Supporting information

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SI Experimental procedures

Lentivirus production and stereotaxic microinjection.

LV-ERβ-shRNA ERβ shRNA (m) Lentiviral Particles, we named it LV-ERβ-shRNA, and its control shRNA Lentiviral Particles (LV) were purchased (Santa Cruz, CA, USA). LV-ERβ-shRNA is a pool of concentrated, transduction-ready viral particles containing 4 target-specific constructs that encode 19-25 nt (plus hairpin) shRNA designed to knock down gene expression. Each vial contains 200 μl frozen stock containing 1.0×10^6 infectious units of virus (IFU) in Dulbecco's Modified Eagle's Medium with 25 mM HEPES pH 7.3.

LV-CREB133-GFP A recombinant lentivirus LV-CREB133-GFP was generated as described previously (1). The plasmid pCMV-CREB133 (Clontech Laboratories, Mountain View, CA, USA) expresses a mutant variant of the human CREB protein (CREB133) that contains a serine to alanine mutation corresponding to amino acid 133 in the mutant mouse CREB protein. This mutation blocks phosphorylation of CREB, thus preventing transcription. The sequence of CREB133 was amplified by PCR from pCMV-CREB133. The primer sequences were: forward, 5´-GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG ACC ATG GAA TCT GGA G-3´; reverse, 5´-TCA CCA TGG TGG CGA CCG GAT CTG ATT TGT GGC AG-3´. The PCR fragments and the pGC-FU plasmid were digested with Age I and then ligated with T4 DNA ligase to produce pGC-FU-CREB133. GFP was fused to the C-terminal of CREB133. The plasmid was used to transform competent DH5α Escherichia coli bacterial strains for identification. Using 100 μl Lipofectamine 2000, 293T cells were co-transfected with 20 μg of the pGC-FU plasmid with a cDNA encoding CREB133, 15 μg of the pHelper 1.0 plasmid, and 10 μg of the pHelper 2.0 plasmid to generate the recombinant lentivirus, LV-CREB133-GFP. After 48 h, supernatant was harvested from 293T cells, filtered at 0.45 μm, and pelleted by ultracentrifugation at $4000 \times g$ for 10-15min at 4°C. After resuspension, serially diluted lentivirus was used to transduce 293T cells; 4 days later, labeled 293T cells were counted to calculate the viral titer (~ 2×10^8 transducing units/ml). As a control, we also generated a lentiviral vector that expresses GFP alone (LV-GFP).

LV-VP16-CREB-GFP VP16-CREB is a constitutively active CREB protein, which binds to CRE sites, regulates transcription of a number of CREB-dependent genes. Plasmid pSK-VP16-CREB was kindly provided by Prof.Angel Barco (Molecular Neurobiology, Instituto de Neurociencias de Alicante). The sequence of VP16-CREB was amplified by PCR from pSK-VP16-CREB. The primer sequences were: forward,5´-GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG GCG TAC AGC CGC G-3´;reverse,5´-TCA CCA TGG TGG CGA CCG GAT CTG ATT TGT GGC AGT AAA GG -3´. The PCR fragments and the pGC-FU plasmid were digested with Age I and then

ligated with T4 DNA ligase to produce pGC-FU-VP16-CREB. GFP was fused to the C-terminal of VP16-CREB. The plasmid was used to transform competent DH5α Escherichia coli bacterial strains for identification. Using 100 µl Lipofectamine 2000, 293T cells were co-transfected with 20 µg of the pGC-FU plasmid with a cDNA encoding VP16-CREB, 15 µg of the pHelper 1.0 plasmid, and 10 µg of the pHelper 2.0 plasmid to generate the recombinant lentivirus, LV-VP16-CREB-GFP. After 48 h, supernatant was harvested from 293T cells, filtered at 0.45 µm, and pelleted by ultracentrifugation at 4000 × g for 10-15min at 4°C. After resuspension, serially diluted lentivirus was used to transduce 293T cells; 4 days later, labeled 293T cells were counted to calculate the viral titer (~ 2×10^8 transducing units/ml). As a control, we also generated a lentiviral vector that expresses GFP alone (LV-GFP). Each milliliter stock of virus containing 2.0×10^8 IFU.

Stereotaxic microinjection LV-ERβ-shRNA, LV, LV-VP16-CREB-GFP, LV-CREB133-GFP or LV-GFP was infused into the hippocampus of mice by stereotaxic microinjection (coordinates: 2.3 mm posterior to bregma, 1.3 mm lateral to the midline,and 2.0 mm below dura) at 5 nl/s.

Culture of hippocampal neurons.

