Delayed Noradrenergic Activation in the Dorsal Hippocampus Promotes the Long-Term Persistence of Extinguished Fear

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Fear extinction has been extensively studied, but little is known about the molecular processes that underlie the persistence of extinction long-term memory (LTM). We found that microinfusion of norepinephrine (NE) into the CA1 area of the dorsal hippocampus during the early phase (0 h) after extinction enhanced extinction LTM at 2 and 14 days after extinction. Intra-CA1 infusion of NE during the late phase (12 h) after extinction selectively promoted extinction LTM at 14 days after extinction that was blocked by the β -receptor antagonist propranolol, protein kinase A (PKA) inhibitor Rp-cAMPS, and protein synthesis inhibitors anisomycin and emetine. The phosphorylation levels of PKA, cyclic adenosine monophosphate response element-binding protein (CREB), GluRI, and the membrane GluRI level were increased by NE during the late phase after extinction that was also blocked by propranolol and Rp-cAMPS. These results suggest that the enhancement of extinction LTM persistence induced by NE requires the activation of the β -receptor/PKA/CREB signaling pathway and membrane GluRI trafficking. Moreover, extinction increased the phosphorylation levels of Erk1/2, CREB, and GluRI, and the membrane GluRI level during the late phase, and anisomycin/emetine alone disrupted the persistence of extinction LTM, indicating that the persistence of extinction LTM requires late-phase protein synthesis in the CA1. Propranolol and Rp-cAMPS did not completely disrupt the persistence of extinction LTM, suggesting that another β -receptor/PKA-independent mechanism underlies the persistence of extinction LTM. Altogether, our results showed that enhancing hippocampal noradrenergic activity during the late phase after extinction selectively promotes the persistence of extinction LTM.

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INTRODUCTION

A novel context (conditioned stimulus (CS)), after being paired with a fearful stimulus (unconditioned stimulus (US)), can alone elicit a fear response. Several hours after conditioning, this fear association is consolidated to become a long-term memory (LTM) (McGaugh, 2000; Mueller and Pilzecker, 1900; Squire and Davis, 1981). Consolidated memory can undergo extinction after repeat exposure to the CS (Herry *et al*, 2010; Lee *et al*, 2006; Pavlov, 1927). Extinction is suggested to be a new CS-no US memory but not the erasure of the original CS-US memory because the extinguished fear can be spontaneously

recovered, renewed, or reinstated (Maren *et al*, 2013; Quirk, 2002). Although extinction memory has been extensively studied, little is known about the cellular and molecular processes that underlie the persistence of extinction LTM.

The hippocampus is one of the most important brain areas that participate in extinction memory (Herry *et al*, 2010), and plays an important role in integrating contextual information (Maren *et al*, 2013; Winocur and Gilbert, 1984). It has been suggested to encode contextual specificity to regulate extinction memory (Ji and Maren, 2007; Sotres-Bayon *et al*, 2012), whereas extinction memory might be encoded in the prefrontal cortex (Milad and Quirk, 2002; Morgan and LeDoux, 1999; Quirk, 2002; Quirk *et al*, 2006; Quirk *et al*, 2000). In the contextual fear memory, the hippocampus is involved in the acquisition, consolidation, and retrieval of contextual fear extinction (de Carvalho Myskiw *et al*, 2013; Kirtley and Thomas, 2010; Szapiro *et al*, 2003; Vianna *et al*, 2001).

The formation of LTM requires new protein synthesis (Bourtchouladze *et al*, 1998; Davis and Squire, 1984; Schafe *et al*, 1999). The inhibition of protein synthesis during the first few hours after learning results in memory loss 24 h

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after learning (Abel *et al*, 1997; Schafe and LeDoux, 2000). Recent evidence suggests that LTM may require other late-phase protein and mRNA synthesis (~ 12 –24 h after learning) to be persistently stored (Katche *et al*, 2013; Medina *et al*, 2008). Disruption of this late-phase process leads to memory loss at 7 days but not 2 days after learning (Bekinschtein *et al*, 2007; Katche *et al*, 2010). Because this late-phase process is similar to consolidation, it is referred to as late consolidation (Medina *et al*, 2008). Several molecules in the hippocampus, including brain-derived neurotrophic factor, extracellular-regulated protein kinases (ERKs), *c-fos*, and Zif268, are involved in late consolidation (Bekinschtein *et al*, 2007; Katche *et al*, 2010; Medina *et al*, 2008; Rossato *et al*, 2009). The persistence of LTM can be disrupted by inhibiting the aforementioned signaling pathways or by stress at 12 h after learning (Yang *et al*, 2013). The noradrenergic system in the hippocampus also contributes to the late consolidation of fear memory (Katche *et al*, 2010; Rossato *et al*, 2009). Norepinephrine (NE) enhances extinction memory that can be blocked by a β -receptor antagonist (Berlau and McGaugh, 2006; Mueller and Cahill, 2010; Mueller *et al*, 2008). The main downstream signaling pathway activated by NE/ β -receptor is the protein kinase A (PKA)/cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) pathway (Kandel, 2012; Seeds and Gilman, 1971; Vitolo *et al*, 2002; Zhang *et al*, 2013). Active PKA also increases the trafficking of AMPA receptor (AMPA) subunit GluR1 to regulate fear extinction (Matsuo *et al*, 2008).

The present study tested the effect of NE in the dorsal hippocampus during the late phase after extinction training on the extinguished fear memory. We then determined whether this effect requires new protein synthesis and whether the β -receptor/PKA/CREB signaling pathway and GluR1 are included in this process. Uncovering the mechanism of extinction LTM persistence will benefit exposure therapy for anxiety-related disorders.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats, weighing 220–240 g, were obtained from the Laboratory Animal Center, Peking University Health Science Center. They were housed in groups of five in a temperature-controlled ($23 \pm 2^\circ\text{C}$) and humidity-controlled ($50 \pm 5\%$) animal facility with free access to food and water. They were kept on a reverse 12 h/12 h light/dark cycle. All 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 Biomedical Ethics Committee for animal use and protection of Peking University.

Surgery

The rats (weighing 280–300 g when surgery began) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Guide cannulae (23 gauge; Plastics One, Roanoke, VA) were bilaterally implanted 1 mm above the CA1 area of the hippocampus (Shi *et al*, 2012; Zhu *et al*, 2012). The coordinates were the following: anterior/posterior, -4.30 mm;

medial/lateral, ± 2.0 mm; dorsal/ventral, -2.0 mm (Paxinos and Watson, 2005). The cannulae were anchored to the skull with stainless-steel screws and dental cement. A stainless-steel stylet blocker was inserted into each cannula to keep it patent and prevent infection (He *et al*, 2011; Li *et al*, 2010). The rats were allowed to recover for at least 1 week after surgery.

