Supporting Information

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SI Materials and Methods

Plasmid Construction. Expression vectors encoding HA–IRBIT, GST–IRBIT, His-tagged IRBIT (His–IRBIT), GFP–IRBIT, GFP–IRBIT truncation mutants, and GST–Homer 3 and HA–Homer 3 were described previously (1–3). The full length of IRBIT fragment was subcloned into the expression vector including internal ribosome entry site (IRES) sequence and mRFP fragment, which was constructed from pIRES2–AcGFP1 vector (Clontech) by replacing AcGFP1 with mRFP. The full-length tyrosine hydroxylase (TH) was amplified by PCR using the primers of 5′-CTAAGCTTATGCCCACCCCCAGCGCCTCCTC-3′ and 5′-GAGAATTCTTAGCTAATGGCACTCAGTGCTTG-3′ (the underlined letters indicate the HindIII and EcoRI sites for cloning, respectively) from a mouse cDNA library and subcloned into pcDNA3.1Zeo(+) vector (Life Technologies). The expression vectors encoding calmodulin and GFP–CaMKIIα were a kind gift from N. Hayashi (4) and Y. Yamagata (5). The cDNA fragments of CaMKIIα were amplified by PCR and subcloned into pGEX-4T-1 vector (Pharmacia) and pFastBac1 vector (Life Technologies). Fluorescence resonance energy transfer (FRET)-based CaMKIIα probe, Camuiα, was constructed by using CaMKIIα fragment, Venus fragment, ECFP fragment, and pEGFP-N1 vector in the same way described in the original report (6).

Antibodies. Rabbit anti-IRBIT antibody (Ab), guinea pig anti-IRBIT Ab, rabbit antiphospho-IRBIT Ser68p/Ser71p Ab, and rabbit antiphospho-IRBIT Ser74p/Ser77p Ab, rabbit anti-Long-IRBIT Ab were described previously (2, 3, 7). Rabbit anti-80K-H Ab and anti-IP_3Rs (KM1112, and 4C11 for IP_3R1, KM1083 for IP_3R2, and KM1082 for IP_3R3) were described previously (8). Rabbit anti-phospho Homer 3 (Ser120p) Ab was described previously (1). Other antibodies used were mouse anti-GFP Ab (B-2, Santa Cruz Biotechnology), rat anti-HA Ab (3F10, Roche), mouse anti–β-actin Ab (AC-15, Sigma), mouse anti-CaMKIIα Ab [6G9, Affinity BioReagents (ABR)], mouse antiphospho CaMKIIα Ab (Thr286p, 22B1; ABR), rabbit anti-Active CaMKII Ab (V1111, Promega), mouse anticalmodulin Ab (05-173, Millipore), mouse anti-tyrosine hydroxylase (MAB318, Millipore), rabbit antiphospho-Ser19 TH (p1580-19, PhosphoSolutions), and antiserotonin (AB938, Chemicon).

Recombinant Proteins. Recombinant CaMKIIα was expressed in Sf9 cells using the Bac-to-Bac system (Life Technologies) and purified using CaM affinity resin (Stratagene). Recombinant GST–CaMKIIα partial or full-length proteins were expressed in *Escherichia coli.* His–IRBIT was expressed in Sf9 cells and purified as described (2). It is notable that His–IRBIT purified from Sf9 cells by Ni-NTA resin (Life Technologies) contained the unidentified endogenous calcium calmodulin dependent kinase. Therefore, we removed the contaminated kinase by CaM affinity resin and confirmed the elimination of contaminated kinase activity by an in vitro kinase assay. *E. coli* IRBIT was expressed as GST–IRBIT and purified with glutathione Sepharose 4B (GE Healthcare). Purified GST–IRBIT was digested by precision protease (GE Healthcare) to eliminate GST–tag. Recombinant Homer 3 was expressed as GST–Homer 3 in *E. coli.* Purified GST–Homer 3 was digested by thrombin to eliminate GST–tag. Recombinant calmodulin was purified from *E. coli* as described previously (4).

Cell Culture. Monkey kidney cell line COS-7 cells and human embryonic kidney cell line HEK-293 cells were obtained from the RIKEN Cell Bank. Cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% (vol/vol) FBS, 50 units/mL penicillin, and 0.05 mg/mL streptomycin (Nakarai). Rat pheochromocytoma cell line PC-12 cells were a kind gift from Yasuo Watanabe (Showa Pharmaceutical University, Tokyo). PC-12 cells were cultured in RPMI medium 1640 (Nakarai) supplemented with 5% (vol/vol) horse serum, 5% (vol/vol) FBS, 50 units/mL penicillin, and 0.05 mg/mL streptomycin.

Kinase Assay in Living Cells. GFP or GFP–CaMKIIα stably expressing HEK-293 cells were transfected with the CaMKIIα-substrate plasmid (HA–Homer 3 or TH) with or without indicated plasmid DNA or siRNAs. After 24 h (for overexpression) or 48 h (for knockdown), transfected cells were washed once in Ca^{2+}-free balanced salt solution [BSS(−): 20 mM Hepes, pH 7.4, 115 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose]. Then, cells were stimulated with 2.5 μM calcium ionophore, 4-BrA23187 in BSS(−). After 2 min, kinase reaction was stopped by liquid nitrogen. Stimulated cells were lysed by SDS/PAGE sample buffer and phosphorylation levels were analyzed by Western blotting with phospho-specific antibodies. PC-12 cells were cultured on glass coverslips and were transfected with GFP or GFP–IRBIT. After 24 h, transfected PC-12 cells were washed once by BSS(−) containing 2 mM $CaCl_2$ [BSS(+)] and stimulated with 50 mM KCl in BSS(+) for 10 min. Nonstimulated cells and stimulated cells were fixed with 4.0% (wt/vol) paraformaldehyde (PFA) in PBS for 10 min. The phosphorylation level of endogenous TH was analyzed by immunostaining using phosho-TH and TH antibodies.

