Human Experiments (Table S1)

Fear Conditioning Acquisition

A total of 161 subjects completed Experiments 1–6. Fifty-four (26 males, age range: 18–29, mean: 23.0), 36 (18 males, age range: 17–29, mean: 22.7), 37 (16 males, age range: 18–31, mean: 22.9), 19 (10 males, age range: 20–27, mean: 23.0) and 15 (8 males, age range: 21–28, mean: 23.3) subjects completed Experiments 1-3 and 5-6 respectively. Twenty-four (10 males, age range: 19–29, mean: 22.8) of the original participants from Experiment 3 participated in Experiment 4. No sex differences were seen in the results of any of the experiments, so the data from males and females were combined. Each participant signed a consent form approved by the Institutional Review Board of Peking University and was paid for their participation.

Subjects were instructed to pay attention to the computer screen and try to determine the relationship between the conditioned stimuli (CSs) and unconditioned stimulus (US; an electric shock). In all of the experiments, the CS+ was paired with the US on a partial reinforcement schedule (38% reinforced). The CS- was not paired with a US. The CSs were presented for 4 s with an interstimulus interval of 8-12 s, during which the participants looked at a fixation point.

In Experiment 1, the procedure included two CSs: blue and yellow squares that were either paired (CS+) or unpaired (CS-) with the US. Acquisition consisted of 10 nonreinforced presentations of each of the CSs, intermixed with an additional six CS+ presentations that co-terminated with electric shock. Two different orders of presentation were used to counterbalance for designations of colored squares (blue or yellow) as the CS+ or CS-. 
In Experiments 2, 3, and 5, two distinct CSs were paired with the same US. The procedure included three CSs: blue, yellow, and red squares (paired CS1+, CS2+ or unpaired CS- with a US). Acquisition consisted of 10 nonreinforced presentations of each of the CSs, intermixed with an additional six CS1+ and six CS2+ presentations that co-terminated with electric shock. Six orders of presentation were used to counterbalance for designations of colored squares (blue, yellow, or red) as the CS1+, CS2+, or CS-.

In Experiment 6, three colored squares (blue, yellow, and red) were used. Each of two squares (CS1+ and CS2+) was paired with one US (US1 [electric shock to the right inner wrist] and US2 [electric shock to the right eyelid] in a counterbalanced designed) on a 38% partial reinforcement schedule. The third square (CS-) was not paired with a US. Acquisition consisted of 10 nonreinforced presentations of each of the CSs, intermixed with an additional six CS1+ and six CS2+ presentations that co-terminated with the electric shock to the right inner wrist or eyelid.

Reactivation and Extinction

In Experiment 1, one day after acquisition, fear memory was reactivated prior to extinction in two groups. The first group (n = 19) underwent extinction after the 10 min period (within the reconsolidation time window). The second group (n = 19) underwent extinction 24 h after reactivation (outside of the reconsolidation time window). The third group (n = 16) was not reactivated but directly proceeded to the 10 min break.

In Experiment 2, one day after acquisition, the effectiveness of a US reminder was compared with a CS1+ reminder. In the first group (n = 18), a weaker electric shock (the same as in Experiment 1) was administered once during reactivation. In the second group (n = 18), the CS1+ was presented once (nonreinforced) during reactivation. All of the participants then underwent extinction after a 10 min break.

In Experiment 3, one day after acquisition, the effectiveness of extinction of either of the CSs after a US reminder was compared with extinction after reminders of the two CSs. In the first group (n = 18), the weaker electric shock (the same as in Experiment 1) was administered once during reactivation. In the second group (n = 19), the CS1+ and CS2+
were presented once each (nonreinforced) during reactivation. The participants then underwent extinction after a 10 min break.

In Experiment 4, extinction (CS1+ and CS2+) occurred 6-7 months after the end of reinstatement testing in Experiment 3. Twenty-four participants were included in the follow-up test (US retrieval group, n = 11; CS retrieval group, n = 13).

In Experiment 5 (n = 15), two weeks later after acquisition, the weaker electric shock (the same as in Experiment 1) was administered once during reactivation. The participants then underwent extinction after a 10 min break.

In Experiment 6 (n = 19), one day after acquisition, the US1 was presented once during reactivation. Half of the participants received the weaker electric shock to the right inner wrist once during reactivation. The other half of the participants received the weaker electrical shock to the right eyelid once during reactivation. The participants then underwent extinction after a 10 min break.

Test

During the spontaneous recovery test and reinstatement test, the CSs were presented for 4 s, followed by an interstimulus interval of 8-12 s, during which the participant looked at a fixation point. The time between the last spontaneous recovery trial and first reinstating US was 30 s. During all of the sessions (i.e., acquisition, reactivation, extinction, and test), the participants were attached to the skin conductance response (SCR) and shock electrodes, and the shock stimulator was set to the “on” position.

Psychophysiological Stimulation and Assessment

Electric shocks were delivered by a constant-current stimulator via a STM 200 stimulator (BIOPAC Systems, Goleta, CA, USA). A stimulating electrode was attached to the right inner wrist or right eyelid. The subjects determined the level of the shock themselves, beginning at a very mild level of shock (5 V) that gradually increased until the shock reached the maximum level that the subjects determined was uncomfortable but not painful (the highest possible level was 50 V) (1; 2). All of the shocks were administered for 200 ms, with a current of 50 pulses per second.
Stimulus presentation was controlled by a computer using E-Prime software. Conditioning was assessed in terms of SCR, which was measured using a Biopac MP150 system and recorded with AcqKnowledge software (BIOPAC Systems, Goleta, CA, USA). The SCRs were acquired from the middle phalanges of the second and third fingers on the left hand using two Ag-AgCl electrodes. AcqKnowledge software was used to analyze SCR waveforms. The level of the SCR response was assessed as the base-to-peak difference in the 0.5-4.5 s window following the onset of a CS (i.e., the blue or yellow square). The SCRs for each participant were converted to standardized T scores and averaged per participant, per condition.