Intracranial Injections

The β -receptor blocker propranolol, protein synthesis inhibitors anisomycin and emetine, PKA inhibitor Rp-cAMPS, and NE were purchased from Sigma (St Louis, MO). Emetine was dissolved in 10% dimethylsulfoxide (DMSO), and the other drugs were dissolved in 0.9% saline. All drugs were freshly prepared before the experiments. The doses of the drugs (10 $\mu\text{g}/\mu\text{l}$ propranolol, 100 $\mu\text{g}/\mu\text{l}$ anisomycin, 25 $\mu\text{g}/\mu\text{l}$ emetine, 1.0 $\mu\text{g}/\mu\text{l}$ Rp-cAMPS, and 0.3 or 1.0 $\mu\text{g}/\mu\text{l}$ NE) were based on previous studies (LaLumiere *et al*, 2003; Lima *et al*, 2009; Moncada *et al*, 2011). The infusion volume of NE was 1.0 μl , and the infusion volume of the other drugs was 0.5 μl to minimize the total infusion volume (Katche *et al*, 2010). The drugs were infused bilaterally over 1 min, and the injection needle was kept in place for an additional 1 min to allow for drug diffusion (Li *et al*, 2008; Lu *et al*, 2005).

Contextual Fear Memory

Contextual fear conditioning was conducted in four identical isolated shock chambers (Beijing Macro Ambition S&T Development, Beijing, China). The contextual fear conditioning procedure was modified from previous studies (Lubin and Sweatt, 2007; Yang *et al*, 2013). The rats were handled for 3 days before conditioning. On the day of the experiments, they were placed into the conditioning chamber and allowed to explore the chamber for 2 min, after which they received an electric footshock (0.8 mA, 1 s). The 2 min/1 s procedure was repeated a total of three times, and the rats were allowed to explore the conditioning chamber for an additional 1 min. After removing the rat from the chamber, the chamber was cleaned with 75% alcohol to eliminate any residual odor. The day after the LTM test, the rats were exposed to the training chamber for 30 min (or 20 min for weak extinction in experiment 2) without any footshock for extinction. Extinction LTM tests 1 and 2 were separately conducted 2 days and 14 days after extinction. After the second extinction LTM test, one shock was given to reinstate extinguished fear (Haaker *et al*, 2013). The reinstatement test was conducted 24 h after the reinstatement footshock. All memory tests were conducted by exposing the rats to the conditioning chamber for 5 min without footshock. All experimental sessions were video-recorded for offline analysis. Freezing behavior was defined as the lack of all movement, with the exception of respiration.

Elevated Plus Maze

The elevated plus maze test was based on our previous studies (Suo *et al*, 2013). Briefly, each rat was first placed in the central zone of the elevated plus maze. The rat was

allowed to freely explore the maze for 5 min, and the entire test was conducted under dim light conditions. The number of entries into and time (in seconds) spent on the open arms were recorded by two independent observers who were blind to the animal groups and sat quietly 2.5 m from the maze.

Locomotor Activity Test

Locomotor activity was measured with an automated video tracking system (DigBehv-LM4, Shanghai Jiliang Software Technology, Shanghai, China) that was used in our previous studies (Sun *et al*, 2013). A monochrome video camera was mounted on top of each chamber. All chambers were connected to a computer that recorded locomotion. The video files (stored on the computer) were analyzed using DigBehv analysis software. Locomotor activity is expressed as the total distance traveled during the 5 min test.

Western Blot Assays

The western blot method was based on our previous studies (Ren *et al*, 2013; Wang *et al*, 2010; Xue *et al*, 2012). All rats were decapitated without anesthesia 1 h after the end of extinction. After decapitation, the brains were rapidly extracted and frozen in -60°C *N*-hexane. The brains were then transferred to a -80°C freezer. Bilateral tissue punches (16 gauge) of the CA1 area 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 being homogenized by an electrical disperser (Wiggenhauer, Sdn Bhd), the homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C to obtain the pellet (P1) that contained nuclei and large debris. The supernatant (S1) was again centrifuged at $10\,000 \times g$ for 30 min at 4°C to generate a crude synaptosomal fraction (P2) and supernatant (S2). The crude synaptosomal membrane pellet (P2) was lysed hypototomically and centrifuged at $25\,000 \times g$ for 30 min at 4°C to generate the synaptosomal membrane fraction (LP1). LP1 was resuspended in the 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 samples (S2 and LP1) were determined using the bicinchoninic acid assay (Beyotime Biotechnology, Jiangsu, China). The samples were further diluted in the HEPES-lysis buffer to equalize the protein concentrations. Protein sample loading buffer (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'*-methylenebisacrylamide resolving gel) for ~ 40 min at 80 V in stacking gel and for ~ 1 h at 120 V in resolving gel. The proteins were electrophoretically transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) at 250 mA for 2.5 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-pPKA

antibody, anti-PKA antibody, anti-Erk1/2 antibody, anti-pErk1/2 antibody, anti-pCREB antibody, anti-CREB antibody, anti-GluR1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-pGluR1 antibody (1:1000; Epitomics (an Abcam company), Burlingame, CA), or anti- β -actin antibody (1:1000; Santa Cruz Biotechnology) 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) 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 then the blots were 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).

Experimental Design

Experiment 1: effect of NE microinfused into the CA1 after extinction on extinction memory. To assess the effect of NE treatment in the CA1 of the dorsal hippocampus on extinction memory, at 1 day after the long-term contextual fear test, rats (12 groups, $n = 8$ –10 per group) were separated into three treatment doses for four time points (0, 6, 12, and 24 h) after extinction. Different groups of rats were microinfused with 0, 0.3, or $1.0\ \mu\text{g}/\mu\text{l}$ per side at different times after extinction, respectively. All rats then underwent following memory tests (Figure 1a).

Experiment 2: distinct effects of NE microinfused into the CA1 at 0 and 12 h after short-term extinction on extinction memory. To assess the differential roles of NE treatment in the CA1 area of the dorsal hippocampus at 0 and 12 h after extinction on extinction memory, short-term extinction training (only 20 min) at 2 days after fear conditioning was performed to obtain weak extinction memory. The $1.0\ \mu\text{g}/\mu\text{l}$ dose of NE was used in the subsequent experiments based on the results of experiment 1. Three groups of rats ($n = 8$ –10 per group) were used: (1) the rats underwent extinction without treatment (Control group), (2) the rats received NE ($1.0\ \mu\text{g}/\mu\text{l}$ per side) into the CA1 at 0 h after extinction (0 h NE group), and (3) the rats received NE ($1.0\ \mu\text{g}/\mu\text{l}$ per side) into the CA1 at 12 h after extinction (12 h NE group). All rats then underwent following memory tests (Figure 2a).

