Supporting Online Material for

Fear Erasure in Mice Requires Synergy Between Antidepressant Drugs and Extinction Training

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Materials and Methods

Animals

Adult male mice C57Bl/6JRcc.Hsd were used in all behavioral experiments. Male B6.129S4-Bdnf^{tm1Jae}/J – heterozygote mice (backcrossed to C57Bl/6J background for more than 10 generations) and bred with C57Bl/6J female mice. Male heterozygous mice and wild-type littermates were used for behavioral testing. Mice were housed individually for 7 days prior to fear conditioning and were 3 months old at the time of fear conditioning. Mice were kept under 12 h light/dark cycle (light on at 6 am). Food and water were available *ad libitum*. All animal procedures were done according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the experimental Animal Ethical Committee of Southern Finland and by the Institutional Animal Care and Use Committees of Columbia University and the New York State Psychiatric Institute.

Drug treatment

Fluoxetine: Mice received fluoxetine (kind gift from Dr. Jukka Sallinen, Orion Pharma, Helsinki, Finland) via drinking water in light-protected tubes. Solutions were prepared fresh every day. Fluoxetine was dissolved in tap water at a concentration of 0.08 mg/ml to achieve an approximately 10 mg/kg per day

dosing unless otherwise stated. We have previously reported that this protocol of chronic fluoxetine administration results in fluoxetine plasma levels within the therapeutic range in humans *(32)*. The treatment continued through all behavioral sessions until sacrifice.

Doxycycline: In the BDNF-virus experiment, Lentiviral Tet-off system was used to shut off the expression of BDNF by doxycycline treatment as described previously (*33*) with minor modifications. Preliminary experiments with additional groups of mice confirmed the effectiveness of the following protocol to temporally regulate the viral expression. Mice started to receive 0.025 % doxycycline mixed with 2 % sucrose in drinking water 2 days before viral injection. In the control groups of mice ("BDNF+Doxycycline" and "Sham+Doxycycline"), this treatment continued throughout the experiments until sacrifice. To induce the virus-mediated BDNF expression in the "BDNF-Doxycycline" group of mice, doxycycline+sucrose solution was replaced by 2 % sucrose in drinking water right after the end of extinction training, 5 days before the fear renewal test to allow sufficient increase in BDNF expression in targeted tissue *(33)*.

Behavior

Freezing behavior was measured with an automatic infrared beam detection system which was placed on sides of chamber of the fear conditioning apparatus. The mouse was considered to be frozen only if it was not moving for at least 3 s and the measure was expressed as a percentage of time spent freezing.

Fear conditioning and extinction took place in two different contexts unless otherwise stated. Fear conditioning context (A) was transparent Plexiglas chamber with metal grids on floor whereas extinction context (B) was black non-transparent Plexiglas chamber with planar floor. Both context A and context B were cleaned before each session with 70 % ethanol and 70 % 2-propanol, respectively.

Experiment 1, "Chronic fluoxetine prior to fear conditioning" (Fig. 1A): Mice were given fluoxetine for 3 weeks before fear conditioning day 1. On day 1, mice were conditioned using 5 pairings of the conditioned stimulus, CS (total CS duration 30 s, 1 Hz, white noise, 80 dB) with the unconditioned stimulus, US (1 s foot-shock 0,6 mA, inter-trial interval: 20-120 s). The US co-terminated with the CS. Freezing level during the first CS, preceding the first US, was taken as baseline freezing during CS. On day 2 and 3, conditioned mice were submitted to extinction training in context B during which they received 12 presentations of the CS on each day (inter-trial interval: 20-60 s). Spontaneous recovery and context-dependent fear renewal were tested 7 days later in context B and context A, respectively, using 4 presentations of the CS (inter-trial interval: 20-60 s).

Experiment 2, "Chronic fluoxetine after fear conditioning" ("Fear Renewal" Protocol I, Fig. 2A): On day 1, mice were conditioned using 5 pairings of the CS (total duration 30 s, 1 Hz, white noise, 80 dB) with the US (1 s foot-shock 0,6 mA, inter-trial interval: 20-120 s). The US co-terminated with the CS. Freezing level during the first CS, preceding the first US, was taken as baseline freezing during CS. Mice were then divided into two groups with equal levels of freezing, one

receiving fluoxetine in drinking water until the end of the experiment and the other receiving tap water. On day 14 and 15, conditioned mice were submitted to extinction training in context B during which they received 12 presentations of the CS on each day (inter-trial interval: 20-60 s). Spontaneous recovery and context-dependent fear renewal were tested 7 days later in context B and context A, respectively, using 4 presentations of the CS (inter-trial interval: 20-60 s).