Generation of IRBIT KO Mice and Mouse Embryonic Fibroblast Cells. Generation of IRBIT KO mice was described previously (9). Briefly, IRBIT KO mice were obtained from the intercross between $IRBIT^{flox/flox}$ mice and EIIa-cre transgenic mice. The accession number of $IRBIT^{flox/flox}$ mice is CDB0669K (www.cdb.riken.jp/arg/mutant%20mice%20list.html). EIIa-cre transgenic mice, which carry the Cre recombinase gene driven by the adenovirus EIIa promoter, were a kind gift from Shigeyoshi Itohara (RIKEN). IRBIT KO mice were backcrossed into C57BL/6J mice for 5–15 generations. Primary MEF cultures were established by standard procedures from embryonic day 13.5 (E13.5) embryos derived from homozygote mating of IRBIT KO mice or littermate wild-type mice. Permanent IRBIT KO or WT cell lines were established from transformed clones arising spontaneously after repeated passage in culture. MEF cells were cultured in Dulbecco's modified essential medium (DMEM) (Nakarai) supplemented with 10% (wt/vol) FBS, 50 units/mL penicillin, and 0.05 mg/mL streptomycin (Nakarai).

FRET and Ca^{2+} Imaging in MEF Cells and Primary Hippocampal Neurons. FRET and Ca^{2+} imaging were performed as described previously (10). Briefly, FRET-based CaMKIIα probe, Camuiα, was transfected into MEF cells with transfection reagents (FuGENE HD; Roche) or cultured hippocampal neurons by calcium phosphate methods. After 18–24 h for MEF cells or 3–5 d for neurons, the transfected cells were used for imaging. After loading the cells with 5 μM Indo-5F AM or Indo-1 AM (DOJINDO), imaging was performed in BSS(−) through an inverted microscope (IX-71; Olympus) with a cooled charge-coupled device camera (ORCA-ER; Hamamatsu Photonics) and a 40× (N.A. 1.35) objective. Sequential excitation of Camuiα and Indo-5F or Indo-1 was performed by using a 450-nm dichroic mirror and two excitation filters (a 425–445-nm filter for Camuiα and a

333- to 348-nm filter for Indo-1). Dual emission at 460–490 nm (for Camuiα and Indo-5F or Indo-1) and >520 nm (for Camuiα) split with a 460- to 490-nm filter, a long-path 520-nm barrier filter, and two 505-nm dichroic mirrors equipped in W-view (Hamamatsu Photonics). Images were acquired at 0.166 Hz. Filters and the camera were controlled using MetaMorph software (Molecular Devices). For MEF cells, 2.5 μM BrA was added by bath application to increase the intracellular Ca^{2+} concentration. For neurons, 25 μM NMDA and 1 μM glycine were added by reflux.

Hippocampal Primary Culture. Hippocampal neurons were prepared from the postnatal 0- to 1-d mice as described previously (8). Briefly, hippocampal cells were dissociated by trypsin treatment and trituration and were plated on polyethyleneimine-coated glass coverslips at a density of 5.0×10^4 cells/cm^2 in 15-mm culture dishes with 1.0 mL of medium. The medium was neurobasal A medium (Life Technologies) containing 2.0% (vol/vol) B-27 supplement (Life Technologies), 0.5 mM L-glutamine (Nacalai), 50 units/mL penicillin, 0.05 mg/mL streptomycin, and 25% (vol/vol) glial conditional medium (Sumitomo Bakelite). After plating, cells were incubated at 37 °C, 5.0% (vol/vol) CO_2/air.

Transfection. Transfection was performed as described previously (8). Briefly, COS-7 cells, HEK-293 cells, PC-12 cells, or MEF cells (2×10^5 cells) were plated in a 35-mm tissue-culture dish or glass bottom dish with 2.0 mL of medium (in some cases, on glass coverslips). After 24 h, transfection was performed using FuGENE HD (Promega) or X-treamGENE HP (Roche Diagnostics) according to manufacturer instructions. For the knockdown experiments, short interfering RNA (20 μM, Stealth siRNA; Life Technologies) and Lipofectamine 2000 (Life Technologies) were used according to manufacturer instructions.

Stable HEK-293 Cell Line Expressing GFP or GFP–CaMKIIα. HEK-293 cells were transfected with GFP or GFP–CaMKIIα expression vector using FuGENE HD transfection reagent (Promega). G418-resistant transformants were selected by 500–700 μg/mL G418. Several clones representing GFP signals at a similar level were obtained by flow cytometry at the Support Unit for Bio-Material Analysis in RIKEN BSI Research Resources Center (RRC). The expression levels of GFP or GFP–CaMKIIα were checked by Western blotting using GFP antibody.

Coimmunoprecipitation and Pull-Down Assay. For immunoprecipitation of exogenous proteins, COS-7 cells or HEK-293 cells expressing HA–IRBIT and GFP–CaMKIIα or Camuiα were washed with PBS and solubilized in TNE buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.0% (wt/vol) Nonidet P-40 (Nonidet P-40, Nakarai), proteinase inhibitor (complete, Roche)]. The homogenate was centrifuged at 20,000 × *g* for 20 min. The supernatant was precleared with Protein-G Sepharose 4B Fastflow (Protein-G, GE Healthcare) and incubated with the appropriate antibodies and Protein-G for 4 h at 4 °C. The beads were then washed four times with TNE buffer, and proteins were eluted by boiling in SDS/PAGE sampling buffer.

