SUPPLEMENTARY INFORMATION

Astrocyte-derived ATP modulates depressive-like behaviors

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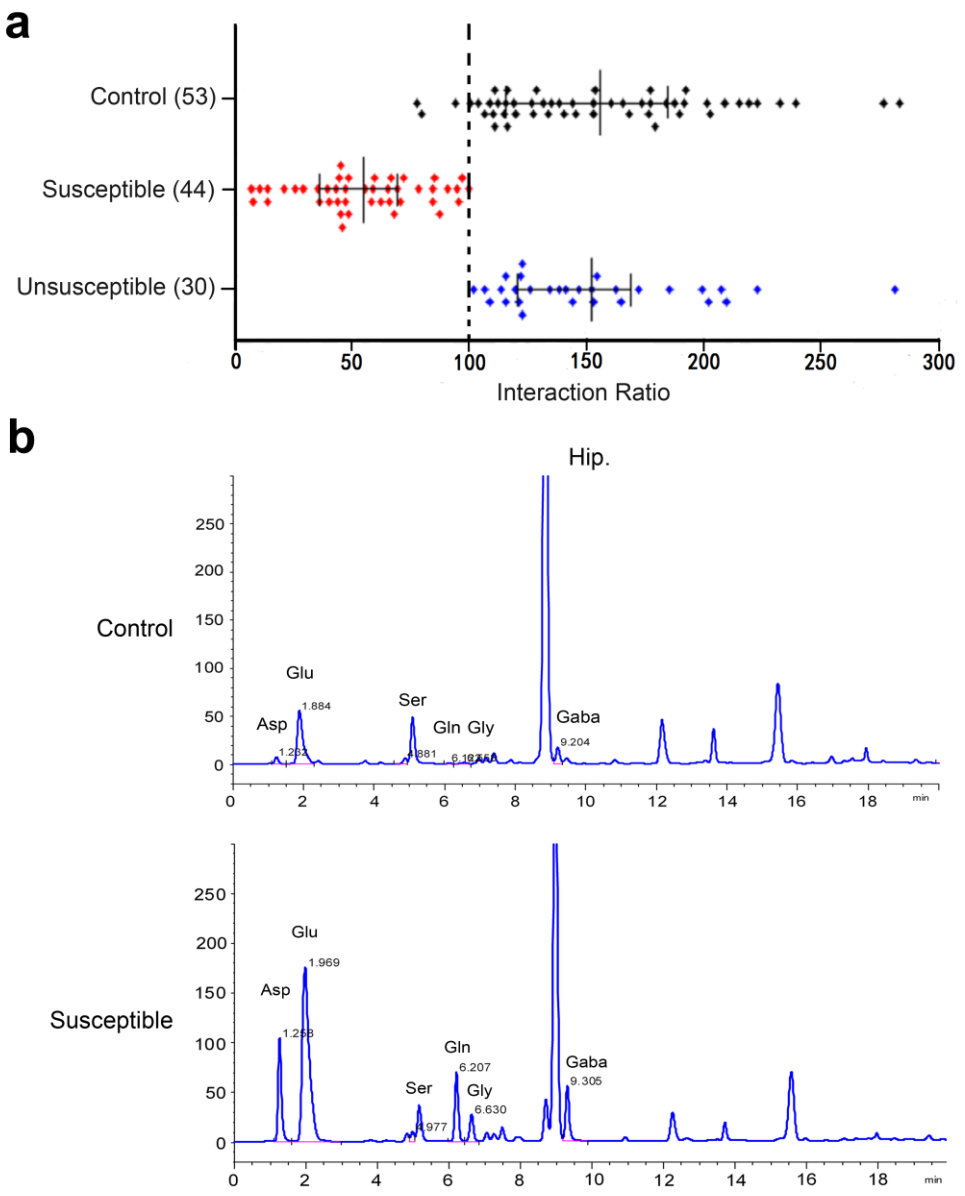
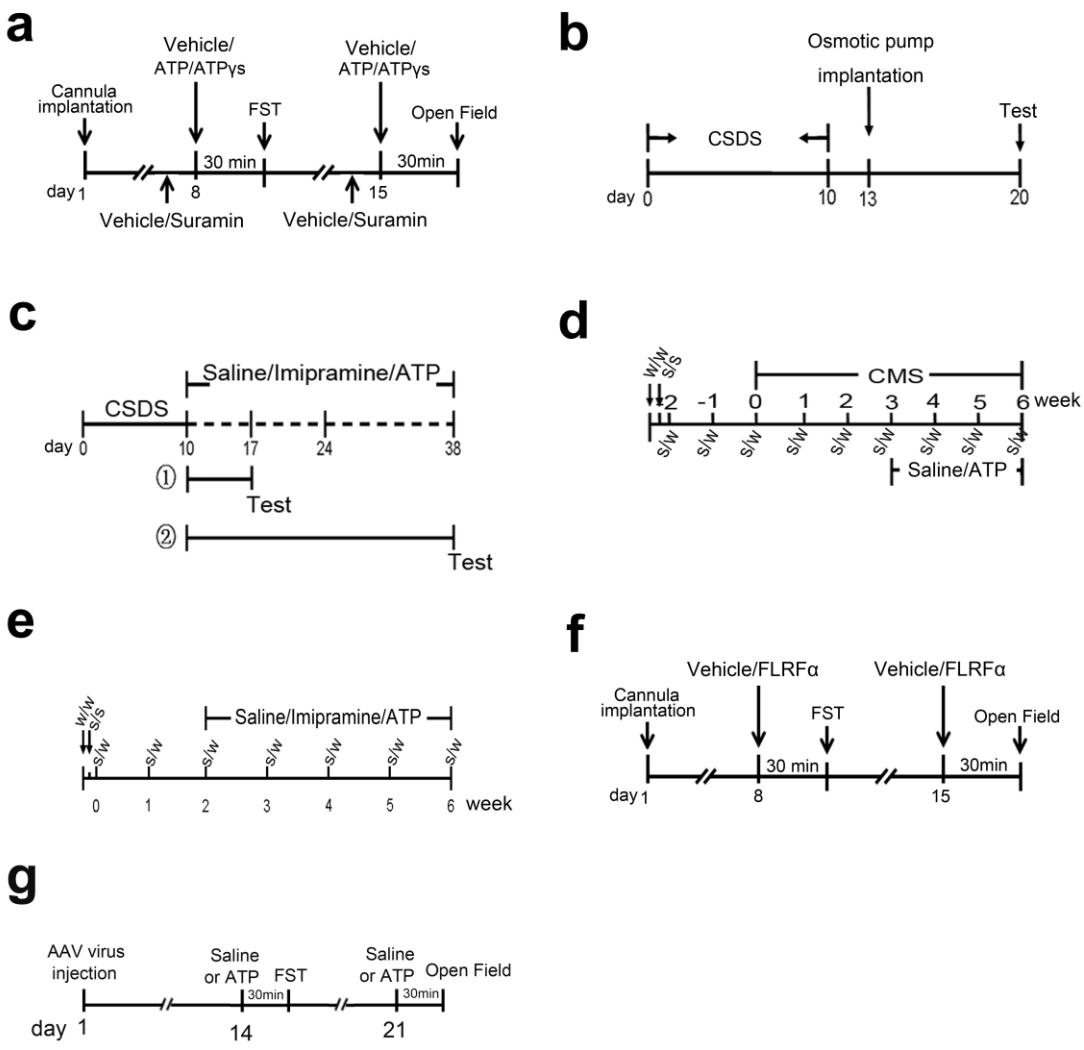
Supplemental Figure 1



Related to Fig. 1a. a. Identification of susceptible and unsusceptible subpopulations. Horizontal scatter plot illustrating the distribution of the interaction ratios for the control, susceptible, and unsusceptible mice following the CSDS protocol (error bars represent mean ± interquartile range). **b.** Representative HPLC chromatograms of aspartate (Asp), glutamate (Glu), serine (Ser), glutamine (Gln), glycine (Gly) and GABA (Gaba) levels in hippocampal (Hip.) slices.