**Statistical Analysis**

For the acquisition and extinction phases, analyses of variance (ANOVAs) were used to compare responding in the first half of the session with responding in the second half. Follow-up *t*-tests were conducted to assess fear acquisition or extinction by comparing responses to the CS+ with responses to the CS- during the second half of the acquisition session. Only subjects that showed successful levels of fear acquisition and extinction were included in the analysis. Therefore, analysis of the acquisition and extinction phases is presented only to verify that there were no differences between the groups before test. Four participants that failed to show evidence of a conditioned response during acquisition (i.e., no more than two SCRs to CS trials were higher than 0.05) and three participants that failed to show evident extinction (i.e., no more than two SCRs to CS trials were lower than 0.10) were excluded. The exclusion of these seven participants did not change our results.

**Rat Experiments**

**Subjects**

Male Sprague-Dawley rats, weighing 220-240 g upon arrival, were obtained from the Laboratory Animal Center, Peking University Health Science Center. They were housed in groups of five in a temperature (23 ± 2°C) and humidity (50 ± 5%) controlled animal facility and maintained on a 12 h/12 h light/dark cycle with free access to food and water.
experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Behavioral Apparatus

All of the procedures were conducted in conditioning chambers, the walls of which were constructed of black polyvinyl chloride. The floor was constructed of stainless steel rods (0.5 cm diameter, 1.0 cm apart) that were used to deliver foot shocks (Shanghai Jiliang Software Technology Co. Ltd, Shanghai, China) (3). The conditioning chamber was enclosed in an acoustic isolation box. A daylight diotron on the training chamber illuminated the chamber during the procedures. The US was an electric foot shock delivered through the stainless steel rods. Behavior was recorded using a video camera mounted within the acoustic isolation box.

Fear Conditioning

In Experiments 7, 8, and 10, the rats were handled for 3 days. On the day of the experiments, they were placed in the conditioning chamber (CS) and allowed to explore it for 2 min, after which they received a 1 s, 1.0 mA electric foot shock (US). The rats received two additional identical foot shocks with an interval of 2 min between each shock. After the last shock, the animals were allowed to explore the chamber for an additional 1 min and then removed from the conditioning chamber. In Experiments 9-11, the two distinct contextual CSs were paired with the same US using the same procedure. After conditioning in the aforementioned chamber (CS1), they were immediately placed in another conditioning chamber with distinct lighting, odor cues, and visual stimuli on the walls (CS2) and conditioned using the same procedure. The rats were then removed and returned to the home cage. For the immediate shock training (Experiment 10), rats were placed in the conditioning chambers and administrated three foot shocks (1 s, 1.0 mA) 5 s later. The rats remained in the chambers for a further 60 s before being removed to home cage (4).

Memory Reactivation

Memory reactivation through CS or US exposure was performed 24 h after fear
conditioning in Experiments 7-10 or 2 weeks after fear conditioning in Experiment 11. CS reactivation was performed by re-exposing the rats to the conditioning context (Experiments 8-11) or one of the two conditioning contexts (Experiments 9-11) without foot shock for 3 min. US reactivation was performed by exposing the rats to a weak foot shock (1 s, 0.3 mA; Experiments 7-11) or a strong foot shock (1 s, 1.0 mA; Experiment 7) in a novel context.

**Extinction**

The rats were exposed to the conditioning chamber for 30 min without receiving any foot shocks. For the procedure with two CSs paired with the same US (Experiments 9 and 10), extinction occurred in only one context (either of the two contexts in a counterbalanced design). Freezing behavior during extinction was measured continuously and separated into 5 min bins for analysis. Extinction long-term memory (LTM) was tested 24 h after extinction training in a 5 min test in the conditioning chamber.

**Reinstatement**

Immediately after the extinction LTM test, the rats received one foot shock (1 s, 1.0 mA) and were then returned to their home cage. Twenty-four hours later, they were placed in the conditioning chamber and tested for fear reinstatement in a 5 min test.

**Spontaneous Recovery Test**

One month after the extinction LTM test, the rats were placed in the conditioning chambers, and fear recovery was assessed in a 5 min test.

**Freezing Behavior**

Freezing behavior was video-recorded during the CS reactivation, extinction, and test sessions for offline analysis using JLbehv-LAG-4 software (Shanghai Jiliang Software Technology Co. Ltd, Shanghai, China).