Hippocampi of postnatal day 0 (P0) ICR mice were removed and placed in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY) containing 1 mM sodium pyruvate and 10 mM HEPES. Then the hippocampal tissues were dissociated in HBSS solution containing 0.125% trypsin solution for 10 min at 37°C. Subsequently, tissues were triturated by repeated passage through a constricted Pasteur pipette. The digestion was stopped with DMEM along with 10% heat-inactivated fetal bovine serum. The dispersed tissues were allowed to settle for 3 min. The supernatant was transferred to a fresh tube and centrifuged at 2000 rpm for 2min. The pellet was resuspended in a neuron-defined culture medium, serum-free neurobasal medium (Gibco), supplemented with B-27, 0.5 mM L-glutamine, 20 IU/ml penicillin and 20 IU/ml streptomycin. The cells were then plated onto 6-well plates coated with poly-D-lysine (100 µg/ml) at 4×10^6 per well. Cell cultures were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Half of the medium was replaced with fresh medium without glutamate every 2-3 days. The purity of neuronal cultures was determined by immunofluorescence using staining with antibody against β-tubulin (1:200; Chemicon International Inc.). The purity of the neurons used to experiments was ~95%.

To investigate dose-dependent effect of E2 on nNOS expression in the cultured hippocampal neurons, we incubated the cultured hippocampal neurons with 10 pM, 10 nM, 10 µM E2 or vehicle for 24 h. It was reported that in the plasma of female rodents E2 concentration was ~20 pM (2). Considering ~50% of the cultured hippocampal neurons from male, we thus chose 10 pM E2 to mimic E2 concentration in the plasma. In the hippocampus E2 was approx. 8 nM for male and 0.5–2 nM for female (2). As ERs are mainly located in neurons in the hippocampus (3), the E2 concentration that neurons in

the hippocampus are exposing to should be higher than the concentration reported (2). We thus chose 10 nM E2 to mimic E2 concentration in neurons of the hippocampus. To test whether E2 overdose affects nNOS expression in the cultured hippocampal neurons, we designed a 10 μM E2 group.

Western blot analysis.

Samples from cultured hippocampal neurons and hippocampal tissues of animals were prepared as described by our previous studies (4). The samples containing equivalent amounts of protein (10 μg) were applied to 8~12 % (8% for nNOS, eNOS; 10% for MR and ERβ, 12% for CREB and p-CREB) acrylamide denaturing gels (SDS-PAGE). The separated proteins were transferred onto nitrocellulose membranes overnight at 4°C. Blotting membranes were incubated with blocking solution (5% nonfat dried milk powder dissolved in TBST buffer (pH 7.5, 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20)) for 1 hour at room temperature, washed three times, and then were incubated with rabbit antiphospho-CREB-ser133 (1:2000; Santa Cruz), or rabbit anti-CREB (1:2000; Sigma-Aldrich), or mouse anti-nNOS (1:1000; Chemicon), or mouse anti-eNOS (1:2000, Chemicon), or rabbit anti-MR (1:3000; Santa Cruz Biotechnology), or mouse anti-ERβ (1:1000; Cell Signaling Technology) in TBST overnight at 4°C. Internal control was carried out using β-actin antibody (1:1000, Sigma-Aldrich). After several washes with TBST buffer, the membranes were incubated for 1 h with horseradish peroxidase-linked secondary antibody (1:10000). The membranes were then processed with enhanced chemiluminescence western blotting detection reagents (Pierce). Chemifluorescence was detected by ChemiDoc™ XRS+ Imaging system (Bio-rad).

Reverse transcription-PCR.

Total RNA was extracted from the hippocampus using Trizol reagent according to the manufacture's instructions (Sigma). The primers for nNOS and β-actin were as follows: For nNOS: forward, 5'-TGT CCT ATA CAG CTT CCA GA-3'; reverse, 5'-CAC GAT GTC ATA TTC CTC CA-3'. For β-actin: forward, 5'-CAC GAT GGA GGG GCC GGA CTC ATC-3'; reverse, 5'-TAA AGA CCT CTA TGC CAA CAC AGT-3'. PCR conditions were 30 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec, and extension at 72 °C for 45 sec. PCR products were separated by electrophoresis through 1.5% agarose gel containing 0.5% μg/ml ethidium bromide and imaged using a BioDoc-IT imaging system (Bio-Rad, Hercules, CA); band intensities were determined using GS-710 calibrated imaging Densitometer (Bio-Rad). The mRNA for β-actin was included in the PCR mixture as a standard.

Novelty suppressed feeding procedure

The novelty suppressed feeding (NSF) procedure is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of brightly lit arena (5). The latency to feed in NSF test has been used to assess the behavioral effects of

antidepressants (6). The NSF test was performed during a 5 min period, as described previously (4). In brief, the testing apparatus consisted of a plastic box (50 × 50 × 20 cm), the floor of which was covered with ~2 cm wooden bedding. Twenty-four hours before behavioral testing, all the food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. Each mouse was placed in a corner of the box, facing the corner, and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches, holding the pellet with its forepaws, and biting the pellet) was timed. Immediately after this test, the animal was transferred to its home cage, and the latency to eat and the amount of food consumed by the mouse in 15 min were measured, serving as a control for change in appetite as a possible confounding factor.

