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Supplementary Information for

Noradrenaline activation of hippocampal dopamine D_1 receptors promotes antidepressant effects

Katsunori Kobayashi, Kisako Shikano, Mahomi Kuroiwa, Mio Horikawa, Wakana Ito, Akinori Nishi, Eri Segi-Nishida, Hidenori Suzuki

Corresponding author: Katsunori Kobayashi
Email: kkatsu-tky@umin.ac.jp

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Materials and Methods

Animals

Male C57BL/6J mice were purchased from Japan SLC or Charles River Japan. Mice were housed in group of up to 4 unless otherwise stated in the institutional standard condition (14:10 light/dark cycle; lights on at 6:00 A.M. through 8:00 P.M.) with ad libitum access to food and water. Animal use and procedures were in accordance with the National Institute of Health guidelines and approved by the Animal Care and Use Committee of Nippon Medical School, Tokyo University of Science and Kurume University.

Electrophysiological analysis

Mice (9 to 14 weeks old) were decapitated under deep isoflurane anesthesia, and both hippocampi were isolated. Transverse slices (380 μm) were cut from the middle part of the hippocampus unless otherwise specified using a tissue slicer (7000smz, Campden Instruments Ltd., Leics., UK) in ice-cold saline (see below). Slices were then incubated for 30 min at 30 ˚C and maintained in a humidified interface holding chamber at room temperature before use. Electrophysiological recordings were made in a submersion-type chamber maintained at 27.0 to 27.5 ˚C and superfused at 2 ml/min with recording saline composed of (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.0; $NaHCO_3$, 26.2; glucose, 11; $CaCl_2$, 2.5; $MgCl_2$, 1.3 (equilibrated with 95% O_2 / 5% CO_2). Field excitatory postsynaptic potentials (EPSPs) arising from the mossy fiber (MF) synapses were evoked by stimulating the dentate granule cell layer with bipolar tungsten electrodes and recorded from the stratum lucidum of the CA3 region using a glass pipette filled with 2 M NaCl. The amplitude of field EPSPs was measured as described (1). A criterion used to identify the MF input was more than 85% block of EPSPs by an agonist of group II metabotropic glutamate receptors, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV, 1 μM). Single electrical stimulation was delivered at a frequency of 0.05 Hz unless otherwise stated. Triple-pulse stimulation at the interval of 200 ms was delivered at 0.033 Hz (1). For recording EPSPs and population spikes (PSs) in the dentate gyrus (DG), the recording electrode was placed in the granule cell

layer, and the medial perforant path was stimulated every 60 s by the electrode placed in the middle third of the molecular layer. For recording EPSPs and PSs in the CA1 region, the recording electrode was placed in the pyramidal cell layer, and the Schaffer collateral/commissural fibers were stimulated by the electrode placed in the stratum radiatum. The initial slope of EPSPs was measured on analysis. The amplitude of PSs was measured as the difference between the negative peak and the average of two positive peaks. In the experiments using SCH23390, slices were maintained in the saline containing SCH23390 (50 nM) more than 1 hour, and then recordings were made in the normal recording saline (1,2). Other antagonists were added in the bath at least 20 min before application of noradrenaline or other agonists. All recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), filtered at 2 kHz and stored in a personal computer via an interface (digitized at 10 kHz). Noradrenaline was purchased from Nacalai Tesque (Kyoto, Japan). Dopamine and propranolol were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Isoproterenol was purchased from Tokyo Chemical Industry (Tokyo, Japan). Fenoterol was purchased from Cayman Chemical (Ann Anbor, Michigan, USA). Serotonin hydrochloride, phentolamine and labetalol were purchased from Sigma-Aldrich. DCG-IV, SKF83566, SCH23390, CGP20712, ICI118551, and nisoxetine were purchased from Tocris Bioscience (Bristol, UK).

Electroconvulsive treatment

Bilateral electroconvulsive treatment (ECT; 25 mA, 0.5 ms delivered at 100 Hz for 1 s) was administered to mice at the age of 9 weeks using a pulse generator (ECT Unit; Ugo Basile, Italy) via moistened ear-clip electrodes. In order to avoid sudden unexpected death associated with ECT-induced immediate seizures, mice were anesthetized with isoflurane (1.5 to 2%). In repeated treatments, ECT was administered 4 times a week for up to 3 weeks. Mice did not show spontaneous seizures in their home cages during the course of treatments. The sham-treated control mice were handled in an identical manner to the ECT-treated mice without the administration of shock.

Phosphorylation analysis

Coronal slices (350 μm) were prepared from male C57BL/6J mice (Charles River Japan), and the regions of the DG or the striatum were dissected from the slices as described (3,4). Each slice was placed in a polypropylene incubation tube with 2 ml fresh Krebs-HCO_3^- buffer containing adenosine deaminase (10 μg/ml). The slices were preincubated at 30 ˚C under constant oxygenation with 95 % O_2/5% CO_2 for 60 min. The buffer was replaced with fresh Krebs-HCO_3^- buffer after 30 min of preincubation. Adenosine deaminase was included during the first 30 min of preincubation. Slices were treated with drugs as specified in each experiment. Noradrenaline was purchased from Nacalai Tesque. Propranolol was purchased from Sigma-Aldrich. SKF83566 was purchased from Tocris Bioscience. Slices were used to determine the levels of protein phosphorylation (3). Slice samples from the DG and striatum were loaded onto 10% SuperSep™ Ace (FUJIFILM Wako Chemicals, Osaka, Japan) or 4-20% Criterion™ TGX™ Precast Gel (Hercules, CA), separated by electrophoresis, and then transferred to nitrocellulose membranes (0.2 μm) (Sigma-Aldrich). Antibodies used for immunoblotting are as follows: phospho-Thr34 DARPP-32, a site phosphorylated by PKA (D27A4, 1:1000 dilution, Cell Signaling Technology, Danvers, MA); phospho-Ser845 GluA1, a site phosphorylated by PKA (p1160–845, 1:250 dilution, PhosphoSolutions, Aurora, CO); phospho-Thr202/Tyr204 ERK, the site phosphorylated by MEK (#9101, 1:5000 dilution, Cell Signaling Technology, Danvers, MA). Antibodies generated against DARPP-32 (C24–5a, 1:20,000 dilution, The Rockefeller University), GulA1 (E-6, 1:250 dilution, Santa Cruz Biotechnology, Dallas, TX) and ERK (#9102, 1:1,000 dilution, New England BioLabs, Beverly, MA) were used to determine the total amount of proteins.