Experiment 3: effect of NE microinfused into the CA1 after extinction on anxiety-like behavior and locomotion. To determine whether NE microinfused into the CA1 after extinction can alter anxiety-like behavior or locomotor activity, the elevated plus maze test and locomotor activity test were performed. Two separate groups of rats ($n = 9$ –10 per group) were microinfused with saline ($1.0\ \mu\text{l}$ per side) or NE ($1.0\ \mu\text{g}/\mu\text{l}$ per side) into the CA1 at 12 h after extinction.

Figure 1 Norepinephrine in the CA1 during the early or late phase after extinction enhanced extinction memory. (a) Timeline for this experiment. Norepinephrine was microinfused into the CA1 subarea of the dorsal hippocampus at 0, 6, 12, and 24 h after extinction. Extinction memory was tested 2 and 14 days later. After the second extinction LTM test, a footshock (0.8 mA, 1 s) was given, and fear reinstatement was tested 1 day later. (b) Representative cannula placement in the CA1 of rat. (c) Norepinephrine at doses of 0.3 and 1.0 $\mu\text{g}/\mu\text{l}$ microinfused into the CA1 at 0 h after extinction enhanced the extinction LTM test 14 days after extinction (both $p < 0.05$), and NE at a dose of 1.0 $\mu\text{g}/\mu\text{l}$ inhibited the reinstatement test ($p < 0.05$). (d) Norepinephrine microinfused into the CA1 at 6 h after extinction did not affect the extinction LTM test or reinstatement test. (e) Norepinephrine at 1.0 $\mu\text{g}/\mu\text{l}$ microinfused into the CA1 at 12 h after extinction enhanced the extinction LTM test 14 days later and NE at a dose of 0.3 and 1.0 $\mu\text{g}/\mu\text{l}$ reduced the subsequent reinstatement test (all $p < 0.05$). (f) Norepinephrine microinfused into the CA1 at 24 h after extinction had no effect on the subsequent extinction LTM test or reinstatement test. The data are expressed as mean $\pm$ SEM ($n = 8-10$ per group). * $P < 0.05$, compared with 0 μg NE group. CFC, contextual fear conditioning; LTM, long-term memory; NE, norepinephrine.

The elevated plus maze test and locomotor activity test were performed 14 days after extinction, consistent with the second extinction LTM test in the other experiments.

Figure 2 Norepinephrine in the CA1 during the late phase after extinction selectively enhanced the persistence of extinction LTM. (a) Timeline for this experiment. Norepinephrine (1.0 $\mu\text{g}/\mu\text{l}$) was microinfused into the CA1 at 0 or 12 h after weak extinction training (only 20 min). Extinction LTM was tested 2 or 14 days later. After the extinction LTM test 2, a footshock (0.8 mA, 1 s) was given, and fear reinstatement was tested 1 day later. A separate group without any drug treatment was used as a control. (b) At 2 days after weak extinction, compared with No NE group, the 0 h NE ($p < 0.05$) but not 12 h NE ($p > 0.05$) group of rats showed reduced freezing time, and both the 0 h NE group and 12 h NE group exhibited lower fear in extinction LTM test 2 and reinstatement test (all $p < 0.05$). These results suggest that NE microinfused into the CA1 at 0 h after extinction enhanced the formation of extinction LTM, whereas NE treatment 12 h after extinction selectively promoted the persistence of extinction LTM. The data are expressed as mean $\pm$ SEM ($n = 8-10$ per group). * $P < 0.05$, compared with control group.

Experiment 4: effect of β -receptor inhibitor on NE-induced enhancement of extinction LTM persistence. To test whether the effect of NE microinfused into the CA1 on the persistence of extinction LTM requires β -receptor activation, the β -receptor antagonist propranolol or saline was infused 30 min before NE microinfusion. Four separate groups of rats were used ($n = 10-11$ per group): (1) the rats were microinfused with saline (0.5 μl per side) into the CA1 at 11.5 h after extinction and then with saline (1.0 μl per side) 30 min later (Saline + Saline group), (2) the rats were microinfused with saline (0.5 μl per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 $\mu\text{g}/\mu\text{l}$ per side) 30 min later (Saline + NE group), (3) the rats were microinfused with propranolol (10–11 $\mu\text{g}/0.5 \mu\text{l}$ per side) into the CA1 at 11.5 h after extinction and then with saline (1.0 μl per side) 30 min later (Propranolol + Saline group), and (4) the rats were microinfused with propranolol (1.0 $\mu\text{g}/0.5 \mu\text{l}$ per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 $\mu\text{g}/\mu\text{l}$ per side) 30 min later (Propranolol + NE group). All rats then underwent following memory tests (Figure 4a).

Experiment 5: effect of PKA inhibitor on NE-induced enhancement of extinction LTM persistence. This experiment assessed the effect of PKA inhibition on the enhanced extinction LTM persistence induced by NE. The

PKA inhibitor Rp-cAMPS or saline was microinfused into the CA1 area 30 min before NE microinfusion. Four separate groups of rats were used ($n = 8$ per group): (1) the rats were microinfused with saline (0.5 μ l per side) into the CA1 11.5 h after extinction and then with saline (1.0 μ l per side) 30 min later (Saline + Saline group), (2) the rats were microinfused with saline (0.5 μ l per side) into the CA1 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Saline + NE group), (3) the rats were microinfused with Rp-cAMPS (0.5 μ g/0.5 μ l per side) into the CA1 11.5 h after extinction and then with saline (1.0 μ l per side) 30 min later (Rp-cAMPS + Saline group), and (4) the rats were microinfused with Rp-cAMPS (0.5 μ g/0.5 μ l per side) into the CA1 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Rp-cAMPS + NE group). All rats then underwent following memory tests (Figure 5a).

Experiment 6: effect of protein synthesis inhibitor on NE-induced enhancement of extinction LTM persistence. This experiment tested whether the enhanced extinction LTM persistence induced by NE requires new protein synthesis. The protein synthesis inhibitor anisomycin was microinfused into the CA1 area 30 min before NE microinfusion. Four separate groups of rats were used ($n = 8$ –10 per group): (1) the rats were microinfused with saline (0.5 μ l per side) into the CA1 at 11.5 h after extinction and then with saline (1.0 μ l per side) 30 min later (Saline + Saline group), (2) the rats were microinfused with saline (0.5 μ l per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Saline + NE group), (3) the rats were microinfused with anisomycin (80 μ g/0.8 μ l per side) into the CA1 at 11.5 h after extinction and then with saline (1.0 μ l per side) 30 min later (Anisomycin + Saline group), and (4) the rats were microinfused with anisomycin (80 μ g/0.8 μ l per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Anisomycin + NE group). All rats then underwent following memory tests. The protein synthesis inhibitor emetine was microinfused into the CA1 area 30 min before NE microinfusion. Four separate groups of rats were used ($n = 10$ per group): DMSO + Saline group; DMSO + NE group; Emetine + Saline group; and Emetine + NE group (Figure 6a).

