SUPPLEMENTARY INFORMATION

Elevation of p11 in Lateral Habenula Mediates Depression-Like Behavior

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SUPPLEMENTARY MATERIALS AND METHODS

Animals

Four transgenic mouse lines were used in this study: **p11-EGFP** mice,[28,30] **D2-Cre** mice,[30-32] **D2-tdT** mice (D2-Cre line crossed with tdTomato line),[28,30] **D1-tdT-D2-eGFP** mice (D1-Cre26 crossed with tdTomato line and D2-eGFP mice).[30] The C57BL/6J mice and tdTomato reporter mice (Rosa26-CAG-tdTomato$^{loxp/+}$, 007908) were purchased from the Jackson Laboratory. We produced the progeny for each line by in vitro fertilization (IVF) and embryo transfer (ET) techniques (Transgenic Facility, The Rockefeller University, New York, NY, USA). All experiments were approved by The Rockefeller University Institutional Animal Care and Use Committee and were performed in accordance with the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in groups of up to five animals on a 12 h dark/light cycle at 22°C and maintained with rodent diet (Picolab, St. Louis, MO, USA) and water available ad libitum. Male mice were used for all experiments.

Restraint stress and antidepressant treatments

The restraint stress treatment was performed as previously described.[28,30,32] Briefly, mice were housed 2 per cage and individually placed head-first into well-ventilated 50 ml polypropylene conical tubes, which were then plugged with a 4.5-cm-long middle tube, and finally tied with a cap of the 50 ml tube. After each session of restraint stress, the mice were returned to their home environment, in which they were housed in pairs in normal plastic cages with free access to food and water. From the day after the last restraint session, imipramine, fluoxetine and escitalopram were administered by a daily I.P. injection (20 mg/kg/day) for up to 4 weeks. Imipramine, fluoxetine and escitalopram were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluoxetine and escitalopram were dissolved in dimethylsulfoxide (DMSO) and then diluted in saline. Imipramine was dissolved in saline. Each drug was finally diluted in 100 µl of 0.9% saline and administered at the dose indicated. Control groups were administered saline.

Viruses

For optogenetic stimulation, AAV5-CaMKIIa-hChR2 (H134R)-EYFP-WPRE-pA (ChR2) and AAV5-CaMKIIa-EYFP (eYFP) viruses were purchased from UNC Vector Core, Chapel Hill, NC, University of North Carolina. For gene silencing, Lenti-GFP-shRNAmir (GFP-shRNA, RHS4348) and Lenti-p11-GFP-shRNAmir (p11-shRNA, VGM5524-99213741) viruses were purchased from Thermo Scientific (Waltham, MA, USA). For Cre-mediated recombination/inversion of the flanked p11 as double-floxed inverse open reading frame (DIO) viruses, AAV vector production of the AAV2 serotype was performed by the University of Pennsylvania vector core.[30] AAV2-EF1a-DIO-eYFP-WPRE-hGH (AAV-DIO-eYFP) was used as the control vector. AAV2-EF1a-DIO-p11-WPRE-hGH (AAV-DIO-p11) vector construction for overexpression of p11 was made by Dr. Jung-Hyuck Ahn (Ewha Womans University, Seoul, South Korea). Double floxed AAV constructs were generated by insertion of the inverted p11 expression cassettes between double lox 2722 and lox P incompatible sites (DIO). In the absence of Cre expression, the p11 or eYFP were not produced. In the presence of Cre expression, the transgene will be FLEXed, leading to the expression of the p11 or eYFP. The titers (genome copies per milliliter) of the AAVs were as follows: $4.04e^{12}$ for AAV2-EF1a-DIO-eYFP-WPRE-hGH (AAV-DIO-eYFP), $3.64e^{12}$ for AAV2-EF1a-DIO-p11-WPRE-hGH (AAV-DIO-p11), $5e^{12}$ for AAV5-CaMKIIa-hChR2 (H134R)-EYFP-WPRE-pA (ChR2) and $6e^{12}$ for AAV5-CaMKIIa-EYFP-WPRE-pA (eYFP).