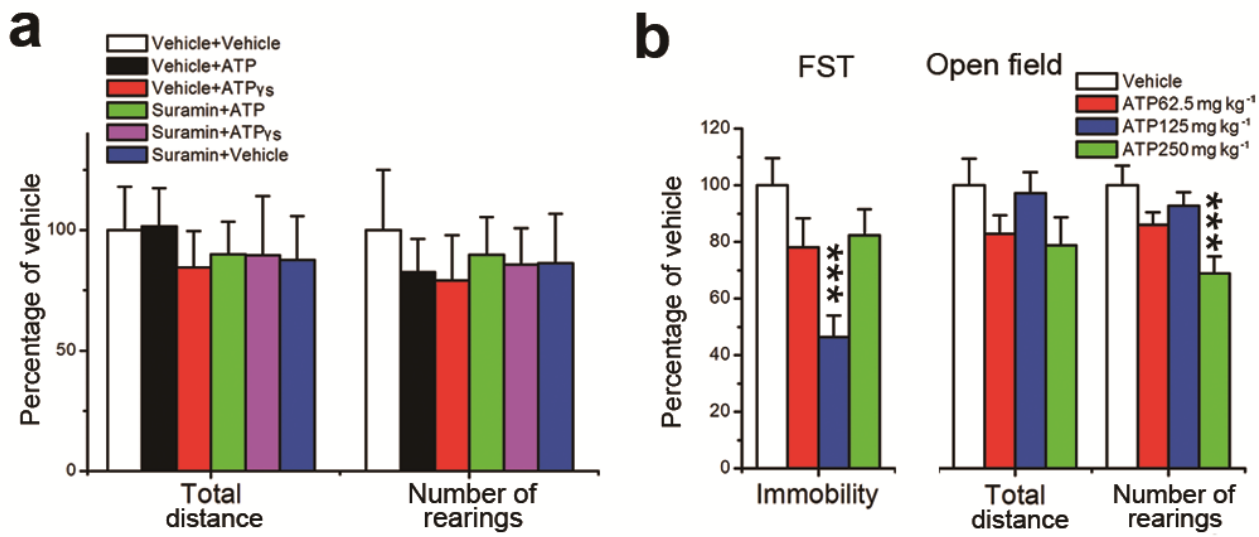
Supplemental Figure 2



Schematic of the experimental designs. a. Stereotaxic injections. A brain infusion cannula was unilaterally implanted in the right cerebral ventricle, mPFC, or hippocampus. Seven days after cannula implantation, free-moving mice were infused with vehicle, suramin (1.5 mM), PPADs (50 uM), BBG (10 μM), NF449 (1μM), or AF-353 (0.1 μM). After 15 minutes, vehicle, ATP (25 μM), or ATPγs (50 μM) was delivered, and the FST was conducted 30 min following the second infusion. Seven days after the FST, a period that was sufficient for behavioral and pharmacological recovery, the mice that received the same schedule of drug infusion were tested

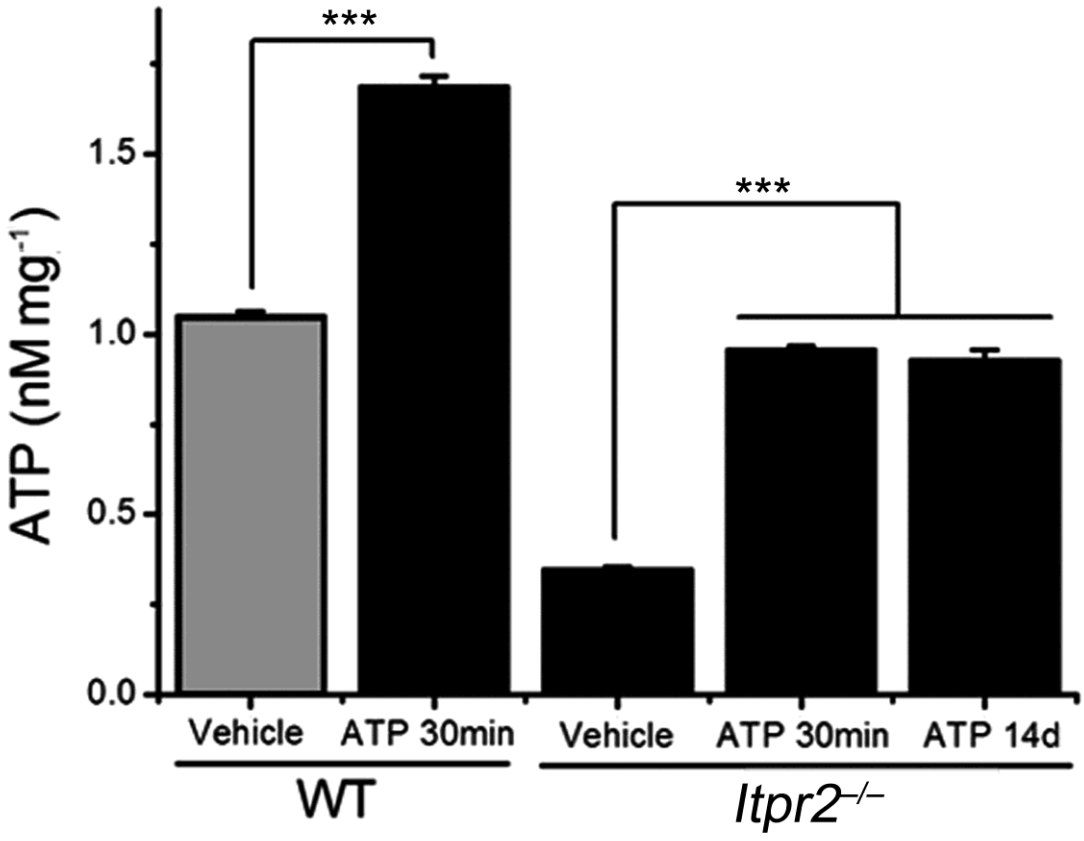
in the open field. **b.** Following the 10-day CSDS protocol, each mouse was implanted with an osmotic pump [containing ACSF, ATP (2.5 mM), ATPγs (5 mM), or FLRFα (0.5 mM)] in the right lateral ventricle, and the behavioral tests were conducted 7 d later. **c.** Following the 10–day CSDS protocol, the mice were divided into 7– and 28–day groups, each of which contained three subgroups, and were given daily injections of ATP (125 mg kg^{-1}, i.p.), imipramine (15 mg kg^{-1}, i.p.) or saline. The long-term behavioral consequences of defeat stress were assessed using the social avoidance test 24 h following the final injection. Mice that were housed in equivalent cages with members of the same strain (cage-mate changed daily) were used as controls. **d.** CMS paradigm. Following habituation to sucrose solution, the coat state assay and the sucrose preference test were conducted weekly. The CMS paradigm began after 3 tests and lasted for 6 weeks. The treatments began at the end of the 3^{rd} week and lasted for 3 weeks. The arrows represent the acclimation tests. **e.** Following sucrose solution habituation, the *Itpr2*$^{-/-}$ mice were given daily injections of ATP (62.5, 125 mg kg^{-1}, i.p.), imipramine or saline for 4 weeks. The state of each mouse's coat and their sucrose preference were analyzed weekly. **f.** FST and i.c.v. injection of FLRFα. Seven days after cannula implantation, the free-moving mice were infused with ACSF (vehicle) or FLRFα (10 μM), and the behavioral test was conducted after 30 min. Seven days after the FST, the mice that received ACSF or FLRFα were tested in the open field. **g.** Protocol for AAV shRNA injections. Viral vectors were delivered bilaterally delivered into the mPFC. Two weeks after the AAV injection, animal was injected with ATP (125 mg kg^{-1}, i.p.) or saline and the FST was conducted 30 min later. Seven days after the FST, mice that received the same schedule of treatment were tested in the open field.

Supplemental Figure 3



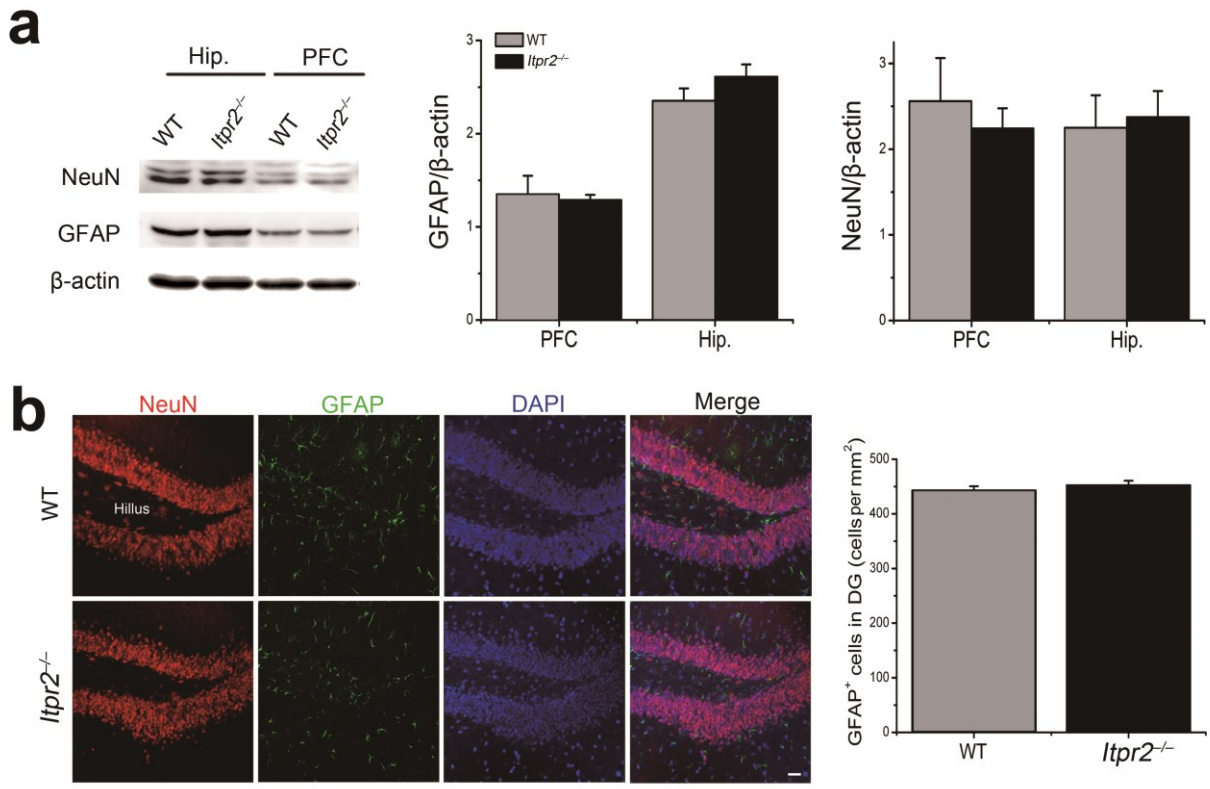
Related to Fig. 1. a. The open field test showing the effect of an infusion of ATP (25 μM), ATPγs (50 μM), or suramin (1.5 mM) on spontaneous locomotor activity (n = 10). **b.** An i.p. injection of ATP at a concentration of 125 mg kg^{-1} induced AD-like effects in the FST without affecting locomotor activity in the open field test (n = 10). LSD *post hoc* test: *** $P < 0.001$.