Experiment 7: effect of NE microinfused into the CA1 on the β -receptor/PKA signaling pathway. In this experiment, we tested whether NE microinfused into the CA1 area at 12 h after extinction alters the expression of pPKA, pErk1/2, pCREB, and pGluR1 (Ser 845, a site phosphorylated by PKA) and membrane expression of GluR1 in the CA1 area. Five groups of rats were used ($n = 8$ per group): (1) the rats were microinfused with saline (1.5 μ l per side) into the CA1 without extinction (No extinction, Saline + Saline group), (2) the rats were microinfused with saline (0.5 μ l per side) into the CA1 at 11.5 h after extinction and then with saline (1.0 μ l per side) 30 min later (Saline + Saline group), (3) the rats were microinfused with saline (0.5 μ l per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Saline + NE group), (4) the rats were microinfused with propranolol (1.0 μ g/0.5 μ l per side) into the CA1 at 11.5 h after extinction

and then with NE (1.0 μ g/ μ l per side) 30 min later (Propranolol + NE group), and (5) the rats were microinfused with Rp-cAMPS (0.5 μ g/0.5 μ l per side) into the CA1 at 11.5 h after extinction and then with NE (1.0 μ g/ μ l per side) 30 min later (Rp-cAMPS + NE group). The rats were decapitated 13 h after extinction (1 h after the NE infusion).

Statistical Analysis

The data are expressed as mean $\pm$ SEM. The statistical analyses were performed using SAS 9.2 software. One-way analysis of variance (ANOVA) was performed to analyze the elevated plus maze and western blot results. To analyze extinction LTM, repeated-measures ANOVA was performed, with Test (last trial of extinction, extinction LTM test 1, extinction LTM test 2, and reinstatement test) as the within-subjects factor (the between-subjects factors are stated in the Results section). The *post hoc* analyses of significant effects in the ANOVAs were performed using the Least Square Means test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

NE in the CA1 Area during the Early or Late Phase after Extinction Enhanced Extinction Memory

In experiment 1, we tested the effect of NE microinfused into the CA1 at different time points (0, 6, 12, and 24 h) after extinction on extinction memory. For each time point (0, 6, 12, and 24 h) after extinction, no significant effects of groups (0, 0.3, and 1.0 μ g NE) were observed in the LTM test, the first trial of extinction, and the last trial of extinction (all $p > 0.05$). For each time point (0, 6, 12, and 24 h) after extinction, the two-way repeated-measures ANOVA, with Test as the within-subjects factor and Dose (0, 0.3, and 1.0 μ g NE) as the between-subjects factor, was conducted to analyze the extinction LTM, separately.

For NE administration at 0 h after extinction, the ANOVA revealed significant effects of Test ($F_{3,14} = 32.96$, $p < 0.01$) and a Test $\times$ Dose interaction ($F_{6,14} = 2.66$, $p < 0.05$) but no effect of Dose ($F_{2,14} = 0.78$, $p = 0.53$). The *post hoc* analysis revealed that the 0.3 and 1.0 μ g NE groups of rats exhibited decreased freezing time in extinction LTM test 2 (0.3 μ g NE: $t = 2.90$, $p < 0.01$; 1.0 μ g NE: $t = 4.21$, $p < 0.01$), and the 1.0 μ g NE group of rats exhibited reduced freezing time in the reinstatement test (0.3 μ g NE: $t = 0.31$, $p = 0.76$; 1.0 μ g NE: $t = 3.49$, $p < 0.01$) compared with the 0 μ g NE group of rats. Moreover, the 0 μ g NE group of rats exhibited increased freezing time in extinction LTM test 2 (spontaneous recovery) compared with the last trial of extinction (0 μ g NE: $t = 3.60$, $p < 0.01$; 0.3 μ g NE: $t = 0.83$, $p = 0.41$; 1.0 μ g NE: $t = 0.56$, $p = 0.58$). However, all groups of rats exhibited increased freezing time in the reinstatement test compared with extinction LTM test 2 (0 μ g NE: $t = 2.33$, $p < 0.05$; 0.3 μ g NE: $t = 5.12$, $p < 0.01$; 1.0 μ g NE: $t = 3.20$, $p < 0.01$), indicating that the original memory was not erased in any of the groups of rats (Figure 1c).

For NE administration at 6 h after extinction, the ANOVA revealed a significant effect of Test ($F_{3,14} = 21.69$, $p < 0.01$) but no effect of Dose ($F_{2,14} = 1.14$, $p = 0.43$) and no Test $\times$ Dose interaction ($F_{6,14} = 0.39$, $p = 0.88$; Figure 1d).

For NE administration at 12 h after extinction, the ANOVA revealed a significant effect of Test ($F_{3,14} = 53.46$, $p < 0.01$) and a Test $\times$ Dose interaction ($F_{6,14} = 2.30$, $p < 0.05$) but no effect of Dose ($F_{2,14} = 1.15$, $p = 0.43$). The *post hoc* analysis revealed that the 1.0 μg NE group of rats exhibited reduced freezing time in extinction LTM test 2 (0.3 μg NE: $t = 0.80$, $p = 0.42$; 1.0 μg NE: $t = 3.77$, $p < 0.01$), and the 0.3 and 1.0 μg NE groups of rats exhibited reduced freezing time in the reinstatement test (0.3 μg NE: $t = 2.07$, $p < 0.05$; 1.0 μg NE: $t = 3.96$, $p < 0.01$) compared with the 0 μg NE group of rats. Only the fear response in the 1.0 μg NE group of rats did not spontaneously recover (0 μg NE: $t = 3.20$, $p < 0.01$; 0.3 μg NE: $t = 2.30$, $p < 0.05$; 1.0 μg NE: $t = 0.09$, $p = 0.93$). Moreover, the fear response was reinstated in all groups of rats (0 μg NE: $t = 5.22$, $p < 0.01$; 0.3 μg NE: $t = 3.96$, $p < 0.01$; 1.0 μg NE: $t = 5.03$, $p < 0.01$; Figure 1e).

For NE administration at 24 h after extinction, the ANOVA revealed a significant effect of Test ($F_{3,14} = 19.75$, $p < 0.01$) but no effect of Dose ($F_{2,14} = 1.00$, $p = 0.46$) and no Test $\times$ Dose interaction ($F_{6,14} = 0.10$, $p = 0.97$; Figure 1f).