Stereotaxic surgery and optogenetic stimulation

As previously described,[30] All stereotaxic injections were carried out on an Angle Two stereotaxic frame for mouse with motorized nanoinjector (Leica, Buffalo Grove, IL, USA). Ten-week-old male mice were anesthetized with ketamine and xylazine and stereotaxically injected with Lenti-GFP-shRNA, Lenti-p11-GFP-shRNA, AAV2-EF1a-DIO-eYFP-WPRE-hGH and AAV2-EF1a-DIO-p11-WPRE-hGH into the medial part of the lateral habenula (AP; -1.92 mm, ML; ± 0.4 mm, DV; -2.67mm from bregma). The total injection volume was 0.5 µl. All injections were performed at a rate of 0.15 µl/min using Hamilton syringes (33 gauge, Reno, NV, USA) and the needle was kept in place for an additional five minutes. After 14 days of injection, depression-like behavioral tests and electrophysiological recording were performed. For optogenetic stimulation of LHb

neurons, after virus injection, a 200-μm-core-diameter optic fiber (Doric Lenses) was implanted into the injection site. After 3 weeks, depression-like behavioral tests were performed with 473 nm laser light (10 mW, 60 Hz, 5 ms, Agilent Technologies, California, CA).[11]

Behavioral assessments

As previously described,[30] all behavioral tests were performed during the light cycle in a dedicated sound-proof behavioral facility by experimenters blind to treatment and genotype information. Mice were brought to the testing room 30 min before the start of each behavioral test and remained in the same room through the test. At all times, sound was masked with 60–65 dB white noise.

Tail suspension test. Mice were suspended individually by their tails. The rod was fixed 50 cm above the surface of a table covered with a safety mat in a sound-isolated room. The tip of the tail was fixed using adhesive Scotch tape; the duration of the test was 5 min. The test session was videotaped and immobility scored by using automated TST/FST analysis software from Clever Systems (Reston, VA, USA).

Forced swim test. In brief, mice were placed in a glass cylinder (height: 30 cm, diameter: 16 cm) containing water at 24 °C and a depth of 14 cm so that they could neither escape nor touch the bottom. Mice were forced to swim for 6 min. The animals were habituated for the first 1 min and behavior was monitored over the next 5 min. A 6 min test session was videotaped and immobility scored by using automated TST/FST analysis software from Clever Systems.

Sucrose preference test. Mice were presented with two water bottles. After habituation for 1 day, mice were given a free choice between two bottles, containing tap water or 2% sucrose solution. To prevent a possible effect of drinking behavior, the left/right location of the bottles was switched every day. The consumption of water and sucrose solution was measured daily for 3 days by weighing the bottles. The sucrose preference was calculated as the ratio of consumed sucrose solution to consumed water.

Novelty-suppressed feeding test. After 24 hours food-deprivation (water was provided ad libitum), mice were assayed by NSF, At the end of this time, a single 2 x 2 cm oval food pellet was placed on a circular piece of white filter paper (150 mm diameter) positioned in the center of the open field (40 x 40 x 40 cm). Each mouse was placed in a corner of the open field. The latency to first bite the lab chow pellet and consumption over 15 min were recorded. Immediately after the mouse began to eat the chow, the tested animal was placed in its home cage alone with a weighed piece of chow for 30 min. At the end of this period, the amount of food consumed was determined by weighing the piece of chow.

Social interaction test. Mice were introduced into the open field contained an empty Plexiglas mesh cage (10 x 6 x 17 cm) at one end of the field for 5 min for habituation. The mouse was then placed back into its home cage for 1 min. The experimental mice were tested for their trajectory in the same open field with a social target animal (an aged aggressive CD1 male mouse) in a mesh cage for 5 min. The video tracking data were used to determine the time spent by the experimental mice in the interaction zone (a 6 cm-wide corridor surrounding the target animal cage) and the corner zone of the open field opposite to the location of the cage (10 x 10 cm). Their movement was monitored for 5 min with an aggressive aged CD1 mouse. We obtained information pertaining to the duration spent in the interaction zone and corner zone, as well as other measures using Ethovision software.

Fecal Boli test. Excreted fecal pellets (0.4 - 0.7 x 0.2 mm) were counted for each 6 min after FST.

Two-way active avoidance test. Mice were placed in the apparatus (Med Associates, St. Albans, Vermont), the guillotine-type door was opened, and the mouse allowed 2 min adaptation and exploration. After 2 min, the mouse was placed in the right compartment, and the test began. Each trial lasted 10 sec with 2 stimuli: first, a light and sound signal of 75dB (conditioned stimulus) was emitted for 5 sec. followed by an electric footshock of 0.3 mA (unconditioned stimulus) for 5 sec. During the trial, the guillotine-type door was

open. It closed when the four paws of the mouse entered the opposite compartment or at the end of the trial. The successive trials started in the side where the mouse was located. The test was comprised of 50 trials. The avoidance responses were measured by avoidance episode: when the mouse crossed during the sound signal; and the latency to escape shock: after the sound signal, the latency to cross the compartment mouse crossed before the electric footshock.

