30} = 3.913$, $P = 0.0309$; no significant effect of drug, $F_{1,15} = 0.0033$, $P = 0.1153$; two-way repeated-measures ANOVA; **Supplementary Fig. 2**). Unpaired t test revealed no significant difference in freezing at 10–15-min time period between groups ($P = 0.0722$). Notably, although it was statistically insignificant, there was a tendency of increasing freezing by capsaicin, suggesting that stimulating CREB neurons was not sufficient for the behavioral recall, but may activate part of the contextual memory trace.

Activating CREB neurons rendered memory labile

When recalled, fear memory often returns to a labile state that requires protein synthesis for re-stabilization, a process known as reconsolidation[10–12]. If activating CREB neurons induces fear memory recall, it may also induce a reconsolidation-like reorganization process. If this were the case, then blocking protein synthesis after activating CREB neurons would impair the memory by disrupting re-stabilization process. Using a previously described approach[12], we first confirmed that memory reconsolidation by an auditory conditioned stimulus was intact in CREB-TR and GFP-TR mice (**Supplementary Fig. 4**). We trained mice for auditory fear conditioning 3 d after CREB-TR or GFP-TR viral vector injection into the lateral amygdala. The next day, right after memory test (test 1), we infused the protein synthesis inhibitor anisomycin or vehicle into the lateral amygdala. The memory was retested (test 2) 24 h after test 1 (**Supplementary Fig. 4a**). The freezing observed during test 2 was significantly decreased compared with observed during test 1 after anisomycin infusion, but not after vehicle, in both CREB-TR–injected (test × drug interaction, $F_{1,17} = 4.173$, $P = 0.0569$, two-way repeated-measures ANOVA; the significant effect of anisomycin was confirmed by Bonferroni *post hoc* test, $P < 0.01$) and GFP-TR–injected mice (test × drug interaction, $F_{1,10} = 14.03$, $P < 0.005$, two-way repeated-measures ANOVA; the significant effect of anisomycin was confirmed by Bonferroni *post hoc* test, $P < 0.001$) (**Supplementary Fig. 4b**), indicating that the memory reconsolidation process occurred normally in CREB-TR– and GFP-TR–injected mice.

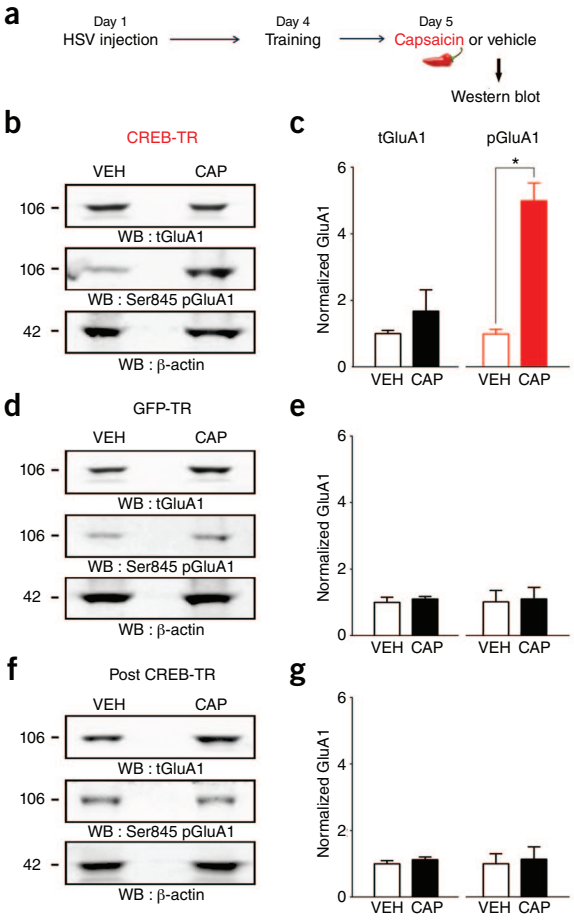

Figure 5 Phosphorylation of GluA1at Ser845 was induced by activating neurons expressing elevated level of CREB. (**a**) Experimental procedure for western blot analysis. Lateral amygdala tissues were collected 30 min after drug infusion (capsaicin or vehicle) and processed for western blot. (**b**–**g**) Representative western blot images (**b**,**d**,**f**) and histograms (**c**,**e**,**g**) showing the normalized level of protein expression of tGluA1 and pGluA1. The protein expression levels of tGluA1 and pGluA1 in lateral amygdala tissues were probed by western blotting. β-actin was included as a loading control. The levels of tGluA1 and pGluA1 were normalized to β-actin for analysis and comparison ($n = 3$, control GFP-TR and post CREB-TR group; $n = 4$, CREB-TR group). Full immunoblots are shown in **Supplementary Figure 8**. $^*P < 0.05$. Data in **c**,**e**,**g** are expressed as mean ± s.e.m.

