Propranolol decreases fear memory retention via modulating the stability of surface GluA1 in the lateral amygdala

Supplementary Material

Supplementary Methods

Surgical procedure and microinjection

Stereotaxic surgery was operated according to our previous studies (Jiang et al., 2013; Luo et al., 2014; Wu et al., 2013) with necessary modification. Briefly, rats were anesthetized with pentobarbital sodium (60 mg/kg) by i.p. injection and placed in a stereotaxic apparatus (Stoelting, USA) with the bregma and posterior in the same level. The body temperature was maintained at 37.0±0.2 ℃ by an electric incandescent lamp. Two stainless-steel guide cannulas (23 gauge, 12 mm long) were bilaterally implanted into the LA region (2.8 mm posterior to the bregma, 5.0 mm lateral to the midline, 5.4 mm below the cranial theca). The cannulas were fixed to the skull with the aid of jeweler screws and dental acrylic resin and dental cement. Rats were allowed to recover from the anesthesia before being returned to their home cages. Rats were given 7 days for recovery before further experiments. The drugs were administrated via intra-LA infusion with a microsyringe (5 μL) connected by a PE-10 polyethylene tubing to a needle (outside diameter = 0.3 mm, 2.0 mm longer than guide cannula). The injection rate was set to 1 μL in 2 min and the volume injected was 1 μL per site. After behavioral experiments, the rats were anaesthetized and sacrificed by decapitation, and brains were removed and cut in 40-μm coronal slices and the placements of the cannula were verified. All animals with an erroneous cannula placement were discarded from statistical analysis.

Adenoviral infection and overexpression

The GluA1 carboxyl terminus (GluA1-C-tail) gene fragment sequence (CGAGTTCTGCTACAAATCCCGTAGCGAGTCGAAGCGGATGAAGGGTTTCT GTTTGATCCCACAGCAATCCATCAATGAAGCCATACGGACATCGACCCTCC CCCGGAACAGTGGGGCAGGAGCCAGCGGAGGAGGCGGCAGTGGAGAGAA TGGCCGGGTGGTCAGCCAGGACTTCCCCAAGTCCATGCAATCCATTCCCTG CATGAGTCACAGTTCAGGGATGCCCTTGGGAGCCACAGGATTGTAA) was obtained as previously described (Shi et al., 2001). The adenovirus was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). GluA1-C-tail adenovirus (CV049) which contains Ubi-driven enhanced green fluorescent protein (EGFP) reporter and cloning restriction sites (NheI/EcoRI) to allow the introduction of GluA1 carboxyl terminus, and control vector adenovirus (GV137) were constructed, amplified and purified to be 10^{11} PFU/mL. 2.0 μL of adenovirus suspension was injected in each side LA at a rate of 0.2 μL/min. Rats were subjected to experiments 36 h after adenovirus injection. The effectiveness of adenoviral transfection was verified by expression of EGFP under fluorescent microscope (IX51WI; Olympus, Tokyo, Japan) and principal LA neurons transfected with adenovirus were visualized under epifluorescent illumination with GFP filter (Olympus) in patch-clamp

experiments.

Pain threshold measurement

Pain threshold was measured according to our previous studies (Li et al., 2012; Wang et al., 2011; Yang et al., 2013). Briefly, rats were placed individually into the conditioning chamber with electric grid. After 3 min of habituation, electric foot shocks (1 s) were applied starting with an intensity of 0.1 mA. The intensity was increased gradually by 0.1 mA (with pauses of 30 s between successive stimuli) until the animal showed the first signs of pain (flinching or jumping), and the corresponding intensity was taken as the pain threshold.

Open-field test

The procedure of open-field test was based on our previous study with minor modifications (Li et al., 2012). Rats were placed individually into the activity chamber ($40\times40\times40$ cm^3) monitored by a video motility system. The 20×20 cm^2 middle part of the activity chamber was defined as central part. The spontaneous motor activity including the total distance travelled, and the anxiety-like behavior activity including the total time spent in the central part during 30 min intervals were monitored and assessed.