Experiment 3, "Chronic fluoxetine after fear conditioning" ("Fear reinstatement" Protocol II, Fig. 2A): All experimental procedures were conducted in the context A. On day 1, mice were conditioned using 5 pairings of the CS (total duration 30 s, 1 Hz, white noise, 80 dB) with the US (1 s foot-shock 0,6 mA, inter-trial interval: 20-120 s). The US co-terminated with the CS. Freezing level during the first CS, preceding the first US, was taken as baseline freezing during CS. Mice were then divided into two groups with equal levels of freezing, one receiving fluoxetine in drinking water until the end of the experiment and the other receiving tap water. On day 14 and 15, conditioned mice were submitted to extinction training during which they received 12 presentations of the CS on each day (inter-trial interval: 20-60 s). Seven days later, mice received 5 unsignaled US and, 24 hours later, fear reinstatement was tested using 4 presentations of the CS (inter-trial interval: 20-60 s). To control for the context specificity of the fear reinstatement test, mice were additionally tested in the new context B two hours later using 4 presentations of the CS (inter-trial interval: 20-60 s), during which mice did not show elevated freezing behavior (data not shown).

Experiment 4 with BDNF$^{+/-}$ mice: Protocol "Chronic fluoxetine after fear conditioning" ("Fear Renewal" Protocol I, Fig. 2A) was used except that the extinction training was extended to 5 sessions to allow the BDNF$^{+/-}$ mice reach similar level of extinction as their wild-type littermates BDNF$^{+/+}$ (Fig. S7B).

Figures 1, 2 and 4 summarize the behavioral data. Every graph represents block of 2 CS.

Immunohistochemistry

Animals were deeply anesthetized, then perfused through the heart with 4% paraformaldehyde in PBS. Brains were removed from the skull, postfixed in the same fixative for 24h at +4°C, cryoprotected in 30%sucrose for 24–48 h and then snap frozen at –80°C. Coronal sections were cut using a cryostat at 30 µm and then kept at -20°C floating in cryoprotectant solution until use. Sections were cleared from cryoprotective solution by washing in PBS and blocked to prevent nonspecific binding. Sections were incubated with one of the primary antibodies for 24h at +4°C in PBS solution containing 0.4% TritonX-100 (PBST). The following primary antibodies were used: 1) mouse anti-parvalbumin (1:5000; Swant, Bellinzona, Switzerland), 2) mouse anti-calbindin (1:5000; Swant, Bellinzona, Switzerland), 3) rabbit anti-calretinin (1:2000; Swant, Bellinzona, Switzerland), 4) mouse anti-PSA-NCAM (1:5000; kind gift from T. Seki (*34*), 5) rabbit anti-KCC2pan (1:2000; kind gift from Dr. C. Rivera (*35*). To count the number of neuronal cells in the basolateral amygdala, mouse anti-NeuN (1:500;

Chemicon, Millipore, Espoo, Finland) was used. Sections were washed then incubated for 1h at RT in PBST with the appropriate secondary antibodies: Alexa 546 goat anti-mouse or anti-rabbit (1:1000; Molecular Probes, Invitrogen, Espoo, Finland). For double immunostaining, sections were next incubated with the biotinylated lectin from *Wisteria floribunda* (WFA, 1:200; Sigma-Aldrich, Helsinki, Finland) for 24h at +4°C followed by washing and incubation with the FITC conjugated streptavidin (1:1000; ZyMED, Invitrogen, Espoo, Finland) for 1h at RT. The sections were washed, mounted on slides and covered with Prolong®Gold anti-fade reagent. To control for the specificity of lectin WFA binding to the primary antibodies, a “no primary antibody” condition was included in every immunostaining experiment.

Quantitative evaluation of immunostaining was performed blind to the treatment group. ImageJ version 1 was used for the immunopositive neurons counts or optical density quantification in the basolateral amygdala, infralimbic cortex and CA1 area of the hippocampus bilaterally using a minimum of 5 sections per hemisphere per animal. Brain structures were identified according to Franklin and Paxinos (1997) (*36*). To evaluate the differences in PSA-NCAM and KCC2 expression, brain regions were delineated using a computer mouse, mean optical densities were measured and results are expressed as percentage of control group.

Electrophysiology

All recordings and data analysis were done blind to the treatment group of the experimental subject. Male mice were given Fluoxetine (0.16 mg/ml) in drinking water from 6 weeks of age for 3 weeks until they were sacrificed. Littermate controls were given only tap water.

Slice preparation. Mice were anesthetized with halothane and then decapitated. The brain was removed and chilled in ice-cold dissection solution (195mM sucrose, 10mM NaCl, 2.5mM KCl, 1mM NaH_2PO_4, 25mM $NaHCO_3$, 10mM glucose, 6mM $MgCl_2$, 0.5mM $CaCl_2$). The cerebellum and the anterior portion of the brain were removed and horizontal brain 400 μm horizontal slices were cut on a vibratome. Slices were immediately transferred to an interface chamber and allowed to recover for at least 1 hr at 31-32°C until use in ACSF (124mM NaCl, 2.5mM KCl, 1mM NaH_2PO_4, 25mM $NaHCO_3$, 10mM glucose, 1mM $MgCl_2$, 2mM $CaCl_2$).