Altogether, these results indicate that NE microinfused into the CA1 area at 0 and 12 h, but not at 6 or 24 h, after extinction significantly enhanced extinction memory, reducing the spontaneous recovery and reinstatement of extinguished fear.

NE in the CA1 Area during the Late Phase after Extinction Selectively Enhanced the Persistence of Extinction LTM

The findings that NE treatment at both 0 and 12 h after extinction enhanced extinction memory prompted us to explore the potentially distinct roles of NE at these two time points on fear extinction. In experiment 2, we tested the differential roles of NE microinfused into the CA1 area during the early phase (0 h) and late phase (12 h) after weak extinction (20 min) on extinction memory. The ANOVA revealed no significant effects of the different treatments (No NE, 0 h NE, and 12 h NE) in the LTM test and the first trial of extinction, and the last trial of extinction (all $p > 0.05$). A two-way repeated-measures ANOVA, with Test as the within-subjects factor and Group (No NE, 0 h NE, and 12 h NE) as the between-subjects factor, was conducted to analyze extinction LTM, revealing a significant effect of Test ($F_{3,14} = 28.19$, $p < 0.01$) and a Test $\times$ Group interaction ($F_{6,14} = 2.32$, $p < 0.05$) but no effect of Group ($F_{2,14} = 0.25$, $p = 0.80$). The *post hoc* analysis revealed that compared with the No NE group of rats, the 0 h NE group of rats exhibited enhanced extinction in extinction LTM test 1 ($t = 2.89$, $p < 0.01$) and extinction LTM test 2 ($t = 3.14$, $p < 0.01$), and the 12 h NE group of rats exhibited enhanced extinction in extinction LTM test 2 ($t = 3.49$, $p < 0.01$) but not extinction LTM test 1 ($t = 0.00$, $p = 1.00$). These results suggest that in the weak extinction protocol, NE treatment at 0 h but not at 12 h after extinction promoted the formation of extinction LTM (tested 2 days after extinction). Fear responses were decreased in the 0 h NE group of rats ($t = 2.92$, $p < 0.01$) and 12 h NE group of rats ($t = 2.02$, $p < 0.05$) compared with the No NE group of rats in the reinstatement test. Moreover, the fear response was reinstated in the 12 h NE group of rats ($t = 2.91$, $p < 0.01$) and nearly reinstated in the 0 h NE group

of rats ($t = 1.86$, $p = 0.07$) compared with the last trial of extinction (Figure 2b). Altogether, these results suggest that NE infusion into the CA1 area during the early phase after extinction enhanced the formation of extinction LTM (tested 2 days after extinction) but selectively enhanced the persistence of extinction LTM (tested 14 days after extinction) during the late phase after extinction (Bekinschtein *et al.*, 2007).

NE in the CA1 during the Late Phase after Extinction Had No Effect on Anxiety-Like Behavior or Locomotion

In experiment 3, we tested whether the enhancement of extinction LTM persistence induced by NE treatment in the CA1 during the late phase after extinction was attributable to alterations in anxiety-like behavior or locomotion. Behavioral tests were performed 14 days after extinction, similar to the second extinction LTM test in the other experiments. For the elevated plus maze test, the one-way ANOVA showed no effects on entries into the open arms ($F_{1,18} = 0.21$, $p = 0.65$), entries into the closed arms ($F_{1,18} = 0.25$, $p = 0.62$), or time spent on the open arms ($F_{1,18} = 0.11$, $p = 0.74$; Figure 3a). For the locomotor activity test, the two-way repeated-measures ANOVA, with Test (every min in the test) as the within-subject factor and Group (saline and NE) as the between-subjects factor, showed no significant effects of Group ($F_{1,17} = 0.04$, $p = 0.84$) and no Test $\times$ Group interaction ($F_{1,17} = 0.22$, $p = 0.65$; Figure 3b). These results indicate that NE treatment in the CA1 during the late phase after extinction had no effect on anxiety-like behavior or locomotion in rats.

Propranolol Blocked the NE-Induced Enhancement of Extinction LTM Persistence

In experiment 4, we tested whether β -receptor inhibition can block the NE-induced enhancement of extinction LTM persistence. No difference was found between groups (Saline + Saline, Saline + NE, Propranolol + Saline, and Propranolol + NE) in the LTM test, the first trial of extinction, or the last trial of extinction (all $p > 0.05$). The two-way repeated-measures ANOVA, with Propranolol (saline and propranolol) and NE (saline and NE) as the between-subjects factors and Test as the within-subjects factor, was conducted to analyze extinction LTM. The ANOVA showed significant effects of Test ($F_{3,52} = 123.35$, $p < 0.01$), NE ($F_{1,52} = 5.96$, $p < 0.05$), and Propranolol ($F_{1,52} = 7.84$, $p < 0.01$) and a Test $\times$ NE $\times$ Propranolol interaction ($F_{3,52} = 3.48$, $p < 0.05$). Follow-up comparisons revealed that compared with Saline + NE group, Propranolol + NE group exhibited increased freezing in both extinction LTM test 2 ($t = 3.95$, $p < 0.01$) and the reinstatement test ($t = 5.17$, $p < 0.01$). Propranolol treatment alone did not affect freezing time in extinction LTM test 2 ($t = 1.80$, $p = 0.08$) or in the reinstatement test ($t = 0.67$, $p = 0.51$; Figure 4b). Altogether, these results indicate that β -receptor inhibition blocked the enhancement of extinction LTM persistence induced by NE treatment in the CA1 during the late phase after extinction.

Figure 3 Norepinephrine in the CA1 during the late phase after extinction had no effect on anxiety-like behavior or locomotion. (a) At 14 days after NE (1.0 $\mu\text{g}/\mu\text{l}$) microinfusion into the CA1, the rats underwent elevated plus maze test and locomotor activity test. Norepinephrine did not affect the numbers of entries into or time spent on the open arms in the elevated plus maze (all $p > 0.05$). (b) Norepinephrine did not affect the total distance traveled in the locomotor activity test ($p > 0.05$). The data are expressed as mean $\pm$ SEM ($n = 9$ –10 per group).