Chronic mild stress and behavioral measures.

The procedure of chronic mild stress (CMS) was designed as described previously (7). In brief, the CMS protocol consists of the sequential application of a variety of mild stressors, including restraint, forced swimming, water and/or food deprivation, and pairing with another stressed animal in wet sawdust, housing in wet sawdust, reversal of the light/dark cycle, housing in constant illumination or darkness each for a period ranging from 10 min to 24 h, in a schedule that lasts for 3 weeks. The schedule is repeated thereafter from week 1. Stress-induced modifications in mice were assessed using immobility time in the tail suspension test (TST) and forced swimming test (FST). The TST and FST were measured as described previously (8). The duration of immobility in the TST and FST was recorded using the Hamilton kinder TS100 on PC computer and Motor-Monitor System SF16R, respectively.

Immunofluorescence

Hippocampal neurons were blocked in PBS containing 3% normal goat serum, 0.3% (w/v) Triton X-100, and 0.1% BSA at room temperature for 1 hour, and incubated with primary antibody at 4°C overnight. The primary antibodies used were mouse anti-nNOS antibody (1:200; Millipore Bioscience Research Reagents) and mouse anti-ERβ (1:200; Cell Signaling Technology). Subsequently, cells were incubated with secondary antibodies goat antirabbit Cy3 (1:200; Chemicon) and goat anti-mouse FITC (1:100; Chemicon) for 2 hours at room temperature. Finally, imaged with a fluorescence microscope (Axio Imager, Zeiss, Oberkochen, Germany), and analyzed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Preparation and maintenance of organotypic hippocampal slice cultures.

Organotypic hippocampal slice cultures (OHSCs) were prepared as described previously (9) with some modification. Briefly, the hippocampus was dissected from decapitated, 15-d-old mouse pups, and cut transversely into 400-μm-thick slices with a McIlwain tissue chopper. The slices were placed on the top of Millicell-CM tissue culture inserts

(0.4 μm pore; Millipore) in six-well plates and maintained at 35°C in a humidified incubator with 5% CO2 for 10–14 d in a culture medium, pH 7.3, composed of 50% MEM, 25% heat-inactivated horse serum, 25% HBSS, and 25 mM HEPES. The medium was changed on the first day after culture and every 3–4 d thereafter.

Total nitric oxide assay

Because NO has an extremely short half-life, we quantified NO by measuring the concentrations of the two stable NO products nitrate and nitrite. Tissues or neurons were collected and analyzed by using the Nitric Oxide Assay Kit according to the protocol of the manufacturer (Beyotime Institute Biotechnology, China). The assay included a process to convert nitrate to nitrite and then to use a Greiss reaction to measure the nitrite concentrations. Absorbance of the samples was measured at 540 nm.

Estrous stages assessment

A full estrous cycle in mice occurs over 4 or 5 days and it can be divided into four stages according to vaginal secretions. In diestrus, the vaginal secretion consists predominantly of leukocytes. In proestrus, there is a predominance of nucleated epithelial cells. It estrus, this stage is distinctively characterized by cornified squamous epithelial cells, which occur in clusters. It metestrus, there is a mix of cell types with a predominance of leukocytes and a few nucleated epithelial and/or cornified squamous epithelial cells. Estrous cycle phase was determined by vaginal smears every morning between 7:00 and 8:00 a.m.

Plasma estradiol and testosterone assay

Blood was obtained from the angulus oculi vessels of mice and collected in heparinized tubes. Plasma was separated from the blood and immediately frozen in liquid nitrogen, and then stored at –80°C. Estradiol and testosterone in plasma was measured by the ACCESS® immunoassay systems (Beckeman-coulter, Inc) according to the instructions of the manufacturer.

Statistical analysis. Data are presented as means ± SEM. Comparisons among multiple groups were made with one-way ANOVA (one factor) or two-way ANOVA (two factors), followed by Scheffe's post hoc test. Comparisons between two groups were made with two-tail Student's t test. Differences were considered significant when $P < 0.05$.