Electrophysiological recording

Preparation of brain slices and protocol for fEPSP recordings were based on our previous studies (Li et al., 2012; Luo et al., 2014). The stimulation intensities were adjusted to produce a fEPSP with 2/3 of the maximal amplitude. After 30 min of baseline recording, LTD was induced by low-frequency stimulation (LFS; 1 Hz for 15 min) at test intensity. Responses were recorded for 60 min following LFS.

Patch-clamp recording was performed according to our previous study with slight modifications (Li et al., 2012). The rats (4- to 5-week-old) were decapitated and the brains quickly moved to ice-cold oxygenated cutting solution containing (in mM): 210 sucrose, 5 $MgSO_4$, 1 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, 11.6 $C_6H_7NaO_6$, 3.1 $C_3H_3NaO_3$ and aerated with 95% O_2-5% CO_2. Coronal brain slices (300 μm) containing LA were cut by a vibratome (VT 1000S, Leica, Wetzlar, Germany) in cutting solution and then transferred to a holding chamber with artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 4 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose and aerated with 95% O_2-5% CO_2. Brain slices were then maintained at 26 ℃ for at least 1 h before recording. The brain slice was transferred to the recording chamber, held submerged and superfused continuously with ACSF at a flow rate of 1-2 ml/min for recording at 26 ℃. Excitatory postsynaptic currents (EPSCs) of principal neurons in LA were recorded by whole-cell patch recording. EPSCs were evoked in the presence of γ-aminobutyric acid $(GABA)_A$ receptor antagonist bicuculine (20 μM). The pipette (input resistance: 3-6 MΩ) solution

contains (in mM) 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA and 5 QX314 (pH 7.35, 285 mOsm). For pharmacologically isolated AMPAR or N-methyl-D-aspartate receptor (NMDAR)-mediated EPSCs, we blocked NMDAR and AMPAR with 100 μM AP5 and 20 μM CNQX, respectively. The cell membrane potential was held at -70 mV. NMDAR-mediated EPSCs were recorded in 0 mM Mg^{2+} ACSF. The cell membrane potential was held at -70 mV and +40 mV for the rectification of AMPAR-medited EPSCs. Data were collected when series resistance fluctuated within 20% of initial values (10-30 MΩ), filtered at 3 kHz and sampled at 10 kHz.

Surface protein cross-linking with BS^3

The surface protein cross-linking was performed according to our previous study with slight modification (Jiang et al., 2013). The coronal slices of amygdala were made as for the electrophysiological recordings. After treatments, the amygdala was dissected and immersed in ice-cold ACSF which was spiked with 1 mM BS^3 immediately after addition of the tissue. Then the tissue was crosslinked for 30 min at 4 ℃ with gentle agitation and this reaction was terminated by quenching with 100 mM glycine (15 min at 4 ℃). The tissue was pelleted by brief centrifugation, resuspended in ice-cold lysis buffer, homogenized rapidly by sonicating for 10 s, and centrifuged (12000 g for 15 min at 4 ℃). The supernatant fraction was aliquoted and stored at -80 ℃ before being used for Western blotting.