Supplemental Figure 4



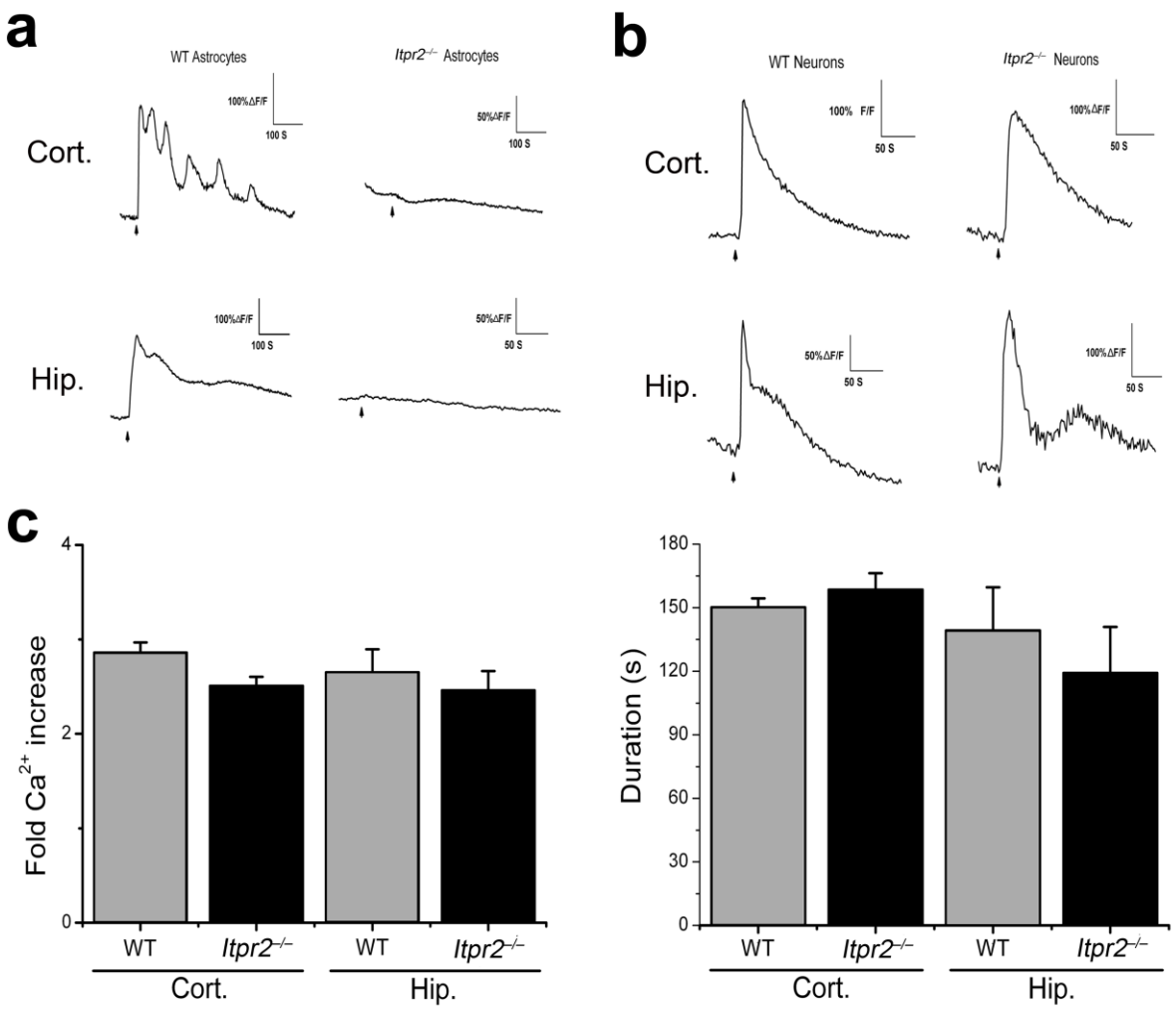
Both acute and chronic injections of ATP increased ATP levels in the brains of *Itpr2*$^{-/-}$ mice and wild type littermates (WT). *Itpr2*$^{-/-}$ mice and control littermates that were injected with saline (vehicle) or ATP (125 mg kg^{-1}, i.p.) were sacrificed 30 min or 14 d later. The hippocampal slices were immediately sectioned and incubated in oxygenized ACSF for 12 min. Measurement of ATP levels indicated that both acute and chronic treatments with ATP reversed the lower ATP levels that were observed in the brains of *Itpr2*$^{-/-}$ mice relative to controls (n = 6). LSD *post hoc* test: *** $P < 0.001$.

Supplemental Figure 5



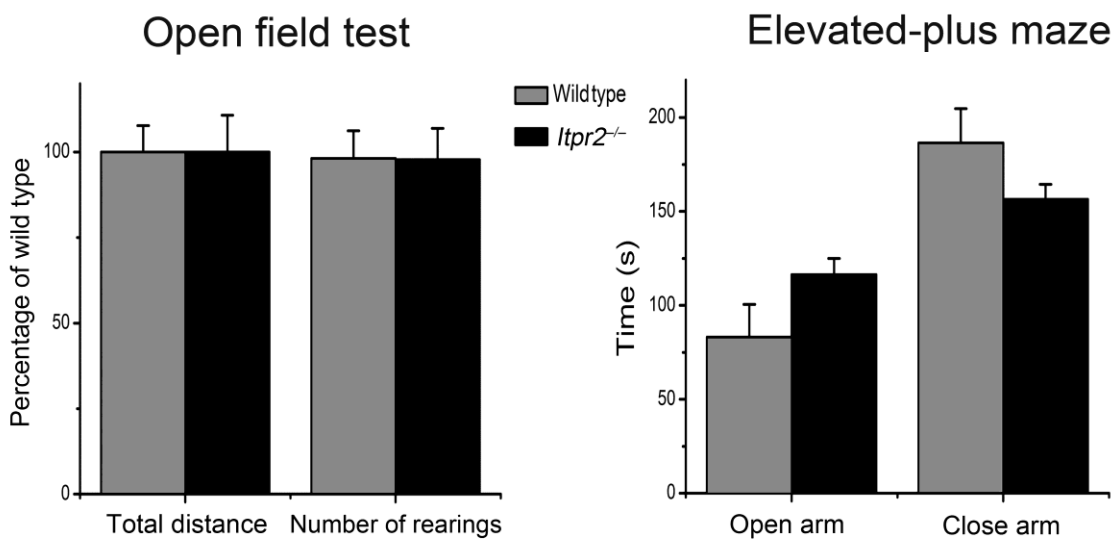
Astrocytic morphology and cell density were independent of the presence of Itpr2. a. Western blots showing expression of GFAP or NeuN in the hippocampus (Hip.) or PFC of 3-month-old *Itpr2*$^{-/-}$ mice (n = 4). **b.** Immunofluorescent staining and quantification showing the morphology or density of GFAP-positive cells (green) in the dentate gyrus of *Itpr2*$^{-/-}$ mice (n = 7). Scale bar, 25 μM.