Plasmids

A plasmid for mammalian expression of mouse dopamine D_1 receptor fused at its N terminal to FLAG peptide was kindly provided by Dr Furuyashiki (5). A plasmid for mammalian expression of mouse β_1 adrenergic receptor fused at its N terminal to 3xHA peptide (pM06 Mm33869) was purchased from Gene Copoeia (Rockville, USA). A plasmid for mammalian expression of mouse β_2

adrenergic receptor fused at its N terminal to 3xHA peptide was generated as fellows. First, we obtained cDNA encoding Adrb2 from the total RNA of mouse brain RNA by reverse transcription-PCR using the two following primers (5'-TTTGAATTCATGGGGCCACACGGGAAC–3' and 5'-TTT CTCGAGTTACAGTGGCGAGTCATTTGTAC– 3'). This PCR fragment was digested with EcoRI and XhoI and ligated to the fragment of pM06 Mm33869 plasmid after EcoRI and XhoI digestion. The sequence of the open reading frames of the plasmid was confirmed by DNA sequencing using the Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA).

Cell culture and transfection

HEK-293T (American Type Culture Collection, Manassas, VA) cells were maintained in Dulbecco's Modified Eagle's Medium (Wako, Japan) supplemented with 5% Equa FETAL (Atlas Biologicals, Inc., Fort Collins, CO, USA) in a humidified atmosphere with 5% CO_2 at 37 ºC. Plasmids were transfected to HEK293T cells with polyethylenimine (PEI) MAX (Polysciences, Inc.). For cAMP assay, 24 hours after DNA transfection, cells were re-plated on 24 well dishes and maintained for 24 hours. For immunoprecipitation assay, cells maintained for 24 hours after DNA transfection.

cAMP measurement

After cells were washed in Krebs HEPES buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 15 mM HEPES, 11 mM D-glucose, 2.2 mM $CaCl_2$, 100 µM) with RO20-1724 (Cayman chemical, Ann Arbor, MI), prewarmed Krebs HEPES buffer containing RO20-1724 and an agonist was applied to the cells at 37 ºC for 30 min. After the buffer was removed, cells were lysed with 0.1 M HCl for 20 min at room temperature. cAMP levels in supernatants were measured using the cAMP Select ELISA Kit (Cayman) according to manufacturer's protocol.

Immunoprecipitation

At 24 hours after transfection, HEK293T cells were resuspended and solubilized in the immunoprecipitation buffer (150 mM NaCl, 2 mM EDTA, 0.5%

NP-40, 20 mM Tris-HCl pH 7.4, 10% Glycerol) with 1% protease inhibitor (Nacalai Tesque) for 20 min at 4 ºC followed by brief sonication on ice. Following centrifugation at 12,000g for 10 min at 4 ºC, supernatants containing 300 µg of proteins were incubated with 6 µg of mouse anti-FLAG antibody (F1804, Sigma-Aldrich) or mouse IgG isotype control (Cat# 5415, Cell Signaling Technology) overnight at 4 ºC. The hippocampal samples were homogenized with a glass Potter homogenizer in the immunoprecipitation buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% NP-40, 2 mM EDTA, 10% Glycerol, 1% TritonX-100) with 1% protease inhibitor (Nacalai). Following centrifugation at 10,000g for 20 min at 4 ºC, supernatants containing 300 µg of proteins were incubated with 2 µg of rabbit anti-β_1 antibody (GTX23546, GeneTex, Inc., Irvine, CA, USA) or rabbit IgG isotype control (GTX35035, GeneTex) overnight at 4 ºC. The immune complex with anti-FLAG or anti-β_1 antibody was precipitated by the Protein A (anti-FLAG antibody) or Protein G (anti-β_1 antibody) magnetic Beads (Dynabeads Protein A/G Stater Pack; DB10015, VERITAS, Tokyo, Japan) according to the manufacture's protocol. Precipitated proteins were eluted in the 4 x SDS buffer (240 mM Tris HCl pH 6.8, 40% Glycerol, 8% SDS, 0.04% BPB, 5% 2-Mercaptoethanol) for 10 min at room temperature. Western blotting was performed as previously described (24) using rat anti-HA antibody (1:1000, Cat 11867431001, Roche), rat anti-D_1 antibody (1:1000, Cat D2944, Sigma-Aldrich) as the first antibodies and horseradish peroxidase-conjugated antibody for anti-rat IgG (1:5000, Cat 112-035-003, Jackson ImmunoResearch Lab) as the second antibody. The signal intensity was calculated by LAS-4000 (GE Healthcare Life Science).