For immunoprecipitaion of endogenous proteins, mouse hippocampi were homogenized with homogenization buffer [10 mM Hepes-NaOH (pH 7.5), 2 mM EDTA, 1 mM 2-mercaptoethanol, 320 mM sucrose, proteinase inhibitor (complete, Roche)] using a Tephrone homogenizer. The homogenate was centrifuged at 1,000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 30 min. The precipitate was solubilized in TNE buffer centrifuged at 20,000 × *g* for 20 min. The supernatant was precleared with Protein-G and was incubated with the appropriate antibodies and Protein-G for 4 h at 4 °C. The beads were then washed four times with TNE buffer, and proteins were eluted by boiling in SDS/PAGE sampling buffer.

For pull-down binding assay, the cell lysate of COS-7 cells expressing GFP–IRBIT truncation proteins were prepared by a similar method for immunopreipitation. The cell lysate was precleared with glutathione Sepharose 4B and incubated with recombinant GST-fusion proteins and glutathione Sepharose 4B for 4 h at 4 °C. The beads were then washed four times with TNE buffer, and proteins were eluted by 25 mM glutathione in TNE buffer and boiled in SDS/PAGE sampling buffer.

For pull-down assay using the recombinant purified proteins, the kinase assay buffer (described in the method for in vitro kinase assay) with 1% (wt/vol) Triton X-100 was used in place of TNE buffer. For elusion assay by excessive amount of Ca^{2+}–CaM, the beads were washed four times and incubated with various amounts of CaM (0, 0.6, 1.2, 3.0, and 6.0 μM) in the kinase assay buffer on ice for 30 min.

Imaging for GFP–CaMKIIα Stable Expressing HEK-293 Cells. Intracellular Ca^{2+} imaging was performed as described previously (8). Briefly mRFP with or without HA–IRBIT was transfected into GFP–CaMKIIα stable expressing HEK-293 cells with transfection reagents (FuGENE HD; Roche). After 18–24 h, cells were used for imaging. After loading the cells with 5 μM Fura-2 AM (DOJINDO), imaging was performed in BSS(−). To increase the intracellular Ca^{2+} concentration, 2.5 μM BrA was added by bath application.

Western Blotting Analysis. Proteins were separated by SDS/PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5.0% (wt/vol) skim milk in PBS containing 0.05% (wt/vol) Tween-20 (PBST) for 1 h and probed with the primary antibody for 1 h at room temperature (RT). After washing with PBST, the membranes were incubated with an appropriate HRP-conjugated secondary antibody, and signals were detected with Immobilon Western Detection Reagents (Millipore).

Immunocytochemistry. Hippocampal primary cultured neurons, PC-12 cells, and MEF cells grown on glass coverslips were washed once with BSS(+) and fixed with 4.0% (wt/vol) PFA in PBS for 10 min. Cells were permeabilized with 0.1% (wt/vol) Triton X-100 in PBS for 5 min and blocked with 1.0% (wt/vol) BSA with 1.0% (vol/vol) normal goat serum (Vector Laboratories) in PBS for 60 min. Cells were then stained with indicated primary antibodies for 60 min at RT or overnight at 4 °C. Following three washes with PBS for 15 min in total, appropriate secondary antibodies (Alexa 488, 594, or Cy5-conjugated goat anti-rabbit IgG, Alexa 488, 594, or Cy5-conjugated goat anti-mouse IgG or Alexa Fluor 488- or 594-conjugated goat anti-guinea pig IgG; Life Technologies) were applied for 60 min at RT. For staining of actin filament, Alexa 594-conjugated phalloidin (Life Technologies) was used. After washing with PBS, the coverslips were mounted with Vectashield containing DAPI (Vector Laboratories) and observed under confocal fluorescence microscopy (FV1000, Olympus).

Histology and Immunohistochemistry. IRBIT KO and their littermate control mice were anesthetized with isoflurane and pentobarbital and transcardially perfused with PBS, then with 4% (wt/vol) PFA in PBS. The brains were dissected from the mice, postfixed in 4% (wt/vol) PFA at 4 °C overnight, and cryoprotected by immersion in 30% (wt/vol) sucrose in PBS overnight at 4 °C. After being embedded in Tissue-Tek OCT compound [50% (vol/vol) compound in 30% (vol/vol) sucrose-PBS; Sakura Finetechnical Co.], the brains were frozen in isopentane chilled in liquid nitrogen. The brains were sectioned parasagittally or coronally at 12-μm thickness with a cryostat (CM1850, Leica Microsystems) at –18 °C to –24 °C. The sections were air dried overnight. For morphological analysis, the sections were stained by hematoxylin-eosin (HE staining) or cresyl violet acetate (Nissl staining) using conventional methods. For

immunohistochemistry, the sections were unmasked by 10 mM hot sodium citrate buffer for 5 min, permeabilized with 0.3% (wt/vol) Triton X-100 in PBS for 10 min, and blocked with blocking solution [1.0% (wt/vol) BSA, 1.0% (vol/vol) normal goat serum (Vector Laboratories) and 0.3% (wt/vol) Triton X-100 in PBS] for 60 min at RT. The sections were then stained with indicated primary antibodies in blocking solution overnight at 4 °C. Following three washes with PBS for 15 min in total, appropriate secondary antibodies [Alexa 488-, or 594-conjugated goat anti-rabbit IgG, Alexa 488, or 594-conjugated goat anti-mouse IgG (Life Technologies)] were applied for 60 min at RT. After washing with PBS, the sections were mounted with Vectashield containing DAPI (Vector Laboratories) and observed under inverted microscope (Nikon) or confocal fluorescence microscopy. For floating immunohistochemistry method, the brains were dissected from mice, postfixed in 4% (wt/vol) PFA at 4 °C overnight, and sectioned parasagittally at 50-μm thickness with a vibrating blade microtome (Leica VT1000 S). The sections were floating with 0.3% (wt/vol) Triton X-100 in PBS more than 60 min and blocked with blocking solution for 60 min at RT. The sections were then stained with indicated primary antibodies in blocking solution 24 h for hippocampal section and 48 h for VTA, cerebellum, and raphe nucleus sections at 4 °C. Following three washes with PBS for 30 min in total, appropriate secondary antibodies were applied overnight at 4 °C. After washing with PBS, the sections were mounted with Vectashield containing DAPI (Vector Laboratories) and observed under confocal fluorescence microscopy.