Next, we examined whether activation of CREB neurons could induce reconsolidation-like change. We infused 1 μM capsaicin into the lateral amygdala of CREB-TR mice instead of presenting an auditory conditioned stimulus, followed by injection of either anisomycin or vehicle. When tested 24 h later, the mice that were administered anisomycin showed a significant decrease in freezing compared with vehicle-treated control mice, suggesting that inhibiting protein synthesis impairs memory (significant effect of drug, $F_{1,59} = 4.292$, $P = 0.0427$; Bonferroni *post hoc*, $P < 0.05$, two-way ANOVA; **Fig. 4a**). This behavioral effect was specific, as anisomycin had no amnesic effect in control groups (CREB, GFP-TR and GFP groups; **Fig. 4a**). Moreover, vehicle injection instead of capsaicin had no effect in CREB-TR ($P = 0.4144$, Student's *t* test) and GFP-TR mice ($P = 0.8908$, Student's *t* test) (**Supplementary Fig. 5**). Simply activating lateral amygdala neurons overexpressing CREB in amygdala network might non-specifically induce the destabilization of a fear memory trace. To test this possibility, we injected CREB-TR virus 24 h after auditory fear conditioning such that neurons injected with CREB-TR were not associated with that fear memory (**Fig. 4b**). Mice infused with either anisomycin or vehicle showed similarly high levels of freezing in response to conditioned stimulus tone ($P = 0.7922$, Student's *t* test; **Fig. 4b**), indicating that simply activating CREB overexpressing neurons in lateral amygdala does not induce the destabilization of established fear memory. Consistent with previous findings[7,25], fear memory was slightly enhanced by CREB overexpression under our experimental conditions. Taken together, our results indicate that activation of CREB neurons by drug renders established fear memory labile, requiring protein synthesis for re-stabilization, like reconsolidation process induced by memory recall.

GluA1 phosphorylation at Ser845 by activating CREB neurons

Phosphorylation of the AMPA-type glutamate receptor subunit 1, GluA1, at Ser845 (a site phosphorylated by PKA) in lateral amygdala synapses is a molecular correlate of fear memory recall that has been shown to be involved in subsequent memory reorganization[26]. The phosphorylation of GluA1 at Ser845 can facilitate activity-dependent enhancement of synaptic strength by regulating the synaptic insertion of GluA1-containing AMPA receptors involved in learning and memory[27–30]. We asked whether activating CREB neurons might also lead to such molecular change. We infused capsaicin or vehicle into the lateral amygdala of CREB-TR mice 1 d after auditory fear conditioning and collected lateral amygdala tissues 30 min after drug infusion (**Fig. 5a**). The expression levels of total GluA1 protein (tGluA1) and the phosphorylated form of GluA1 at Ser845 (pGluA1) were probed by western blotting. For quantification analysis, the levels of tGluA1 and pGluA1 were normalized to control β-actin. The pGluA1 expression was substantially increased after capsaicin infusion compared with vehicle control ($P < 0.01$, Student's *t* test), without a significant change in tGluA1 expression ($P = 0.3808$, Student's *t* test) (**Fig. 5b**,**c**).

This molecular change induced by drug was specific to the CREB-TR group; it was not observed in GFP-TR ($P = 0.5837$, tGluA1; $P = 0.8625$, pGluA1; Student's *t* test) and post CREB-TR control groups ($P = 0.4005$, tGluA1; $P = 0.7896$, pGluA1; Student's *t* test) (**Fig. 5d**–**g**). As a control, we also examined whether there were any differences in the level of pGluA1 and tGluA1 across different groups (CREB-TR, GFP-TR and post CREB-TR) under control conditions, no drug or vehicle injection. There were no significant differences in tGluA1 ($F_{2,12} = 0.03857$, $P = 0.9623$, one-way ANOVA for no drug injection; $F_{2,12} = 0.005297$, $P = 0.9947$, one-way ANOVA for vehicle injection) and pGluA1 levels ($F_{2,12} = 0.06262$, $P = 0.9396$, one-way ANOVA for no drug injection; $F_{2,12} = 0.1608$, $P = 0.8533$, one-way ANOVA for vehicle injection) across different groups in both no drug and vehicle injection conditions (**Supplementary Fig. 6**), suggesting that GluA1 phosphorylation at Ser845 is specific to activation of CREB neurons. Together, these results suggest that activating neurons with elevated CREB expression at the time of fear learning is sufficient to induce a key molecular change associated with memory recall and subsequent reconsolidation in lateral amygdala synapses: phosphorylation of GluA1 at Ser845.

Multiple reactivations of CREB neurons strengthened memory

Multiple reactivation of a memory trace or internal replay of neuronal activity may be important for strengthening a previously acquired memory[13–18,31,32]. Given that fear memory was reactivated by activation of CREB neurons, we reasoned that the same manipulation may lead to memory strengthening. To explore this idea, we reactivated CREB neurons by drug once per day over 3 d after auditory fear conditioning. We trained mice for auditory fear conditioning 3 d after bilateral CREB-TR viral vector injection into the lateral amygdala and cannula implantation using a relatively weak foot shock (0.4 mA). Mice in one group were administered 1 μM capsaicin once per day over three consecutive days