Supplemental Figure 6



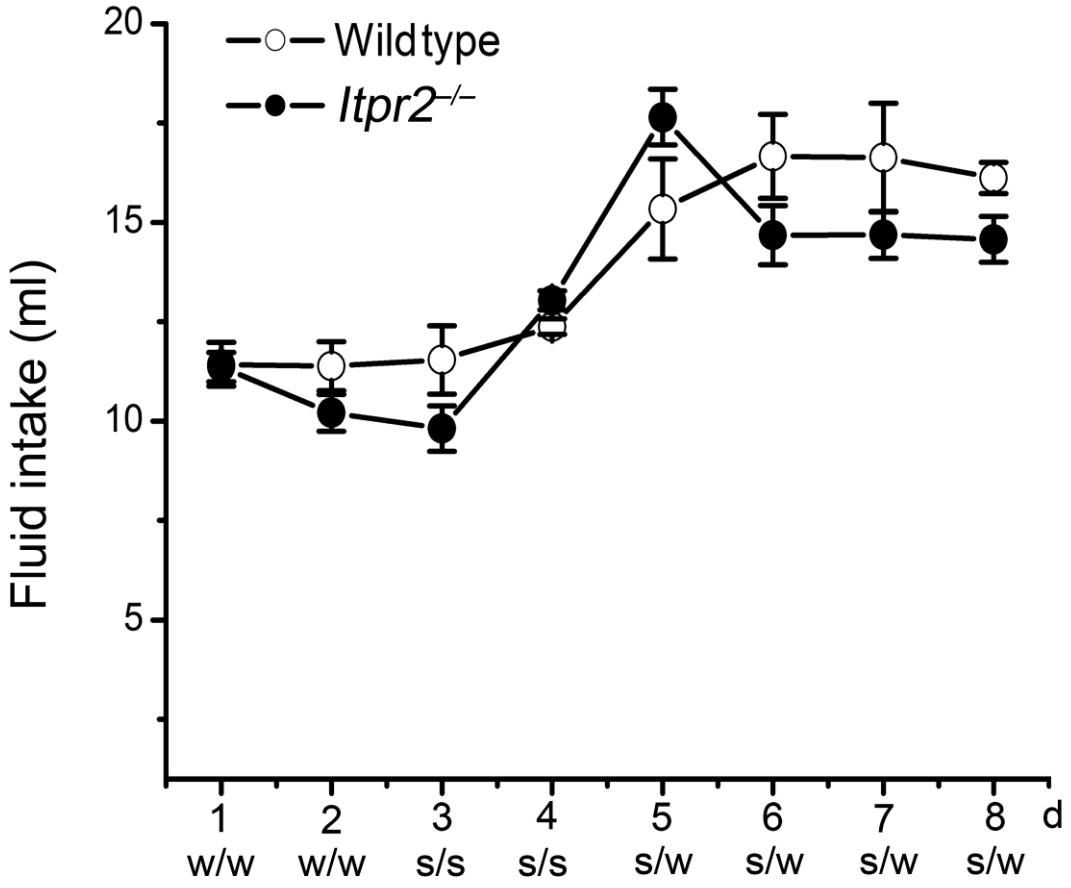
Astrocytes from *Itpr2*$^{-/-}$ mice lack increases in Gq-linked GPCRs [Ca^{2+}]$_i$. Representative Ca^{2+} traces from cultured astrocytes (**a**) and neurons (**b**) with internal solutions that contained Fluo–4 Ca^{2+} indicator. The traces were obtained in response to the application of a Gq GPCR- agonist cocktail (50 μM DHPG, 10 μM histamine, and 10 μM carbachol). **c.** The amplitude and duration of IP_3R-mediated [Ca^{2+}]$_i$ responses in neurons. Hip., hippocampal; Cort., cortical.

Supplemental Figure 7



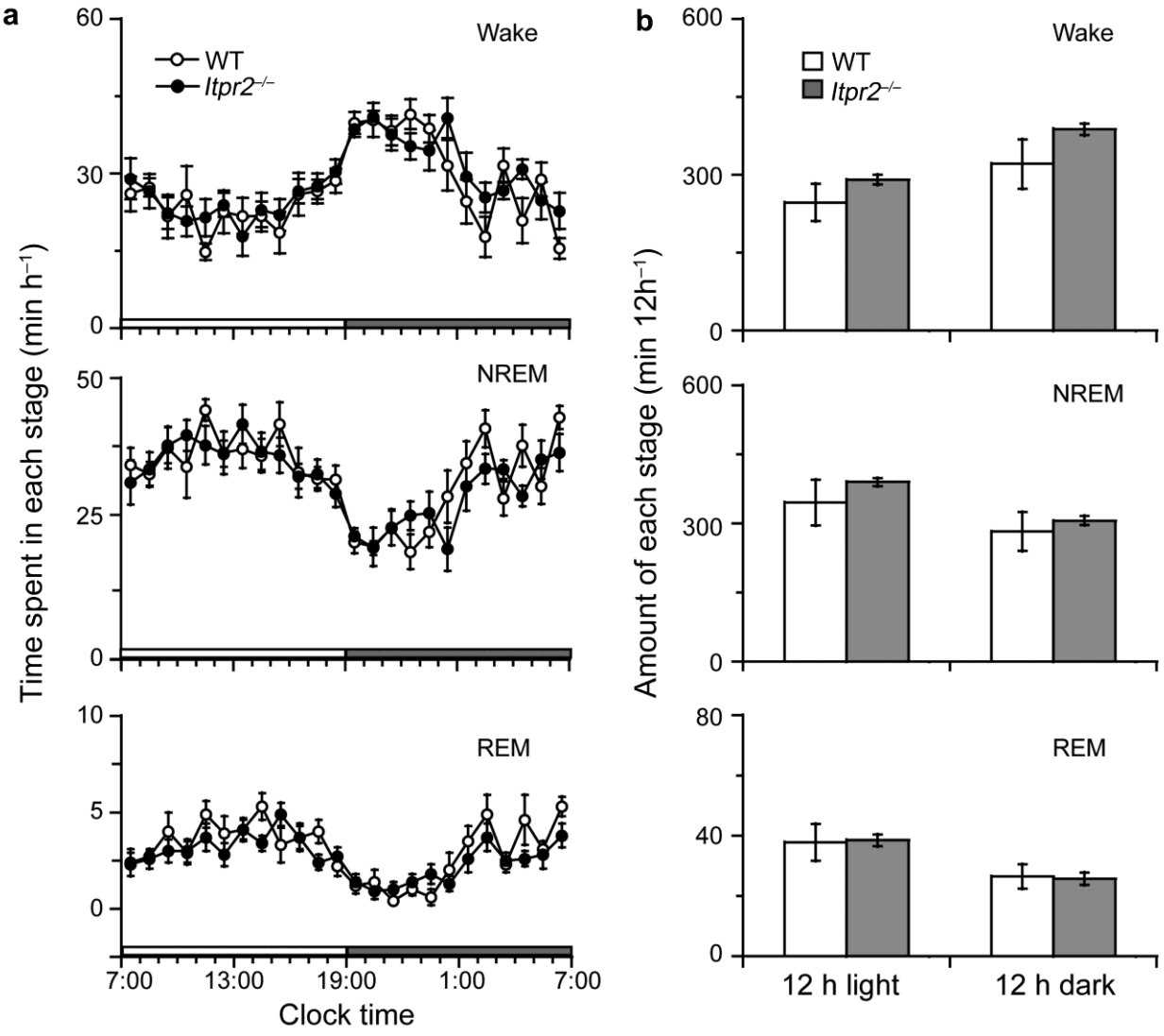
The behaviors of *Itpr2*$^{-/-}$ mice in the open field and elevated-plus maze tests. a. The open field test showing the total distance and number of rearing in *Itpr2*$^{-/-}$ mice and WT (n = 11). **b.** The elevated-plus maze test showing the time spent in the open arm and the closed arm in *Itpr2*$^{-/-}$ mice and WT (n = 10).

Supplemental Figure 8



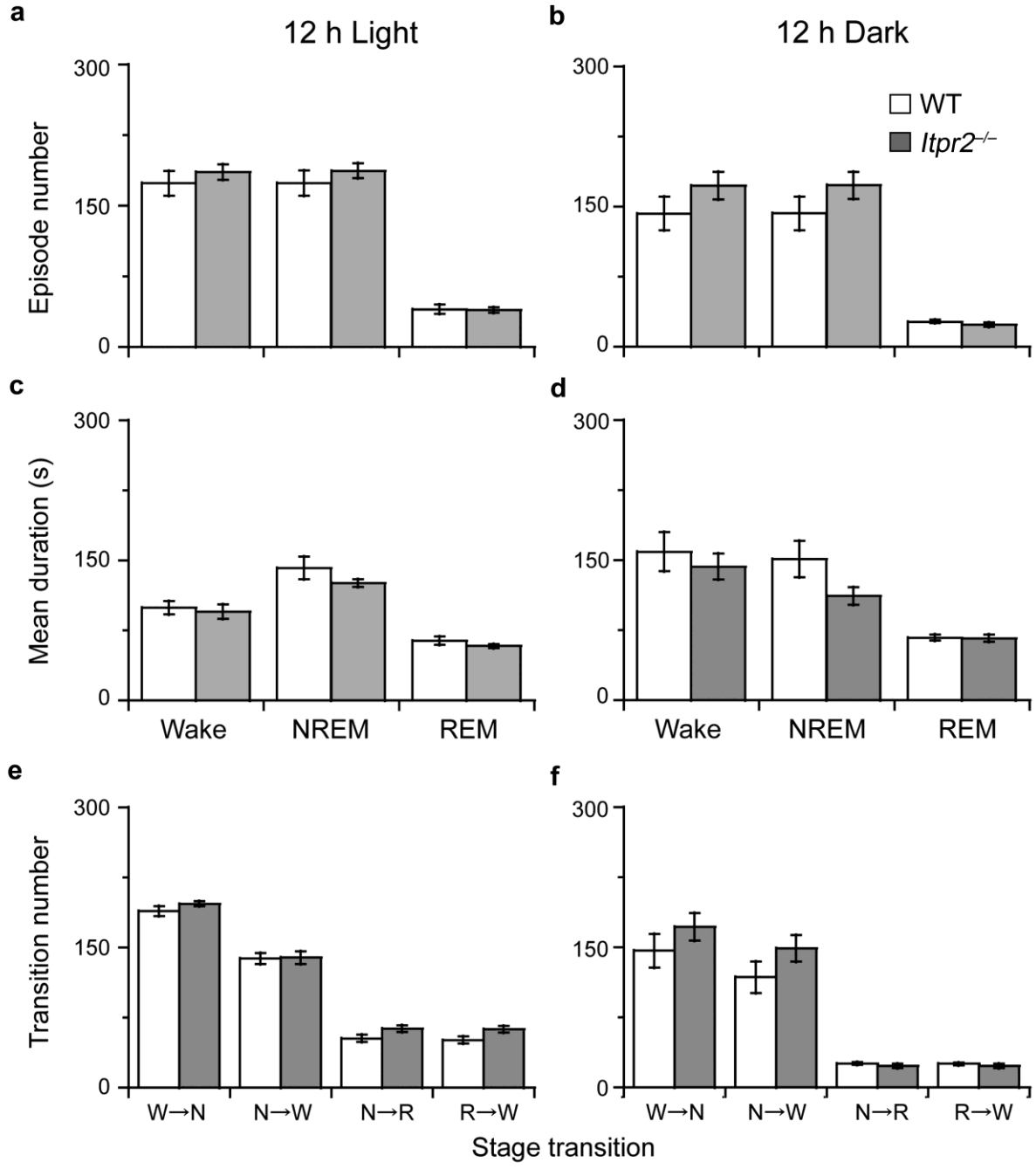
The total intake (water + sucrose solution) was measured throughout the sucrose preference test in *Itpr2*$^{-/-}$ mice and wild type littermates (Repeated measures, n = 11). w/w, water/water; s/s, sucrose/ sucrose; s/w, sucrose/water.