Enrichment of synaptic and extrasynaptic membrane fractions

Separation of synaptic and extrasynaptic fractions was performed according to previous reports (Goebel-Goody et al., 2009; Li et al., 2011) with minor modifications. In brief, the amygdala slices were prepared as for electrophysiological experiment and treated with NE (10 μM for 10 min) after recovery. Then the slices were harvested in ice-cold sucrose homogenization buffer (20 μL per slice) containing the following (in mM): 320 sucrose, 10 Tris (pH7.4), 1 Na_3VO_4, 5 NaF, 1 EDTA and 1 EGTA. Slices were then homogenized in a glass grinding vessel using a rotating Teflon pestle (2000 rpm) with at least 20 passes to create a Dounce homogenate. The homogenate was centrifuged (1000 g for 10 min at 4 ℃) to remove nuclei and incompletely homogenized material. The supernatant was centrifuged at 10000 g, 4 ℃ for 15 min, and the resulting pellet was resuspended in 60 μL of sucrose buffer using a pipette directly in the microfuge tube with 30 pulses. The resuspension solution was then subjected to detergent extraction by adding 8 volume of Triton X-100 buffer (final=0.5% v/v) containing the following reagents (in mM): 10 Tris (pH7.4), 1 Na_3VO_4, 5 NaF, 1 EDTA and 1 EGTA. This suspension was incubated at 4 ℃ for 20 min with gentle rotation and then centrifuged at 32000 g, 4 ℃ for 20 min. The pellet was the postsynaptic density-associated (or synaptic) fraction and the supernatant was the Triton-soluble (or extrasynaptic) fraction. Protein concentrations were determined

using Coomassie blue protein-binding assay (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China) before Western blot analysis.

Western blotting

Protein samples of the amygdala were prepared for western blot analysis. The performance was based on our previous protocol with minor modifications (Jiang et al., 2013). Briefly, amygdala slices from each rat were homogenized in ice-cold lysis buffer. Protein concentrations were examined by Coomassie blue protein-binding assay. Then protein samples (30 or 50 μg) were separated by 10% or 6% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, the transferred membranes were incubated overnight at 4 ℃ with primary antibodies: anti-β-actin (1:3000) (Upstate Biotechnology, Lake Placid, NY, USA); anti-phospho-CaMKII (1:400) and anti-CaMKII (1:500) (Cell Signaling, San Francisco, CA, USA); anti-phospho-ERK1/2 (1:1000) and anti-ERK1/2 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-PKA C (Thr197) (1:800) and anti-PKA C (1:1000) (Cell Signaling); anti-phospho-PKC (1:800) and anti-PKC (1:1000) (Cell Signaling); anti-phospho-GluA1 (Ser831) (1:400), anti-phospho-GluA1 (Ser845) (1:400) and anti-GluA1 (1:500) (Cell Signaling); anti-phospho-GluA2 (Tyr876) (1:400) and anti-GluA2 (1:1000) (Cell Signaling); anti-GluN2A (1:500) and anti-GluN2B (1:500) (Santa Cruz Biotechnology). Following three washes with TBST, membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000) in TBST with 1% BSA for 1 h at room temperature, and reacted with enhanced chemiluminescence reagents (Super Signal West Pico; Pierce Chemical Co., Rockford, IL, USA). Images were scanned and captured with Micro Chemi (DNR Bio-imaging systems, Jerusalem, Israel) and the optical density of the bands was determined using Scion Image software (Fredrick, MD, USA). All assays were performed at least three times, and the results are presented as percentage of control after normalization.

References

Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, Browning MD (2009) Phospho-regulation of synaptic and extrasynaptic N-methyl-d-aspartate receptors in adult hippocampal slices. Neuroscience 158:1446-1459.

Jiang B, Wang W, Wang F, Hu ZL, Xiao JL, Yang S, Zhang J, Peng XZ, Wang JH, Chen JG (2013) The stability of NR2B in the nucleus accumbens controls behavioral and synaptic adaptations to chronic stress. Biol Psychiatry 74:145-155.

Li S, Jin M, Koeglsperger T, Shepardson NE, Shankar GM, Selkoe DJ (2011) Soluble Abeta oligomers inhibit long-term potentiation through a mechanism involving excessive activation of extrasynaptic NR2B-containing NMDA receptors. J Neurosci 31:6627-6638.