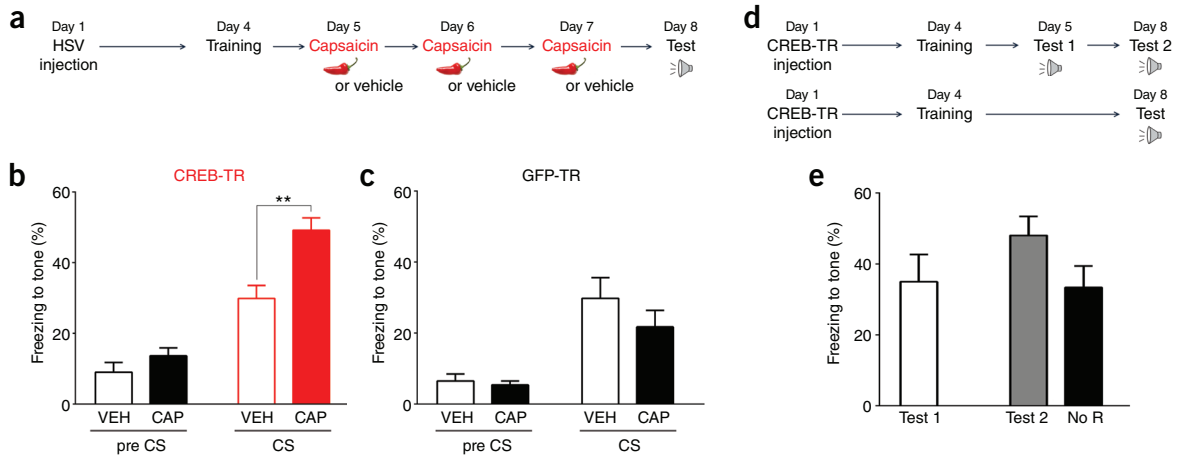

Figure 6 Fear memory was strengthened after multiple reactivations of neurons expressing elevated CREB. (**a**) Behavior experimental procedure. Mice were injected with viral vectors and trained for auditory fear conditioning with relatively weak shock (0.4 mA). Capsaicin was locally infused into bilateral lateral amygdala once per day over three consecutive days in the mice's home cage. Vehicle was infused as a control. (**b**,**c**) Histograms showing the results of the behavior experiment. The percentage of freezing induced by tone presentation during testing is shown. Mice were injected with either CREB-TR ($n = 10$, vehicle; $n = 14$, capsaicin; **b**) or GFP-TR ($n = 14$, vehicle; $n = 15$, capsaicin; **c**) viral vector. Baseline freezing (pre CS) was determined by measuring freezing for 2 min right before tone conditioned stimulus presentation. (**d**) Behavior experimental procedure. In the first group (top), mice were tested at two different time points after training for each mouse: 1 d (test 1) and 4 d (test 2) after training ($n = 12$). In the second group (bottom; no reactivation, No R), mice were tested once 4 d after training without any drug infusion ($n = 7$). (**e**) Histogram showing the results of the behavior experiments. All mice were injected with CREB-TR and trained for auditory fear conditioning with weak shock (0.4 mA). $^{**}P < 0.01$. Data in **b**,**c**,**e** are expressed as mean ± s.e.m.

in their home cage, whereas mice in the other group were administered vehicle as a control (**Fig. 6a**). During testing, the capsaicin group showed a significantly higher level of freezing than the control vehicle group ($P < 0.01$, Student's t test; **Fig. 6b**), indicating that fear memory was strengthened by multiple reactivations of CREB neurons.

To determine whether this memory strengthening was specific, we conducted a control experiment with GFP-TR–injected mice. We found no significant effect of capsaicin compared with vehicle control in this condition ($P = 0.2835$, Student's t test; **Fig. 6c**), suggesting that the fear memory enhancement was specific to the activation of CREB neurons and was not a result of simply increased repeated activity of any subset of lateral amygdala neurons.

Given that we tested mice 4 d after training, it is possible that fear memory was weakened naturally during this time course and that capsaicin prevents this weakening, rather than strengthening the memory. To test this possibility, we performed a control experiment using CREB-TR–injected mice. First, we measured freezing at two different time points after training for each mouse, 1 d (test 1) and 4 d (test 2) after training, and compared freezing levels at test 1 to those observed at test 2. Second, we measured freezing 4 d after training without any drug administration (no reactivation, No R; **Fig. 6d**). We found no significant difference in the freezing levels between tests 1 and 2 ($P = 0.1909$, paired t test; **Fig. 6e**) and the freezing in the No R group was comparable with that observed during test 2 ($P = 0.1006$, unpaired t test; **Fig. 6e**). These results indicate that fear memory was maintained during the time course and that single reactivation by a conditioned stimulus had no memory enhancing effect. Notably, the freezing level observed with no drug injection was also similar to that observed with vehicle injection in the control group (**Fig. 6b**,**c**), suggesting that vehicle injection into the amygdala did not cause a substantial decrease of fear memory expression in our condition.

A previous study found that memory is strengthened by the multiple reactivations of memory trace with unreinforced natural conditioned stimulus under the conditions that do not induce extinction[18]. We wondered whether we would evoke similar memory strengthening by exposing the mice to a natural conditioned stimulus tone under the condition parallel to drug-induced reactivations. To test this possibility, we reactivated fear memory trace by natural conditioned stimulus instead of drug. We injected CREB-TR viral vector and, 3 d later, we trained mice for auditory fear conditioning. During the reactivation session, one group of mice (three conditioned stimuli) was exposed to a single 10-min tone per day over 3 d in their home cage and the control group (no conditioned stimulus) stayed in the home cage without conditioned stimulus presentation. During testing, mice in the three conditioned stimuli group exhibited a significantly higher level of freezing compared with control mice ($P < 0.05$, unpaired t test; **Supplementary Fig. 7**). This result suggests that memory strengthening occurred naturally by the conditioned stimulus tone presentation under the conditions parallel to drug-induced reactivation. Taken together, our data indicate that multiple reactivations of CREB neurons strengthen auditory fear memory and that this memory strengthening is specific to the activation of CREB neurons, supporting the notion that internal reactivation of a memory trace can contribute to strengthening that memory during memory incubation.