Calbindin expression analysis

The expression analysis of Calbindin protein in the DG by Western blotting was performed as previously described (6) using mouse anti-Calbindin-D-28k antibody (1:3000, C9848, Sigma-Aldrich) and anti-β actin antibody (1:3000, MA5-15739, Thermo Fisher Scientific). The signal intensity was calculated by LAS-4000 (GE Healthcare Life Science).

Home cage activity monitoring and stress procedure

Mice (8 weeks of age) were singly housed in the cage equipped with an infrared video camera at the top, and horizontal activity in the cage was continuously monitored as described (7). The nocturnal activity (activity during the dark period) was evaluated in quantitative analyses. To assess the effects of chronic restraint stress, mice were placed in a 50 ml conical tube with breathing holes for 4 h a day. The stress procedure started after recording the baseline activity level and lasted for 4 weeks. This stress protocol was based on the previous study that demonstrated augmenting effects of a D_1 receptor agonist on behavioral effects of fluoxetine (4). To assess the effects of wheel running combined with other treatments, the baseline activity level was monitored before placement of a plastic running wheel (15 cm in diameter). The wheel running activity was not monitored. During the 4th week of treatments, the running wheel was removed for 24 h to monitor the horizontal activity.

Behavioral experiments

Mice were transferred to a behavioral testing room and allowed to acclimatize to the environment of the room for at least 1 h 30 min before starting behavioral tests. Room temperature was kept at 23 ± 0.5 ˚C. All tests were performed between 15:00 P.M. and 17:00 P.M. The open-field test and tail suspension test were performed as described (7,8). Two tests were sequentially performed on different days at the end of the period of chronic restraint stress. Only one test was conducted for each mouse in a day. In the tail suspension test, one mouse climbed the tail during the test and was excluded from the analyses.

Drug treatment

Desipramine hydrocholoride (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in drinking water and orally applied at a dose of 30 mg/kg/day for 4 weeks. SKF83566 was also orally applied at a dose of 1 mg/kg/day. Mice were singly housed. The drug solutions were prepared every day, and the concentrations of drugs were determined for individual mice on the basis of the water consumption during the preceding 24 h and the body weight measured every other day. Since desipramine reduced water intake, saccharin (0.2%) was included in the desipramine solution in most cases to

keep water intake comparable to the baseline. Saccharin itself has no effects on electrophysiological properties, behavior, or molecular marker expression assessed in the present study (7,9).

Statistics

All data are presented as means ± SEM. Statistical analyses were performed using GraphPad Prism version 7.01. Experiments with two groups were compared with unpaired two-tailed Student’s t test unless otherwise specified. Differences from the specific values were assessed using one sample t test. Experiments with more than two groups were subjected to one-way ANOVA or two-way ANOVA, followed by the Tukey’s, Bonferroni's or Dunnett’s test. Statistical significance was set at $P < 0.05$.