Extracellular field recordings. Extracellular field recordings were made in an interface chamber at 31-32°C in ACSF (2 ml/min) with recording electrodes (≈ 2-3 MΩ) filled with ACSF. Stimulation was with a concentric bipolar stimulating electrode (tip diameter 0.2 mm, FHC). Data were acquired by pClamp9 software, using an Axoclamp-2 amplifier and Digidata 1320A. To record evoked fEPSPs in lateral amygdala, the recording electrode was positioned in the lateral nucleus of the amygdala caudal to the stimulating electrode placed in the external capsule. fEPSPs were quantified by measuring their peak amplitude. At the beginning of the recording period, input-output curves were acquired by stimulating in 3V

increments. For subsequent assaying of paired pulse ratio (PPR) and long-term potentiation (LTP) the stimulus intensity was adjusted to evoke a half-maximal fEPSP and all test pulses and tetani were delivered at this intensity. 20-30 minute baselines were recorded and post-induction responses were normalized to the final 10 min. LTP was induced by 2 trains of 1 sec at 100 Hz separated by a 30 sec interval. The average of the last 5 responses of the 1 hour monitoring period was taken as the LTP size.

Messenger RNA analysis

Animals were anesthetized and killed by decapitation, the basolateral amygdala, hippocampi and medial prefrontal cortices were dissected, immediately frozen on dry ice and kept at -80^{o}C. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. Total RNA was treated with DNAse I mix, then mRNA was reverse transcribed using oligo(dT) primer and SuperScript III Reverse Transcriptase mix. The amount of cDNA was quantified using real-time PCR. The following primers were used to amplify specific cDNA regions of the transcripts: BDNF total (5'- GAAGGCTGCAGGGGCATAGACAAA -3' and 5' – TACACAGGAAGTGTCTATCCTTATG -3'), common reverse primer for all BDNF exons (5'- ACCGAAGTATGAAATAACCATAGTAAG -3') and forward primers for BDNF 1 (5'- CAAGACACATTACCTTCCTGCATCT -3') and BDNF 4 (5'- TGTTTACTTTGACAAGTAGTGACTGAA -3'), nomenclature according to Aid et al. (2007) *(37)*; finally, a control PCR was performed with the

primers for a housekeeping gene *gapdh* (5'- GGTGAAGGTCGGTGTGAACGG -3' and 5'- CATGTAGTTGAGGTCAATGAAGGG -3'). DNA amplification reactions were run in triplicate at least two independent times in the presence of SYBR-Green. Ct values from each sample were obtained using the LightCycler 480 software. Relative quantification of template was performed as described previously *(38)* using ΔΔCt method, with cDNA data being normalized to the control *gapdh level*. The control reactions without Reverse Transcriptase were also performed. BDNF exon 4 expression was not influenced by the experimental procedures in any brain area investigated.

Lentiviral production and stereotaxic injection

Time-specific BDNF overexpression in the basolateral amygdala was achieved using injection of lentivirus regulatable by doxycycline Tet-off system (*33*). To produce viral particles, the vector plasmid pTK431-BDNF, the packaging plasmid pΔNR and the envelope plasmid pMDG-VSV-G (ratio 4:3:1) were cotransfected into HEK293T cells as described previously (*33*). The viral particles were collected by ultracentrifugation and resuspended in MEM. Virus titer was determined using p24 antigen ELISA as 0.21 mg/ml of p24 and viral solution was kept at -80°C in small aliquots.

Pilot experiments were performed to determine the stereotaxic coordinates of the basolateral amygdala: bregma -1.7, lateral ±3.6 and ventral -4.0 according to the Allen atlas (http://www.brain-map.org/). Mice were anesthetized with isoflurane

and placed in a stereotaxic frame. Bilateral injection into the basolateral amygdala was performed using a 10 µl syringe with a stainless steel needle. On each brain side, 500 nl of the virus were infused at a speed of 3 nl/s. The needle was kept in place for 8 minutes after the infusion to improve the penetration of the viral solution into the tissue. As a control for the infection, additional mice were injected with the viral diluent solution (Sham) using the same protocol. The analgesic carprofen (5 mg/kg) was administered subcutaneously. After the surgery, mice were returned to their home cages and left to recover for 2 weeks.

BDNF riboprobes production and in situ hybridization

Identification of the viral infection sites was performed by *in situ* hybridization using DIG labeled BDNF probes on free floating sections. After analysis, two infected animals were removed from the group BDNF+Doxycycline because of detectable levels of BDNF-virus expression not completely blocked by doxycycline. One animal from the BDNF-Doxycycline group was removed because of an incorrect infection site. A Sham+Doxycycline group was included as a negative control for BDNF-virus infected sites.

BDNF riboprobes. The riboprobes were made using DIG-labeling kit following the manufacture's protocol. Briefly, the BDNF coding region of the mouse (nucleotides 224-734, see *39*) was cloned into pGEM vector. The anti-sense or sense riboprobes were made and labeled with DIG by *in vitro* transcription of the

cloned insert using T7 or SP6 RNA-polymerases. The DIG-labeling efficiency was verified by dot-blot technique.