**Western Blot**

In Experiment 10, the western blot method was based on our previous studies (5; 6). After decapitation, the rats’ brains were rapidly extracted and frozen in -60°C N-hexane and then transferred to a -80°C freezer. Bilateral tissue punches (16 gauge) of the dorsal hippocampus were placed in a 1.5 ml microtube that contained ice-cold homogenization
buffer (0.32 M sucrose, 4 mM HEPES, 1 mM EDTA, 1 mM EGTA, and protease/phosphatase inhibitors cocktail, pH 7.4). After homogenization by an electrical disperser (Wiggenhauser, Sdn Bhd), the homogenate was centrifuged at 1000 × g for 10 min at 4°C to obtain the pellet (P1) that contained nuclei and large debris. The supernatant (S1) was again centrifuged at 10,000 × g for 30 min at 4°C to generate a crude synaptosomal fraction (P2) and supernatant (S2; the cytosolic fraction). The crude synaptosomal membrane pellet (P2) was lysed hypo-osmotically and centrifuged at 25,000 × g for 30 min at 4°C to generate the synaptosomal membrane fraction (LP1). The S2 and LP1 were separately resuspended in HEPES-lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, and protease/phosphatase inhibitors cocktail, pH 7.4). The protein concentrations of all of the samples (S2 and LP1) were determined using the bicinchoninic acid assay (Beyotime Biotechnology, Jiangsu, China). The samples were further diluted in HEPES-lysis buffer to equalize the protein concentrations. Loading buffer (4×; 16% glycerol, 20% mercaptoethanol, 2% sodium dodecyl sulfate [SDS], and 0.05% bromophenol blue) was added to each sample (3:1, sample:loading buffer) before boiling for 3 min. The samples were cooled and subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide/0.27% N,N’-methylene-bisacrylamide resolving gel) for approximately 40 min at 80 V in stacking gel and approximately 1 h at 120 V in resolving gel. The proteins were electrophoretically transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) at 250 mA for 2 h. The membranes were washed with TBST (Tris-buffered saline plus 0.05% Tween-20, pH 7.4) and then dipped in blocking buffer (5% bovine serum albumin [BSA] in TBST) overnight at 4°C. The next day, the membranes were incubated for 1 h at room temperature on an orbital shaker with anti-GluR1 antibody, anti-GluR2 antibody, anti-pPKA antibody, anti-PKA antibody, anti-pCREB antibody, anti-CREB antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pErk1/2 antibody, anti-Erk1/2 antibody, anti-pP70s6k antibody, anti-P70s6k antibody, anti-BDNF antibody (1:1000; Cell Signaling Technology, Boston, MA, USA), and β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST plus 5% BSA.
After three 5 min washes in TBST buffer, the blots were incubated for 45 min at room temperature on a shaker with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG for β-actin and GluR1 and goat anti-rabbit IgG for the others; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:5000 in blocking buffer. The blots were then washed three times for 5 min each in TBST and incubated with a layer of Super Signal Enhanced chemiluminescence substrate (Detection Reagents 1 and 2, 1:1 ratio; Applygen Technologies, Beijing, China). Excess mixture was dripped off before the blots were wrapped with a clean piece of plastic wrap (no bubbles between blot and wrap), and the blots were then exposed to X-ray film (Eastman Kodak Company) for 5-60 s. Band intensities were quantified using Quantity One software (version 4.4.0, Bio-Rad, Hercules, CA, USA).

**Specific Experiments**

**Experiment 7: To investigate the appropriate US intensity to reactivate fear memory in rats**

Three groups of rats (n = 8 per group) were used to investigate the role of the relative US intensity used to reactivate fear memory before extinction in the US retrieval-extinction procedure. One day after fear conditioning, the rats were divided into three groups: 1) Group 1, rats received extinction training without a US reminder (No US), 2) Group 2, rats were given a weak US (1 s, 0.3 mA) 10 min before extinction training (Weak US), and 3) Group 3, rats were given a strong US (1 s, 1.0 mA) 10 min before extinction training (Strong US). Freezing behavior in extinction was measured continuously and separated into 5 min bins for analysis.

**Experiment 8: To investigate the effects of extinction within the US-triggered reconsolidation window on fear reinstatement and spontaneous recovery in rats**

Five groups of rats (n = 6-7 per group) were used in the reinstatement experiment to investigate the effects of CS- or US-triggered reconsolidation-extinction on fear memory. One day after fear conditioning, the rats were divided into five groups: 1) Group 1, rats received extinction training without a memory reminder (NoR), 2) Group 2, rats were given a CS reminder 10 min before extinction training (CS-10 min), 3) Group 3, rats were given a CS reminder 24 h before extinction training (CS-24 h), 4) Group 4, rats were given a US reminder
10 min before extinction training (US-10 min), and 5) Group 5, rats were given a US reminder 24 h before extinction training (US-24 h). The intensity of all US reminders was weaker than the one used during conditioning (1 s, 0.3 mA). Twenty-four hours later, all of the groups were assessed for the retention of extinction (Extinction LTM test). Immediately after the test, the rats were given one foot shock (1 s, 1.0 mA) in the conditioning chamber. Memory was tested again 24 h later (Reinstatement test).

For the spontaneous recovery experiment, the conditioning, reconsolidation-extinction, and extinction LTM procedures were the same as in the reinstatement experiment (five groups, n = 10-12 per group). No reinstatement shock was administered after the extinction LTM test. One month later, the rats were tested for fear recovery.

Experiment 9: To investigate whether interfering with the reconsolidation of one fear-predictive cue affects the fate of another associated cue using this US retrieval-extinction procedure in rats

Rats were divided into eight groups to investigate the effect of CS extinction training within either CS- or US-triggered reconsolidation on the reinstatement and spontaneous recovery of fear associated with two CSs. For the reinstatement experiment, the rats were conditioned to two distinct contexts (CS1 and CS2) on the conditioning day. The next day, the rats were exposed to one of the two CSs in a counterbalanced design or the US to reactivate the conditioning memory. They then underwent extinction (i.e., exposure to the reactivated context for 30 min). The rats were divided into four groups (n = 9-12 per group): 1) Group 1, rats received extinction training for CS1 without a memory reminder (NoR), 2) Group 2, rats were given a CS1 reminder 10 min before extinction training for CS1 (CS-10 min), 3) Group 3, rats were given a US reminder 10 min before extinction training for CS1 (US-10 min), and 4) Group 4, rats were given a US reminder 24 h before extinction training for CS1 (US-24 h). All of the US reminders had a weaker intensity than the one used during conditioning (1 s, 0.3 mA). The fear responses to CS1 and CS2 were tested the next day (Extinction LTM test). Immediately after the test, the rats were given one foot shock (1 s, 1.0 mA) in the extinction chamber. Twenty-four hours later, the fear responses to CS1 and CS2 were tested again.
For the spontaneous recovery experiment (four groups, \( n = 10-12 \) per group), the conditioning, reconsolidation-extinction, and extinction LTM procedures were the same as in the reinstatement experiment. No reinstatement shock was administered after the extinction LTM test. One month later, the rats were tested for spontaneous recovery of the fear responses to CS1 and CS2.