Locomotion test. Locomotor activity was measured in the open field of a Plexiglas chamber (40 x 40 x 40 cm). Each mouse was placed in the corner of the open field, and locomotion was recorded for the indicated period for 30 min. An automated Superflex software (Accuscan Instruments, Columbus, OH, USA) was used to measure the total distance traveled across a session. The measures were automatized using two rows of infrared photocells placed 20 and 50 mm above the floor, spaced 31 mm apart. Photocell beam interruptions were recorded on a computer using the Superflex software (Accuscan Instruments).

Immunohistochemistry

As previously described,[30] Brains were perfused transcardially with cold PBS, followed by 4% paraformaldehyde (PFA) and postfixed in the same solution overnight at 4°C. The brains were coronally cut into 40 μm-thick sections with a vibratome (VT 1000S, Leica). Free-floating sections were washed three times with 0.1 M PBS containing 0.1% Triton X-100 in PBS-T, pH 7.4, for 15 min each time and permeabilized with PBST in 2% normal goat serum, 2% normal horse serum, and 2% BSA for 1 h. After blocking, sections were incubated with the primary antibodies diluted in the blocking buffer. The immunohistochemistry was done using the following antibodies: anti-p11 (goat polyclonal, 1:200, R&D systems, Minneapolis, MN, USA), anti-eGFP (chicken polyclonal, 1:500, Abcam, Cambridge, MA, USA), anti-c-fos (rabbit polyclonal, 1:500, Santa Cruz, Dallas, TX, USA), anti-CaMKII (rabbit polyclonal, 1:500, Santa Cruz, Dallas, TX, USA) and anti-VGAT (rabbit polyclonal, 1:500, SYSY, Göttingen, Germany). After 24 hours incubation, sections were washed, and incubated with Alexa-fluor-conjugated secondary antibodies (1:500, Invitrogen, Carlsbad, CA, USA). Slices were washed three

more times in PBS-T for 15 min each and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) onto microscope slides. All the sections were examined under a Zeiss LSM710 confocal microscope or wide-field fluorescence microscope (Zeiss, Jena, Germany). All histology findings were confirmed in at least five different animals.

Cell counting

The number of eGFP, tdTomato, p11, CaMKII or VGAT-immunolabeled neurons was quantified with ImageJ software (NIH). Three to five coronal sections per animal were quantified and averaged for each animal. Fluorescence images for obtaining the medial part of the LHb were acquired using a Zeiss LSM710 confocal microscope with a ×40/0.50 NA objective (45176.65 um^2; 262144 pixels). Background autofluorescence was accounted for by applying an equal cut-off threshold to all images. All imaging and analyses were performed blind to the experimental conditions.

Western blot analysis

As previously described,[30] tissue samples were obtained from the Hb of the mice. Tissue samples were homogenized in RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Complete-EDTAfree; Roche) and a phosphatase inhibitor cocktail (PhosStop, Roche). Protein concentrations were determined using a BCA assay (Thermo Scientific). 30 μg of protein were denatured in Laemmli sample buffer at 95 °C for 5 min and separated by SDS-PAGE using 4–20% Tris-glycine gel (Life Technologies, Gaithersburg, MD, USA). After transfer of proteins to nitrocellulose membranes, blots were blocked in 5% non-fat milk for 1 h at room temperature and incubated with the respective primary antibody at 4 °C overnight. Primary antibodies were as follows: anti-p11 (goat polyclonal, 1:500, R&D systems), and c-fos (rabbit polyclonal, 1:2000, Santa Cruz, Dallas, TX, USA). anti-β actin (mouse monoclonal, 1:5000, Abcam, Cambridge, MA, USA). Primary antibodies were detected using either HRP-linked donkey anti–goat IgG (1:2000, Santa Cruz) or HRP-linked sheep anti–mouse IgG (1:10000, GE Healthcare, Madison, WI, USA) together with Western Lightning Plus-ECL (Perkin Elmer, Norwalk, CT, USA). Signals were quantified with ImageJ software (NIH).