Figures

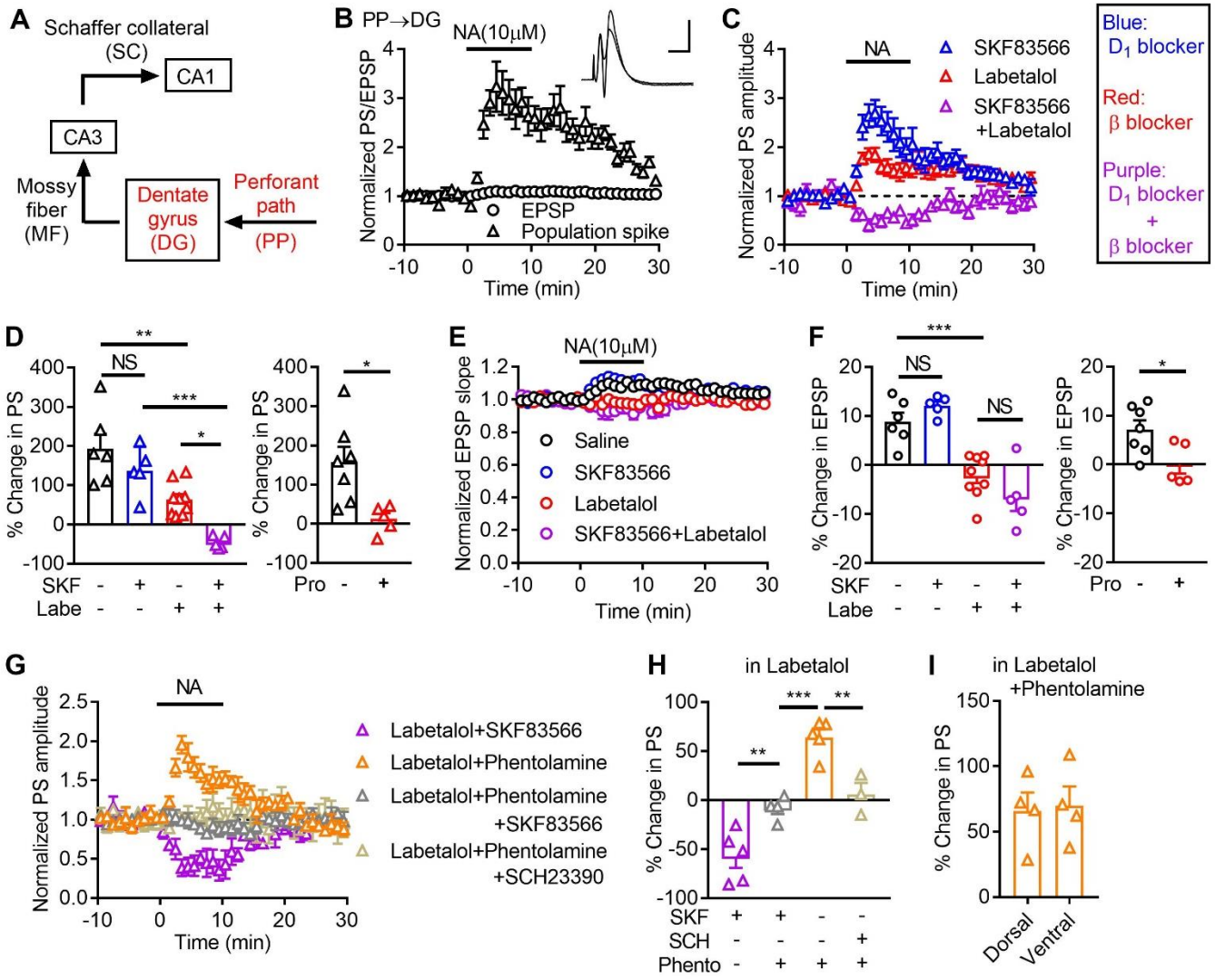

Fig. S1. Noradrenaline facilitates spike generation in the dentate gyrus via D_1 receptor activation. (A) Schematic diagram of hippocampal excitatory circuit highlighting perforant path (PP) to dentate gyrus (DG) connection. (B) Effects of noradrenaline (NA) on field EPSPs and population spikes (PSs) evoked by perforant path stimulation in DG. Sample traces show averaged field potentials before and during NA application. Scale bars: 10 ms, 0.5 mV. (C) Effects of D_1 and β receptor antagonists on NA-induced potentiation of PSs. (D) Summary of antagonist effects on NA-induced changes in PS. Labe: labetalol (20 μM). Pro: propranolol (1 μM). Left: two-way ANOVA (SKF effect, $F_{1,21}$ = 11.69, P = 0.0026; Labe effect, $F_{1,21}$ = 41.37, P < 0.0001) followed by Tukey's

test (NS: not significant, *P = 0.0156, **P = 0.0022, ***P = 0.0004). Right: Student's t test (t_{10} = 2.973, *P = 0.014). (E) Effects of NA on PP-DG EPSPs in the presence and absence of D_1 and β receptor antagonists. (F) Summary of effects of D_1 and β receptor antagonists on NA-induced potentiation of perforant path EPSPs. Left: two-way ANOVA (Labe effect, $F_{1,21}$ = 63.32, P < 0.0001; SKF effect, $F_{1,21}$ = 0.08101, P = 0.7787) followed by Tukey's test (NS: not significant, ***P = 0.0008). Right: Student's t test (t_{10} = 2.602, *P = 0.0264). (G) Effects of D_1 receptor antagonists on NA-induced potentiation of PSs in the presence of labetalol and/or phentolamine. (H) Summary of effects of D_1 receptor antagonists on NA-induced potentiation of PSs in the presence of labetalol and/or phentolamine. One-way ANOVA ($F_{3,13}$ = 30.68, P < 0.0001) followed by Bonferroni's test (**P < 0.01, ***P = 0.0004). (I) Comparison of NA-induced potentiation of PSs mediated by D_1 receptors between dorsal and ventral parts of the hippocampus.