References

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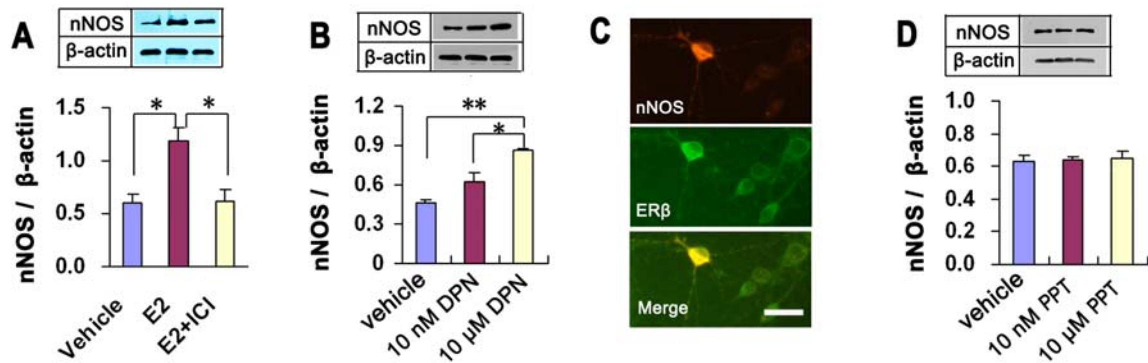

Fig. S1 Estrogen up-regulates nNOS expression in the hippocampus via ERβ. (**A**) Immunoblots showing nNOS levels in the cultured hippocampal neurons treated by 10 nM E2 with or without 1 μM ICI180,720 (a nonselective ERs antagonist) for 24 h (n = 4). Means ± SEM, **P* < 0.05. (**B**) Immunoblots showing nNOS levels in the cultured hippocampal neurons treated by 10 nM or 10 μM DPN (an ERβ selective agonist) for 24 h (n = 3). Means ± SEM, **P* < 0.05; ***P* < 0.01. (**C**) Representative immunofluorescence imaging showing the co-localization of nNOS and ERβ in the cultured hippocampal neurons. Scale bar = 20 μm. (D) Immunoblots showing nNOS levels in the cultured hippocampal neurons treated by 10 nM or 10 μM PPT (an ERα selective agonist) for 24 h (n = 3). Means ± SEM.