Quantitative RT-PCR

As previously described,[30] reverse transcription was performed with 1 μg of total RNA using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) with Oligo dT primer according to the manufacturer's protocol. 10 ng of cDNA was used for each qPCR reaction and all samples were run in triplicate. qPCR was carried out using an Applied Biosystems 7900HT system (Foster City, CA, USA). Taqman Universal PCR Master Mix (Life Technologies, Foster City, CA, USA) was used for all analyses. Taqman gene expression assays (FAM) from Life Technologies were as follows: p11 (Mm00501457_m1) and Gapdh (Mm99999915_g1). All data were normalized to TaqMan Rodent GAPDH Control, and relative expression levels between conditions were calculated by the comparative CT (2-ΔΔCT) method.

Electrophysiological recordings

The whole-cell patch-clamp recording technique was used to measure spontaneous action potentials and synaptic currents in LHb neurons. Mouse slices (300 μm) were positioned in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus, Tokyo, Japan) and visualized with a x40 water-immersion lens and recorded with the Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA). Tight seals (2-10 GΩ) were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole-cell configuration was obtained. The access resistances ranged from 10 to 15 MΩ and did not change more than 10% during the recording.

Spontaneous action potentials were recorded in the whole-cell current clamp mode. The external solution was regular ACSF (in mM: in mM: 130 NaCl, 26 $NaHCO_3$, 1 $CaCl_2$, 5 $MgCl_2$, 3 KCl, 10 glucose, 1.25 NaH_2PO_4). The internal solution contained (in mM): 20 KCl, 100 K-gluconate, 10 HEPES, 4 Mg-ATP, 0.5 Na2GTP, and 10 Na-phosphocreatine. The inter-spike membrane potential was -65 mV with a small (< 50 pA) adjusting current (negative DC). Synaptic currents (sEPSC and sIPSC) were recorded in the whole-cell voltage-clamp mode (clamped at -70mV). The external solution was regular ACSF. The internal solution for EPSC contained (in mM): 130 Cs-methanesulphonate 10 CsCl, 4

NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2 QX-314, 12 phosphocreatine, 5 MgATP, 0.5 Na_2GTP, pH 7.2-7.3, 265-270 mosM. The internal solution for IPSC contained (in mM): 100 CsCl, 30 N-methyl-D -glucamine (NMG), 10 HEPES, 4 NaCl, 1 $MgCl_2$ 5 EGTA, 2 QX-314, 12 phosphocreatine, 5 MgATP, 0.5 Na_2GTP, pH 7.2-7.3, 265-270 mOsM. GABAAR antagonist Bicuculline (10 μM) was added in sEPSC recordings and AMPAR antagonist DNQX (50 μM) was added in sIPSC recordings. Data analyses were performed with Clampfit (Axon instruments, Molecular Devices), Mini Analysis Program (Synaptosoft, Decatur, GA, USA), Kaleidagraph software (Albeck, Synergy Software, Reading, PA, USA) and Prism software (GraphPad).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Optogenetic stimulation of LHb induces depression-like behaviors and the activation of p11-expressing LHb neurons. (**a**-**c**) Depression-like behaviors as measured by avoidance responses (**a**) and latency to escape shock (**b**) in two-way active avoidance test (AAT), and immobility time in tail-suspension test (TST, **c**) in mice after LHb injection of AAV-ChR2-eYFP (ChR2) or AAV-eYFP (eYFP) with (ON) or without (OFF) optogenetic stimulation of LHb (n = 6, eYFP+ON and eYFP+OFF; n = 7, ChR2+ON and ChR2+OFF). * $P < 0.05$, ** $P < 0.01$, one-way ANOVA. (**d**) Locomotor activity in the 4 groups. (**e**, **f**) Western blots (**e**) and quantification (**f**) showing the expression of c-fos protein in the 4 groups (n = 6 per group). * $P < 0.05$, one-way ANOVA. (**g**) Immunofluorescence images (low- and high-magnifications) illustrating the co-localization of p11 (blue) with ChR2 (green) and c-fos (red) in the medial part of the LHb neurons from ChR2-injected mice with optogenetic stimulation (ChR2+ON). Scale bar, 20 μm (upper panel) and 5 μm (bottom panel). The percentage of neurons expressing c-fos, ChR2, or p11 is indicated in Venn diagrams (arrows: the percentage of co-localized neurons). Data presented as means ± s.e.m.

Supplementary Figure S2. Chronic stress induces the selective reduction of synaptic inhibition in LHb neurons. (**a**) Bar graphs showing the amplitude and frequency of sponteneuous inhibitory postsynaptic current (sIPSC) in LHb neurons from control (CON) and stressed (RST) mice (n = 11, CON; n = 12, RST). (**b**) Representative sIPSC traces in CON vs. RST mice. (**c**) Bar graphs showing the amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSC) in LHb neurons from CON vs. RST mice (n = 35, CON; n = 35, RST). (**d**) Representative sEPSC traces in CON vs. RST mice. * $P < 0.05$, one-way ANOVA. Data are presented as means ± s.e.m.