Supplemental Figure 9



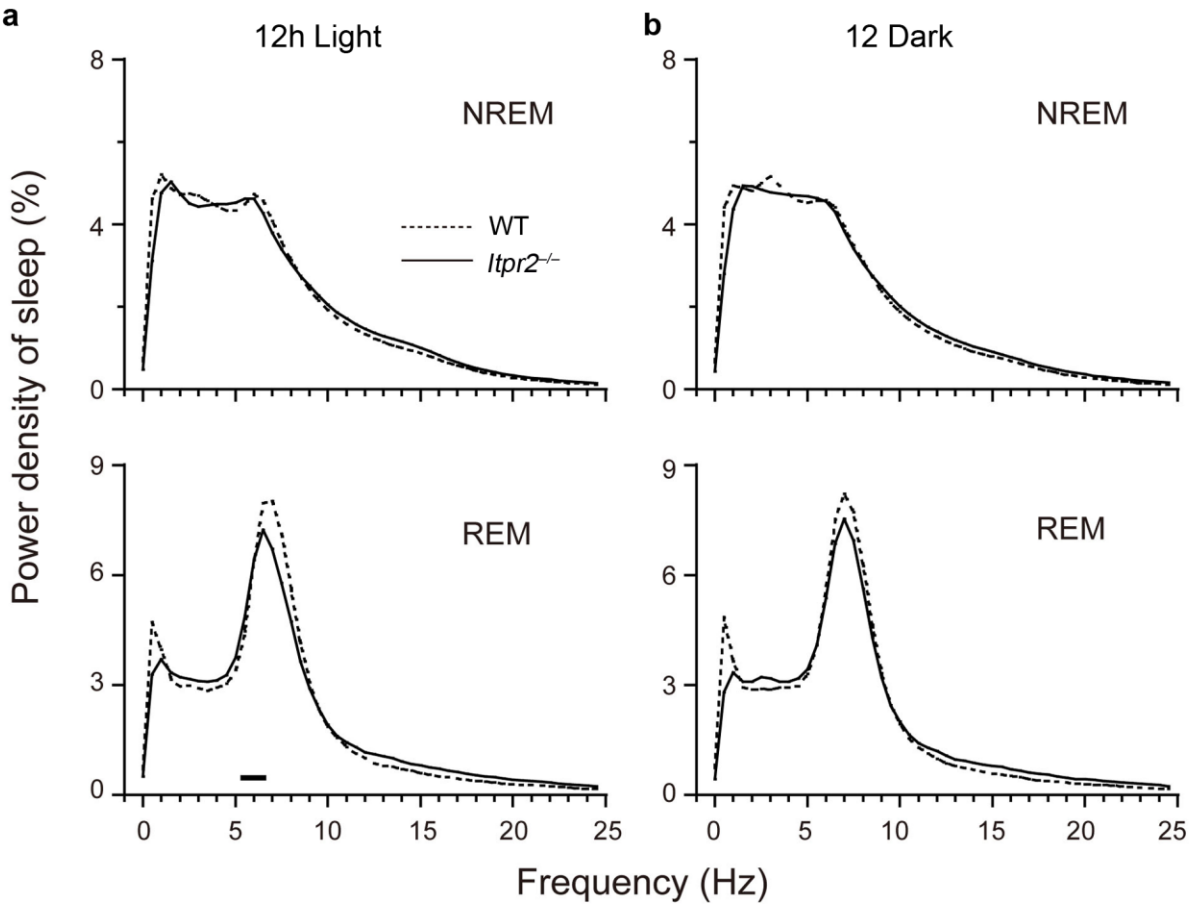
Baseline sleep architecture is unaffected in *Itpr2*$^{-/-}$ mice. a. Time course changes in wakefulness, NREM, and REM sleep. Each circle represents the mean hourly duration of each stage. The horizontal open and filled bars on the X-axes indicate the 12 h light and 12 h dark periods, respectively. There were no differences in the sleep-wake profiles between WT and *Itpr2*$^{-/-}$ mice under baseline conditions (Repeated measures ANOVAs). **b**. The total time spent in wakefulness, NREM, and REM sleep during the 12 h dark and 12 h light phases. WT, $n = 8$; *Itpr2*$^{-/-}$, $n = 10$.

Supplemental Figure 10



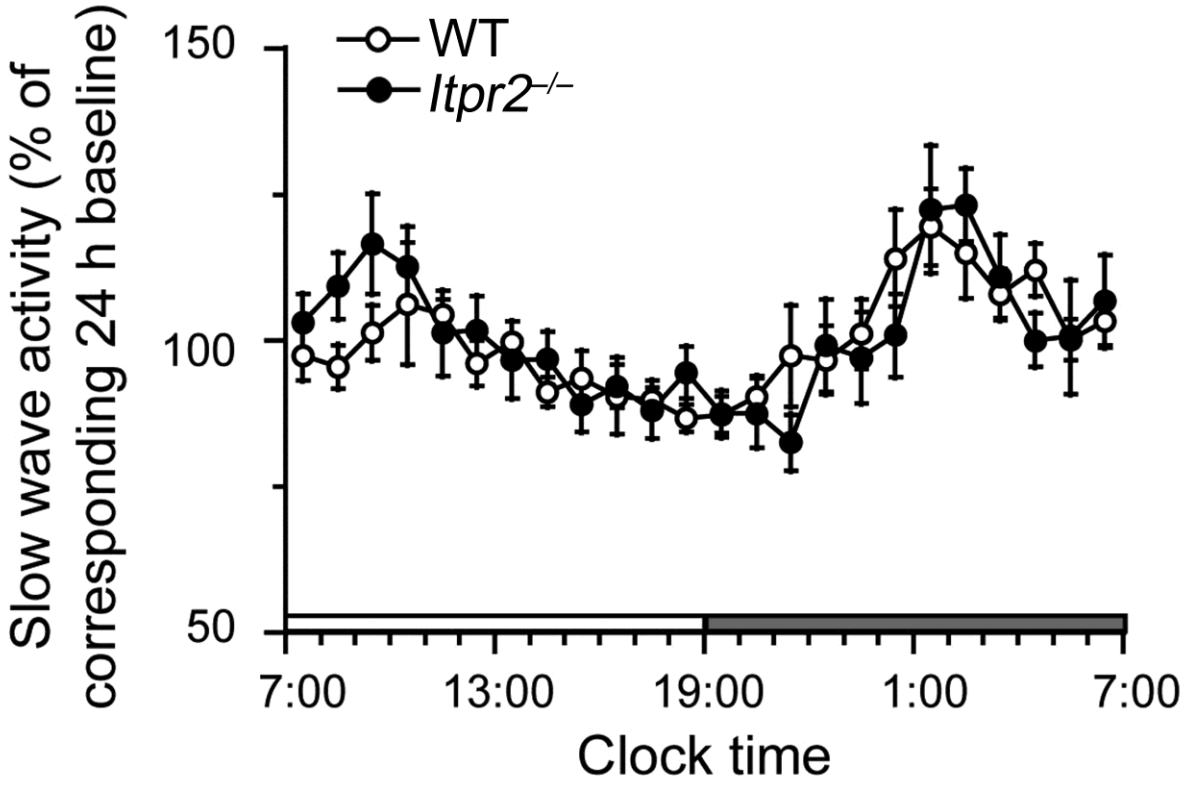
The lack of *Itpr2* had no effect on number of episodes, mean durations or stage transitions during the 12 h light and dark phases. W, wakefulness; N, NREM; R, REM. WT, $n = 8$; *Itpr2*$^{-/-}$, $n = 10$.