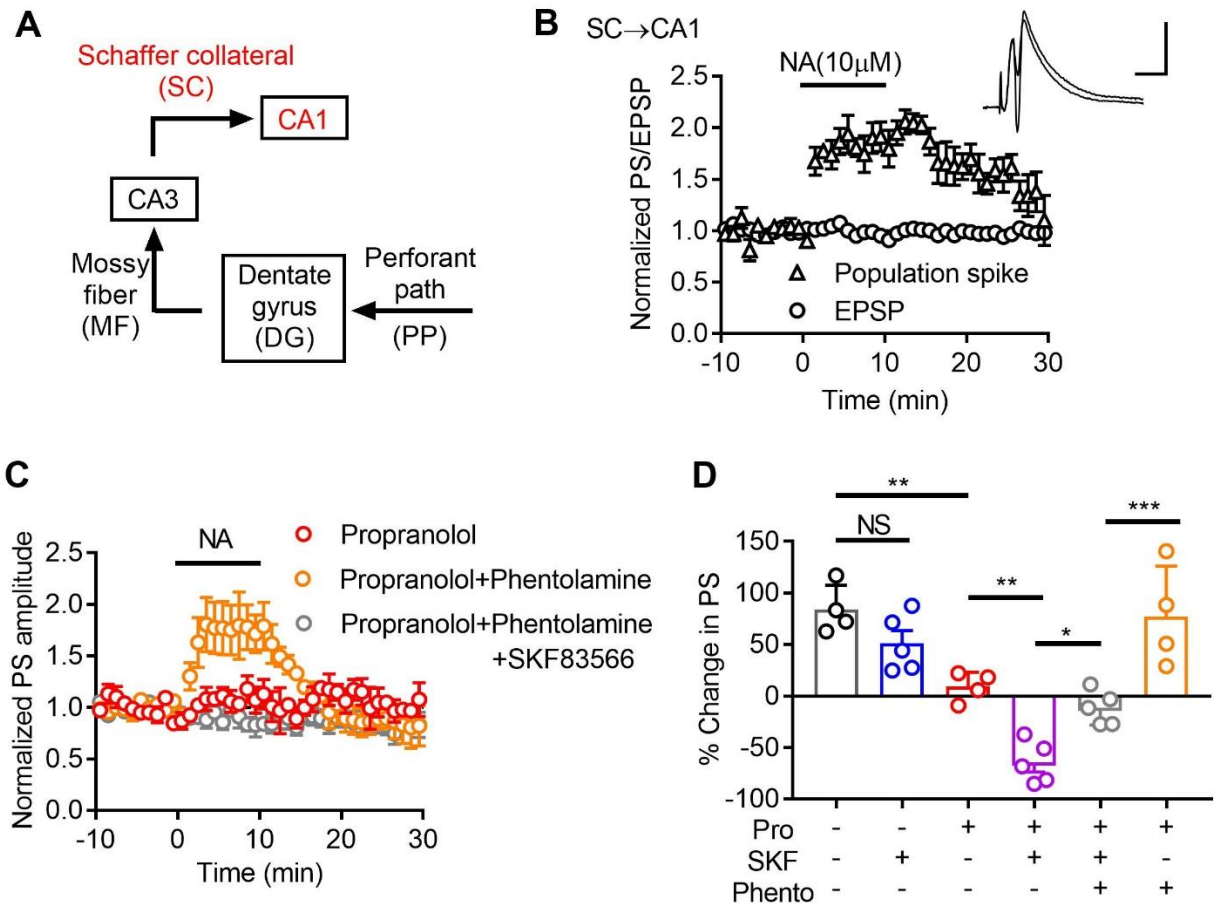

Fig. S2. Effects of noradrenaline on EPSPs and population spikes evoked by Schaffer collateral stimulation in the CA1 region. (A) Schematic diagram of hippocampal excitatory circuit highlighting Schaffer collateral to CA1 connection. (B) Effects of NA on EPSPs and population spikes (PSs) evoked by Schaffer collateral/commissural fiber stimulation in CA1. Sample traces shows averaged field potentials before and during NA application. Scale bars: 10 ms, 0.5 mV. (C) Effects of propranolol (Pro, 10 μM), phentolamine (Phento, 20 μM) and SKF83566 (SKF, 200 nM) on NA-induced potentiation of PSs. (D) Summary of effects of adrenergic and dopamine D_1 receptor antagonists on NA-induced potentiation of PSs. One-way ANOVA ($F_{5,21} = 20.76$, $P < 0.0001$) followed by Bonferroni's test (NS: not significant, *P = 0.0264, **P < 0.005, ***P = 0.0004).

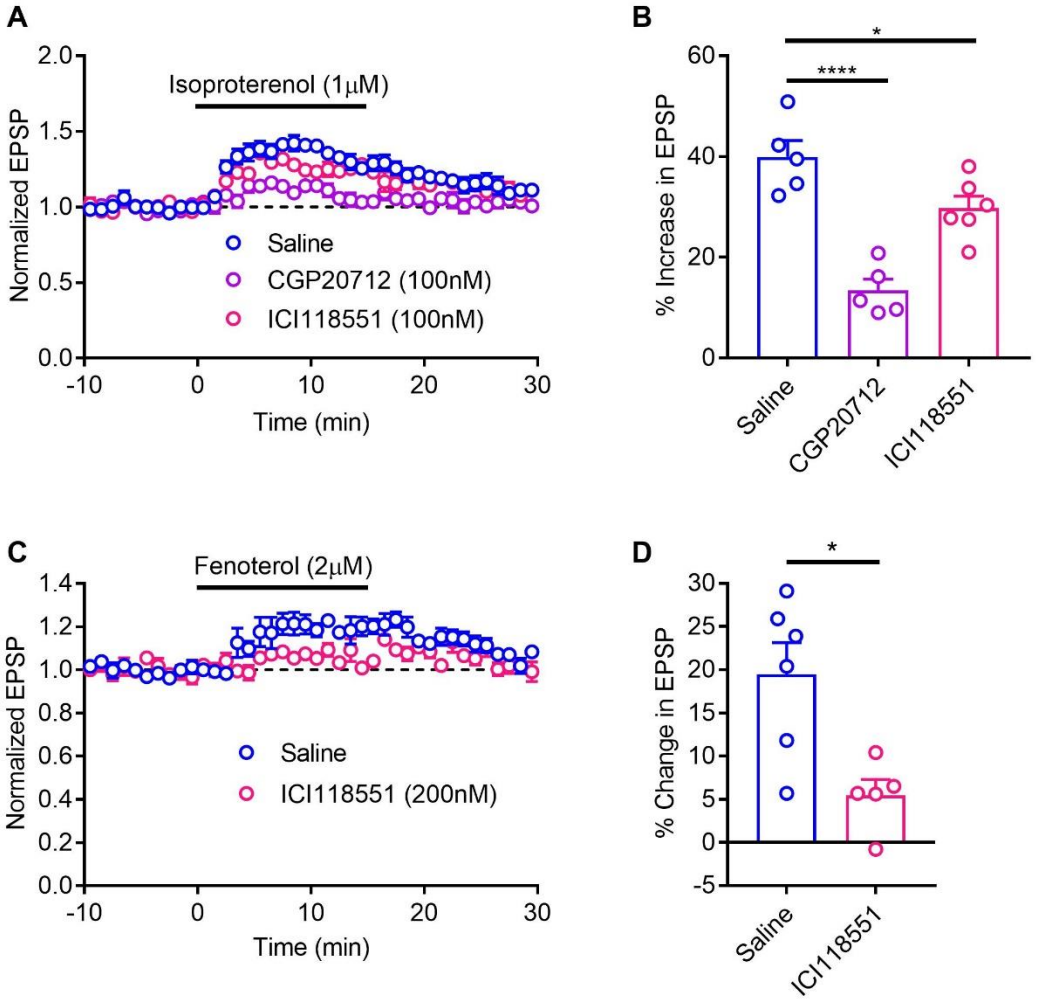

Fig. S3. Synthetic β receptor agonists can potentiate MF synaptic transmission via β_2 receptors in naive mice. (A) Effects of the β receptor agonist isoproterenol on MF synaptic transmission recorded in normal saline or in β receptor antagonists. (B) Summary of effects of β receptor antagonists on isoproterenol-induced potentiation. One-way ANOVA ($F_{2,13}$ = 24.15, P < 0.0001) followed by Dunnett's test (*P = 0.0295, ****P = 0.0001). (C) Effects of the β_2 receptor-specific agonist fenoterol on MF synaptic transmission. (D) Summary of effects of the β_2 antagonist on fenoterol-induced potentiation (t_9 = 3.204, *P = 0.0108).