**Experiment 10: To investigate the effect of US retrieval on alterations of plasticity-related proteins (PRPs) in the dorsal hippocampus in fear-conditioned rats**

Some PRPs, such as GluR1, GluR2, PKA, ERK and p70s6k, have been identified to mediate the CS-triggered reconsolidation (7-10). Thus to determine the possible mechanisms of US- and CS-triggered fear reconsolidation, we evaluated these PRPs alterations in the dorsal hippocampus after US or CS retrieval in rats. The rats were divided into 10 groups (\( n = 6 \) per group) to investigate the PRPs alterations induced by the CS- or US-triggered fear reactivation. On the first day, the rats were exposed to one context (no conditioning), conditioned with one context (one-CS conditioning), or conditioned with two contexts (two-CS conditioning) separately. Long-term memory was tested 24 h later. The day after the LTM test, the rats were grouped as described below. Rats that were not subjected to conditioning were divided into three groups: 1) Group 1, rats received no CS or US reactivation (NoR), 2) Group 2, rats received CS reactivation (CSR), and 3) Group 3, rats received US reactivation (USR).

Rats that were subjected to one-CS conditioning were divided into three groups: 4) Group 4, rats received no CS or US reactivation (NoR), 5) Group 5, rats received CS reactivation (CSR), and 6) Group 6, rats received US reactivation (USR). Rats that were subjected to two-CS conditioning were divided into four groups: 7) Group 7, rats received no CS or US reactivation (NoR), 8) Group 8, rats received CS1 reactivation (CS1R), 9) Group 9, rats received CS2 reactivation (CS2R), and 10) Group 10, rats received US reactivation (USR).

All of the rats were decapitated 10 min after CS or US reactivation for Western blot analysis.

To confirmed the PRPs alterations are restricted within the reconsolidation time window, three groups of rats were included to compare the molecular levels 10 min and 24 h after US
reactivation \( (n = 6 \text{ per group}) \): 1) Group 1, rats received no CS or US reactivation (NoR), 2) Group 2, rats received US reactivation and were decapitated 10 min later (USR-10 min), and 3) Group 3, rats received US reactivation 24 h later (USR-24 h).

To ensure that these PRPs alterations were caused by the US-induced memory retrieval rather than the aversiveness of the US, rats were administrated three foot shocks (1 s, 1.0 mA) immediately after being placed in the training chambers in a control experiment. Three groups of rats were included \( (n = 6 \text{ per group}) \): 1) Group 1, rats underwent one-CS training and received no CS or US reactivation (Training-NoR), 2) Group 2, rats underwent one-CS training and received no US reactivation (Training-USR), and 3) Group 3, rats received immediate foot shocks and received US reactivation (Immediate shock-USR). All of the rats were decapitated 10 min after CS or US reactivation.

Experiment 11: To investigate the effects of the US retrieval-extinction procedure on the reinstatement and spontaneous recovery of remote fear memory in rats

Rats were divided into six groups to investigate the effect of CS extinction training within either a CS- or US-triggered reconsolidation time window on the reinstatement and spontaneous recovery of remote fear associated with two CSs. For the reinstatement experiment, the rats were conditioned with two distinct contexts (CS1 and CS2) on the conditioning day. After 2 weeks, the rats were exposed to one of the two CSs in a counterbalanced design or a weaker US reminder (1 s, 0.3 mA) to reactivate the conditioning memory. They then underwent extinction (i.e., exposure to the reactivated context for 30 min). The rats were divided into three groups \( (n = 8-9 \text{ per group}) \): 1) Group 1, rats received extinction training for CS1 without a memory reminder (NoR), 2) Group 2, rats were given a CS1 reminder 10 min before extinction training for CS1 (CS-10 min), and 3) Group 3, rats were given a US reminder 10 min before extinction training for CS1 (US-10 min). The fear responses to the CS1 and CS2 were tested the next day (Extinction LTM test). Immediately after the test, the rats were given one foot shock (1 s, 1.0 mA) in the extinction chamber. Twenty-four hours later, the fear responses to the CS1 and CS2 were tested again (Reinstatement test).
For the spontaneous recovery experiment (three groups, \( n = 9-10 \) per group), the conditioning, reconsolidation-extinction, and extinction LTM procedures were the same as in the reinstatement experiment. No reinstatement shock was administered after the extinction LTM test. One month later, the rats were tested for the spontaneous recovery of fear responses to the CS1 and CS2.

**Statistical Analysis**

The data are expressed as mean ± SEM. The statistical analysis was performed using repeated-measures ANOVA for Experiments 7, 8, 9, and 11, and one-way ANOVA for Experiment 10. For the extinction phase, repeated-measures ANOVAs were used to compare responding across the 5 min bins. One-way ANOVAs were used for the extinction LTM test to ensure that fear was the same in all groups before the reinstatement/spontaneous recovery manipulation. Repeated-measures ANOVAs were then used to compare responding in the extinction LTM test and reinstatement or spontaneous recovery test to determine the permanence of extinction. A significant effect was followed by post hoc least significant difference tests, to compare each group with the control group. Follow-up \( t \)-tests were conducted to test for return of fear. The between-subjects and within-subjects factors are stated in the results. Values of \( p < .05 \) were considered statistically significant.