Monoamine Content Analysis by HPLC. IRBIT KO and their littermate control mice were anesthetized with isoflurane and killed. The brain was dissected from mouse and samples were collected manually. Samples were homogenized by a Tephrone homogenizer and were deproteinized with 0.2 M perchloric acid on ice 30 min, and centrifuged at 20,000 × *g* for 15 min. Supernatant was collected and mixed with sodium acetate (pH 3.0) and monoamine contents were measured by HPLC–ECD system (EICOMPAK SC-5ODS/ECD–300, EICOM, +750 mV vs. Ag/AgCl).

Behavior Analysis. All behavioral tests were carried out with male IRBIT KO mice and their wild-type littermates that were 12–13 wk old at the start of the testing (KO, $n = 20$; WT, $n = 20$). Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 AM) with access to food and water ad libitum. Behavioral tests were performed between 9:00 AM and 6:00 PM except for the home cage social interaction test, as described previously (11–14).

Neuromuscular strength test. Neuromuscular strength was assessed with the forelimb grip strength test and wire hang test. Forelimb grip strength (in newtons) was measured by pulling a mouse by the tail while its forepaws hung onto a wire grid attached to a spring balance (O'Hara & Co.). In the wire hang test, mice were placed on a wire mesh, which was then inverted slowly. Latency to fall from the wire mesh was recorded with a 60-s cutoff time.

Hot plate test. Mice were placed on a hot plate (Columbus Instruments) set at 55.0 ± 0.1 °C to evaluate sensitivity to a painful stimulus. The latency to the first fore- or hindpaw response was recorded with a 15-s cutoff time. The paw response was defined as either a foot shake or a paw lick.

Rotarod test. To evaluate motor coordination and balance, mice were placed on rotating drums (3 cm diameter) of an accelerating rotarod (UGO Basile). The speed of the rotarod accelerated from 4 to 40 rpm over a 300-s period. Latency to fall off the rotarod was recorded with a 300-s cutoff time for three trials per day over 2 consecutive days.

Tail suspension test. Mice were suspended 30 cm above the floor of a white plastic chamber in a visually isolated area by adhesive tape placed less than 1 cm from the tip of the tail. Images were captured at one frame per second for 10 min. Similar to the Porsolt forced swim test, immobility was calculated using ImagePS software.

Light/dark transition test. The apparatus consisted of a cage (21 × 42 × 25 cm) divided into two sections of equal size by a partition with a door (O'Hara & Co.). One chamber was brightly illuminated (390 lx), whereas the other chamber was dark (2 lx). Mice were placed into the dark chamber and allowed to move freely between the chambers for 10 min. The distance traveled (in centimeters), latency to first enter the lit chamber (in seconds), time spent in each chamber (in seconds), and number of transitions were recorded automatically using ImageLD software.

Elevated plus maze test. The maze apparatus consisted of two open arms (25 × 5 cm) with a 3-mm-high ledge, two enclosed arms of the same size with 15-cm-high transparent walls, and a center square (5 × 5 cm) connecting arms (O'Hara & Co.). The arms and center square were made of white plastic plates and were elevated 55 cm above the floor. Mice were placed into the central square facing a closed arm and allowed to explore the maze for 10 min. The distance traveled (in centimeters), number of arm entries, entries into open arms (percentage), and time spent in open arms (percentage) were calculated automatically using ImageEP software.

Porsolt forced swim test. Mice were placed in a cylinder (20-cm height, 10-cm diameter, O'Hara & Co.) filled with water (21–23 °C) up to a height of 7.5 cm. Images were captured at one frame per second for 10 min. For each pair of successive frames, the amount of area (in pixels) within which the mouse moved was measured. The amount of area below and above a threshold indicates that the mouse was "immobile" and "moving," respectively. Immobility lasting for less than 2 s was not included in the analysis. Data acquisition and analysis were performed automatically using ImagePS software.

Open field test. Each subject was placed in the corner of an open field apparatus (40 × 40 × 30 cm; Accuscan Instruments), which was illuminated at 100 lx at the center (20 × 20 cm). Total distance traveled (in centimeters), vertical activity (rearing measured by counting the number of photobeam interruptions), time spent in the center (in seconds), and beam-break counts for stereotypic behaviors were recorded. Data were collected for 120 min.

Social interaction test in home cage. Two mice of the same genotype that had been housed separately were placed together in a cage (25 × 15 × 23.5 cm) containing an infrared videocamera, which was attached to the top of the cage (O'Hara & Co.). Their social behavior and locomotor activity were monitored for 7 d through an infrared videocamera and recorded by a computer. Images from each cage were captured at a rate of one frame per second. Social interaction was measured by counting the number of particles detected in each frame: two particles indicated that the mice were not in contact with each other, and one particle indicated contact between the two mice. Locomotor activity was also measured by quantifying the number of pixels that changed between each pair of successive frames. Data acquisition and analysis were performed automatically using ImageHA software.