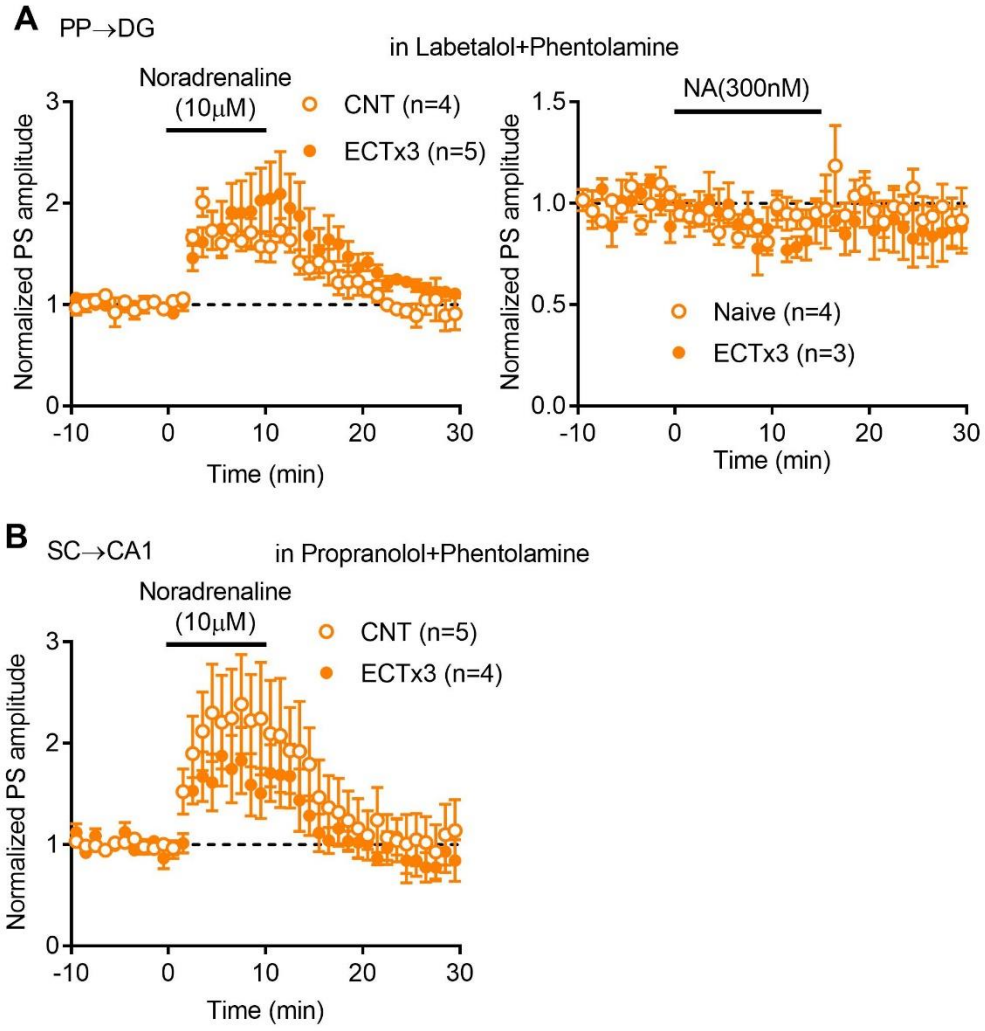

Fig. S4. Effects of ECT on D_1 receptor-dependent potentiation of population spikes. (A) Effects of ECT on D_1 receptor-mediated component of NA-induced PS potentiation in DG. The D_1 component was pharmacologically isolated using labetalol and phentolamine. Left: PS potentiation by 10 μM noradrenaline showed significant time × treatment interaction (two-way repeated measure ANOVA: $F_{7,49} = 3.729$, $P = 0.0026$). Right: Submicromolar noradrenaline in the presence of nisoxetine had no detectable effects on PSs even after ECT. (B) Effects of ECT on D_1 receptor-mediated component of NA-induced PS potentiation in CA1.