**Supplemental Results**

**Extinction 10 Min after Strong US Exposure Did Not Extinguish Fear in Rats (Figure S1)**

Three groups of rats were used to investigate US intensity: No US, Weak US, and Strong US. A repeated-measures ANOVA, with the between-subjects factor US intensity and within-subjects factor Test (first bin and last bin of extinction), revealed significant main effects of Test \( [F_{(1,21)} = 32.85, p < .05] \) and Group \( [F_{(2,21)} = 10.66, p < .05] \) and a significant Test × Group interaction \( [F_{(2,21)} = 6.05, p < .05] \). The post hoc analysis showed that fear responses
were extinguished in the No US and Weak US groups (all \( p < .05 \)) but not Strong US group (\( p > .05 \)). These results indicate that extinction training after strong US reactivation did not extinguish the fear response in rats.

**Extinction 10 Min after US Exposure Prevented Spontaneous Recovery and Reinstatement of Extinguished Fear in Rats (Figure S2)**

Five groups of rats were used to investigate reinstatement: NoR, US-10 min, US-24 h, CS-10 min, and CS-24 h. The repeated-measures ANOVA, with the between-subjects factor Group and within-subjects factor Time Bin (six 5 min bins), demonstrated that the groups exhibited similar extinction, with a significant effect of Time Bin \([F_{(1,25)} = 48.02, p < .05]\) but no effect of Group and no Group \(\times\) Time Bin interaction (all \( p > .05 \)). The analysis of the extinction LTM test using a one-way ANOVA, with Group as the between-subjects factor, revealed no significant differences between groups (\( p > .05 \)). A reinstatement test was conducted 24 h after reinstatement shocks were administered in the conditioning chamber. The repeated-measures ANOVA, with Group as the between-subjects factor and Test (extinction LTM test and reinstatement test) as the within-subjects factor, showed significant main effects of Test \([F_{(1,25)} = 38.00, p < .05]\) and Group \([F_{(4,25)} = 12.12, p < .05]\) and a significant Test \(\times\) Group interaction \([F_{(4,25)} = 3.13, p < .05]\). Follow-up \( t \) tests compared the fear response between the extinction LTM and reinstatement tests. Reinstatement was found in the NoR, US-24 h, and CS-24 h groups (all \( p < .05 \)) but not in the US-10 min or CS-10 min group (all \( p > .05 \)). These results indicate that extinction training during the US- or CS-triggered reconsolidation blocked the reinstatement of fear memory in rats.

For the rats in the spontaneous recovery test, the analyses used for the extinction and extinction LTM tests were the same as in the reinstatement analysis. The analysis demonstrated that the groups exhibited similar extinction, with a significant effect of Time Bin \([F_{(1,49)} = 223.66, p < .05]\) but no effect of Group and no Group \(\times\) Time Bin interaction (all \( p > .05 \)). The analysis of the extinction LTM test using a one-way ANOVA, with Group as the
between-subjects factor, revealed no significant differences between groups ($p > .05$). The repeated-measures ANOVA, with Group as the between-subjects factor and Test (extinction LTM test and spontaneous recovery test) as the within-subjects factor, revealed significant main effects of Test ($F_{(1,49)} = 67.55, p < .05$) and Group ($F_{(4,49)} = 6.04, p < .05$) and a significant Test $\times$ Group interaction ($F_{(4,49)} = 5.06, p < .05$). Follow-up t tests showed that spontaneous recovery occurred in the NoR, US-24 h, and CS-24 h groups (all $p < .05$) but not in the US-10 min or CS-10 min group (all $p > .05$). These results indicate that extinction during the US- or CS-triggered reconsolidation time period blocked the spontaneous recovery of fear memory in rats. Altogether, the reinstatement test and spontaneous recovery results suggest that US-triggered reconsolidation-extinction permanently disrupted fear memory in rats, similar to CS-triggered reconsolidation-extinction.

**Either CS1 or CS2 Extinction During the US-triggered Reconsolidation Time Period Permanently Disrupted Fear Conditioning for Both the CS1 and CS2 in Rats (Figure S3)**

This experiment investigated the effect of extinction of either of the CSs during the US-triggered reconsolidation time period on the fear response to all CSs. Reinstatement and spontaneous recovery were independently measured, with four groups for each measurement (NoR, US-10 min, US-24 h, and CS-10 min).

For the rats used in the reinstatement test, repeated-measures ANOVA, with the between-subjects factor Group and within-subjects factor Time Bin (six 5 min bins), revealed that the groups exhibited similar extinction, with a significant effect of Time Bin ($F_{(1,37)} = 211.26, p < .05$) but no effect of Group and no Group $\times$ Time Bin interaction (all $p > .05$). The CS1-CS2 extinction LTM tests were analyzed using repeated-measures ANOVA, with Group as the between-subjects factor and Test (CS1 extinction LTM and CS2 extinction LTM) as the within-subjects factor. This analysis revealed significant main effects of Group ($F_{(3,37)} = 6.61, p < .05$) and Test ($F_{(1,37)} = 78.05, p < .05$) and a significant Group $\times$ Test interaction ($F_{(3,37)} = 4.41, p < .05$). The post hoc analysis revealed a significant difference between the US-10 min...
group and other groups (all \( p < .05 \)). Follow-up \( t \) tests showed no significant difference in responding to the CS1 and CS2 was found in the US-10 min group (\( p > .05 \)). The reinstatement test was analyzed using repeated-measures ANOVA for the CS1 and CS2 separately, with Group as the between-subjects factor and Test (extinction LTM test vs. reinstatement test) as the within-subjects factor. In the CS1 reinstatement test, the analysis revealed significant main effects of Test \( [F_{(1,37)} = 65.72, p < .05] \) and Group \( [F_{(3,37)} = 6.31, p < .05] \) and a significant Group \( \times \) Test interaction \( [F_{(3,37)} = 7.84, p < .05] \). Follow-up \( t \) tests showed that reinstatement was found in the NoR and US-24 h groups (all \( p < .05 \)) but not in the CS-10 min or US-10 min group (all \( p > .05 \)). For the CS2, the pattern of results in the reinstatement test matched the results after CS1 was extinguished, suggesting that the extinction of CS1 in the US-triggered reconsolidation time window caused a permanent decrease in responding to CS2, and the other groups exhibited no decreased responding to be reinstated. The analysis revealed a significant effect of Group \( [F_{(3,37)} = 13.35, p < .05] \) but no significant effect of Test and no Group \( \times \) Test interaction (all \( p > .05 \)). These results indicate that extinction of either of the two CSs in the US-triggered reconsolidation time period decreased responses to both CSs and blocked reinstatement in response to both cues.