***In situ* hybridization.** Brain sections were prepared as described in the *Immunohistochemistry* section of the *Materials and Methods* except all solutions were made using DEPC-treated water. Sections were cleared of cryoprotective solution by washing in 0.1M phosphate buffer pH7.0. Once cleared the sections were prehybridized in the hybridization buffer (50% formamide, 0.3M NaCL, 20mM Tris-HCL pH8.0, 5mM EDTA pH8.0, 10% dextran sulphate, 1x Denhardt's solution, 0.5mg/ml yeast tRNA, 100mM Dithiothreitol) at 62^{o}C for 1 h. Hybridization was performed at 62^{o}C for 24 hours on a rotating platform using the same buffer with the addition of either BDNF anti-sense or sense probes at a concentration of 120 ng/ml. After stringent washing at 65^{o}C the slides were incubated with an anti-DIG antibody and proceeded to color development with NBT/BCIP substrate following the manufacture's protocol). After the color reaction the sections were rinsed, mounted on slides and air dried. The sections were cleared using a series of methanol / ethanol / xylene and finally covered with VectaMount™ mounting medium. In addition to the sense-riboprobe control, the following controls were performed to validate the *in situ* hybridization results: 1) competition hybridization with an excess concentration of unlabeled anti-sense riboprobe and 2) no anti-DIG antibodies control.

Statistical analysis

All values reported in the text, table and figures represent mean ± SEM. For the comparison between two groups Control and Fluoxetine, Student's unpaired two-tailed *t*-test was used. Statistical analyses of the behavioral tests were performed using repeated-measures ANOVA followed by Student's paired or unpaired two-tailed *t*-test. For the post-hoc matching analysis, the subjects with exactly matching the freezing levels at the "Acquisition" time point in Control and Fluoxetine groups were selected. A *P*-value < 0.05 was considered statistically significant.

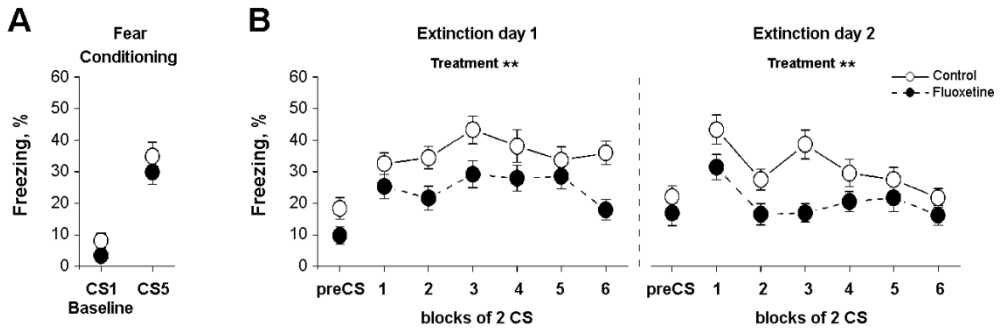

Fig. S1

Chronic fluoxetine treatment prior to fear conditioning did not affect the freezing levels on the fear conditioning day (A). Neither baseline freezing during the first CS1 preceded the first US, nor freezing during the last CS5 were significantly changed. (B) Fluoxetine accelerated the extinction training in the Extinction Context B on the next two days, $^{**}P < 0.01$: Extinction Day 1 $F_{(1,63)} = 7.43$, $P = 0.008$, Extinction Day 2 $F_{(1,63)} = 8.12$, $P = 0.006$ vs. Control group, two-way repeated measures ANOVA. Both Control and Fluoxetine groups (n = 31-34/group) exhibited similar levels of fear acquisition (extinction day 1, first block of 2 CS). In the end of extinction training (extinction day 2, last block of 2 CS), both groups decreased their freezing levels ($P < 0.01$, Control; $P < 0.05$, Fluoxetine, two-tailed paired *t*-test), but there was no significant difference between groups at this time point.