Supplemental Figure 11



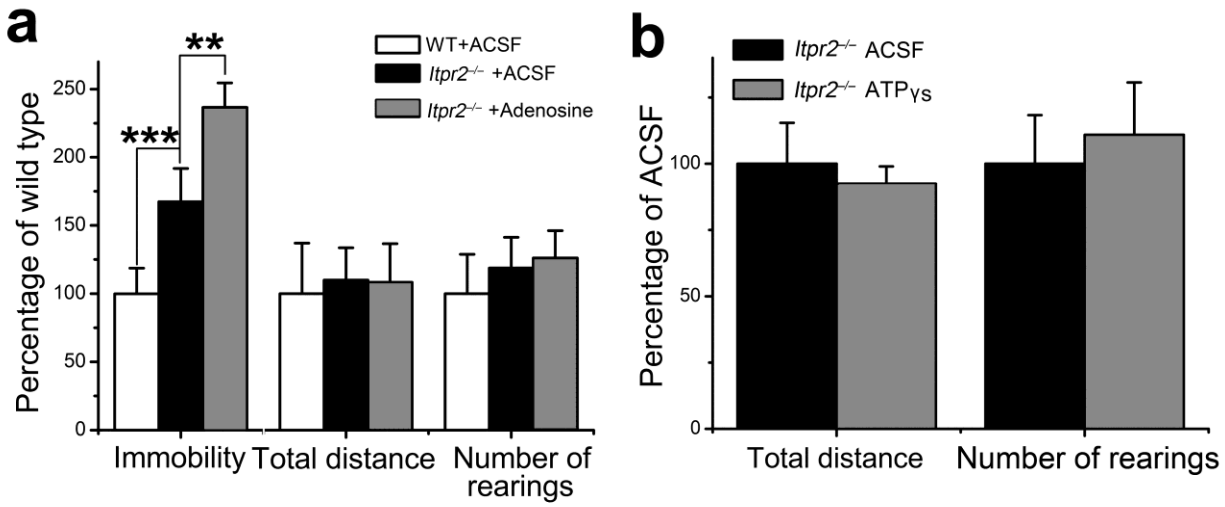
The EEG power density during sleep across the 12 h light and dark phases. The power of each 0.5 Hz bin was first averaged across the sleep stages individually and then normalized across all of the sleep stages by calculating the percentage of the total power of each bin (0–24.5 Hz) for the individual animals. The horizontal bars indicate where a significant difference ($P < 0.05$) was observed relative to WT littermates as determined by one-way ANOVA (WT, $n = 8$; *Itpr2*$^{-/-}$, $n = 10$).

Supplemental Figure 12



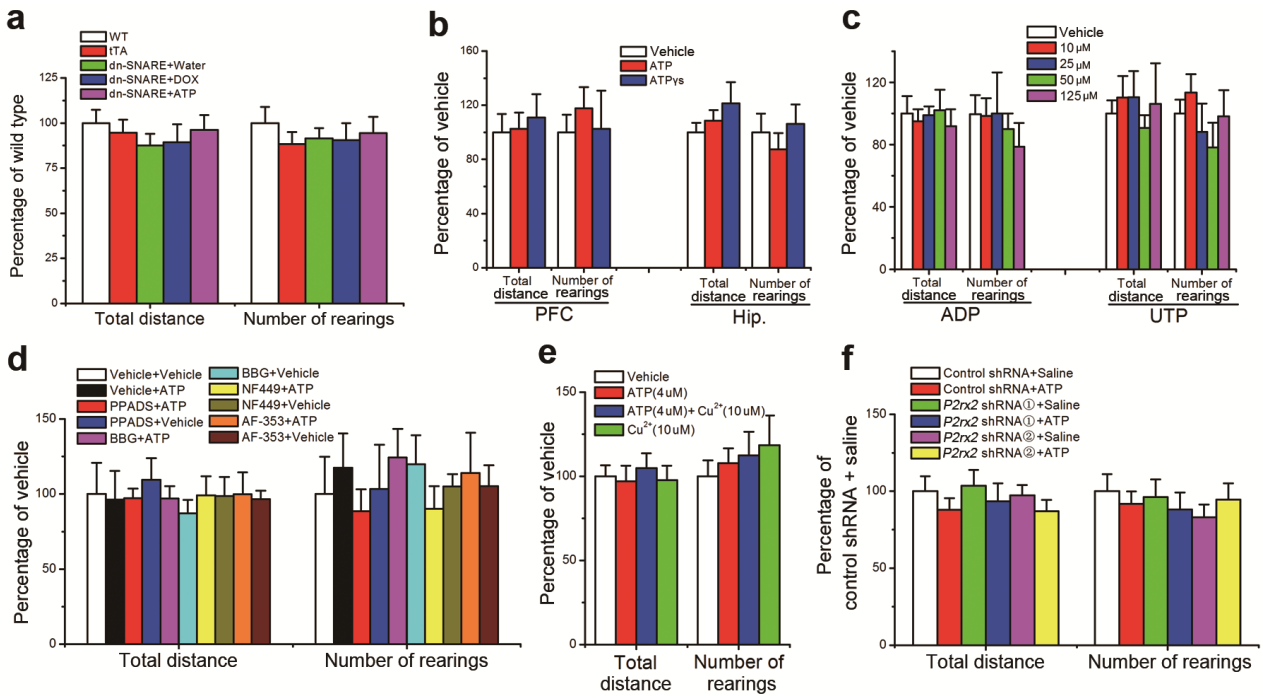
Slow wave activity of NREM in WT and *Itpr2*$^{-/-}$ mice. EEG power density between 0.5 and 4 Hz is given for each mouse as a percentage of the mean of the 24 h baseline. The horizontal open and filled bars on the X-axes indicate the 12 h light and 12 h dark periods, respectively. There was no difference in SWA between the two genotypes by repeated measures ANOVA (WT, $n = 8$; *Itpr2*$^{-/-}$, $n = 10$).

Supplemental Figure 13



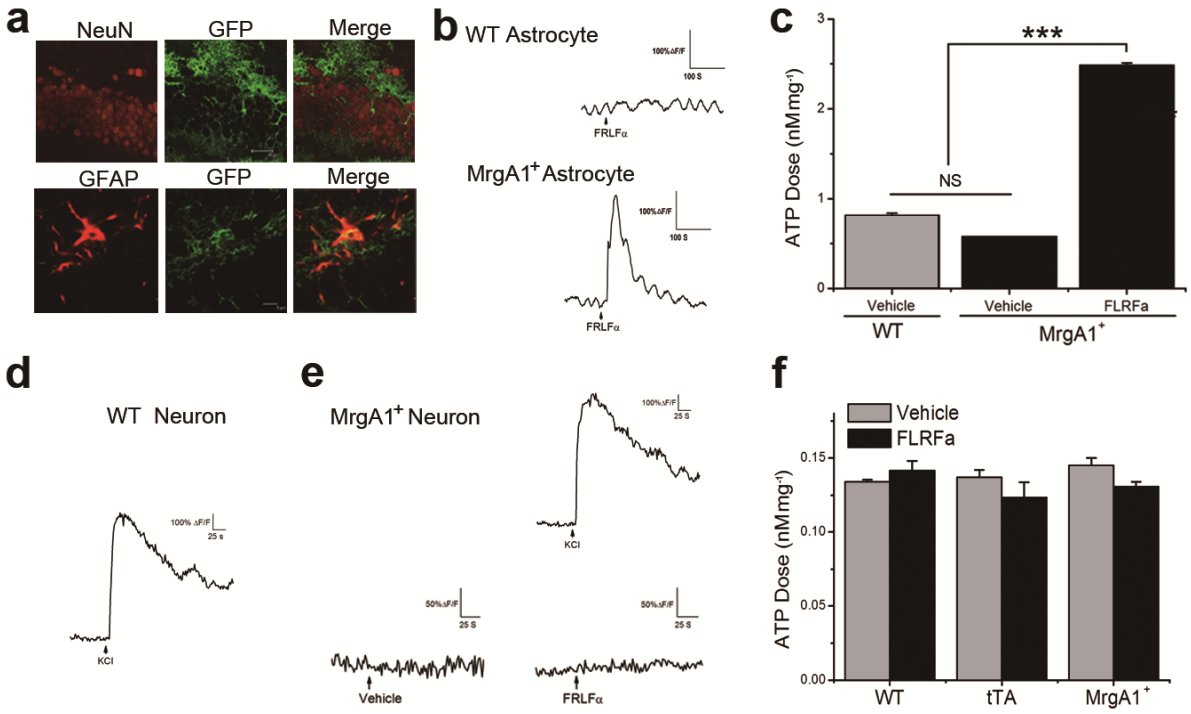
Related to Fig. 3. a. Quantification of the immobility for the WT and *Itpr2*$^{-/-}$ mice infused with adenosine or ACSF (i.c.v) (n = 8). In the open field test, there was no difference in the total distance and number of rearing among the three groups (n = 6–8). **b.** The effect of ATPγs infusion on locomotor activity in *Itpr2*$^{-/-}$ mice. Seven days following cannula implantation, naïve *Itpr2*$^{-/-}$ mice were infused with ACSF or ATPγs (50 μM) and were subjected to the open field test for 5 min following the infusion (n = 10). LSD *post hoc* test: *P < 0.05; ** P < 0.01.