Social interaction test in a novel environment. Two mice of the same genotype that were previously housed in different cage were placed in a box together (40 × 40 × 30 cm, O'Hara & Co.) and allowed to explore it freely for 10 min. The total duration of contacts (in seconds), number of contacts, total duration of active contacts (in seconds), mean duration of contacts (in seconds), and distance traveled (in centimeters) were recorded. Behavior of the mice was defined as an "active contact" when they contacted each other and the distance they traveled was longer than 10 cm. Data acquisition and analysis were performed automatically using ImageSI software.

Image analysis. The application programs for behavioral data acquisition and analysis (ImageLD, ImageEP, ImageSI, ImagePS, and ImageHA) were created on the platform of National Institutes of Health Image (rsb.info.nih.gov/nih-image/) and ImageJ (rsb.info.nih.gov/ij/) by Tsuyoshi Miyakawa.

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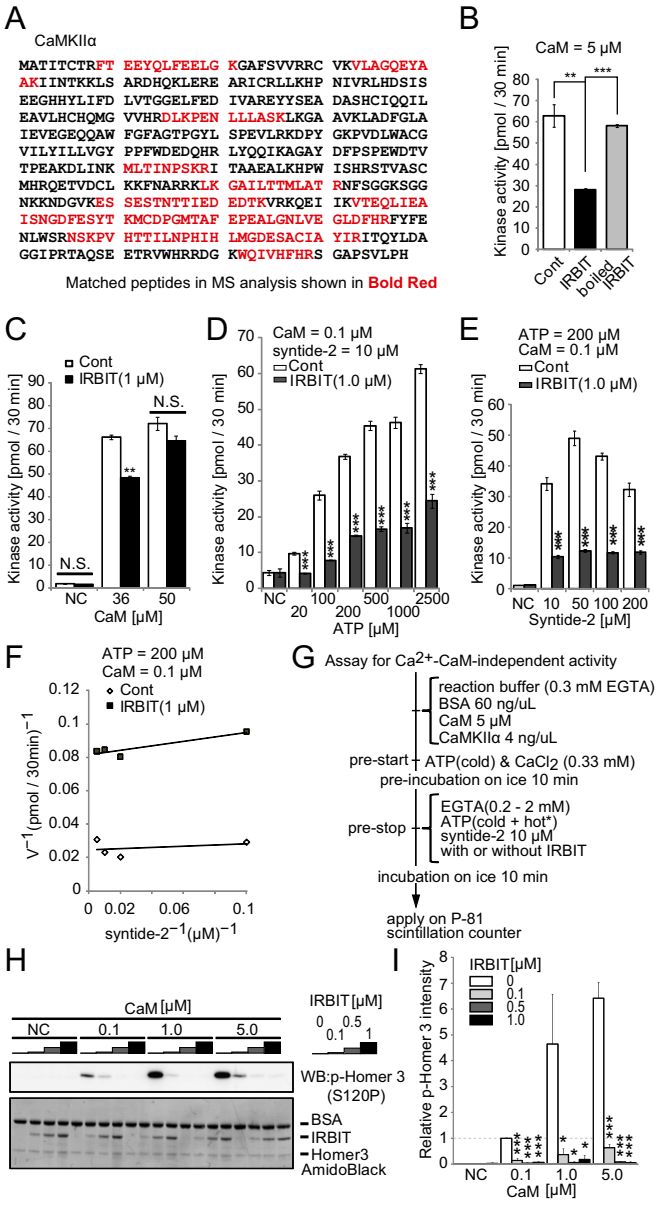

Fig. S1. In vitro CaMKIIα kinase assay. (*A*) Schematic illustration of CaMKIIα peptides (bold red), identified by mass-spectrometry analysis. (*B*) In vitro CaMKIIα kinase assay with or without IRBIT (1 μM) or boiled IRBIT (1 μM). (*C*) Effect of excess of Ca^{2+}–CaM on kinase activity with IRBIT. NC (negative control): kinase assay without CaM. (*D*) Effect of IRBIT on the ATP-dependency of CaMKIIα activity. (*E*) Effect of IRBIT on the substrate dependency of CaMKIIα activity. (*F*) The double-reciprocal analysis of results in *E*. (*G*) Flowchart of Ca^{2+}–CaM-independent CaMKIIα kinase activity assay. (*H*) Purified Homer 3 protein was phosphorylated by CaMKIIα with or without IRBIT. Phosphorylation of Homer 3 was detected with antiphospho-Homer 3 antibody. (*I*) Quantitative analysis of phospho-Homer 3 in *H*. $n = 3$. ATP: 200 μM.