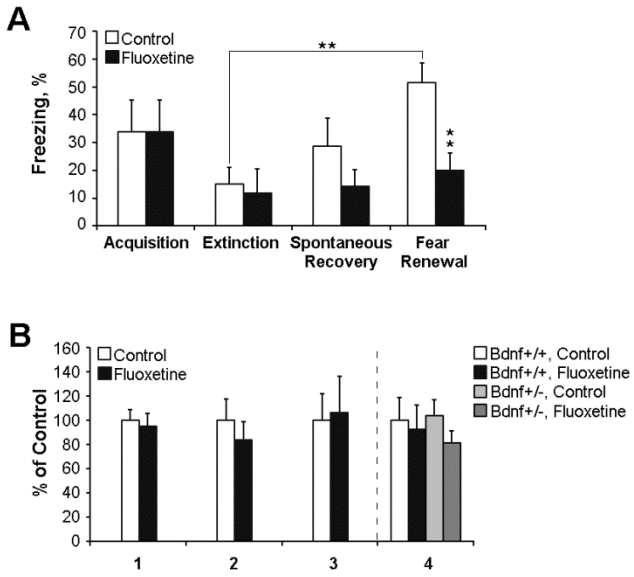

Fig. S2

(A) Post-hoc matching analysis in the experiment 1 “Chronic fluoxetine treatment prior to fear conditioning”. Control and Fluoxetine groups were matched selecting pairs of animals with exactly the same freezing levels at the “Acquisition” time point. One week after successful extinction training, Fluoxetine matched group of mice had no more fearful memory in the fear renewal test. N = 6/group. $^{**}P < 0.01$. (B) Chronic fluoxetine did not affect locomotor activity during the “Acquisition” time point, first block of 2 CS on the first extinction days of experiments 1-4 (see Materials and Methods for a description of the experiments). Locomotor activities of the Fluoxetine groups are presented as % of distance traveled by Control groups in the experiments 1-3 and by “$BDNF^{+/+}$, Control” group in experiment 4.

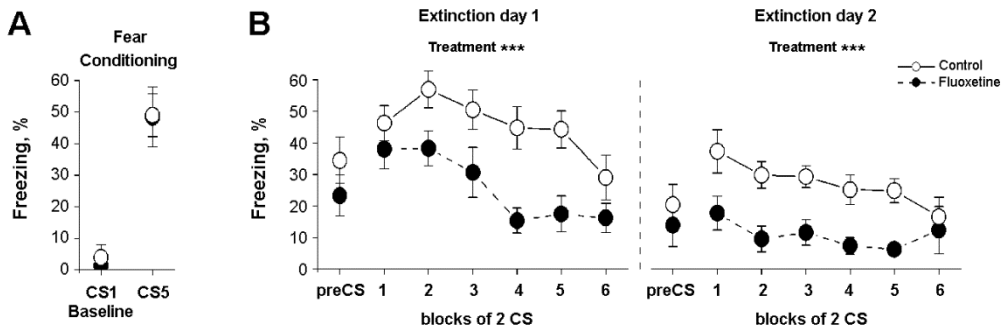

Fig. S3

(A) For Fear Renewal experiment 2 “Chronic fluoxetine treatment after the fear conditioning”, mice were divided into two groups with equal freezing levels during the fear conditioning day. Baseline freezing during the first CS1 preceded the first US. (B) Fluoxetine accelerated the extinction training in the Extinction Context B two weeks later, ***$P < 0.001$: Extinction Day 1 $F_{(1,18)} = 16.58$, $P = 0.0007$, Extinction Day 2 $F_{(1,18)} = 26.58$, $P < 0.0001$ vs. Control group, two-way repeated measures ANOVA. Control and Fluoxetine groups (n = 10/group) exhibited similar levels of fear acquisition (extinction day 1, first block of 2 CS). At the end of extinction training (extinction day 2, last block of 2 CS), both groups had decreased freezing levels ($P < 0.01$ for both groups, two-tailed paired *t*-test), but there was no significant difference between the groups at this time point.

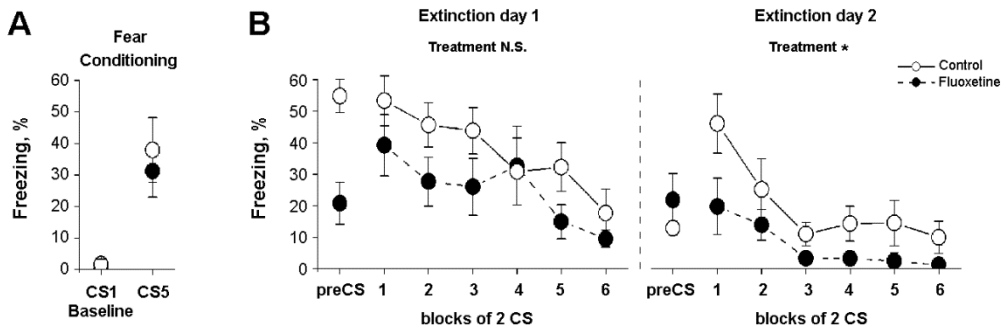

Fig. S4

(A) For Fear Reinstatement experiment 3 “Chronic fluoxetine treatment after the fear conditioning”, mice were divided into two groups with equal freezing levels during the fear conditioning day. Baseline freezing during the first CS1 preceded the first US. (B) Fluoxetine slightly accelerated the extinction training in the Fear Context A two weeks later, N.S. - non-significant, Extinction Day 1 $F_{(1,14)} = 2.11$, $P = 0.17$, Extinction Day 2 $F_{(1,14)} = 7.35$, $P = 0.017$ vs. Control group, two-way repeated measures ANOVA. Control and Fluoxetine groups (n = 8/group) exhibited similar levels of fear acquisition (extinction day 1, first block of 2 CS). At the end of extinction training (extinction day 2, last block of 2 CS), both groups had decreased freezing levels ($P < 0.0001$, Control; $P < 0.01$, Fluoxetine, two-tailed paired *t*-test), but there was no significant difference between the groups at this time point.