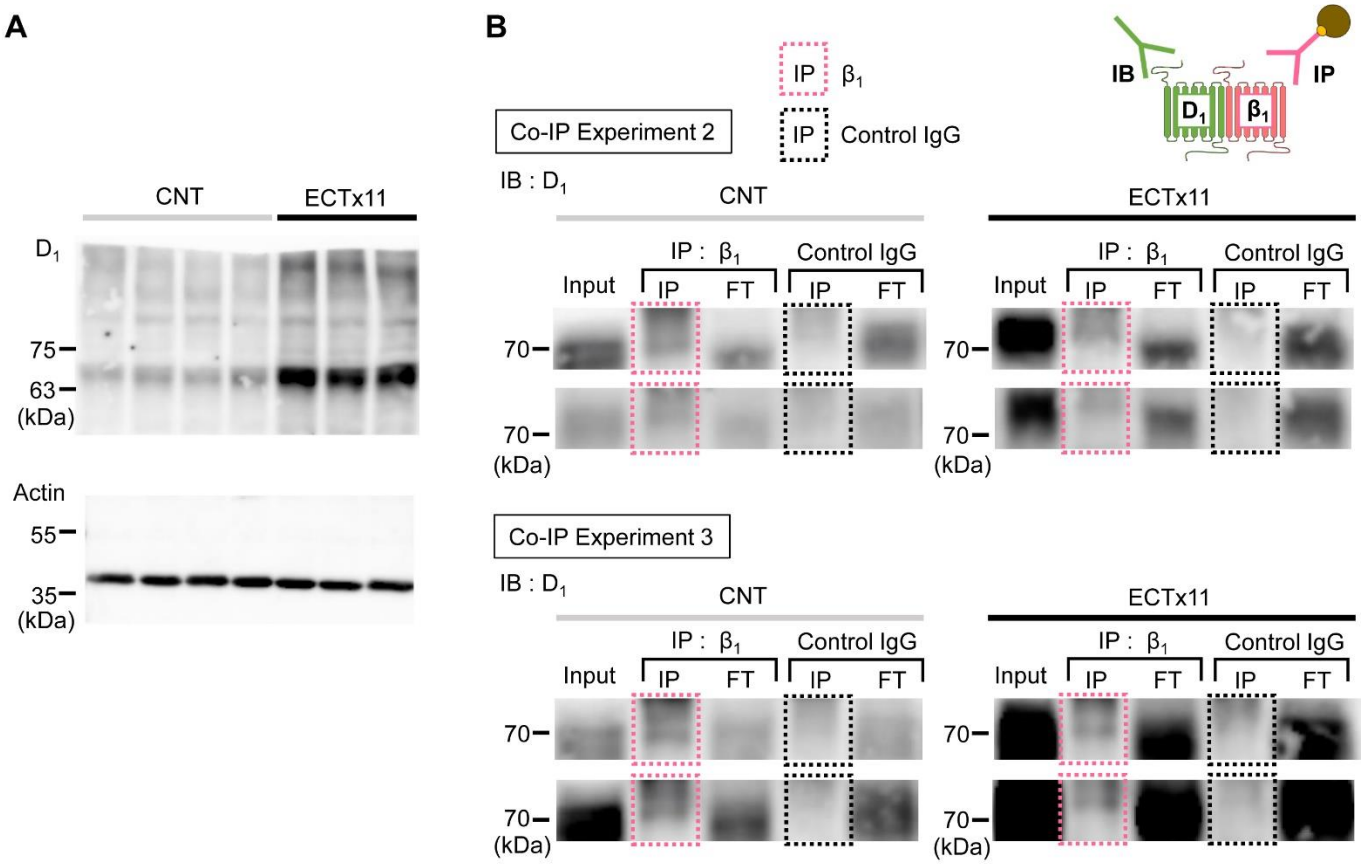

Fig. S5. Hippocampal D_1 receptor expression and co-immunoprecipitation with β_1 receptors. (A) Immunoblots with D_1 or actin antibodies in control mice and mice treated with 11 times of ECT (ECTx11). (B) Co-immunoprecipitation (Co-IP) of hippocampal D_1 and β_1 receptors in control and ECTx11-treated mice. FT: flow through.

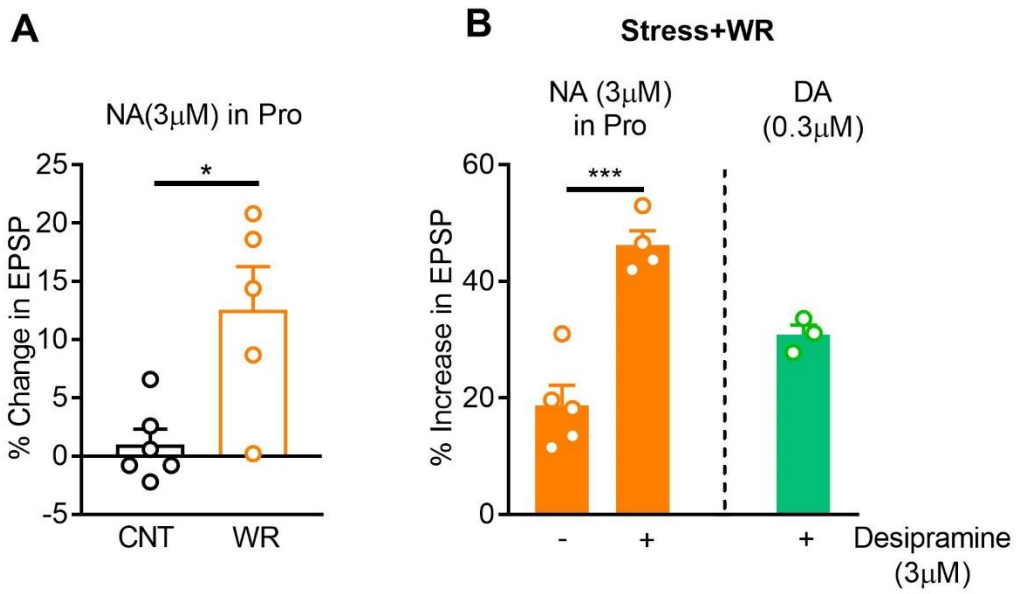

Fig. S6. D_1 receptor-dependent synaptic potentiation induced by low micromolar noradrenaline in mice subjected to wheel running. (A) Effects of NA (3 μM) in the presence of propranolol on MF synaptic transmission in control mice and mice subjected to wheel running (WR) ($t_9 = 3.165$, *P = 0.0115). (B) MF synaptic potentiation by low concentrations of NA and dopamine (DA) in mice subjected to chronic restraint stress and wheel running. Desipramine applied in the bath significantly increased NA-induced synaptic potentiation in the presence of propranolol ($t_7 = 6.25$, ***P = 0.0004).