DISCUSSION

It has been thought that neurons activated during learning are recruited to form a memory trace and that later reactivation of them may lead to recall of that memory. Using an optogenetic technique, a previous study reported that reactivation of dentate gyrus neurons that are active during contextual fear memory training is sufficient to drive the recall of that memory[33]. Several studies have suggested the possibility that only a portion of neurons that receive necessary sensory inputs, and are therefore activated during learning, may be selected to be incorporated into the memory trace. However, no study to date has targeted such a specific ensemble of neurons and determined whether artificially activating these selected neurons is sufficient to recall a particular memory. In addition, although lateral amygdala has been known to be a key brain site in which fear memory is permanently stored, whether just activating specific sets of neurons in lateral amygdala can induce recall of a fear memory remains unknown. Here we found, to the best of our knowledge for the first time, that artificially activating a subset of lateral

amygdala neurons expressing elevated levels of CREB at the time of fear conditioning is sufficient for the recall of that fear memory. Moreover, we also found that the same manipulation was sufficient to induce a reconsolidation-like reorganization process and strengthening of the fear memory. Our results establish a direct link between the activation of a defined subset of neurons in the lateral amygdala and the recall of a fear memory and its subsequent modifications.

The fear memory recall induced by drug-mediated neuronal activation was highly specific. It was induced only if lateral amygdala neurons that had elevated CREB at the time of fear conditioning were reactivated. Thus, vehicle infusion or capsaicin without TRPV1 expression did not induce freezing. Activating a similar number of GFP-TR–infected lateral amygdala neurons did not induce freezing, even in the case of strong fear memory. Freezing by drug was not simply a result of the activation of neurons overexpressing CREB, as observed in the post CREB-TR group. Notably, CREB overexpression may increase the overall excitability of lateral amygdala network, and stimulating any set of lateral amygdala neurons in such condition could induce freezing. We tested this possibility using dual injection of GFP-TR and CREB, but did not observe freezing in response to drug administration, thereby excluding the possibility. These control data strongly support our conclusion that fear memory recall is specifically induced by drug-mediated stimulation of lateral amygdala neurons with elevated CREB.

Our data suggest that freezing induced by drug represents the activation of auditory fear memory, but not the activation of either non-memory–related traces produced by non-associative processes during training or contextual fear memory. No induction of freezing by drug in mice trained with the immediate shock training procedure excluded the possibility that activation of CREB neurons during training produced non-associative plasticity in the lateral amygdala and that later activation of the same set of neurons induced non-memory–related freezing. During auditory fear conditioning, context memory was also formed in parallel. In fact, our previous study showed that ablation of neurons overexpressing CREB after auditory fear conditioning impaired both auditory fear memory and contextual fear memory[8]. Using an unpaired conditioning procedure, we examined whether activation of CREB neurons is sufficient to induce recall of context memory. Although there was a slight increase of freezing by drug, mice did not exhibit significant levels of freezing. Thus, activating CREB neurons by drug was not sufficient for the recall of contextual fear memory. Nevertheless, it is possible, although less likely, that both auditory and contextual fear memory were activated by drug in mice trained for auditory fear conditioning.

Although significant freezing was observed by activating CREB neurons, its level was relatively low compared with that usually evoked by natural auditory conditioned stimulus cue. It is possible that the neuronal population size that we manipulated was too small to reach the level of amygdala circuit activity that is required for full expression of a fear memory, or that activation of other brain regions in fear circuitry might also be necessary[33,34]. Alternatively, the precise activity pattern, which we did not control in this study, might be critical for the full expression of the stored fear memory. The artificial stimulation by drug in our experimental condition evoked a relatively small increase of spike firing rate in the lateral amygdala cells that we recorded, likely as a result of the nature of drug-mediated response *in vivo*, such as drug diffusion and receptor activation properties. This relatively weak activation could contribute to a low level of freezing induced by drug during memory recall test. Given that we only measured drug-mediated spike firing changes in untrained mice, it is also possible that the artificial stimulation by drug might induce a larger increase in the spike firing rate in conditioned mice.

The natural auditory stimulus evokes a rapid increase of spike firing in a population of lateral amygdala cells. The *in vivo* unit recording reveal that the increase of firing rate typically start at 10–20 ms after tone onset, peak within 100 ms of tone onset and that the number of cells that show tone-evoked spike firing peak at 20–30 ms after tone onset[35–37]. However, the artificial stimulation by drug in our study induced much slower responses in lateral amygdala (**Fig. 2b**). The firing rate in lateral amygdala cells slowly increased and peaked approximately 5 min after drug infusion. The slow response time course in firing rate by drug fits well with the drug-induced freezing response that we observed during memory recall test. The mice started to show freezing 5–10 min after drug infusion (**Fig. 3b**), and this time point matches well with the time point at which lateral amygdala neurons close to drug infusion site started to show peak increase of firing rate. As the drug diffused in the lateral amygdala, thereby stimulating more lateral amygdala neurons, freezing level was further increased such that mice showed slowly increasing freezing over the time course, at least up to 10–15 min after drug infusion (**Fig. 3b**). Thus, it seems that the stimulation of more CREB neurons was correlated with more freezing in our condition.