For the rats used in the spontaneous recovery test, the analysis of extinction and the extinction LTM test was the same as in the reinstatement test. The analysis of extinction responding revealed that the groups exhibited similar extinction, with a significant effect of Time Bin \( [F_{(1,40)} = 327.58, p < .05] \) but no effect of Group and no Group \( \times \) Time Bin interaction (all \( p > .05 \)). The analysis of the CS1-CS2 extinction LTM tests revealed significant main effects of Group \( [F_{(3,40)} = 3.89, p < .05] \) and Test \( [F_{(1,40)} = 54.32, p < .05] \) and a significant Group \( \times \) Test interaction \( [F_{(3,40)} = 4.60, p < .05] \). The post hoc analysis revealed a significant difference between the US+10 min group and other groups (all \( p < .05 \)). Follow-up \( t \) tests showed no significant difference in responding to CS1 and CS2 was found in the US+10 min group (\( p > .05 \)). The spontaneous recovery tests were analyzed using repeated-measures
ANOVA for the CS1 and CS2 separately. For the CS1 spontaneous recovery test, the analysis revealed a significant main of Test \( F_{(1,40)} = 13.92, p < .05 \) and a significant Group × Test interaction \( F_{(3,40)} = 3.01, p < .05 \). Follow-up t tests showed significant spontaneous recovery in the NoR and US-24 h groups (all \( p < .05 \)) but not in the US-10 min or CS-10 min group (all \( p > .05 \)). For CS2, the pattern of results in the spontaneous recovery test matched the results after CS1 was extinguished, suggesting that the extinction of CS1 in the US-triggered reconsolidation time window caused a permanent decrease in responding to CS2, and the other groups had no decreased responding to spontaneously recover. The analysis revealed a significant effect of Group \( F_{(3,40)} = 17.57, p < .05 \) but no effect of Test and no Group × Test interaction (all \( p > .05 \)). These results indicate that extinction of either of the two CSs in the US-triggered reconsolidation time period decreased responses to both CSs and blocked spontaneous recovery in response to both cues.

Altogether, the reinstatement test and spontaneous recovery test results suggest that the US reconsolidation-extinction procedure disrupted the memories conditioned with that US in rats.

**Effect of US Reactivation on Molecular Activation in the Dorsal Hippocampus in Fear-Conditioned Rats (Figure S4)**

Molecular alterations, including membrane GluR1 and GluR2 and cytosolic pPKA, pErK1/2, p70s6K, pCREB, and BDNF levels, in the dorsal hippocampus were evaluated in rats. Three groups of rats conditioned with no CS were used: No reactivation (NoR), CS reactivation (CSR), and US reactivation (USR). The one-way ANOVA revealed no significant effects on any of the molecular parameters (all \( p > .05 \)). Three groups of rats conditioned with one CS were used: No reactivation (NoR), CS reactivation (CSR), and US reactivation (USR). The one-way ANOVA revealed significant effects on GluR1 \( F_{(2,17)} = 9.33, p < .05 \), GluR2 \( F_{(2,17)} = 12.53, p < .05 \), pPKA \( F_{(2,17)} = 18.99, p < .05 \), pP70s6k \( F_{(2,17)} = 12.54, p < .05 \), and pCREB \( F_{(2,17)} = 20.30, p < .05 \) but not pErK1/2 \( F_{(2,17)} = 0.20, p > .05 \) or BDNF \( F_{(2,17)} = 0.22, \)
The post hoc analysis indicated that CS reactivation significantly reduced membrane GluR1 and GluR2 expression and increased cytosolic pPKA, p70s6K, and pCREB levels, which were augmented by US reactivation (all $p < .05$). Four groups of rats conditioned with two CSs were used: No reactivation (NoR), CS1 reactivation (CS1R), CS2 reactivation (CS2R), and US reactivation (USR). The one-way ANOVA revealed significant effects on GluR1 ($F_{(3,23)} = 14.74, p < .05$), GluR2 ($F_{(3,23)} = 8.69, p < .05$), pPKA ($F_{(3,23)} = 10.72, p < .05$), pP70s6k ($F_{(3,23)} = 10.35, p < .05$), and pCREB ($F_{(3,23)} = 11.53, p < .05$) but not pErK1/2 ($F_{(3,23)} = 0.01, p > .05$) or BDNF ($F_{(3,23)} = 0.33, p > .05$). The post hoc analysis indicated that both CS1 and CS2 reactivation significantly reduced membrane GluR1 and GluR2 expression and increased cytosolic pPKA, p70s6K, and pCREB levels, which were augmented by US reactivation (all $p < .05$).

We then tested whether these molecular alterations induced by US reactivation would persist to 24 h after US reactivation, when the extinction can not disrupt fear memory anymore. Three groups of rats conditioned with one CS were used: No reactivation (NoR), US reactivation-10 min (USR-10 min), and US reactivation-24 h (USR-24 h). The one-way ANOVA revealed significant effects on GluR1 ($F_{(2,17)} = 6.67, p < .05$), GluR2 ($F_{(2,17)} = 5.28, p < .05$), pPKA ($F_{(2,17)} = 12.95, p < .05$), pP70s6k ($F_{(2,17)} = 4.37, p < .05$), and pCREB ($F_{(2,17)} = 3.98, p < .05$). The post hoc analysis indicated that the levels of GluR1, GluR2, pPKA, p70s6K, and pCREB altered by US reactivation recovered to the same levels in the NoR group of rats 24 h later (all $p > .05$). These results suggest that the PRPs alterations induced by US reactivation may be involved in the effect of US-retrieval extinction on fear memory.