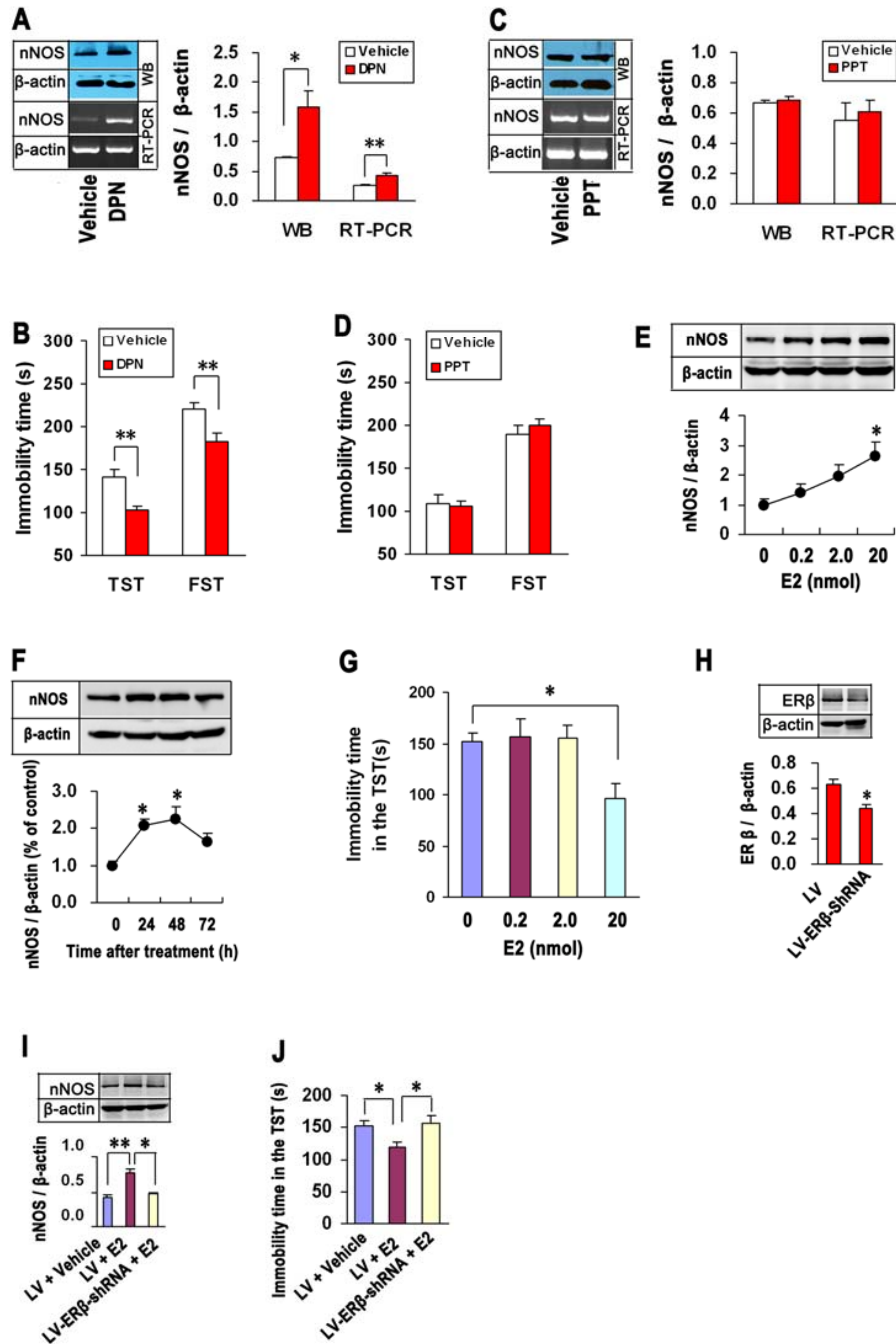

Fig. S2 ERβ activation regulates nNOS expression in the hippocampus and depressive behaviors. (**A-B**) nNOS protein levels and mRNA levels in the hippocampus (n = 4) (**A**) and immobility in the TST and FST (n = 9-10) (**B**) of the OVX-female mice treated with DPN. DPN (20 nmol, 2μl) was delivered by microinjection into the bilateral hippocampi at d 7 after OVX surgery. Hippocampal nNOS levels were measured and immobility was assessed at 48 h after DPN treatment. Means ± SEM, $*P < 0.05$; $**P < 0.01$. (**C-D**)

nNOS protein levels and mRNA levels in the hippocampus (n = 4) (**C**) and immobility in the TST and FST (n = 10-12) (**D**) of the OVX-female mice treated with PPT. PPT (20 nmol, 2μl) was delivered by microinjection into the bilateral hippocampi of mice at d 7 after OVX surgery. Hippocampal nNOS levels were measured and immobility was assessed at 48 h after PPT treatment. (**E**) Immunoblots showing nNOS levels in the hippocampus of the OVX-female mice treated with 0, 0.2, 2 or 20 nmol E2 (intrahippocampal microinjection) at 48 h after treatment (n = 5). Means ± SEM, $*P < 0.05$; compared with 0 nmol. (**F**) Immunoblots showing nNOS levels in the hippocampus of the OVX-female mice treated with 20 nmol E2 at 0, 24, 48 or 72 h after treatment (n = 6). Means ± SEM, $*P < 0.05$; compared with 0 h. (**G**) Immobility time in the TST of the OVX-female mice treated with 0, 0.2, 2 or 20 nmol E2 (intrahippocampal microinjection) 48 h after treatment (n = 8-11). Means ± SEM, $*P < 0.05$. (**H**) Immunoblots showing ERβ level in the hippocampus of the OVX-female mice treated with LV-ERβ-shRNA, a lentiviral vector containing the shRNA of ERβ (n = 3). LV-ERβ-shRNA (2μl) was delivered by microinjection into the bilateral hippocampi of mice at d 7 after OVX surgery. ERβ levels were measured 7 d after LV-ERβ-shRNA infection. Means ± SEM, $*P < 0.05$. Compared with LV group. (**I-J**) Immunoblots showing nNOS levels in the hippocampus (n = 3) (**I**) and immobility time in the TST (n = 9-12) (**J**) of the OVX-female mice treated by E2 with LV-ERβ-shRNA or LV vector. LV-ERβ-shRNA or control lentiviral vector (1 μl) was delivered by microinjection into the bilateral hippocampi of mice at d 7 after OVX surgery. E2 (20 nmol, 1 μl) was delivered by microinjection into the bilateral hippocampi of mice 7 d after LV-ERβ-shRNA or LV vector infection. nNOS expression and immobility were assessed at 48 h after E2 treatment. Means ± SEM, $*P < 0.05$.

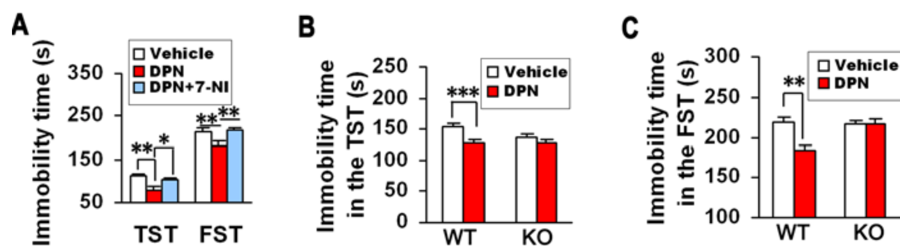

Fig. S3 nNOS is required for ERβ-mediated behavioral effects. (**A**) Immobility time in the TST and FST of the OVX-female mice treated with DPN alone (20 nmol, 1 μl) or in combination with 7-NI (10 nmol, 1 μl) (n = 14-15). DNP was delivered by microinjection into the bilateral hippocampi of mice at d 7 after OVX surgery. Intrahippocampal microinjection of 7-NI was performed at 30 min before DPN treatment. Means ± SEM, $*P < 0.05$; $**P < 0.01$. (**B-C**) Immobility time in the FST (**B**) and TST (**C**) of the nNOS KO and WT (n = 10-11) female mice treated with intrahippocampal DPN (20 nmol, 2 μl). Immobility time was assessed at 48 h after DNP microinjection. Means ± SEM, $**P < 0.01$, $***P < 0.001$.

