Zic2 hypomorphic mutant mice as a schizophrenia model and ZIC2 mutations identified in schizophrenia patients

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Supplementary information

Supplementary Methods and Materials

Animals

Mutant mice heterozygous for the *Zic2*^{kd} allele (*Zic2*$^{kd/+}$) were described previously (1-3). *Zic2*^{kd} was generated by the insertion of the neomycin resistant cassette into an intron of mouse *Zic2*, resulting in the 20% of the wild-type allele (1). The mice were maintained by Research Resource Center in RIKEN Brain Science Institute, were generated from the 129/SvJ ES cell line, and were backcrossed to the C57BL/6J background more than 12 times. All animal experiments were carried out according to the RIKEN guidelines for animal experiments. The mice were housed under the

standard 12-h light/dark cycles with lights on at 8:00. The behavioral experiments were carried out between 10:00 and 18:00 with the exception of the resident-intruder test. In total, 50 pairs of *Zic2*$^{kd/+}$ and control wild-type (*Zic2*$^{+/+}$) male mice from 8 wk to 24 wk of age were subjected to behavioral analyses. The control wild-type mice were age-matched to the *Zic2*$^{kd/+}$ mice, and derived from their littermates. Most morphological analyses were performed soon after the behavioral experiments with the exception of newborn stage analysis. BALB/c mice for resident-intruder test were purchased from Nihon SLC, Shizuoka, Japan.

Home cage activity measurement

Spontaneous activity of mice in their home cage was measured using a 24-ch ABsystem 4.0 (Neuroscience, Tokyo, Japan). Cages were set individually into the stainless steel compartments of the negative breeding rack (JCL, Tokyo, Japan). A piezoelectric sensor was placed on the ceiling of each compartment to detect the movements of the mice.

Open field test

The open field test was performed by using a four-channel open field system in a small sound-proof room [185 × 185 × 225 cm (height[H])]. Each open field was made of

white plastic [50 × 50 × 40 (H) cm], and was illuminated by light emitting diodes (LEDs; 70 lux at the center of the field). The behavior of each mouse was monitored by a charge-coupled device (CCD) camera fixed to the ceiling of the rack containing the open fields. During the measurements, lights in the sound-proof room were turned off and an electronic fan was turned on both for ventilation and as a source of background noise (35 dB). To start the open field test, mice were introduced individually at one corner of the field and were then allowed to move freely for 15 min. The indices measured were the distance traveled, and the period of time in the central area of the field, which was 30% of the total field, expressed as a percentage of the total period of time in the field. Data were collected every 1 min and were analyzed using ImageJ OF4 program (O'Hara, Tokyo, Japan).

Morris water maze test

A circular maze made of white plastic (1 m diameter, 30 cm depth) was filled with water at 22–23°C to a depth of about 20 cm. Water was colored by white paint so that mice could not see the platform or other cues under the water. The platform was 19 cm high, 10 cm in diameter and was placed 1 cm below the surface of water. There were some landmark cues outside the maze that were visible to the mice within the maze including

a calendar, a figure, and a plastic box. The movements of mice in the maze were recorded and analyzed with ImageJ WM (O'Hara). Mice were subjected to 6 trials of the water maze test in 1 session per day for 4 consecutive days (days 1–4). Each trial was initiated by placing an individual mouse into the water facing the outer edge of the maze at 1 of 4 designated starting points quasi-randomly. The submerged platform remained in a constant position for each mouse throughout testing. Each trial was terminated when the mouse reached the platform, and the latency and distance swum were measured. The cut-off time for each trial was 60 s, and mice that did not reach the platform within 60 s were removed from the water and placed on the platform for 30 s before being toweled off and placed back into their home cage. The inter-trial interval was about 6 min. After the 4 days of training, we conducted the probe test on day 5. In the probe test, the platform was taken away, and each mouse was placed into the water at a point opposite the position of the target platform, and allowed to swim within the maze for 60 s. We measured the distance swum to reach the platform, the number of crossings the position of the target platform and the other three platforms, and the period of time in each of the four quadrants. On day 6, we performed a reverse test by repeating the same experiment as in day 1–4 except that the hidden platform was placed in the opposite quadrant to that used for the training sessions from days 1 to 4.