Supplemental Figure 14



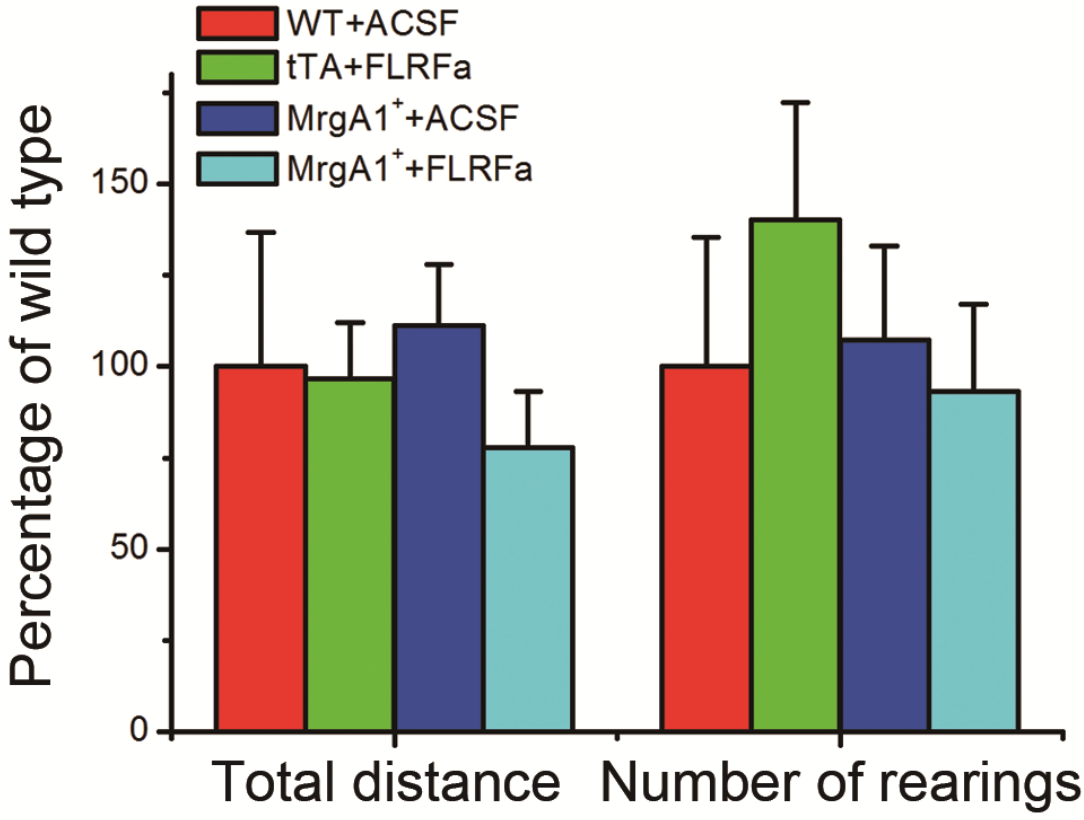
Open field tests. a. i.p. injection of ATP had no effect on dn-SNARE mice in the open field test (n = 10). **b.** mPFC infusion of ATP or ATPγs had no effect on locomotor activity (n = 8). **c.** mPFC infusion of ADP (n = 9) or UTP (n = 9) had no effect on locomotor activity. **d.** mPFC infusions of P2X antagonists had no effect on locomotor activity (n = 9). **e.** mPFC injection of Cu^{2+} had no effect on locomotor activity (n = 10). **f.** For the AAV-*P2rx2* shRNA experiments, no differences were found in the open field test (n = 10).

Supplemental Figure 15



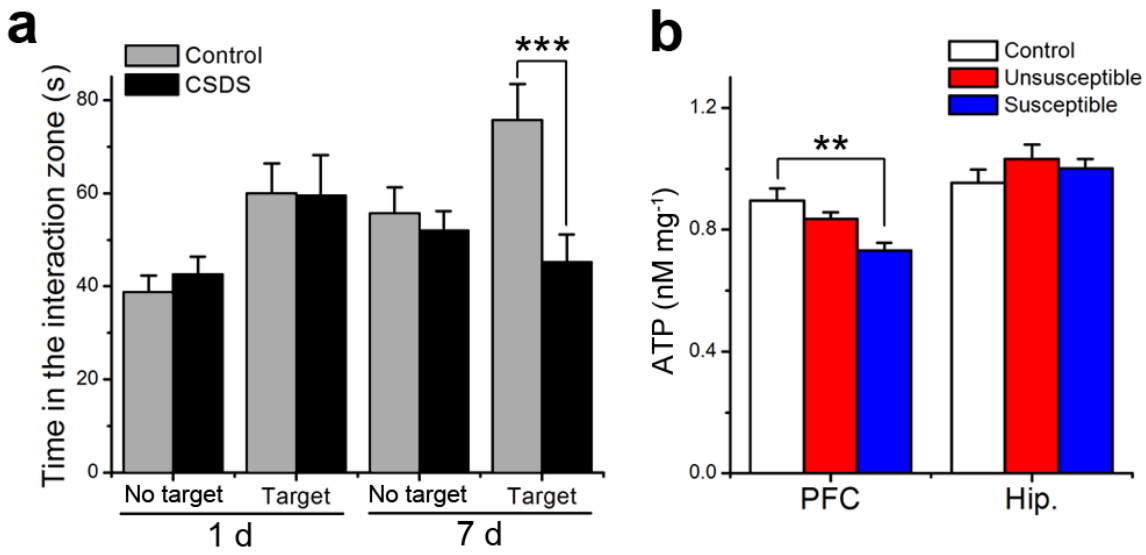
FLRFα stimulation induced transient increases in the amplitude of Ca^{2+} waves and in ATP release from MrgA1^{+} transgenic mouse astrocytes. a. Representative confocal images revealed that astrocytes (scale bar, 5 μm), but not neurons (scale bar, 25 μm), expressed GFP-tagged MrgA1^{+} receptors. **b.** Sample Ca^{2+} traces from cultured MrgA1^{+} astrocytes and WT astrocytes. The cells were filled with an internal solution that contained Fluo-4 Ca^{2+} indicator and were treated with FLRFα (5 μM). **c.** FLRFα stimulation induced a dramatic increase in ATP levels in the culture medium of MrgA1^{+} astrocytes (n = 8). **d.** $[Ca^{2+}]_i$ rises immediately following the addition of KCl (65 mM) in WT neurons. **e.** MrgA1^{+} neurons did not respond to FLRFα treatment. **f.** ATP measurement showing the ATP levels in the cultured medium of MrgA1^{+} neurons stimulated by vehicle or FLRFα (n = 9). LSD *post hoc* test: *** $P < 0.001$; NS, not significant.

Supplemental Figure 16



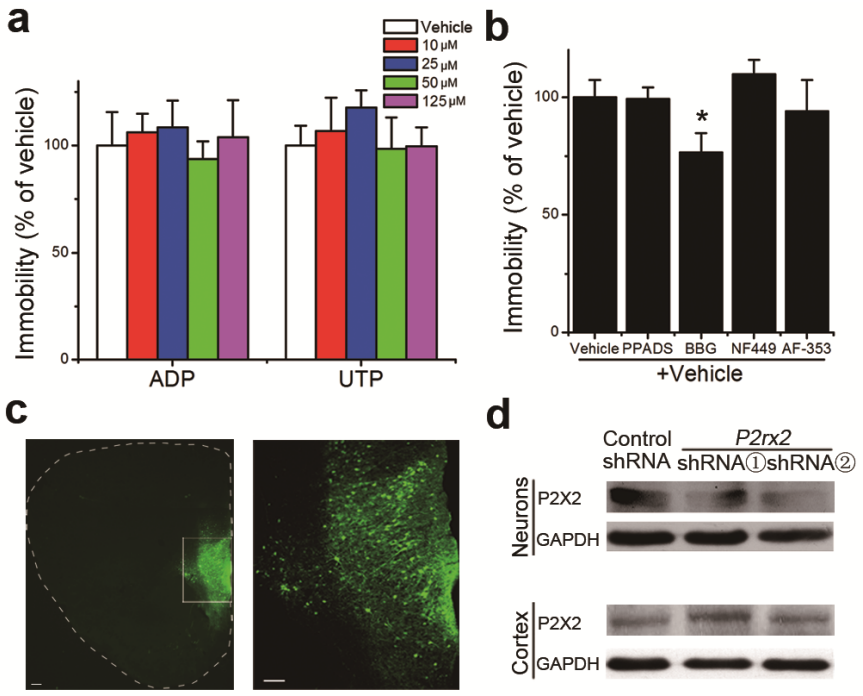
The infusion of FLRFα (i.c.v.) had no effect on spontaneous locomotor activity. No differences were observed in the open field test (n = 8).