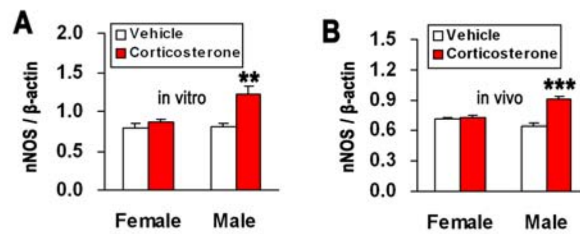

Fig. S4 Glucocorticoid up-regulates nNOS expression in male not in female (Representative immunoblots were shown in **Fig. 3B**). (**A**) Summarized nNOS protein levels in the corticosterone- or vehicle-treated organotypic hippocampal slice cultures from male and female mice (n = 4). Means ± SEM, $^{**}P < 0.01$. (**B**) Summarized nNOS protein levels in the hippocampus of male or OVX-female mice treated with intrahippocampal corticosterone- or vehicle (n = 4). Means ± SEM, $^{***}P < 0.001$, *vs* vehicle.

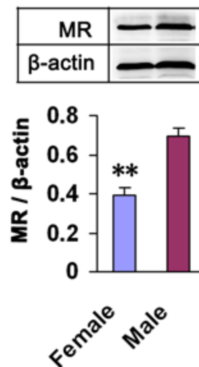

Fig. S5 Immunoblots showing MR levels in the hippocampus of male and OVX-female mice (n = 4). Means ± SEM, $^{**}P < 0.01$, *vs* male.

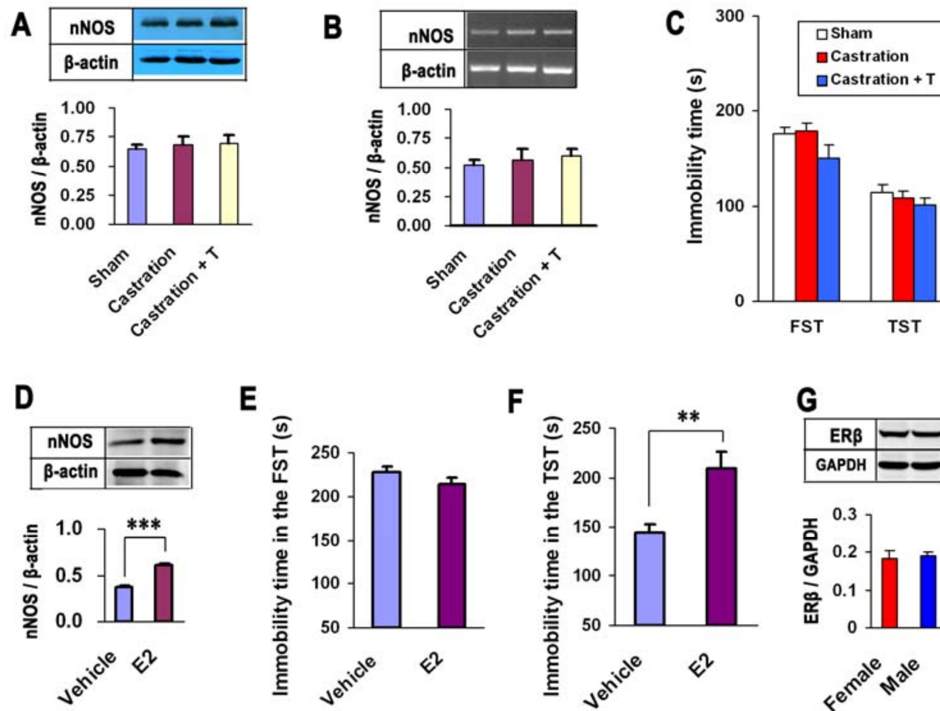

Fig. S6 Effects of gonadal hormone on hippocampal nNOS expression and depressive behaviors. (**A**) Immunoblots showing nNOS levels in the hippocampus of the male mice subjected to castration and treated with testosterone or vehicle (n = 4). (**B**) nNOS mRNA levels in the hippocampus of the male mice subjected to castration and treated with testosterone (n = 3). mRNA were measured by RT-PCR. (**C**) Immobility time in the TST and FST in the male mice subjected to castration and treated with testosterone or vehicle (n = 10-11). Testosterone (160 μg/kg/d, s.c.) was administrated for 7 days starting at 2 h after surgery. T: Testosterone. (**D**) Immunoblots showing nNOS levels in the hippocampus of male mice treated by E2 (160 μg/kg/d, s.c.) for 7 days (n = 3). (E-F) Immobility time in the FST (**E**) and TST (**F**) of male mice treated by E2 (160 μg/kg/d, s.c.) for 7 days (n = 11). (**G**) Immunoblots showing ERβ levels in the male and female hippocampus (n = 4-5). Means ± SEM, $**P < 0.01$, $***P < 0.001$.