Activating lateral amygdala neurons with elevated level of CREB was sufficient not only for recall, but also for modifications of a fear memory, reconsolidation-like change, strengthening of memory and related molecular changes. To the best of our knowledge, this is the first demonstration that artificially activating a specific ensemble of lateral amygdala neurons is sufficient to induce modifications of a previously acquired fear memory. The way that we manipulated the activity of neurons by drug without re-exposure to external behavioral cues may mimic the internal activity replay process in the brain during memory incubation, sleep or free recall. This internal activity replay has been thought to contribute to strengthening a previously acquired memory[13–15,17,31,32]. Moreover, it was shown that multiple reactivations of a memory trace by unreinforced conditioned stimulus also strengthen the memory by reconsolidation mechanism[16,18]. Thus, it is most likely that the memory strengthening by drug in our experiment resulted from multiple reactivations of established fear memory. Consistent with this hypothesis, we also found similar memory strengthening by natural conditioned stimulus under parallel conditions with drug-mediated reactivation.

The neurons with elevated levels of CREB that were manipulated in our study may be a small portion of the entire broad fear memory trace. However, activating just these neurons was sufficient for the recall of a fear memory and modification of it. Thus, a subset of neurons in lateral amygdala with elevated level of CREB than their neighbors at the time of fear learning may be more than just a small portion of fear memory trace, instead representing key components of the fear memory trace such that reactivation of this specific ensemble of neurons alone is sufficient for the recall of memory and its modifications.

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

J.-H.H. designed and directed the study. J.K. and J.-H.H. designed the HSV vector and the imaging, western blot and memory reconsolidation experiments. S.A.J. and J.-H.H. designed the memory strengthening experiment. J.-T.K. and J.-H.H. designed the *in vivo* recording and memory recall experiments. J.K. performed HSV vector construction, virus packaging, imaging, western blot and the memory reconsolidation experiment. J.K. and H.-S.K. performed the memory strengthening experiment. J.-T.K. performed *in vivo* recording and the memory recall experiment. J.K. and J.-H.H. analyzed the imaging, western blot and memory reconsolidation experiments. J.-T.K. and J.-H.H. analyzed the *in vivo* recording and memory recall experiments. J.K., H.-S.K. and J.-H.H. analyzed the memory strengthening experiments. J.K., J.-T.K., H.-S.K., S.A.J. and J.-H.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. 129/C57Bl/6 hybrid background mice (2–3 months old, 23–35 g) were group housed (four mice per cage) on a 12-h light/dark cycle at a constant temperature of 22 ± 1 °C with 40–60% humidity. Food and water were available *ad libitum* throughout the experiment. All procedures were approved by the Animal Ethics Committee at the Korea Advanced Institute of Science and Technology.

HSV vectors. We used a HSV vector with a dual promoter system for efficient coexpression of CREB and TRPV1 in infected neurons. The expression of CREB is driven by the constitutive virus immediate-early gene promoter IE 4/5, and the expression of TRPV1 is driven by the cytomegalovirus (CMV) promoter. Four different HSV vector constructs were used: HSV-CREB-TRPV1, HSV-GFP-TRPV1, HSV-CREB and HSV-GFP. The HSV-CREB-TRPV1 construct was generated by inserting PCR-amplified cDNA encoding N-terminally GFP-tagged CREB and PCR-amplified cDNA encoding rat TRPV1 at sites cut by specific restriction enzymes. EGFP was used as an expression marker. The HSV-GFP-TRPV1 construct was generated similarly using GFP cDNA instead of a GFP-CREB fusion construct. HSV vectors were chosen for increasing CREB function in lateral amygdala neurons because HSV is naturally neurotropic[38]. Previous research has established that similar infusion of a CREB vector increases both CREB levels and function[39–41]. Tagging the N terminus of CREB with EGFP does not interfere with CREB functional activity[42].

HSV packaging. HSV packaging was performed as previously described[43]. Briefly, host cells (2-2 cell line containing the IE2/ICP27 gene) were transfected with the recombinant HSV vectors and packaged using a replication-defective helper virus (with a 5dl 1.2 deletion). This mixture was passaged three times in 2-2 cells. The resultant virus was purified on a sucrose gradient, pelleted and resuspended in 10% sucrose (wt/vol). The average titer of recombinant virus stocks was typically 5.0×10^7 infectious units/ml.

Western blot. The level of TRPV1 protein expression in the lateral amygdala of wild-type and HSV-injected (CREB-TR and GFP-TR) mice was determined by dissecting lateral amygdala tissues from these mice and sectioning them (400 μm thick) using a Vibratome. Strong and specific fluorescence of the expression marker GFP in the lateral amygdala was confirmed under a fluorescence microscope before dissection. The dissected tissue was washed twice with cold phosphate-buffered saline (PBS) and then lysed in 40 μl of cold PLC lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Nappi, 100 mM NaF). Total protein was determined using the Bradford assay. Proteins (50 μg per lane) were resolved by sodium dodecyl sulfate-PAGE (SDS-PAGE) and transferred to PVDF (polyvinylidene difluoride) membranes using a Bio-Rad turbo system. After blocking with TN-TX buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.2% Triton X-100). containing 3% bovine serum albumin (BSA, wt/vol), membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody to TRPV1 (1:1,000). β-actin, a loading control, was detected using a mouse monoclonal antibody to β-actin (1:4,000). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat antibody to rabbit IgG or to mouse IgG, followed by enhanced chemiluminescence (ECL Western Blotting Detection System, Amersham).