Supplemental Figure 17



One-day CSDS did not induce social avoidance behaviors. a. Adult C57BL/6J mice subjected to 7–day CSDS paradigm exhibited social avoidance behaviors (control $n = 12$; CSDS $n = 30$). However, mice subjected to 1–day CSDS behaved similarly to the nondefeated mice when a caged aggressor was introduced into the test area (control $n = 12$; CSDS $n = 15$). **b**. ATP measurement showing ATP levels in the PFC or hippocampus in mice subjected to 7–day CSDS ($n = 6$). LSD *post hoc* test: ** $P < 0.01$; *** $P < 0.001$.

Supplemental Figure 18



Related to Fig. 4. a. Quantification of the immobility for the mice infused with ADP or UTP into the mPFC ($n = 9$). **b.** mPFC infusion of BBG (10 μM) decreased the total duration of immobility in the FST, whereas PPADS (50 μM), NF449 (1μM) or AF-353 (0.1μM) did not ($n = 9$). **c.** AAV injection site into the mPFC (left, scale bar, 200 μm) with image of eGFP expression 2 weeks after injection of AAV- *P2rx2* shRNA (right, scale bar, 100 μm). **d.** P2X2 western blots. For neuron transfection, primary cultured cortex neurons (DIV 9) were transfected with AAVs expressing *P2rx2* shRNAs or control shRNA and cells were harvested 3 days for western blot. Both *P2rx2* shRNAs induced a robust deletion of endogenous P2X2 receptors (Top). We also detected the level of P2X2 receptor in the cerebral cortex (excluded mPFC) of animals injected with AAV-shRNAs. No differences were observed among the three groups (Bottom), indicating that mPFC infusion of AAV- *P2rx2* shRNAs specifically knockdown the level of P2X2 receptor in the mPFC. LSD *post hoc* test: * $P < 0.05$.

Supplementary Methods

Immunofluorescence. The brain tissue was prepared as described previously [1]. Briefly, *Itpr2*$^{-/-}$ mice (10–12 weeks of age) were deeply anesthetized and sacrificed by intracardial perfusion with heparinized 0.9% saline followed by ice-cold 4% paraformaldehyde. The brains were removed and post-fixed with 4% paraformaldehyde overnight and then incubated with a 30% sucrose solution in PBS at 4 °C for 2 d. Using a microtome (Leica CM 1850), coronal sections (40 μm) were taken from the brains every 240 μm, spanning the entire hippocampus (six sections total). Following a 2–hour incubation in a blocking buffer that contained 10% normal goat serum and 0.3% triton X–100, the sections were incubated with primary antibodies. The antibodies that were used included polyclonal rabbit anti-GFAP (1:500, Millipore) and monoclonal mouse anti-NeuN (1:200, Millipore). The sections were incubated with these antibodies overnight at 4 °C. The secondary antibodies, including Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen), were applied for 1 h at room temperature. The sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories Inc.) and visualized using a confocal laser scanning system (Leica DM IRE2). The density of GFAP^{+} cells in the hilus of the DG was measured at a magnification of 400 ×.

Western blot analysis. Western blot analysis was performed according to a protocol that is routinely used in our laboratory [2]. Briefly, mice (10–12 weeks) were sacrificed following anesthetization. Both of the hippocampi, the PFC (for the detection of P2X2 receptors, samples were extracted from mPFC 2 weeks after AAV injection) and cortex (excluding the PFC) were rapidly dissected and homogenized in ice-cold buffer [20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$,

0.2 mM EDTA and 0.1 M NaCl], which was supplemented with a 0.2 mM dithiothreitol and a protease inhibitor cocktail (P8340, Sigma-Aldrich). Subsequently, NaCl was added to a final concentration of 0.45 M, and the homogenate was centrifuged at 10,000 × g for 30 min. The supernatants were collected and mixed with equal volumes of homogenization buffer, which contained 40% (v/v) glycerol. The mixtures were then stored at –80°C. The samples were subjected to electrophoresis on SDS-10% polyacrylamide gels and were transferred to PVDF membranes using standard procedures. The membranes were blocked with 5% non-fat milk powder dissolved in Tris-buffered saline tween–20 (TBST). The membranes were incubated overnight at 4 °C with monoclonal mouse anti-GFAP (1:1,000, Cell Signaling), monoclonal mouse anti-NeuN (1:200, Millipore), polyclonal rabbit anti-P2X2 (1:1,000, Neuromics) polyclonal rabbit anti-GAPDH (1:800, Goodhere) or monoclonal mouse anti-β-actin (1:5,000, Abmart) antibodies. This incubation was followed by incubation with secondary horseradish peroxidase-conjugated antibodies and an enhanced chemiluminescence detection solution (GE Healthcare). The immunofluorescent signal was detected using a FluorChem™ SP Quantitative Imaging System (Cell Biosciences). Protein abundance was quantified by analyzing the western blot bands using FluorChem™ SP software. The optical densities (OD) were normalized to the OD values of the corresponding β-actin or GAPDH bands on the same membranes.

Cell culture. Primary astrocytes were isolated from the hippocampus or cortex of postnatal day 1 mice and were prepared using a modified established protocol [3,4]. Briefly, after washing in D-Hanks medium, the tissue was transferred to a 50 ml Falcon tube containing 0.5 ml D-Hanks medium. The tissue was gently dissociated using a pair of sterile operating scissors and incubated

with 0.25% trypsin (Gibco) in 0.5 mM EDTA at 37 °C for 10 min. Ten milliliters of DMEM/F12 (HyClone) containing 10% FBS (Invitrogen) and 1% penicillin and streptomycin (HyClone) (culture medium) was added to inhibit the trypsin, and the cell suspension was filtered through a 75 μm filter. The filtrate was centrifuged at 200 × *g* for 5 min, and the pellet was resuspended in 10 ml of culture medium. The cells were placed into a culture flask at a density of 5×10^6 cells $5ml^{-1}$. Following incubation in a 5% CO_2/95% air, humidified incubator at 37 °C for 7–10 d, the flask was shaken at 250 rpm for 6 h at 37 °C to isolate the microglial cells. Following this step, the culture medium was refreshed, and the flask was returned to the shaker for 18 h to isolate oligodendrocytes. Under these conditions, the majority of the cells were GFAP or S-100b positive astrocytes. Pure primary hippocampal and cortical neurons were prepared according to a protocol that is routinely performed in our laboratory [5].

Calcium imaging. Dye-filled astrocytes and neurons were visualized using a Leica DM IRE2 confocal microscope. Cells that were cultured on glass bottom dishes (MatTek Corporation) were placed for 30 min in a HBSS solution (37.93 mM NaCl, 4.17 mM $NaHCO_3$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 0.338 mM Na_2HPO_4, 5.56 mM D-Glucose and 1.3 mM $CaCl_2$, pH 7.4) that contained a concentration of 6–8 μM of Fluo–4/AM (Invitrogen). After the unincorporated dye was carefully washed off, the dishes were treated with the Gq-linked GPCR agonist cocktail [containing 50 μM (RS)-3,5-dihydroxyphenylglycine (DHPG), 10 μM histamine, and 10 μM carbachol, with or without 1 μM tetrodotoxin (TTX)], FLRFα (5 μM), or KCl (65 mM) when the $[Ca^{2+}]_i$ baseline was stable. The variations of fluorescence intensity over time were recorded in both astrocytes and neurons using LCS software by placing analyses boxes over individual

cellular compartments. All of the experiments were conducted at room temperature (20 °C). Increases in the fluorescence intensity over baseline were calculated for each trace and are reported as $\Delta F/F_0$.

Sleep recordings and vigilance state analysis. *Itpr2*$^{-/-}$ mice and control littermates were anesthetized and chronically implanted with electroencephalography (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings. The implant consisted of two stainless steel screws (1 mm diameter) that were inserted through the skull into the cortex (AP, + 1.0 mm, mediolateral, - 1.5 mm from Bregma) and served as EEG electrodes. Two insulated stainless steel, Teflon-coated wires were placed bilaterally into both of the trapezius muscles to serve as EMG electrodes. All of the electrodes were attached to a micro-connector and affixed to the skull using dental cement. The EEG and EMG recordings were performed by means of a slip ring, which was designed such that the behavioral movement of the mice would not be restricted. Following a 10–day recovery, the mice were housed individually in transparent barrels and habituated to the recording cable for 3–4 d prior to the polygraphic recordings. To examine the spontaneous sleep-wakefulness cycles, each animal was recorded for 24 h, beginning at 7:00 A.M. (the onset of the light period). The animals then entered the pharmacological phase of the study, in which sleep-wakefulness parameters were recorded for 48 h. The data that were collected during the first 24 h also served as baseline comparison data for the 2nd experimental day. First, the cortical EEG and EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz). The signals were then digitized at a sampling rate of 128 Hz and recorded using SLEEPSIGN software (Kissei Comtec), as previously described [6,7]. When completed, the polygraphic

recordings were automatically scored off-line in 10 s epochs using SLEEPSIGN software as wakefulness, rapid eye movement (REM) sleep, and non-REM (NREM) sleep according to standard criteria [6,7]. Lastly, the defined sleep-wake stages were examined visually and corrected if necessary. Differences between the *Itpr2*$^{-/-}$ mice and the control littermates with respect to 1) the time course of sleep-wake cycle 2) the time spent awake and 3) the time spent in NREM and REM sleep in the light/dark phases were determined.

Supplementary References

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