Li YK, Wang F, Wang W, Luo Y, Wu PF, Xiao JL, Hu ZL, Jin Y, Hu G, Chen JG (2012) Aquaporin-4 deficiency impairs synaptic plasticity and associative fear memory in the

lateral amygdala: involvement of downregulation of glutamate transporter-1 expression. Neuropsychopharmacology 37:1867-1878.
Luo Y, Wu PF, Zhou J, Xiao W, He JG, Guan XL, Zhang JT, Hu ZL, Wang F, Chen JG (2014) Aggravation of seizure-like events by hydrogen sulfide: involvement of multiple targets that control neuronal excitability. CNS Neurosci Ther 20:411-419.
Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 105:331-343.
Wang W, Wang F, Yang YJ, Hu ZL, Long LH, Fu H, Xie N, Chen JG (2011) The flavonoid baicalein promotes NMDA receptor-dependent long-term potentiation and enhances memory. Br J Pharmacol 162:1364-1379.
Wu WN, Wu PF, Zhou J, Guan XL, Zhang Z, Yang YJ, Long LH, Xie N, Chen JG, Wang F (2013) Orexin-A activates hypothalamic AMP-activated protein kinase signaling through a Ca(2)(+)-dependent mechanism involving voltage-gated L-type calcium channel. Mol Pharmacol 84:876-887.
Yang J, Li MX, Luo Y, Chen T, Liu J, Fang P, Jiang B, Hu ZL, Jin Y, Chen JG, Wang F (2013) Chronic ceftriaxone treatment rescues hippocampal memory deficit in AQP4 knockout mice via activation of GLT-1. Neuropharmacology 75C:213-222.