HSV-infected areas of lateral amygdala tissue were dissected under fluorescence microscope. The obtained tissue extracts were washed twice with cold PBS containing 1 mM sodium orthovanadate and then lysed in 50 μl of cold PLC lysis buffer. Lysates were clarified by centrifugation in a microcentrifuge for 10 min at 4 °C. Serially diluted supernatants were boiled in SDS sample buffer and subjected to SDS-PAGE using 10% polyacrylamide gels. Resolved proteins were electrophoretically transferred to Immobilon-P (Millipore) membranes. After blocking in TN-TX buffer containing 3% BSA, CREB protein was detected using rabbit polyclonal antibody to CREB antibody (1:2,000). GAPDH, used as a loading control, was detected using a rabbit polyclonal antibody to GAPDH (1:4,000). Primary antibodies were detected using HRP-conjugated goat antibody to rabbit IgG, followed by enhanced chemiluminescence.

HSV-infected areas of lateral amygdala tissue were dissected under fluorescence microscope and processed as described above. After blocking in TN-TX buffer containing 3% BSA (for antibody to phosphotyrosine blots) or skimmed milk, tGluA1 and pGluA1 proteins were specifically detected using a rabbit polyclonal antibody to GluA1 S845 (1:1,000) and mouse antibody to GluA1 (1:1,000), respectively. β-actin was also detected as a loading control. Primary antibodies were detected using HRP-conjugated goat antibody to rabbit IgG or to mouse IgG, as appropriate, followed by enhanced chemiluminescence. Blots were visualized using a Fuji-film LAS-3000 System. The levels of tGluA1 and pGluA1 proteins were normalized to β-actin for data analysis.

Cell-counting analysis. The percentage of TRPV1-positive cells in the lateral amygdala was quantified by manually counting $TRPV1^+$ cells from brain sections immunostained with antibody to TRPV1. The anterior-posterior order of sections was carefully reconfirmed under a microscope, and each section (40 μm thick) was assigned a corresponding anterior-posterior value. For cell counting, 14 sections were obtained from each mouse brain ($n = 4$ mice per group) for each experimental group (CREB-TR, CREB, GFP-TR and GFP). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) to obtain total cell numbers. The percentage of $TRPV1^+$ cells in a given lateral amygdala section was obtained by calculating the ratio of the total number of $TRPV1^+$ cells to the total number of DAPI-stained cell nuclei. The percentages of $TRPV1^+$ cells were 17.317 ± 1.121% ($n = 4$) in the CREB-TR group, 0.185 ± 0.090% ($n = 4$) in the CREB group, 17.017 ± 0.883% ($n = 4$) in the GFP-TR group, and 0.288 ± 0.207% ($n = 4$) in the GFP group (**Fig. 1d**).

From each group, 9–12 sections were taken from each mouse brain ($n = 4$ mice per experimental group), fixed, and immunostained using an antibody to TRPV1. The order of sections was carefully reconfirmed under a confocal microscope, and each section was assigned a corresponding anterior-posterior value. Co-expression of CREB (or GFP) with TRPV1 in a given brain section was quantified by manually counting the total number of $CREB^+$) (or GFP^+), $TRPV1^+$ and double-positive $CREB^+$ $TRPV1^+$ or GFP^+ $TRPV1^+$ cells in the corresponding confocal microscopic image. The colocalization percentage in a given lateral amygdala section was calculated as the percentage ratio of the total number of $CREB^+$ $TRPV1^+$ or GFP^+ $TRPV1^+$ double-positive cells to the total number of $CREB^+$ or GFP^+ cells, respectively. The percentages of co-expressing cells were 68.1 ± 1.9% ($n = 4$) for the CREB-TR group, 1.4 ± 0.4% ($n = 4$) for the CREB-only group, 68.1 ± 5.8% ($n = 4$) for the GFP-TR group, and 1.7 ± 0.4% ($n = 4$) for the GFP-only group (**Fig. 1d**). It should be noted that most, but not all, $CREB^+$ or GFP^+ cells coexpressed TRPV1. All cell counts were conducted in a blinded manner.

Immunohistochemistry. Brains were sliced coronally (40 μm) on a cryostat and prepared for immunohistochemistry. After blocking step, sections were incubated with rabbit polyclonal antibody to TRPV1 (1:500) overnight at 4 °C. Alexa Fluor 568–conjugated goat antibody to rabbit IgG (1:2000) was used as a secondary antibody. The GFP signal was visualized as an expression marker. Each section was imaged and analyzed using a ZEISS LSM 710 upright confocal laser-scanning microscope and Zen 2009 software.