Rp-cAMPS Blocked the NE-Induced Enhancement of Extinction LTM Persistence

In experiment 5, we tested whether the PKA inhibitor Rp-cAMPS can block the NE-induced enhancement of extinction LTM persistence. No difference was found between groups (Saline + Saline, Saline + NE, Rp-cAMPS + Saline, and Rp-cAMPS + NE) in the LTM test, the first trial of extinction, or the last trial of extinction (all $p > 0.05$). The two-way repeated-measures ANOVA, with Rp-cAMPS (saline and Rp-cAMPS) and NE (saline and NE) as the between-subjects factor and Test as the within-subjects factor, was conducted to analyze extinction LTM. The ANOVA revealed significant effects of Test ($F_{3,43} = 105.33$, $p < 0.01$) and NE ($F_{1,43} = 4.65$, $p < 0.05$), a Test $\times$ NE $\times$ Rp-cAMPS interaction ($F_{3,43} = 2.99$, $p < 0.05$), and a trend toward a significant effect of Rp-cAMPS ($F_{1,43} = 3.69$, $p = 0.06$). Follow-up comparisons revealed that Rp-cAMPS treatment alone did not affect freezing time in extinction LTM test 2 ($t = 0.67$, $p = 0.50$) or the reinstatement test ($t = 0.34$, $p = 0.73$). However, compared with Saline + NE group, Rp-cAMPS + NE group exhibited increased freezing in extinction LTM test 2 ($t = 5.10$, $p < 0.01$) and the reinstatement test ($t = 1.95$, $p = 0.05$; Figure 5b). Altogether, these results indicate that the PKA inhibitor blocked the enhancement of extinction LTM persistence induced by NE treatment in the CA1 during the late phase after extinction.

Figure 4 Propranolol blocked the NE-induced enhancement of extinction LTM persistence. (a) Timeline for this experiment. Norepinephrine (1.0 $\mu\text{g}/\mu\text{l}$) was microinfused into the CA1 at 12 h after extinction training, and the β -receptor blocker propranolol was microinfused into the CA1 30 min before. Extinction LTM was tested 2 or 14 days later. After the extinction LTM test 2, a footshock (0.8 mA, 1 s) was given, and fear reinstatement was tested 1 day later. (b) Norepinephrine microinfused into the CA1 at 12 h after extinction enhanced the extinction LTM test 14 days after extinction and the reinstatement test ($p < 0.05$) that were blocked by propranolol ($p < 0.05$). Propranolol microinfused alone did not affect the extinction LTM test or reinstatement test (both $p > 0.05$). The data are expressed as mean $\pm$ SEM ($n = 10$ –11 per group). * $P < 0.05$ compared with Saline + Saline group.

Persistence of Extinction LTM Requires Late-Phase Protein Synthesis in the CA1

In experiment 6, we tested whether the protein synthesis inhibitors anisomycin and emetine can block the NE-induced enhancement of the persistence of extinction LTM. No difference was found between groups (Saline + Saline, Saline + NE, Anisomycin + Saline, and Anisomycin + NE) in the LTM test, the first trial of extinction, or the last trial of extinction (all $p > 0.05$). The two-way repeated-measures ANOVA, with Anisomycin (saline and anisomycin) and NE (saline and NE) as the between-subjects factor and Test as the within-subjects factor, was conducted to analyze extinction LTM. The ANOVA showed significant effects of Test ($F_{3,46} = 96.71$, $p < 0.01$), NE ($F_{1,46} = 22.26$, $p < 0.01$), Anisomycin ($F_{1,46} = 16.57$, $p < 0.01$), and a Test $\times$ NE $\times$ Anisomycin interaction ($F_{3,46} = 3.98$, $p = 0.01$). Follow-up comparisons revealed that anisomycin treatment alone increased freezing time in extinction LTM test 2 ($t = 2.19$, $p < 0.05$) and completely blocked the NE-induced decrease in freezing in extinction LTM test 2 ($t = 6.47$, $p < 0.01$) and the reinstatement test ($t = 3.68$, $p < 0.01$; Figure 6b). Furthermore, another protein synthesis inhibitor, emetine, also blocked the persistence of extinction LTM and prevented the extinction-enhancing effect of NE (all $p < 0.05$; Figure 6c). Altogether, these results indicate that not only the enhancement of extinction LTM persistence

Figure 5 Rp-cAMPS blocked the NE-induced enhancement of extinction LTM persistence. (a) Timeline for this experiment. Norepinephrine ($1.0 \mu\text{g}/\mu\text{l}$) was microinfused into the CA1 at 12 h after extinction training, and the PKA inhibitor Rp-cAMPS was microinfused into the CA1 60 min before. Extinction LTM was tested 2 or 14 days later. After the extinction LTM test 2, a footshock (0.8 mA , 1 s) was given, and fear reinstatement was tested 1 day later. (b) Norepinephrine microinfused into the CA1 at 12 h after extinction enhanced the extinction LTM test 14 days after extinction and the reinstatement test ($p < 0.05$) that were blocked by Rp-cAMPS ($p < 0.05$). Rp-cAMPS treatment alone did not affect the extinction LTM test or reinstatement test (both $p > 0.05$). The data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with Saline + Saline group.

induced by NE treatment, but also the late consolidation of extinction required new protein synthesis in the CA1 during the late phase after extinction.

NE Activated the β -Receptor/PKA Signaling Pathway in the CA1 during the Late Phase after Extinction

In experiment 7, we tested whether NE microinfusion into the CA1 during the late phase after extinction can activate the β -receptor/PKA signaling pathway. The rats were decapitated 13 h after extinction (1 h after NE infusion). The one-way ANOVA was conducted to analyze the effect of NE on the expression of membrane GluR1, cytosolic GluR1, PKA, Erk1/2, and CREB. The ANOVA showed a significant effect for membrane GluR1 expression ($F_{4,39} = 5.95$, $p < 0.05$). The *post hoc* analysis indicated that extinction training increased membrane GluR1 expression ($p < 0.05$; Figure 7b). The ANOVA revealed a significant effect of the level of pGluR1 (Ser 845, a site phosphorylated by PKA; $F_{4,39} = 3.57$, $p < 0.05$) but not tGluR1 ($F_{4,39} = 0.25$, $p = 0.91$). The *post hoc* analysis indicated that pGluR1 was increased by NE that was blocked by propranolol and Rp-cAMPS ($p < 0.05$; Figure 7c and d). The ANOVA showed a significant effect for pPKA level ($F_{4,39} = 3.89$, $p < 0.05$) but not total PKA level ($F_{4,39} = 0.05$, $p = 1.00$). The *post hoc* analysis showed that NE treatment increased pPKA level that was blocked by propranolol and Rp-cAMPS (all $p < 0.05$; Figure 7e and f). The ANOVA showed a significant effect for pErk1/2 level

Figure 6 The persistence of extinction LTM requires late-phase protein synthesis in the CA1. (a) Timeline for this experiment. Norepinephrine ($1.0 \mu\text{g}/\mu\text{l}$) was microinfused into the CA1 at 12 h after extinction training, and the protein synthesis inhibitor anisomycin was microinfused into the CA1 at 1 h before. Extinction LTM was tested 2 or 14 days later. After the extinction LTM test 2, a footshock (0.8 mA , 1 s) was given, and fear reinstatement was tested 1 day later. (b) Anisomycin blocked the effect of NE on extinction LTM test 2 and the reinstatement test ($p < 0.05$), suggesting that the effect of NE on extinction LTM required new protein synthesis ($p < 0.05$). Moreover, anisomycin alone significantly promoted fear recovery in extinction LTM test 2, suggesting a protein synthesis-dependent process during the late phase after extinction ($p < 0.05$). (c) Emetine disrupted extinction LTM in test 2 and prevented the extinction-enhancing effect of NE ($p < 0.05$). The data are expressed as mean $\pm$ SEM ($n = 8-10$ per group). * $P < 0.05$ compared with Saline + Saline group or DMSO + Saline group.