Supplementary Figure legends

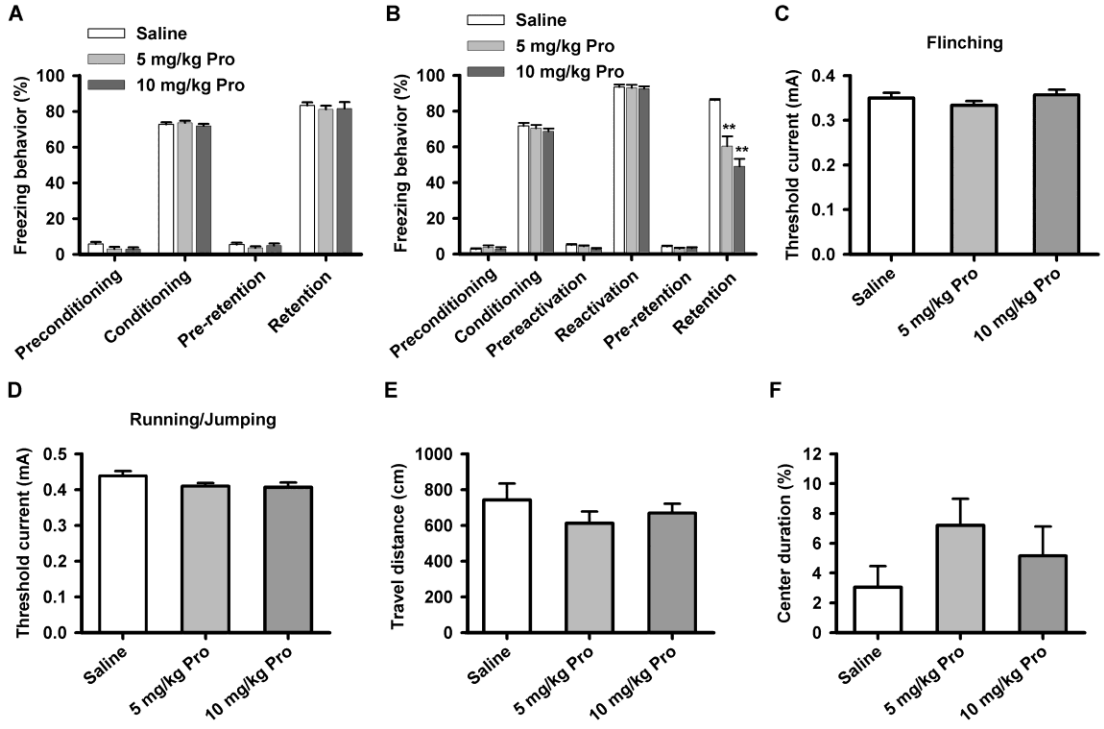

Figure S1. There are no differences in baseline behaviors between saline and propranolol-treated rats. (A) Saline (n=8), 5 mg/kg (n=8) and 10 mg/kg (n=8) propranolol-treated rats exhibited similar freezing behavior before and during conditioning training, and before and during consolidation test. (B) Saline (n=8), 5 mg/kg (n=8) and 10 mg/kg (n=8) propranolol-treated rats exhibited similar freezing

behavior before and during conditioning training, and before and during reactivation. (C) Thresholds of shock intensities for flinching. (D) Thresholds of shock intensities for running/jumping. No difference was observed among saline (n=12), 5 mg/kg (n=12) and 10 mg/kg (n=12) propranolol-treated rats. (E) Total distance moved and (F) the percentage of time spent in central part during 30 min intervals in the open-field test. No difference was observed among saline (n=10), 5 mg/kg (n=10) and 10 mg/kg (n=10) propranolol-treated rats. **P<0.01 vs saline.

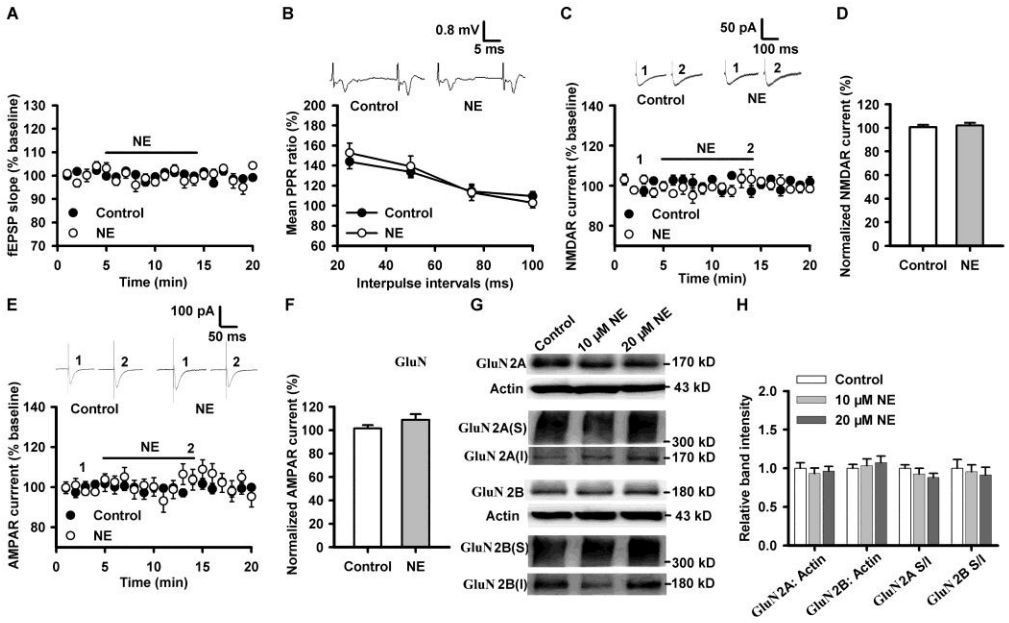

Figure S2. Negative effects of NE on glutamatergic synapse transmission. (A) The basal fEPSP was not affected by NE in LA slices of rats (n=6 slices from 4 rats). (B) Paired-pulse ratio recording. Insets are representative fEPSPs evoked by paired-pulses delivered 25 ms apart (n=6 slices from 4 rats). (C) No difference in basal NMDAR-current was observed between control slices (n=7 from 4 rats) and slices treated with NE (n=7 from 4 rats). Insets are representative NMDAR-currents recorded from experiments. (D) Bar graphs showing the mean normalized amplitude of control and NE-treated slices. (E) No difference in basal AMPAR-current was observed in control slices (n=6 from 4 rats) and slices treated with NE (n=10 from 5 rats). Insets are representative AMPAR-currents recorded from experiments. (F) Bar graphs showing the mean normalized amplitude of control and NE-treated slices. (G) Representative images of western blotting. (H) Histogram showing the total and surface expression of NMDAR subunits GluN2A and GluN2B were not affected by 10 μM NE for 10 min (n=8).