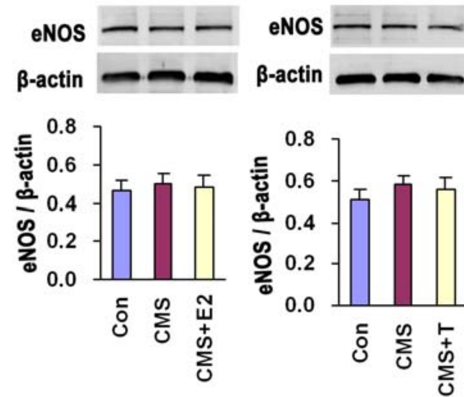

Fig. S7 CMS with or without gonadal hormone treatment has no effect on eNOS expression. Immunoblots showing eNOS levels in the hippocampus of the female mice exposed to CMS for 21 d and treated with E2 (160 μg/kg/d, s.c.) or vehicle (Left) (n = 3), and eNOS levels in the hippocampus of the male mice exposed to CMS for 21 d and treated with testosterone (160 μg/kg/d, s.c.) or vehicle (Right) (n = 3). T: Testosterone. E2: estradiol. Means ± SEM.

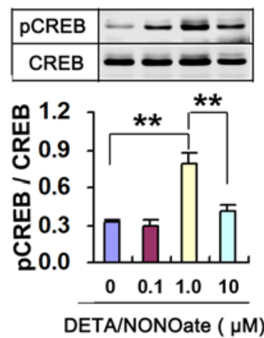

Fig. S8 Immunoblots showing pCREB levels in the cultured hippocampal neurons incubated with DETA/NONOate (n = 4). Means ± SEM, $**P < 0.01$.