To confirm these molecular alterations induced by US reactivation were not due to the aversiveness of the US, three groups of rats were included: One-CS training and No retrieval (Training-NoR), One-CS training and US retrieval (Training-USR), and Immediate shock exposure and US retrieval (Immediate shock-USR). The one-way ANOVA revealed significant effects on GluR1 ($F_{(2,17)} = 4.16, p < .05$), GluR2 ($F_{(2,17)} = 3.89, p < .05$), pPKA ($F_{(2,17)} = 4.55, p < .05$), pP70s6k ($F_{(2,17)} = 4.84, p < .05$), and pCREB ($F_{(2,17)} = 4.75, p < .05$). The post hoc analysis indicated that the levels of GluR1, GluR2, pPKA, p70s6K, and pCREB were not
altered in the immediate shock-USR group of rats compared with the training-NoR group of rats (all $p > .05$).

Altogether, these results indicate that the PRPs alteration in the dorsal hippocampal induced by US reactivation was stronger than CS reactivation in fear-conditioned rats, which may underlie the molecular mechanism of the US reconsolidation-extinction procedure.

**Extinction in the US-Triggered Reconsolidation Time Period Permanently Disrupted Remote Fear Memory in Rats (Figure S5)**

This experiment investigated the effect of extinction in the US-triggered reconsolidation time period on fear responses 2 weeks after fear conditioning. Reinstatement and spontaneous recovery were independently measured, with three groups for each measurement (NoR, US-10 min, and CS-10 min).

For the rats used in the reinstatement test, repeated-measures ANOVA, with the between-subjects factor Group and within-subjects factor Time Bin (six 5 min bins), revealed that the groups exhibited similar extinction, with a significant effect of Time Bin [$F_{(1,23)} = 80.00$, $p < .05$] but no effect of Group and no Group $\times$ Time Bin interaction (all $p > .05$). The CS1-CS2 extinction LTM tests were analyzed using repeated-measures ANOVA, with Group as the between-subjects factor and Test (CS1 extinction LTM and CS2 extinction LTM) as the within-subjects factor. The analysis revealed significant main effects of Group [$F_{(2,23)} = 3.90$, $p < .05$] and Test [$F_{(1,23)} = 21.02$, $p < .05$] and a significant Group $\times$ Test interaction [$F_{(2,23)} = 3.55$, $p < .05$]. The post hoc analysis revealed a significant difference between the US-10 min group and other groups (all $p < .05$). Follow-up t tests showed no significant difference was found in responding to CS1 and CS2 in the US-10 min group ($p > .05$). The reinstatement test was analyzed using repeated-measures ANOVA for the CS1 and CS2 separately, with Group as the between-subjects factor and Test (extinction LTM test vs. reinstatement test) as the within-subjects factor. In the CS1 reinstatement test, the analysis revealed significant main effects of Test [$F_{(1,23)} = 17.71$, $p < .05$] and Group [$F_{(2,23)} = 3.67$, $p < .05$] and a significant
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Group × Test interaction \([F_{(2,23)} = 4.53, p < .05]\). Follow-up \(t\) tests showed that reinstatement was found in the NoR and CS-10 min groups (all \(p < .05\)) but not in the US-10 min group \((p > .05)\). For CS2, the pattern of results in the reinstatement test matched the results after CS1 was extinguished, suggesting that the extinction of CS1 in the US-triggered reconsolidation time window caused a permanent decrease in responding to CS2, and the other groups had no decreased responding to be reinstated. The analysis revealed a significant effect of Group \([F_{(2,23)} = 8.28, p < .05]\) but no significant effect of Test or Group × Test interaction (all \(p > .05\)). These results indicate that US-extinction presented 2 weeks after fear conditioning decreased responding to both CSs and blocked reinstatement in response to both cues.

For the rats used in the spontaneous recovery test, the analysis of extinction and the extinction LTM test was the same as in the reinstatement test. The analysis of extinction responding revealed that the groups exhibited similar extinction, with a significant effect of Time Bin \([F_{(1,25)} = 39.58, p < .05]\) but no effect of Group and no Group × Time Bin interaction (all \(p > .05\)). The analysis of the CS1-CS2 extinction LTM tests revealed significant main effects of Group \([F_{(2,23)} = 3.83, p < .05]\) and Test \([F_{(1,25)} = 18.17, p < .05]\) and a significant Group × Test interaction \([F_{(2,23)} = 3.95, p < .05]\). The post hoc analysis revealed a significant difference between the US-10 min group and other groups (all \(p < .05\)). Follow-up \(t\) tests showed no significant difference was found in responding to CS1 and CS2 in the US-10 min group \((p > .05)\). The spontaneous recovery tests were analyzed using repeated-measures ANOVA for the CS1 and CS2 separately. For the CS1 spontaneous recovery test, the analysis revealed a significant main of Test \([F_{(1,25)} = 25.37, p < .05]\) and a significant Group × Test interaction \([F_{(2,25)} = 3.49, p < .05]\). Follow-up \(t\) tests indicated significant spontaneous recovery in the NoR and CS-10 min groups (all \(p < .05\)) but not in the US-10 min group \((p > .05)\). For CS2, the pattern of results in the spontaneous recovery test matched the results after CS1 was extinguished, suggesting that the extinction of CS1 in the US-triggered reconsolidation time window caused a permanent decrease in responding to CS2, and the
other groups had no decreased responding to spontaneously recovery. The analysis revealed a significant effect of Group \( F_{(2,25)} = 10.20, p < .05 \) but no effect of Test and no Group \( \times \) Test interaction (all \( p > .05 \)). These results indicate that US-extinction presented 2 weeks after fear conditioning decreased responding to both CSs and blocked spontaneous recovery in response to both cues.