($F_{4,39} = 3.73$, $p < 0.05$) but not total Erk1/2 level ($F_{4,39} = 0.32$, $p = 0.87$). The *post hoc* analysis showed that extinction training increased pErk1/2 levels ($p < 0.05$) that were unaffected by NE, propranolol, and Rp-cAMPS (all $p > 0.20$; Figure 7g and h). The ANOVA showed a significant effect for pCREB level ($F_{4,39} = 5.10$, $p < 0.05$) but not total CREB level ($F_{4,39} = 0.24$, $p = 0.91$). The *post hoc* analysis indicated that extinction training increased pCREB level ($p < 0.05$). NE treatment augmented the increase in pCREB level that was blocked by propranolol and Rp-cAMPS (all $p < 0.05$; Figure 7i and j). Altogether, these results indicate that NE in the CA1 during the late phase after extinction activated the PKA/CREB signaling pathway and increased membrane GluR1 trafficking.

Figure 7 NE activated the β -receptor/PKA signaling pathway in the CA1 during the late phase after extinction. (a) Representative western blots. (b) Extinction training significantly increased synaptosomal membrane GluR1 expression in the CA1 during the late phase of extinction ($p < 0.05$). NE augmented the alteration of GluR1 that was blocked by propranolol and Rp-cAMPS (both $p < 0.05$). (c) The level of pGluR1 (Ser 845) in the CA1 during the late phase of extinction was increased by NE that was prevented by propranolol and Rp-cAMPS (all $p < 0.05$). (d) NE had no effect on total GluR1 level ($p > 0.05$). (e) NE increased pPKA level in the CA1 during the late phase of extinction that was blocked by propranolol and Rp-cAMPS (all $p < 0.05$). (f) NE had no effect on total PKA level ($p > 0.05$). (g) pErk1/2 was only increased by extinction ($p < 0.05$) and unaffected by NE, propranolol, and Rp-cAMPS (all $p > 0.05$). (h) Extinction did not affect total Erk1/2 level ($p > 0.05$). (i) NE promoted the increase in pCREB level in the CA1 induced by extinction training ($p < 0.05$). Propranolol and Rp-cAMPS blocked the enhanced expression of pCREB (both $p < 0.05$) but had no effect on the increase in pCREB level induced by extinction training (both $p > 0.05$). (j) NE had no effect on total CREB level ($p > 0.05$). The data are expressed as mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$ compared with Extinction + Saline + Saline group; # $p < 0.05$ compared with No Extinction + Saline + Saline group. pGluR1, phosphorylated GluR1; tGluR1, total GluR1; pPKA, phosphorylated PKA; tPKA, total PKA; pErk1/2, phosphorylated Erk1/2; tErk1/2, total PKA; pCREB, phosphorylated CREB; tCREB, total CREB.

DISCUSSION

In the present study, we investigated the effect of NE treatment in the dorsal hippocampus during the late phase after extinction on extinguished fear memory. The results showed that microinfusion of NE into the CA1 area at 12 h after extinction enhanced the persistence of extinction LTM. Pretreatment with the β -receptor antagonist propranolol, PKA inhibitor, and protein synthesis inhibitor blocked the effect of late-phase NE treatment on the persistence of extinction LTM. The expressions of pPKA, pCREB, pGluR1, and membrane GluR1 but not pErk1/2 were increased by NE treatment that were blocked by pretreatment with propranolol or the PKA inhibitor in the CA1.

Role of Hippocampal NE in the Persistence of Extinction LTM

Extinguished fear will recover after a few weeks or months, suggesting that the extinction memory is too weak to be persistently stored. Several studies have investigated ways to enhance fear extinction (Abraham *et al*, 2012; Berlau and McGaugh, 2006; McGaugh *et al*, 1990; Mueller *et al*, 2008). Treatment with D-cycloserine, a partial agonist of NMDA receptors at the glycine modulatory site, enhanced extinction memory (Bouton *et al*, 2008; Mao *et al*, 2008; Walker *et al*, 2002). NE infusion into the infralimbic cortex improved extinction (Mueller *et al*, 2008), and this is consistent with the present results in which NE infusion into the CA1 immediately after extinction enhanced extinction consolidation. In addition, extinction has been used as a behavioral strategy to disrupt memory reconsolidation after retrieval in many types of memories (Costanzi *et al*, 2011; Flavell *et al*, 2011; Monfils *et al*, 2009; Xue *et al*, 2012). Nevertheless, even if these methods effectively promote extinction, none of them is specific to the persistence of extinction LTM.

Recent findings suggest that the persistence of long-term fear memory may require a late consolidation process that engages the activation of several molecules, synthesis of new proteins (Katche *et al*, 2013; Parfitt *et al*, 2012a, b), and circadian oscillation of the hippocampal cAMP/MAPK/CREB transcriptional pathway (Eckel-Mahan *et al*, 2008). In the present study, using short extinction training, we found that NE treatment immediately after extinction promoted extinction LTM 2 days later, but NE treatment during the late phase after extinction promoted extinction LTM at 2 weeks but not 2 days later. These results indicate that NE treatment immediately after extinction promoted the formation of extinction LTM, but NE treatment during the late phase after extinction selectively enhanced the persistence of extinction LTM. Moreover, the extinction memory promoted by NE during the late phase could be reinstated, indicating that NE did not impair the original memory. NE treatment during the late phase after extinction also did not affect anxiety-like behavior or locomotor activity in rats. Considering that NE is an important molecule in the brain that affects the sleep/wake cycle, a potential interaction may exist between NE and sleep in the late consolidation process of extinction memory. In the inhibitory avoidance model, the late consolidation of LTM has been shown to be independent of the time of day when training is conducted

(Rossato *et al*, 2009). Nevertheless, the late consolidation of extinction memory may also be different from the original fear memory, and this needs to be investigated in future studies. Altogether, these results suggest that extinction engages a late consolidation process in the dorsal hippocampus that could be modulated by NE and specifically determines the persistence of extinction LTM.