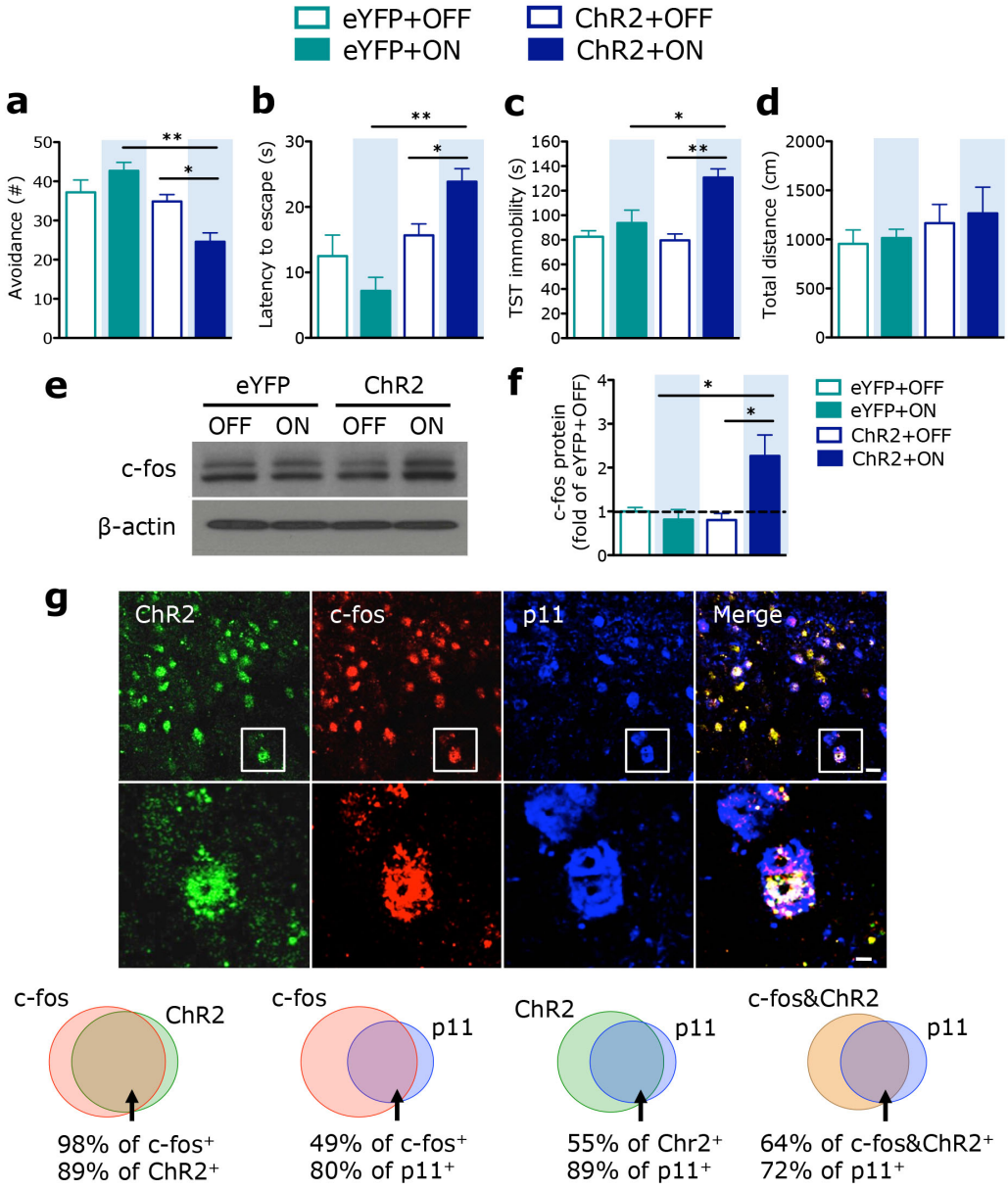
Supplementary Figure S3. Knockdown of p11 in LHb blocks stress-induced reduction of synaptic inhibition. (**a**) Bar graphs showing the amplitude and frequency of sISPC in LHb neurons from RST mice injected with p11 shRNA (RST+sh-p11) or GFP shRNA (RST+sh-GFP) (n = 8, RST+sh-GFP; n = 14, RST+sh-p11). (**b**) Representative sIPSC traces of RST+sh-p11 vs. RST+sh-GFP mice. (**c**) Bar graphs

showing the amplitude and frequency of sEPSC in LHb neurons from RST+sh-GFP vs. RST+sh-p11 mice (n = 6, RST+sh-GFP; n = 7, RST+sh-p11). (**d**) Representative sEPSC traces of RST+sh-p11 vs. RST+sh-GFP mice. * $P < 0.05$, one-way ANOVA. Data are presented as means ± s.e.m.