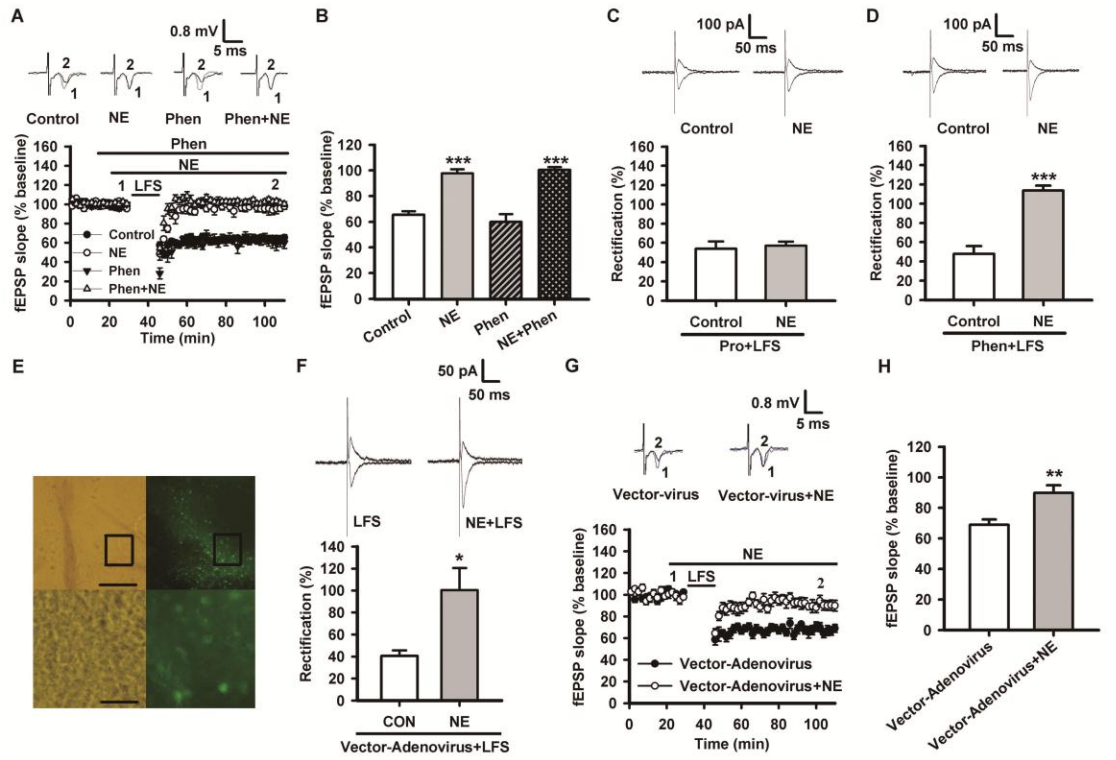

Figure S3. Activation of β-AR and phosphorylation of GluA1-C terminus mediate NE-induced upregulation of AMPAR function in LA. (A) Time course of the fEPSP from control (n=12 slices from 7 rats), NE (n=7 slices from 4 rats), Phen (n=7 slices from 4 rats) and Phen+NE-treated slices (n=7 slices from 4 rats). Inset: The representative fEPSPs recorded in individual slices before (1) and 60 min after (2) LFS in control, NE, Phen or Phen+NE-treated slices. (B) The histogram showing the level of LTD at 60 min after LFS in control, NE, Phen or Phen+NE-treated slices. (C) Inset: Example of evoked AMPAR-mediated synaptic responses recorded at -70 mV and +40 mV from Pro and LFS-treated, or Pro and NE+LFS-treated slices. Average rectification values ($I_{-70\,mV}/I_{+40\,mV}$) for LFS (n=5 from 4 rats) or NE+LFS (n=6 from 3 rats)-treated slices. Values are normalized to control cells. (D) Inset: Example of evoked AMPAR-mediated synaptic responses recorded at -70 mV and +40 mV from Phen and LFS-treated, or Phen and NE+LFS-treated slices. Average rectification values ($I_{-70\,mV}/I_{+40\,mV}$) for LFS (n=5 from 4 rats) or NE+LFS (n=5 from 4 rats)-treated slices. Values are normalized to control cells. (E) Representative images of transmitted light (left) and fluorescence (right) of a coronal section of the right LA at 36 h after injection of adenovirus. Fluorescence indicates successful infection of adenovirus. The corresponding enlarged images of the upper were shown at the lower. Scale bars in the images represent 200 (upper) and 50 μm (lower). (F) Inset: Example of evoked AMPAR-mediated