Antibodies. We used antibodies to Trpv1 receptor (Millipore, AB9554), GluA1 s845 (Novus, NB300-171), total GluA1 (Santa Cruz, SC-13152), CREB (Cell signaling, 9197), GAPDH (Abcam, ab9485), actin (Sigma, A3853), HRP-conjugated antibody to rabbit IgG (Bio-rad, 172-1019), HRP-conjugated antibody to mouse IgG (Sigma, A4416) and Alexa Fluor 568–conjugated goat antibody to rabbit IgG (Invitrogen, A-11011).

Surgery. Mice were anesthetized by intraperitoneal injection of pentobarbital (83 mg per kg of body weight) and placed in a stereotaxic frame. A glass micropipette containing an HSV solution was positioned on the targeted lateral amygdala brain region (anteroposterior = –1.7 mm, mediolateral = ±3.45 mm, dorsoventral = –4.2 mm from bregma, according to ref. 44). HSV solutions were bilaterally injected locally into the lateral amygdala at a rate of 0.1 μl min^{-1} for 15 min (total, 1.5 μl per side). Micropipettes were left in place for an additional 10 min to ensure diffusion. After HSV injection, cannulae were chronically implanted into the lateral amygdala (anteroposterior = –1.7 mm, mediolateral = ±3.45 mm, dorsoventral = –3 mm from bregma). Unless otherwise specified, mice were trained 3 d following surgery, a time at which transgene expression is maximal using this HSV vector system[29].

Intra–lateral amygdala infusions. Capsaicin solutions (M2028, Sigma) were prepared by dissolving in 100% ethanol and then diluting 1:1,000 with PBS to produce a final concentration of 1 μM. Capsaicin solutions were infused into each side of the lateral amygdala at a rate of 0.2 μl min^{-1} using an infusion pump.

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Anisomycin solutions were prepared by dissolving anisomycin (A9789, Sigma) in 1 N HCl and then diluting 1:1 in artificial cerebrospinal fluid to produce a final concentration of 125 μg μl^{-1}. Anisomycin solutions were infused into the lateral amygdala at a rate of 0.2 μl min^{-1} using an infusion pump.

***In vivo* recording.** One or two days after virus injection, mice were anesthetized with pentobarbital (83 mg per kg, intraperitoneal) and placed in a stereotaxic frame. A tungsten electrode (5 MΩ) attached to a glass pipette filled with 1 μM capsaicin solution was positioned at the same lateral amygdala location where the virus was injected. Recordings were amplified 10^4 times and band-pass filtered at 300 Hz (low) and 10 kHz (high) using a DAM80 differential amplifier (World Precision Instruments), and digitized at 25 kHz. Spike firings were processed and monitored by using Spike2 acquisition software (Cambridge Electronic Design). Once the recordings were stable at the target area, 10-min baseline spike firings were recorded and followed by a 1 μl infusion of 1 μM capsaicin for 5 min. Electrode placement was confirmed post mortem. Data were stored and analyzed off-line. To determine whether capsaicin increases firing rate of lateral amygdala neurons injected with TRPV1, we chose those units with relatively low baseline firing rate for further drug treatment experiment based on baseline firing rate during recording procedure. Single-unit isolation was achieved by matching spike waveforms to a template using Spike2 analysis program. In this analysis, each data point of a spike is compared against a template. If sufficient points fall within the template, the spike is matched. If not, the spike forms a new template under the template building algorithm. After all templates are confirmed from a recording, spikes from the recording are re-sorted to the corresponding template, and each group is considered as a single-unit. Single unit isolation process was further refined using principle component analysis based on peak amplitude and spike width. In order to verify that the isolated units were in fact single units, we examined inter-spike interval (ISI) histogram and confirmed that all isolated units do not have ISI values of ≤ 1 ms. Firing frequency of each unit was binned into one minute and standardized to the baseline (z-score). For CREB-TR and GFP-TR groups, single-unit responses that satisfied the following three criteria were considered to be capsaicin-induced responses: (1) The average z-score from 5 to 15 min after capsaicin administration was greater than one s.d. above baseline. (2) The increased firing frequency returned to baseline over time. (3) The response was reversible. Based on the criteria for capsaicin-induced response, we calculated the percentage of cells that satisfied the criteria in each group: 43.6% (17/39) for CREB-TR group, 44.1% (15/34) for GFP-TR group, 10.3% (3/29) for CREB alone group, and 6.7% (3/45) for GFP alone group. The z-score of 2 cells among 3 cells that satisfied the criteria in both CREB and GFP alone groups was just slightly above borderline, one s.d. above baseline. Thus, for the comparison, we pooled data for CREB and GFP alone groups. For the data analysis of *in vivo* single unit recording, we only included animals that showed successful expression of viral vectors on-targeted into the lateral amygdala with recording electrode well positioned on the virus infected sites. For GFP-TR and CREB-TR groups, we could not find drug-responsive units in lateral amygdala of animals that showed off-target or very low-level of expression of viral vectors and also when recording electrode position was not well matched with virus infected areas in the lateral amygdala. The recording data from these animals were not included for the data analysis.