After memory retrieval (short- or long-term retrieval), memory undergoes reconsolidation or extinction. The late reconsolidation process after retrieval in contextual fear memory has been demonstrated in at least two papers. The late reconsolidation of contextual fear memory requires protein synthesis in the BLA in mice (Nakayama *et al*, 2013). In our previous study, we demonstrated that cold water stress disrupted the late reconsolidation of contextual fear memory in rats (Yang *et al*, 2013). The hippocampus, which we focused on in the present study, is involved in both reconsolidation and extinction of contextual fear memory, but its role in these two processes is distinct. First, extinction suppresses the expression of fear, but short-term retrieval, which induces reconsolidation, has no such effect. Second, some opposing molecular mechanisms in the hippocampus underlie these two processes. For example, hippocampal CREB and NF- κ B are activated by reconsolidation but not by extinction (de la Fuente *et al*, 2011; Mamiya *et al*, 2009), suggesting that some specific mechanism determines the memory process after retrieval.

Mechanism That Underlies the Role of Hippocampal NE in the Persistence of Extinction LTM

The enhancement of NE in the formation of fear memory and extinction has been extensively studied (McGaugh, 2004; Mueller and Cahill, 2010). As a stress hormone, NE promotes the stressful fear memory but does not appear to promote the nonstressful extinction memory. Moreover, treatment with the β -receptor antagonist propranolol alone did not affect extinction memory, suggesting that β -receptor activation is not involved in the consolidation of extinction (Cain *et al*, 2004; Rodriguez-Romaguera *et al*, 2009). Electrophysiological studies have demonstrated that β -receptor activation induced by NE could not induce hippocampal long-term potentiation but lowered the subthreshold stimulus response to long-term potentiation (Gelinas and Nguyen, 2005; Katsuki *et al*, 1997; Thomas *et al*, 1996; Tully *et al*, 2007). The enhancing effect of NE on extinction may be attributable to an influence on emotional arousal and attention (Mason, 1983) or the promotion of the intrinsic excitability of neurons responsible for extinction memory (Mueller and Cahill, 2010). Our results showed that propranolol blocked the enhanced persistence of extinction LTM induced by NE but had no effect when administered alone, indicating that β -receptor activation itself did not participate in the late consolidation of extinction memory.

The roles of PKA in the hippocampus in fear learning and extinction are distinct (Tronson *et al*, 2012). Fear conditioning activates the PKA signaling pathway that is significantly inhibited by extinction (Isiegas *et al*, 2006; Li *et al*, 2009; Moita *et al*, 2002; Schafe *et al*, 1999). Genetic inhibition of PKA activity or pharmacological inhibition of the anchoring of PKA to A-kinase anchoring proteins facilitated extinction learning (Nijholt *et al*, 2008). Our

results showed that the inhibition of PKA by Rp-cAMPS blocked the enhancing effect of NE on the persistence of extinction LTM and activation of the β -receptor-dependent PKA/CREB signaling pathway induced by NE. The cAMP analog Rp-cAMPS might also affect the activity of other cAMP substrates, including cyclic nucleotide-gated ion channels and cAMP hydrolases and cyclic nucleotide phosphodiesterases, two groups of molecules that interact with PKA (Moorthy *et al*, 2011; Rochais *et al*, 2004). Nevertheless, in the present study, we showed that the phosphorylation levels of both PKA and GluR1 (Ser 845, a site phosphorylated by PKA) were inhibited by Rp-cAMPS, suggesting that PKA is at least one of the main molecules that mediate the effect of NE on the persistence of extinction LTM. These results demonstrate that NE enhances the persistence of extinction LTM in a β -receptor/PKA/CREB-dependent manner.

Protein kinase A is well known to regulate AMPAR trafficking to the postsynaptic membrane by phosphorylating the AMPAR (Crombag *et al*, 2008; Nayak *et al*, 1998). The β_2 receptor activation can stimulate the PKA-dependent phosphorylation of the GluR1 subunit of AMPAR associated with the β_2 receptor and then increase membrane GluR1 expression at postsynaptic sites (Joiner *et al*, 2010). Several studies have suggested that extinction that induces AMPAR trafficking is distinct in different brain areas. In the amygdala, surface GluR1 was upregulated by fear startle training, whereas D-cycloserine-facilitated extinction reversed this alteration (Mao *et al*, 2008). In the CA1 area of the hippocampus, both contextual fear learning and extinction increased GluR1 expression in mushroom-type spines (Matsuo *et al*, 2008). In the present study, we found that membrane GluR1 in the CA1 was also increased in the late consolidation of extinction memory that was augmented by NE. The increase in membrane GluR1 expression induced by NE was consistent with the elevation of pGluR1 (Ser 845) level that was blocked by propranolol and the PKA inhibitor, indicating that the increase in membrane GluR1 expression depended on PKA activation. Nevertheless, propranolol and the PKA inhibitor did not completely block the extinction-induced increase in membrane GluR1, suggesting other PKA-independent GluR1 trafficking mechanisms.

Although previous studies showed that the activation of PKA in the hippocampus is critical for the late consolidation of fear learning (Rossato *et al*, 2009), the present results demonstrated that the β -receptor/PKA signaling pathway does not underlie the late consolidation of extinction memory. Interestingly, the inhibition of protein synthesis during the late phase after extinction significantly reduced the persistence of extinction LTM, suggesting that β -receptor/PKA-independent new protein synthesis is needed for the late consolidation of extinction. Several molecules in the hippocampus have been shown to be involved in both fear conditioning and fear extinction, including cyclin-dependent kinase-5, protein kinase C, PKA, and Erk1/2 (Orsini and Maren, 2012; Tronson *et al*, 2012). Among these kinases, only Erk1/2 is activated in both processes (Atkins *et al*, 1998; Fischer *et al*, 2007; Irvine *et al*, 2005; Kimura *et al*, 2008; Tronson *et al*, 2012). Moreover, Erk1/2 is critical for the late consolidation of fear memory (Bekinschtein *et al*, 2007; Wan *et al*, 2010). In the present study, we found

that Erk1/2 was activated in the late phase after extinction and was unaffected by NE. We infer that new protein synthesis in the dorsal hippocampus in the late consolidation of extinction memory may depend on the activation of Erk1/2, but this still needs further investigation.

Concluding Remarks

In summary, NE treatment in the dorsal hippocampus enhanced the late consolidation of extinction to promote the persistence of extinction LTM that was dependent on the activation of the β -receptor/PKA/CREB signaling pathway and membrane GluR1 trafficking. The specific modulation of the late consolidation of extinction may provide more precise strategies to selectively regulate the persistence of extinction LTM that may ultimately help optimize exposure therapy for posttraumatic stress disorder or anxiety-related disorders.

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The authors declare no conflict of interest.

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