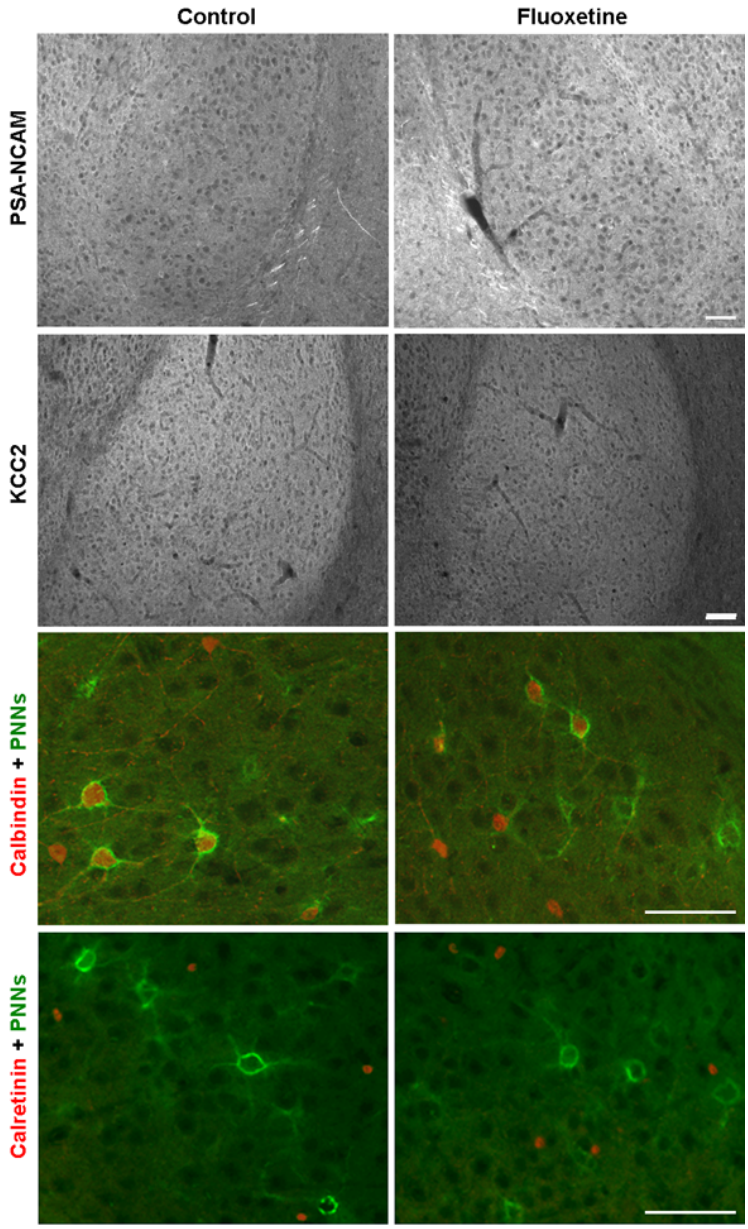

Fig. S5

Representative immunofluorescence staining images of markers specific for immature (PSA-NCAM) or mature (KCC2) neurons in the basolateral amygdala of the control and chronic fluoxetine treated animals. Chronic fluoxetine did not affect the number of the calbindin-positive interneurons with PNNs in the basolateral amygdala. Essentially no colocalization of calretinin-positive interneurons with PNNs was found in either group of animals. Scale bar, 100 μm.

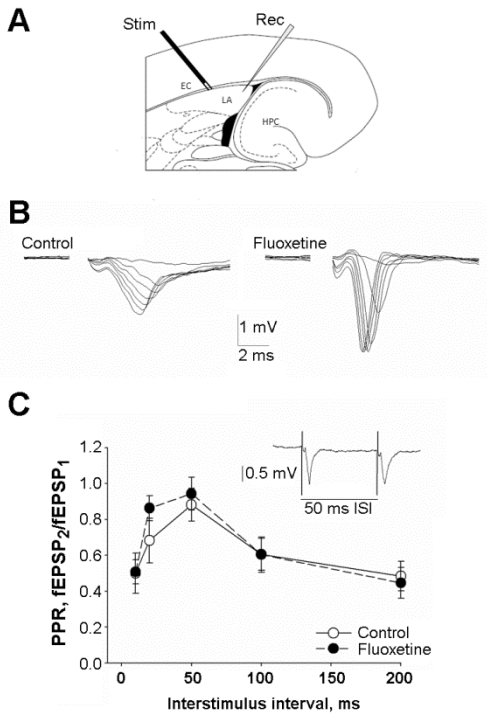

Fig. S6

(A) Schematic illustration of recording configuration showing stimulating electrode placed on the external capsule (EC) and recording electrode in the lateral amygdala (LA). (B) Example traces showing fEPSPs evoked by incremental stimulus intensities (6-24V). Left, control, right, fluoxetine-treated. (C) Paired pulse ratios at all interstimulus intervals tested were not affected by fluoxetine treatment.