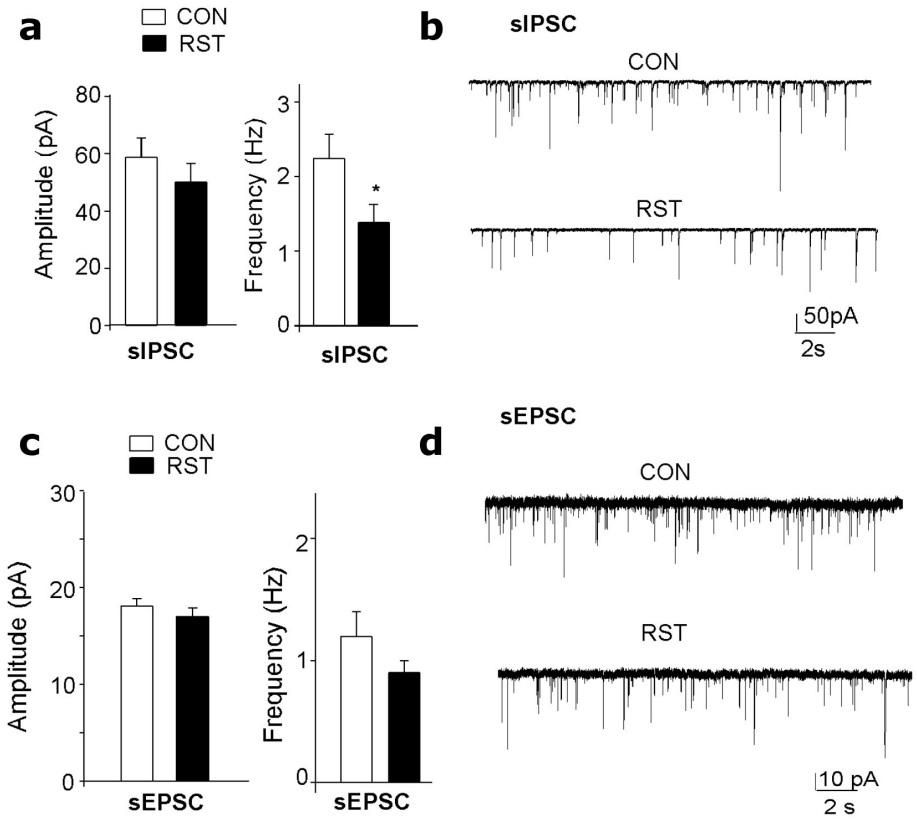
Supplementary Figure S4. p11 is expressed in glutamatergic and GABAergic LHb neurons. (**a**) Immunofluorescent images showing the expression of p11 (green) in $CaMKII^+$ (red) LHb neurons. Scale bar, 10 μm. (**b**) Immunofluorescent images showing the expression of p11 (green) in $VGAT^+$ (red) LHb neurons. Scale bar, 10 μm.

Supplementary Figure S5. p11 is expressed in $D2R^+$ LHb neurons. (a) Immunofluorescent images showing the co-localization of p11 (blue) with D2 (D2-eGFP; green), but not D1 (D1-tdT; red), in LHb neurons from D1-tdT x D2-eGFP mice. Scale bar, 10 μm. (**b**) Immunofluorescent images showing the co-localization of p11 (green) with D2 (red), but not VGAT (blue), in LHb neurons from D2-tdT mice. Scale bar, 10 μm.

Supplementary Figure S1.



Supplementary Figure S2.