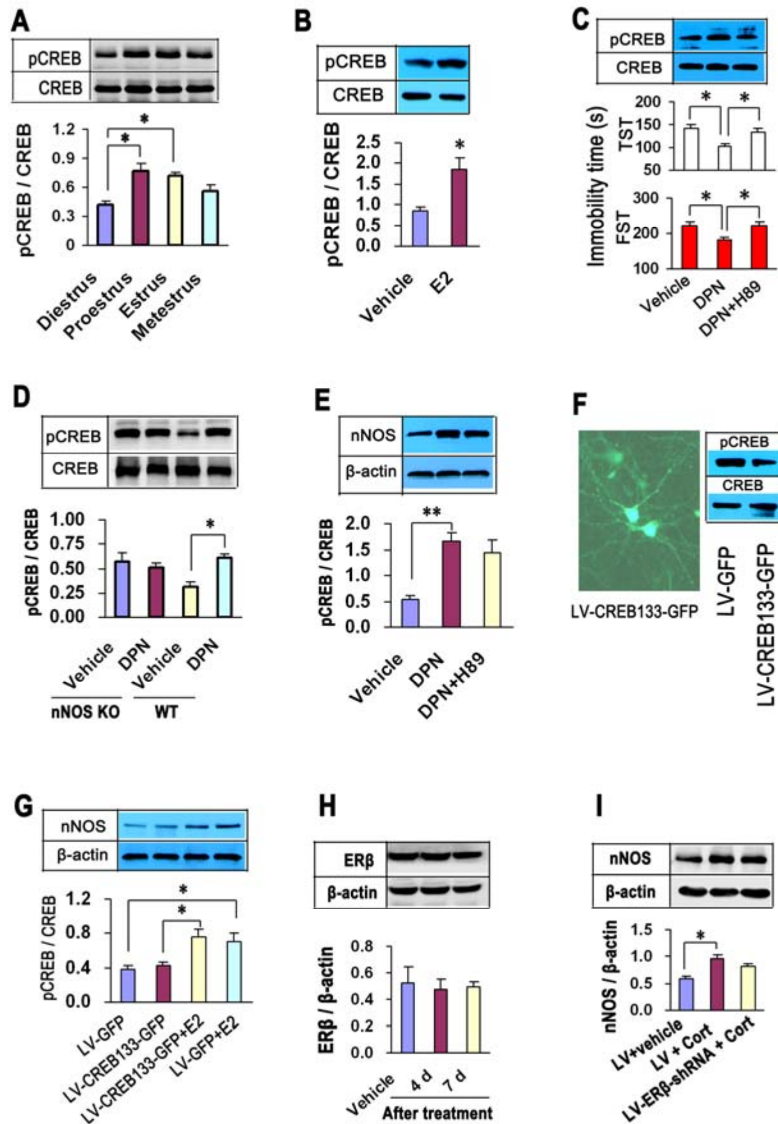

Fig. S9 Estrogen promotes CREB activation via up-regulating hippocampal nNOS expression. (**A**) Immunoblots showing pCREB levels in the hippocampus of the female mice within an oestrous cycle (n = 3). Means ± SEM, **P* < 0.05. (**B**) Immunoblots showing pCREB levels in the hippocampus of the OVX-female mice treated with E2 (160 μg/kg/d, s.c.) or vehicle for 7 d starting at 2 h after surgery (n = 4). CREB and pCREB levels in the hippocampus were measured at 24 h after the last injection of E2. Means ± SEM, **P* < 0.05, vs vehicle. (**C**) pCREB levels in the hippocampus revealed by immunoblots (n = 3) and immobility in the TST and FST (n = 9-10) of the female mice treated by DPN with or without H89, a PKA inhibitor. DPN (20 nmol, 1 μl) was delivered into bilateral hippocampi by microinjection at 30 min after intrahippocampal H89 microinjection (10 nmol, 1 μl). pCREB levels in the hippocampus and immobility time in the TST and FST were measured at 48 h after DPN treatment. Means ± SEM, **P* < 0.05. (**D**) Immunoblots showing pCREB levels in the hippocampus of the nNOS KO

and WT mice treated with intrahippocampal DPN (20 nmol, 2 μl) (n = 3). pCREB levels were measured at 48 h after DNP microinjection. Means ± SEM, *P < 0.05. (**E**) Immunoblots showing nNOS levels in the hippocampus of the female mice treated by DPN with or without H89 (n = 4). DPN (20 nmol, 1 μl) was delivered into bilateral hippocampi by microinjection at 30 min after intrahippocampal H89 microinjection (10 nM, 21 μl). nNOS levels in the hippocampus were measured at 48 after DPN treatment. Means ± SEM, **P < 0.01. (**F**) A representative fluorescence image showing cultured hippocampal neurons infected with LV-CREB133-GFP vectors (left) and representative immunoblots showing CREB and pCREB expressions in the cultured hippocampal neurons (right). (**G**) Immunoblots showing nNOS levels in the cultured hippocampal neurons infected by LV-CREB133-GFP or its control LV-GFP and treated with E2 (n = 4). After infecting with LV-CREB133-GFP and LV-GFP vectors for 5 d, the cultured hippocampal neurons were with incubated with 10 nM E2 for 24 h. E2: estradiol. Means ± SEM, *P < 0.05. (**H**) Immunoblots showing ERβ levels in the hippocampus of male mice treated with corticosterone (40 mg/kg/d, s.c.) for 4 or 7 days (n = 5). (**I**) Immunoblots showing nNOS levels in the hippocampus of male mice treated by corticosterone (40 mg/kg/d, s.c.) for 7 days with LV-ERβ-shRNA or LV vector (n = 5). LV-ERβ-shRNA or control lentiviral vector (2 μl) was delivered by microinjection into the bilateral hippocampi of mice. Corticosterone treatment started at 2 h after LV-ERβ-shRNA microinjection. Cort: corticosterone. Means ± SEM, *P < 0.05.

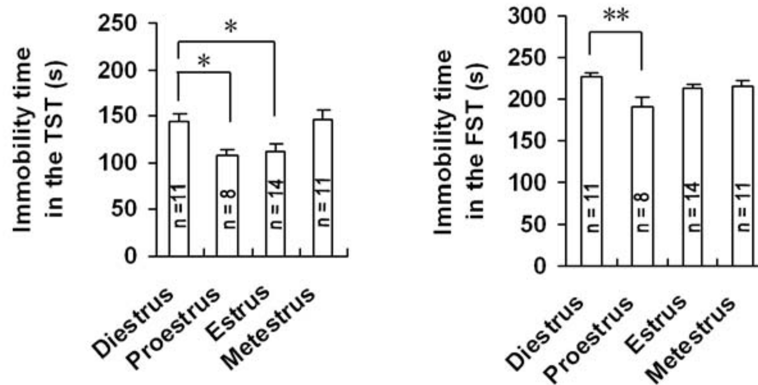

Fig. S10 Association of estrogen fluctuation with depression-like behaviors. Immobility time in the TST (left) and FST (right) was measured in the female mice being during estrous cycle. Means ± SEM, *P < 0.05; **P < 0.01.