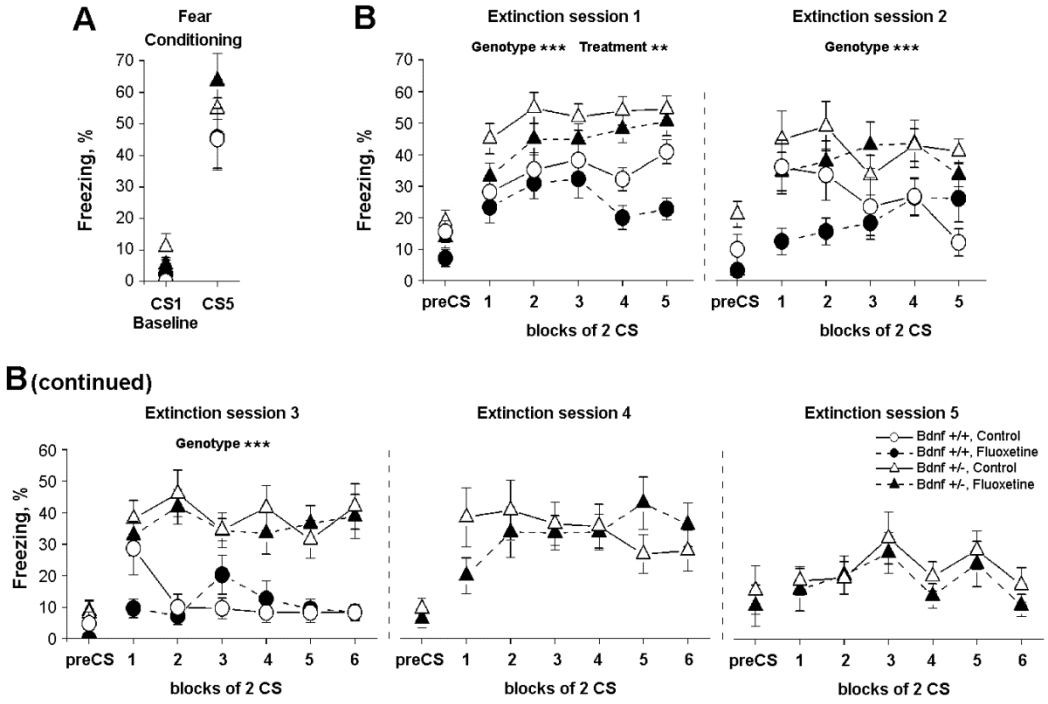

Fig. S7

(A) Both BDNF$^{+/+}$ and BDNF$^{+/-}$ littermates showed significant fear learning on the fear conditioning day. (B) Impaired extinction in the BDNF$^{+/-}$ mice was not improved even in the presence of chronic fluoxetine. Chronic fluoxetine treatment was given after fear conditioning and extinction training was performed two weeks later. N = 10-11/group, *$P < 0.05$, ***$P < 0.001$: Extinction session 1: main effect of genotype $F_{(1,37)} = 38.84$, $P < 0.0001$, main effect of treatment $F_{(1,37)} = 8.73$, $P = 0.004$; Extinction session 2: main effect of genotype $F_{(1,37)} = 23.24$, $P < 0.0001$; Extinction session 3: main effect of genotype $F_{(1,37)} = 60.60$, $P < 0.0001$, two-way repeated measures ANOVA. Circles: Wild-type mice; triangles: BDNF$^{+/-}$ mice; open symbols: vehicle; closed symbols: fluoxetine.