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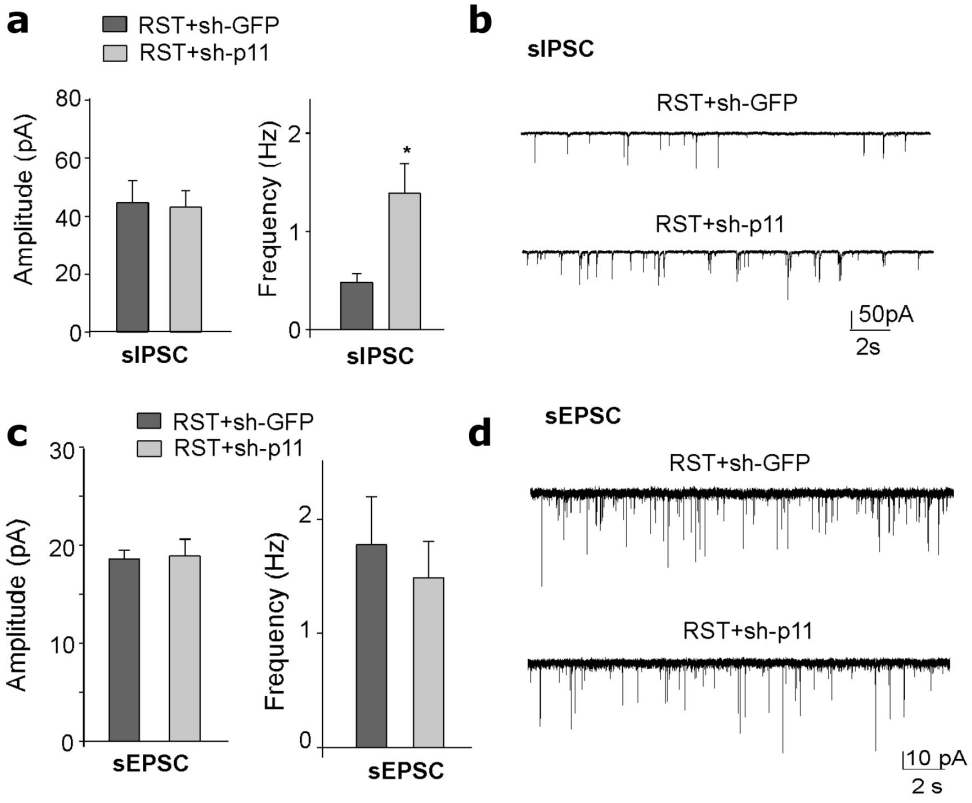

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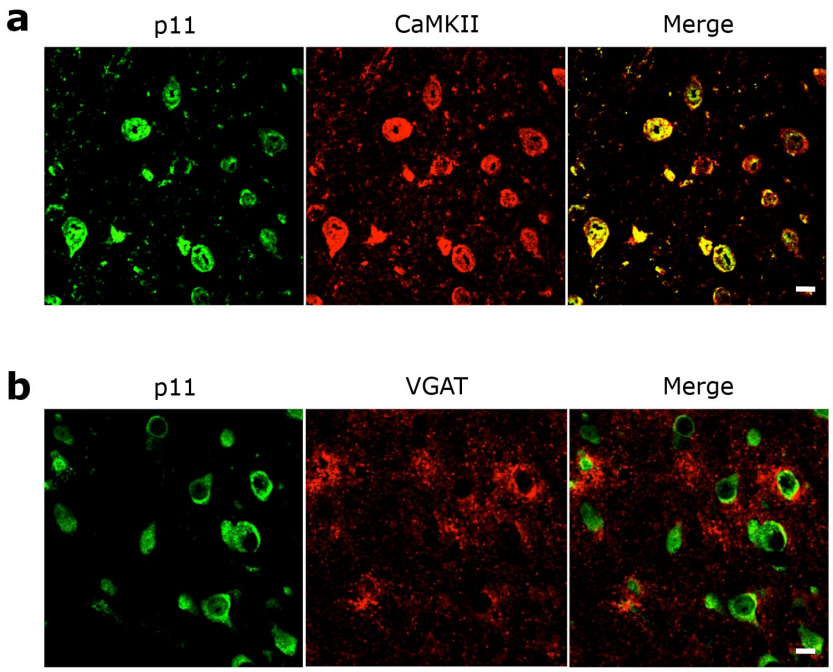