Auditory fear conditioning. Mice were trained 3 d after surgery. Training consisted of placing mice in a conditioning chamber (Coulbourn Instruments) and, 2 min later, presenting the conditioned stimulus (2,800-Hz, 85-dB tone lasting 30 s) that co-terminated with an unconditioned stimulus (0.4- or 0.5-mA shock for 2 s). Mice were kept in the chamber for an additional 30 s after shock delivery. Fear memory tests were conducted 24 h after training. Mice were placed in a context-shifted testing chamber. After establishing a 2-min baseline freezing level (pre–conditioned stimulus), the tone was presented for 3 min and freezing behavior in response to the conditioned stimulus was monitored. Freezing behavior was assessed via automated procedures using FreezFrame software (Actimetrics). For unpaired conditioning, shock was presented first at 120 s and tone conditioned stimulus was followed with 60-s interval during training. For immediate shock experiment, mice were placed in the training chamber and foot shock was immediately presented. Right after shock delivery, mice were removed from the conditioning chamber. For the sake of clarity, baseline (pre–conditioned stimulus) freezing scores for most of the experiments were not presented. After behavioral testing, transgene expression was confirmed by histological analysis under a fluorescence microscope. The mice that showed strong and restricted expression of transgenes in the lateral amygdala were included in the data analysis. For every mouse used in the behavior experiments, we obtained the brain sections covering whole amygdala, and GFP signal, an expression marker of HSV viral constructs, was monitored from those brain sections to confirm the virus targeting and expression level. Mice were included for the data analysis if the majority of the infected neurons were localized into the lateral amygdala, and if the virus infected approximately 10–20% of lateral amygdala cells. Although most of the animals included showed highly confined viral expressions in the lateral amygdala, in few cases we observed a few additional infected cells in dorsal or ventral endopiriform cortex, which is located just lateral to the lateral amygdala and/or in small part of caudate putamen along the injection track. These animals were also included in our data analysis. The animals that were excluded typically showed off-target expressions such that the majority of infected cells were located at part of endopiriform cortex or caudate putamen outside lateral amygdala. In some of these off-target animals, we observed few viral infected cells in lateral amygdala, approximately less than 1% of lateral amygdala cells. In rare cases, we observed no or only very few number of viral-infected cells in the brain. These animals were also excluded.

Capsaicin-induced freezing. 2 d after virus injection and cannula implantation, mice were handled and habituated with internal cannula insertion. 3 d after surgery, mice were trained for auditory fear conditioning with 0.5–mA shock, as described above. 24 h later, vehicle (PBS) or 1 μM capsaicin was loaded into the injection cannula connected to a Hamilton syringe and micro pump and simultaneously infused bilaterally into the lateral amygdala for 5 min (total infusion volume, 1 μl per side). Immediately after the end of drug administration, injection cannulae were removed and mice were quickly placed in a context-shifted testing chamber. Behavioral responses after drug infusion were monitored by video camera for 20 min, and then conditioned stimulus–induced freezing responses were measured during a 1-min conditioned stimulus tone presentation.

Protein synthesis inhibition after capsaicin administration. Mice were injected with GFP, CREB, GFP-TR and CREB-TR vectors and implanted with a cannula. 3 d later, mice were trained for auditory fear conditioning. The next day, mice in their home cage were locally infused with 1 μl of capsaicin (1 μM) three times at 10-min intervals unilaterally into the lateral amygdala. 10 min after the end of capsaicin infusion, 1 μl of anisomycin (125 μg μl^{-1}) or vehicle was locally infused into the lateral amygdala. The next day, fear memory was re-tested. Mice were placed in a context-shifted testing chamber, and conditioned stimulus–induced freezing responses were monitored after presenting a 3-min conditioned stimulus tone.

Reconsolidation induced by a conditioned stimulus tone. 3 d after HSV vector injection, mice were trained for auditory fear conditioning. The next day, fear memory was retrieved by presenting a 3-min conditioned stimulus tone. Immediately after fear memory testing, mice were locally infused (unilaterally) with 1 μl of anisomycin (125 μg μl^{-1}) or vehicle in the lateral amygdala. 24 h after training and drug infusion, mice were re-tested for fear memory, during which a conditioned stimulus tone was presented for 3 min and conditioned stimulus-induced behavioral freezing responses were measured.

Multiple reactivations by capsaicin administration. 3 d after surgery, mice were trained for auditory fear conditioning with a relatively weak shock (0.4 mA), a stimulus chosen to avoid a "ceiling effect". Starting the next day after training, mice received a bilateral 1 μl infusion of capsaicin (1 μM) or vehicle into the lateral amygdala once a day over three consecutive days in their home cage. 24 h after the last drug administration, mice were tested.

Multiple reactivations by auditory conditioned stimulus. 3 d after surgery and cannulae implantation, mice were fear conditioned (0.4 mA), Starting the next day after training, mice were exposed to a 10-min conditioned stimulus, once a day over three consecutive days in their home cage. 24 h after last presentation of a 10-min conditioned stimulus tone, mice were placed in the testing chamber. After establishing a 2-min baseline freezing level (pre–conditioned stimulus), mice received a conditioned stimulus tone for 3 min and their freezing behavior was measured as a memory index.

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Erratum: Memory recall and modifications by activating neurons with elevated CREB

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In the version of this article initially published online, the *P* value in Figure 4a was given as $^{**}P < 0.01$ instead of $^{*}P < 0.05$ and the *y* axis of Figure 1e was labeled "Colocalized cells (%)" instead of "Normalized TRPV1." The errors have been corrected for the print, PDF and HTML versions of this article.