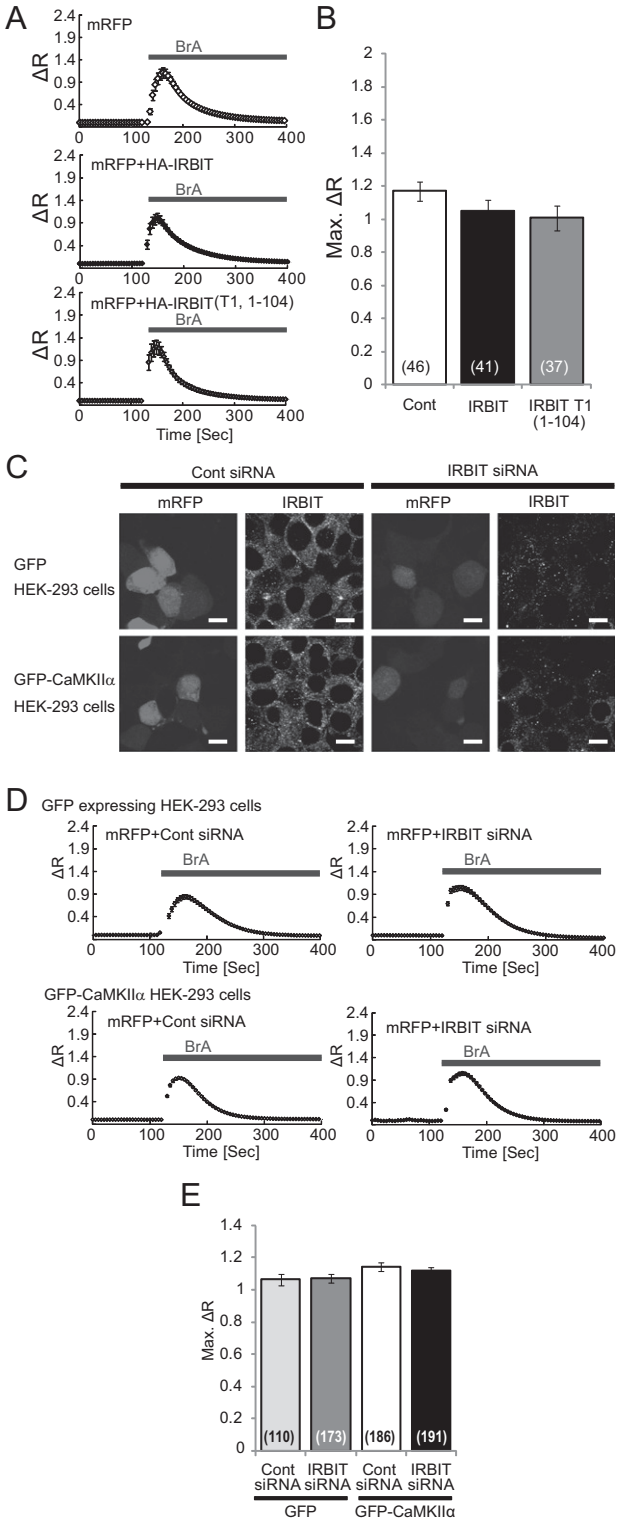

Fig. S2. (*A*) The calcium increase induced by BrA (Ca^{2+}-ionophore, *BrA*-23187, 2.5 μM) was not affected by overexpression of IRBIT or the IRBIT T1 fragment. Representative Ca^{2+} changes after BrA stimulation in GFP–CaMKIIα cells. (*B*) Quantitation of Ca^{2+} peak amplitude in *A*. Results are shown as the mean ± SEM of at least three independent experiments. Total cell numbers were indicated in each bar. Peak amplitude of Ca^{2+} responses (Max. ΔR, delta fura-2 ratio) that were expressed as the averaged amplitude of 0–50 s are equal to zero. (*C*) GFP or GFP–CaMKIIα cells were transfected with mRFP and control siRNA or IRBIT siRNA. After 48 h, cells were fixed and stained by IRBIT antibody. (Scale bar, 10 μm.) (*D*) The magnitude of BrA-induced increase in intracellular calcium was not affected by IRBIT knockdown. Representative Ca^{2+} changes after BrA (2.5 μM) stimulation in the GFP or GFP–CaMKIIα cells. (*E*) Quantitation of Ca^{2+} peak amplitude. Results are shown as the mean ± SEM of at least three independent experiments. The total cell numbers were indicated in each bar. Peak amplitude of Ca^{2+} responses (Max. ΔR, delta fura-2 ratio) that were expressed as the averaged amplitude of 0–50 s are equal to zero.