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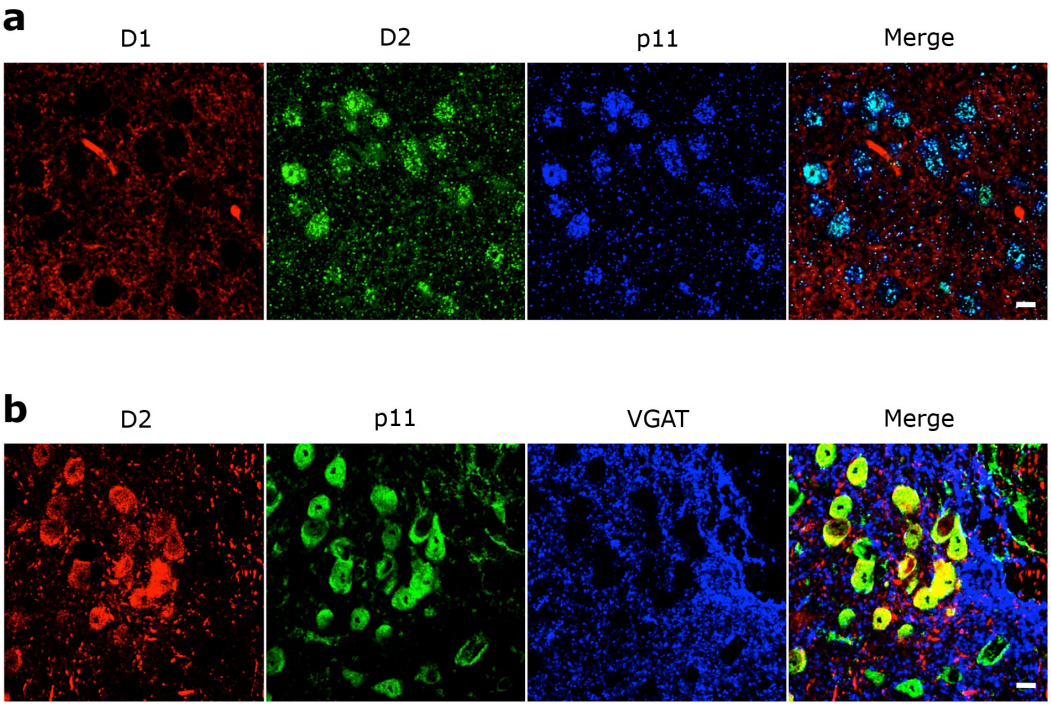

SUPPLEMENTARY TABLES

Supplementary Table S1. Quantification of the co-localization of p11, D2 and CaMKII in the medial part of the LHb neurons.

%	$p11^+$	$D2^+$	$CaMKII^+$	$Triple^+$
Total $p11^+$	**100 ± 11.4**	**74.6 ± 6.8**	**90.6 ± 12.4**	**68.6 ± 7.4**
Total $D2^+$	**92.3 ± 8.7**	**100 ± 8.3**	**95.7 ± 7.2**	**88.9 ± 10.2***
Total $CaMKII^+$	**75.0 ± 16.1**	**50.0 ± 5.6**	**100 ± 9.5**	**48.6 ± 5.5**

Red: Percentage of $D2^+$ neurons in total p11-expressing cells.

Green: Percentage of $p11^+$ cells in total D2-expressing neurons.

Blue: Percentage of $CaMKII^+$ neurons in total p11-expressing cells or D2-expressing neurons.

*: Percentage of $CaMKII^+$ and $p11^+$ neurons in total D2-expressing neurons.

Supplementary Table S2. Quantification of the co-localization of p11, D2 and VGAT in the medial part of the LHb neurons.

%	$p11^+$	$D2^+$	$VGAT^+$	$Triple^+$
Total $p11^+$	**100 ± 3.0**	**72.8 ± 3.8**	**12.0 ± 2.0**	**3.0 ± 1.4**
Total $D2^+$	**86.7 ± 3.5**	**100 ± 3.5**	**5.1 ± 2.6**	**3.6 ± 1.7**
Total $VGAT^+$	**28.8 ± 11.6**	**13.3 ± 6.8**	**100 ± 16.77**	**11.1 ± 5.35**

Red: Percentage of $D2^+$ neurons in total p11-expressing cells.

Green: Percentage of $p11^+$ cells in total D2-expressing neurons.

Supplementary Table S3. Quantification of the co-localization of p11, D1 and D2 in the medial part of the LHb neurons.

%	$p11^+$	$D1^+$	$D2^+$	$Triple^+$
Total $p11^+$	**100 ± 9.1**	**1.4 ± 0.5**	**79.3 ± 9.0**	**0.0 ± 0.0**
Total $D1^+$	**5.6 ± 2.2**	**100 ± 15.5**	**5.6 ± 3.0**	**0.0 ± 0.0**
Total $D2^+$	**90.2 ± 10.4**	**1.6 ± 0.8**	**100 ± 8.6**	**0.0 ± 0.0**

Red: Percentage of $D2^+$ neurons in total p11-expressing cells.

Green: Percentage of $p11^+$ cells in total D2-expressing neurons.