synaptic responses recorded at -70 mV and +40 mV from vector-adenovirus-infected and LFS or NE+LFS-treated slices. Average rectification values ($I_{-70\,mV}/I_{+40\,mV}$) for LFS (n=5 from 5 rats) or NE+LFS (n=5 from 5 rats)-treated slices. Values are normalized to control cells. (G) Time course of the fEPSP from vector infection (n=12 slices from 7 rats) or vector infection+NE (n=7 slices from 4 rats)-treated slices. Inset: The representative fEPSPs recorded in

individual slices before (1) and 60 min after (2) LFS in vector infection or vector infection+NE-treated slices. (H) The histogram showing the level of LTD at 60 min after LFS. *P<0.05, **P<0.01, ***P<0.001 vs control or vector-adenovirus.

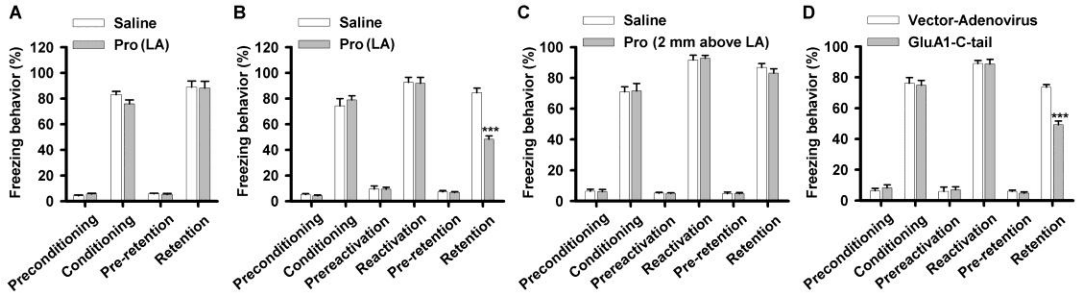

Figure S4. Neither propranolol nor overexpression of GluA1-C-tail in LA influences fear acquisition, consolidation or reactivation of rats. (A) Saline (n=7) and propranolol (n=9)-treated rats exhibited similar freezing behavior before and during conditioning training, and before and during consolidation test. (B) Saline (n=7) and propranolol (n=7)-treated rats exhibited similar freezing behavior before and during conditioning training, and before and during reactivation. (C) Saline (n=7) and propranolol (n=8)-treated rats exhibited similar freezing behavior before and during conditioning training, and before and during reactivation. (D) Vector-adenovirus (n=8) and overexpression of GluA1-C-tail-treated groups (n=9) exhibited similar freezing behavior before and during conditioning training, and before and during reactivation. ***P<0.001 vs saline or vector-adenovirus-treated rats.