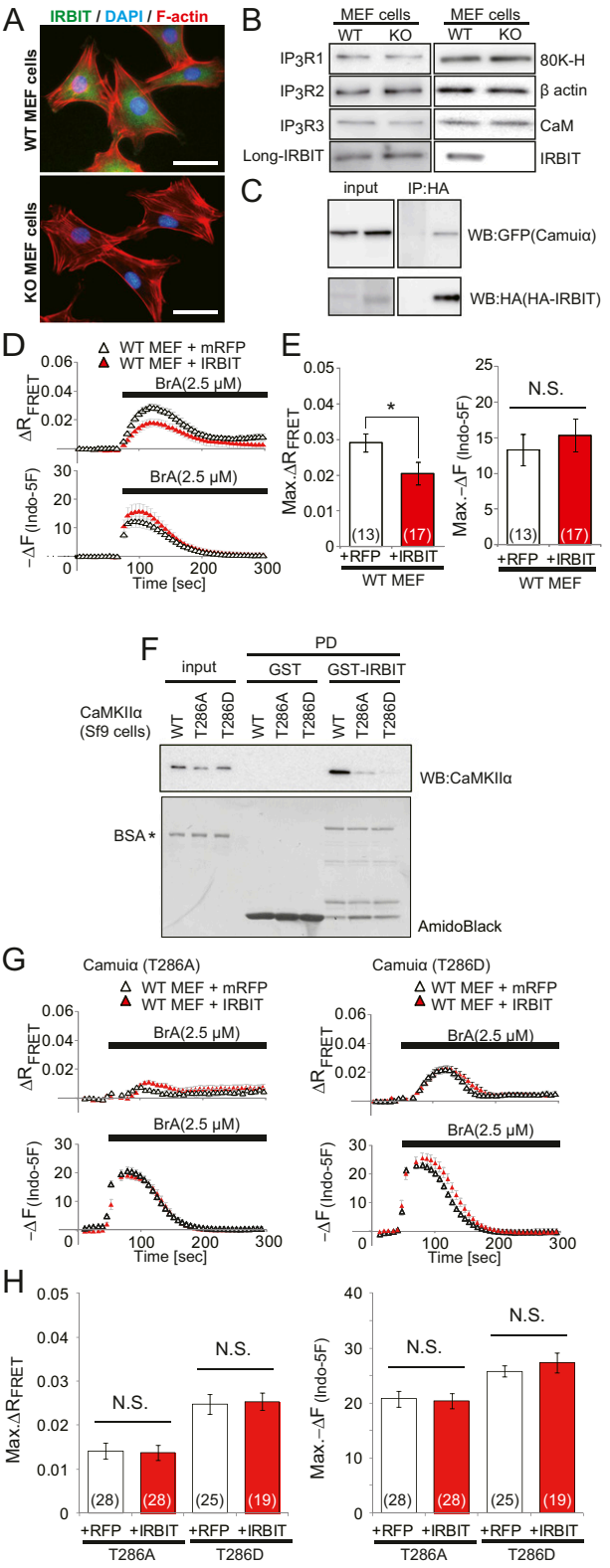

Fig. S3. (*A*) IRBIT WT or KO MEF cells were stained with anti-IRBIT antibody (green), 4,6- diamidino-2-phenylindole (DAPI; blue), and phalloidin (red). (Scale bar, 50 μm.) (*B*) Lysates of IRBIT WT or KO MEF cells were analyzed by Western blotting (WB) with the antibodies indicated. (*C*) HA–IRBIT interacted with FRET-based CaMKIIα probe, Camuiα. Lysate (input) and immunoprecipitation (IP) samples were analyzed by WB with the indicated antibodies. (*D*) Simultaneous recording of FRET-based CaMKIIα probe and Ca^{2+} change in WT MEF cells transiently expressing mRFP or IRBIT–IRES–mRFP after 2.5 μM BrA stimulation. (*Upper*) Representative FRET changes are shown. (*Lower*) Representative Ca^{2+} responses ($-\Delta F_{Indo\text{-}5F}$) are shown. (*E*) Quantitation of FRET and Ca^{2+} peak amplitude. Results are shown as the mean ± SEM of at least three independent experiments. Total cell numbers are indicated in each bar. Peak amplitude FRET (Max. ΔR_{FRET}) and Ca^{2+} responses (Max. $-\Delta F_{Indo\text{-}5F}$), expressed as the averaged amplitude of 0–50 s, are equal to zero. (*F*) The point mutation of Thr286A or

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Thr286D attenuated interaction between IRBIT and CaMKIIα. (*G*) Simultaneous recording of FRET-based CaMKIIα mutant probe (T286A or T286D) and Ca^{2+} change in WT MEF cells transiently expressing mRFP or IRBIT–IRES–mRFP after 2.5 μM BrA stimulation. (*Upper*) Representative FRET changes are shown. (*Lower*) Representative Ca^{2+} responses ($-\Delta F_{Indo\text{-}5F}$) are shown. (*H*) Quantitation of FRET and Ca^{2+} peak amplitude. Results are shown as the mean ± SEM of at least three independent experiments. Total cell numbers are indicated in each bar.

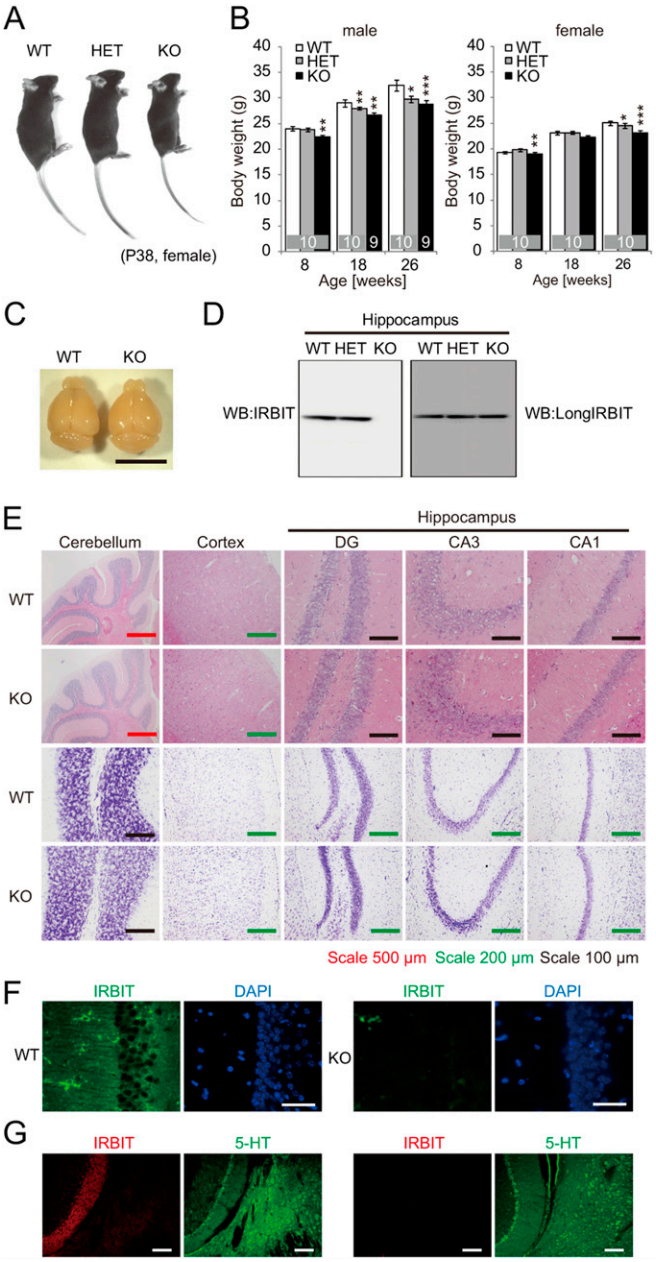

Fig. S4. Basic properties of IRBIT KO mice. (*A*) IRBIT KO mice showed slight dwarfism. WT, wild type; KO, IRBIT knockout mice; HET, heterozygous mice. (*B*) Body weight of IRBIT KO mouse. (*C*) Appearance of IRBIT KO mouse brain. (Scale bar, 10 mm.) (*D*) Protein expression level of IRBIT in IRBIT KO mouse and littermate. (*E*) IRBIT KO mice showed normal histological structures in adult brain sections examined by HE staining and Nissl staining. DG, dentate gyrus, CA3, Cornu Ammonis 3; CA1, Cornu Ammonis 1. (*F*) Immunostaining of IRBIT (green) and DAPI staining (blue) in hippocampus CA1. (Scale bar, 50 μm.) (*G*) Immunostaining of IRBIT (red) and serotonin (5-HT, green) in the cerebellum and raphe nucleus. (Scale bar, 100 μm.)