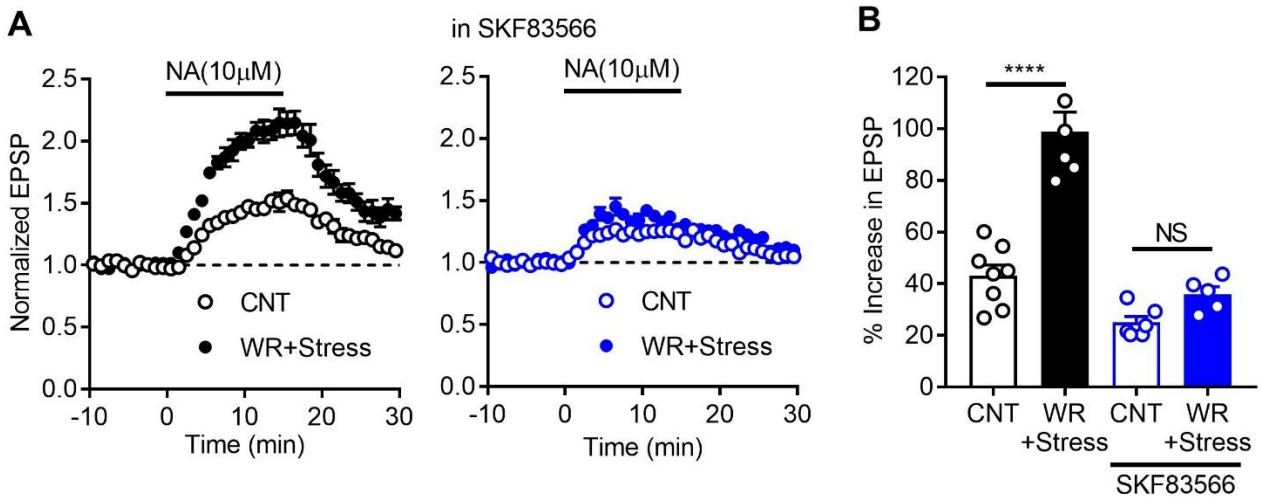

Fig. S7. Chronic stress combined with wheel running enhances noradrenaline-induced synaptic potentiation mediated by D_1 receptors. (A) Effects of chronic restraint stress and WR on NA-induced potentiation of MF synaptic transmission in normal saline (left) and SKF83566 (right). (B) Summary of effects of stress and WR. Two-way ANOVA (WR+stress effect, $F_{1,21} = 46.69$, $P < 0.0001$; SKF83566 effect, $F_{1,21} = 68.97$, $P < 0.0001$; Interaction, $F_{1,21} = 21.11$, $P = 0.0002$) followed by Bonferroni's test (NS: not significant, ****$P < 0.0001$).

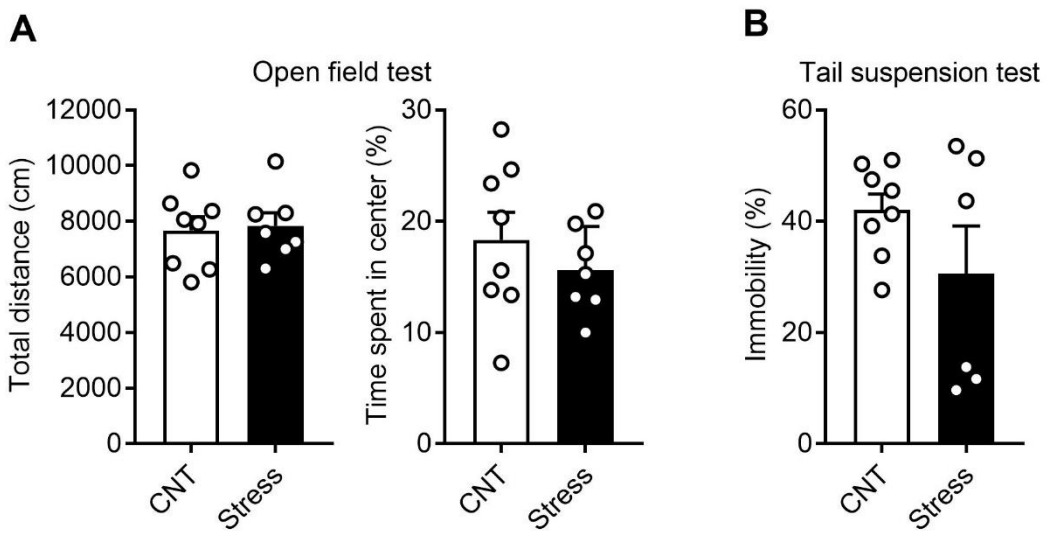

Fig. S8. Effects of chronic restraint stress on anxiety- and depression-related behavior in novel environments. (A) No significant effects of chronic stress on locomotor activity or anxiety-related behavior in the open-field test. Behavior of mice in the open-field arena was monitored for 20 min. (B) No significant effects of chronic stress on depression-related behavior in the tail suspension test. Behavior of mice was monitored for 6 min and immobile time was measured during the last 5 min.

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