Altogether, the reinstatement test and spontaneous recovery test results suggest that the US reconsolidation-extinction procedure disrupted remote fear memories conditioned with that US in rats.
## Table S1. Acquisition, retrieval, extinction, and test of human fear conditioning

<table>
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<th>Experiment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 2/3</th>
<th>Day 3/4</th>
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<td></td>
</tr>
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<td>10 CS+</td>
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</tr>
<tr>
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<td>10 CS-</td>
<td>5 CS-</td>
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<td>10 CS1+</td>
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<tr>
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<td>10 CS-</td>
<td>5 CS-</td>
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</tbody>
</table>

CS, conditioned stimulus; US, unconditioned stimulus.
Figure S1. Extinction 10 min after strong US exposure did not extinguish fear in rats. Rats underwent extinction training 10 min after one US presentation (Weak US: 1 s, 0.3 mA; Strong US: 1 s, 1.0 mA). The responses to the CS in the first bin of extinction in all of the groups were similar, but responding to the CS in the last bin of extinction was low in the No US group and Weak US group but not in the Strong US group. *p < .05, comparison between first bin of extinction and last bin of extinction. The data are expressed as mean ± SEM (n = 8 per group). CS, conditioned stimulus; US, unconditioned stimulus.
Figure S2. Extinction 10 min after US exposure prevented spontaneous recovery and reinstatement of extinguished fear in rats. (A) Experimental design and timeline. Rats underwent contextual fear conditioning (three 1 s, 1.0 mA shocks; 2 min intertrial interval). (B) Data from the reinstatement groups. (C) Data from the spontaneous recovery groups. Freezing was reinstated or spontaneously recovered in the NoR, US-24 h, and CS-24 h groups but not in the US-10 min or CS-10 min group. *p < .05, comparison between the extinction LTM test and reinstatement test or spontaneous recovery test (within-group). The data are expressed as mean ± SEM (n = 6-12 per group). NoR, no retrieval; CS, conditioned stimulus; US, unconditioned stimulus; LTM, long-term memory.
Figure S3. Either CS1 or CS2 extinction in the US-triggered reconsolidation time period permanently disrupted fear conditioning in response to both CS1 and CS2 in rats. (A) Experimental design and timeline. (B) Data from the reinstatement groups. (C) Data from the spontaneous recovery groups. Responding to CS1 was low for all groups in the extinction LTM test, but responding to CS2 was only low in the US-10 min group in the extinction LTM test. This pattern was maintained for CS2 in the reinstatement and spontaneous recovery tests, whereas reinstatement and the spontaneous recovery of responding to CS1 occurred in the NoR and US-24 h groups but not in the CS-10 min or US-10 min group. *p < .05, comparison between CS1 extinction LTM and CS2 extinction LTM; †p < .05, comparison between the extinction LTM test and reinstatement or spontaneous recovery test (within-group). The data are expressed as mean ± SEM (n = 9-12 per group). NoR, no retrieval; CS, conditioned stimulus; US, unconditioned stimulus; LTM, long-term memory.
**Figure S4.** US reactivation induced stronger PRPs alterations than CS reactivation in the dorsal hippocampus in fear-conditioned rats. (A) CS or US presentation did not alter PRPs expression in no-training rats (all $p > .05$). (B) CS reactivation decreased membrane GluR1 and GluR2 expression and increased cytosolic pPKA, pP70s6K, and pCREB levels but did not affect pErk1/2 or BDNF levels in the dorsal hippocampus in rats conditioned to one CS. The molecular alterations were augmented by US reactivation compared with the CSR group. (C) Both CS1 and CS2 reactivation decreased membrane GluR1 and GluR2 expression and increased cytosolic pPKA, pP70s6K, pCREB levels but did not alter pErk1/2 or BDNF levels in the dorsal hippocampus in rats conditioned to two CSs. The PRPs alterations were augmented by US reactivation compared with the CS1R and CS2R groups. (D) The PRPs alterations induced by US reactivation recovered to normal levels 24 h later. (E) US reactivation did not alter the related molecular levels in the rats that underwent immediate foot shock exposure. *$p < .05$, comparison with NoR group; # $p < .05$, comparison with CSR, CS1R, and CS2R groups. The data are expressed as mean ± SEM ($n = 6$ per group). NoR, no reactivation; CS, conditioned stimulus; CSR, CS reactivation; US, unconditioned stimulus; USR, unconditioned reactivation; LTM, long-term memory; PRP, plasticity-related proteins.
Figure S5. Extinction in the US-triggered reconsolidation time period permanently disrupted remote fear memory in rats. (A) Experimental design and timeline. (B) Data from the reinstatement groups. (C) Data from the spontaneous recovery groups. Responding to CS1 was low for all groups in the extinction LTM test, but responding to CS2 was only low in the US-10 min group in the extinction LTM test. This pattern was maintained for CS2 in the reinstatement and spontaneous recovery tests, whereas reinstatement and the spontaneous recovery of responding to CS1 occurred in the NoR and CS-10 min groups but not in the US-10 min group. #p < .05, comparison between CS1 extinction LTM and CS2 extinction LTM; *p < .05, comparison between the extinction LTM test and reinstatement or spontaneous recovery test (within-group). The data are expressed as mean ± SEM (n = 8-10 per group). NoR, no retrieval; CS, conditioned stimulus; US, unconditioned stimulus; LTM, long-term memory.
Supplemental References