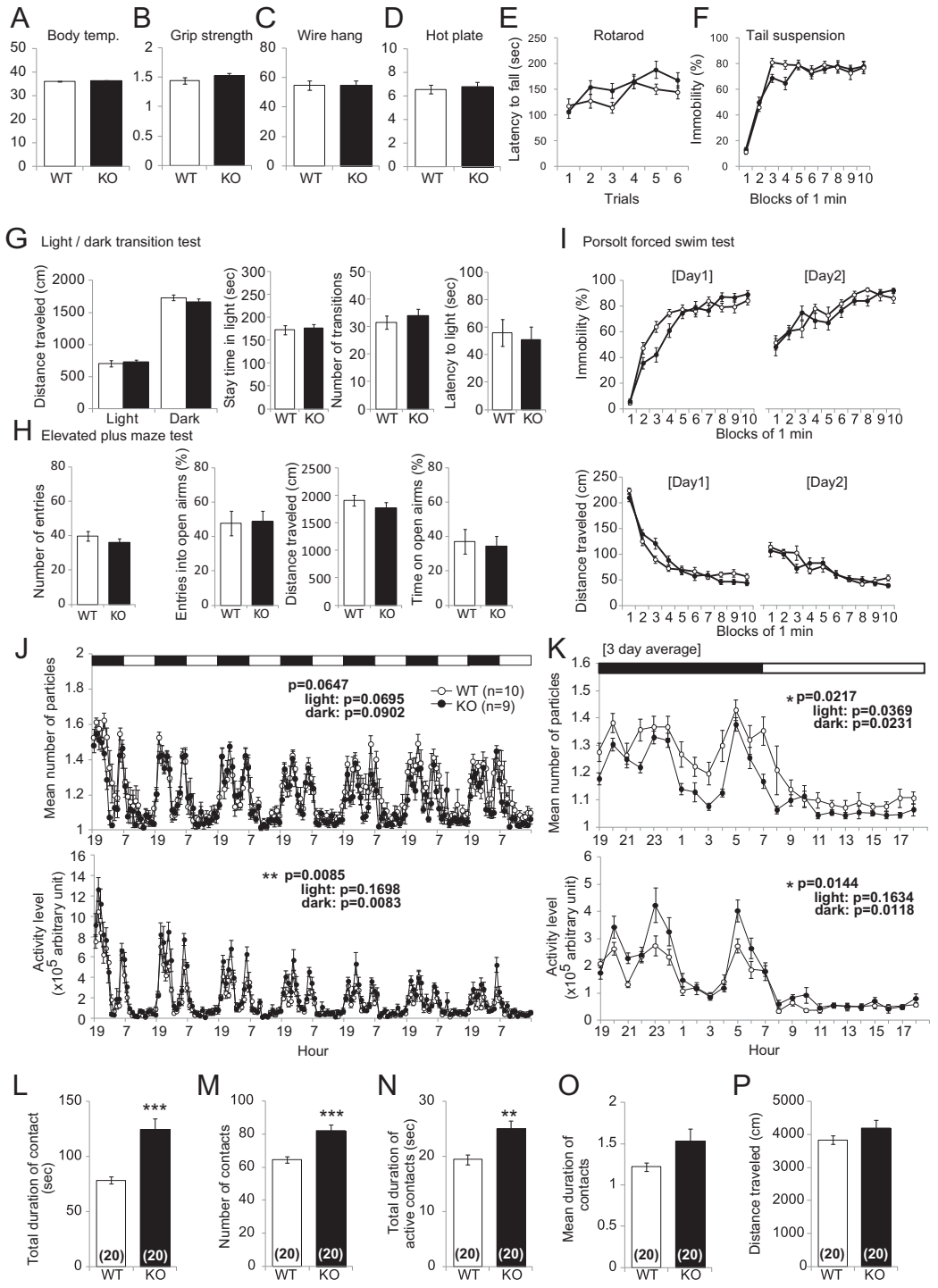

Fig. S5. Behavior analysis of IRBIT KO mouse. (*A*) Body temperature. (*B*) Grip strength. (*C*) Wire hang test. (*D*) Hot plate analysis. (*E*) Rotarod performance test. (*F*) Tail suspension test. (*G*) Light/dark transition test. (*H*) Elevated plus maze test. (*I*) Porsolt forced swim test. Results are shown as the mean ± SEM. $n = 20$. (*J*) Social interaction and activity levels of WT and IRBIT KO mouse in home cage: mean number of particles detected (*Upper*) and activity level (*Lower*) were recorded over 7 d. (*K*) The averaged graph of mean number of particles detected (*Upper*) and activity level (*Lower*). (*L*–*P*) Social interaction test in a novel environment (one-chamber social interaction test): total duration of contact (*L*), number of contacts (*M*), total duration of active contact (*N*), mean duration of contacts (*O*), and distance traveled (*P*) are recorded. Mean ± SEM. $n = 20$.