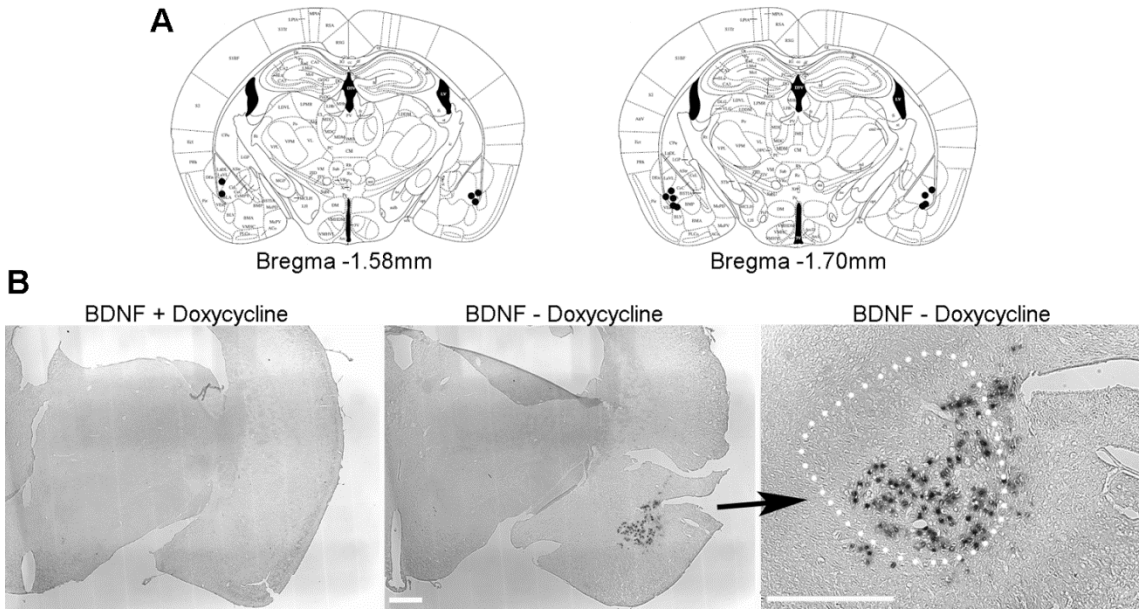

Fig. S8

(A) Centers of sites of the BDNF-lentiviral injections in the “BDNF-Doxycycline” group identified by in situ hybridization with BDNF-riboprobes. (B) Representative images of in situ hybridizations with BDNF-riboprobe showing that the cells expressing the BDNF virus were mostly confined within the basolateral amygdala (indicated by the white dotted line). Bar 500 μm.

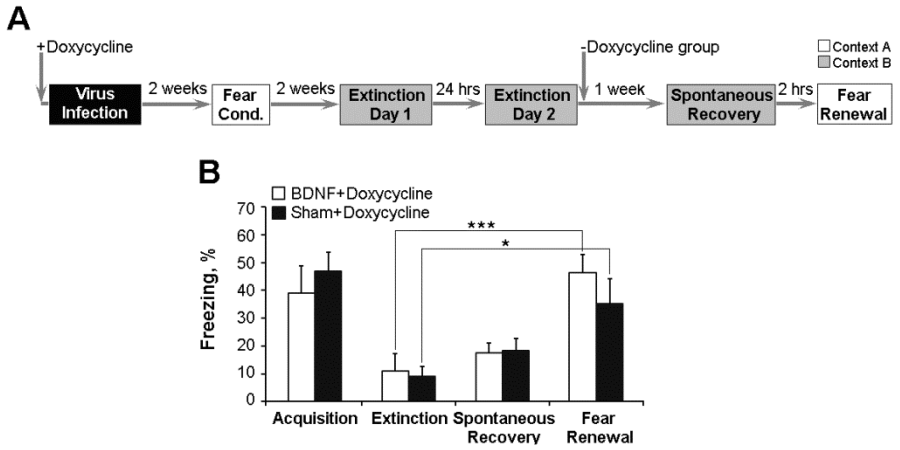

Fig. S9

(A) Protocol of combined BDNF-expressing virus injection into BLA with Fear Renewal experiment. BDNF expression was blocked under "+Doxycycline" state and induced under "-Doxycycline" state. (B) Both control groups (n = 4-6/group), BDNF+Doxycycline with blocked BDNF overexpression and Sham+Doxycycline, had similar levels of fear renewal one week after extinction: ***P < 0.001, BDNF+Doxycycline group; *P < 0.05, Sham+Doxycycline group vs. respective extinguished level of freezing, two-tailed paired t-test.

Table S1. Markers of neuronal plasticity in the fear memory circuitry.

Marker / Brain area	Control	Fluoxetine
Number of cells / section		
PNN+		
BLA	22.13±1.45	24.38±2.14
CA1	26.78±1.69	28.67±0.46
IL	56.47±5.72	58.33±6.29
PV+		
BLA	15.18±1.29	11.80±1.43
CA1	25.53±1.66	22.19±0.70 #
IL	21.86±2.30	23.25±3.19
PV+PNN+		
BLA	9.46±0.85	6.71±0.83 *
CA1	21.78±1.28	20.03±0.51
IL	7.75±0.74	6.42±1.45
% PV+PNN+ / all PNN+		
BLA	42.81±2.88	27.42±2.40 **
CA1	81.55±1.52	69.87±1.49 ***
IL	13.95±1.03	10.48±1.65 #
% PV+PNN+ / all PV+		
BLA	62.81±3.42	58.01±4.17
CA1	85.51±1.39	90.34±0.97 *
IL	37.04±4.45	26.73±4.71
Optical density, % of Control		
PSA-NCAM		
BLA	100±3.95	125.31±8.63 *
CA1	100±8.10	91.12±15.91
IL	100±12.96	83.20±13.24
KCC2		
BLA	100±6.04	50.16±13.75 *
CA1	100±9.71	56.71±12.71 *
IL	100±13.16	73.95±18.82
Additional markers:		
Number of cells / section in BLA		
% CB+PNN+ / all PNN+	39.68±1.26	37.50±3.38
% CR+PNN+ / all PNN+	0.89±0.26	0.55±0.28
NeuN	323.86±16.79	315.51±8.38

PNN+, perineuronal nets positive; PV+, parvalbumin positive; CB+, calbindin positive; CR+, calretinin positive. Data are presented as mean ± SEM, n = 6 animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, #$0.05 < P < 0.1$ vs. Control group, two-tailed unpaired *t*-test.

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