Classical fear conditioning

The classical fear conditioning test consisted of three parts; a conditioning trial (day 1), a context test trial (day 2), and a cued test trial (day 3). Fear conditioning was carried out in a clear plastic chamber equipped with a stainless steel grid floor [34 × 26 × 30 (H) cm]. A CCD camera was fixed to the ceiling of the chamber and was connected to a video monitor and computer. The grid floor was wired to a shock generator. White noise (65 dB) was supplied from a loudspeaker as an auditory cue (conditioned stimulus, CS). An unconditioned stimulus (US) consisting of foot-shock (0.3 mA, 2 s) was administered at the end of each 30 s CS period. The conditioning trial consisted of a 2 min exploration period followed by two CS-US pairings separated by 1 min each. Twenty-four hours after the conditioning trial, a context test was performed in the same conditioning chamber for 5 min in the absence of the white noise. A cued test was performed 24 h later in an alternative context with distinct cues; the test chamber was different from the conditioning chamber in terms of brightness (almost 0–1 Lux), color (white), floor structure (no grid), and shape (triangular). The cued test consisted of a 2 min exploration period (no CS), to evaluate the nonspecific contextual fear, followed by 2 min CS period (no foot shock), to evaluate the acquired cued fear. The rate of freezing

response (immobility excluding respiration and heartbeat) of the mice was measured as an index of fear memory. Data was analyzed using the ImageJ FZ2 (O'Hara).

Y-maze test

The Y-maze test was performed as described elsewhere (4). Briefly, each arm of the Y-maze was 40 cm long, 3 cm wide at the bottom and 10 cm wide at the top, and 12 cm high. Each mouse was placed at the end of a fixed arm as the start arm, and was allowed to move freely though the maze during the 5-min test session. The sequence and total number of arm entries during the observation period was recorded manually. An alternation was defined as entry into all three arms on consecutive choices. The number of maximum alternations was defined as the total number of arms entered minus 2, and the percentage alternation was calculated as (actual alternations/maximum alternations) × 100. The arms were cleaned between each test.

Resident-intruder test

Group-reared mice were transferred to a new cage with a wire top and bedding material to a depth of 1 cm, and kept in isolation for 5 days before the test. The test was carried out in a dark phase (0:30 to 2:30) in a chamber that keeps the cage under dim (infrared)

light at 25°C. On the test day, the cage top was changed to transparent plexiglass. Video cameras were set in place 5 min before introducing an intruder mouse (4-wk-old male BALB/c). For video recording of animal behavior, two cameras were placed on opposite sides of the cage at positions more than 10 cm from the cage: one in a horizontal position and the other placed to view the entire cage field. The intruder mice were allowed to acclimatize to a similar environment in another cage for 5 min. Video recording was initiated once the intruder mice had been gently placed in a vacant spot in the cage of the resident mice. The behaviors of the resident mouse were recorded for 10 min. The duration and number of times the resident mice spent sniffing, in active contact with, and in pursuit and attack with the intruder mice were measured by observers who were blinded to the genotypes of the mice.

Social dominance tube test

A wild type and a *Zic2*$^{kd/+}$ mice were placed in a head-to-head position first at the opposite ends of a clear plexiglass tube (3.4 cm inner diameter, 30 cm in length) in which two shutter plates were inserted at a distance of 13 cm from each end. The tests were begun by removing the shutters and ended when one mouse completely retreated from the tube. The mouse that retreated first was designated as the loser, and the

remaining mouse was judged as the winner. The maximal test time was set to 2 min. A judgment was not made if both mice remained within the tube at the end of the testing period. Sixty-six tests using 14 wild type and 11 *Zic2*$^{kd/+}$ mice were performed. Each mouse confronted three different combatants per day with intervals of at least 30 min.

Magnetic resonance imaging (MRI) based volumetric analysis

MRI images of the adult male mice were acquired by subjecting anesthetized mice to an MRI scan using a vertical bore 9.4-T Bruker AVANCE 400WB imaging spectrometer (Bruker BioSpin, Rheinstetten, Germany). Animals were anesthetized with 3% and 1.5% isoflurane in air (2 L/min flow rate) for induction and maintenance, respectively. MRI images were obtained by using the FISP-3D protocol of Paravision software 5.0, by setting the following parameter values: Effective TE = 4.0 ms, TR = 8.0 ms, Flip angle = 15 degree, Average number = 5, Acquisition Matrix = 256 × 256 × 256, FOV = 25.6 × 25.6 × 25.6 mm. Fifteen pairs of 13–14 wk-old *Zic2*$^{kd/+}$ and *Zic2*$^{+/+}$ males were subjected to MRI based volumetric analysis. Manual measurements were made on the 3-dimensional (3D) MRI data to calculate total brain volume, hippocampus volume and lateral ventricle volume using the InsightITK-Snap software (5). Regional volumetric changes were measured by voxel-based morphometry (VBM) using the Statistical

Parametric Mapping (SPM) software package (http://www.fil.ion.ucl.ac.uk/spm/software/) for MATLAB (Mathworks, Natick, MA, USA) for pilot survey (data not shown).

Histology and immunostaining

For histological examination, the mice were transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). Dissected brains were immersed in the same solution at 4°C for 12–24 h for fixation. The tissues were then cryoprotected in 20% sucrose in phosphate buffered saline for 12–24 h, embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), frozen, and sectioned (10 μm). For the morphometric analysis, serial coronal sections were prepared, and analyzed at the following positions: +0.74 to +1.10 for the septum, diagonal band, striatum and the motor cortex; −0.34 to −0.82 for the substantia innominata, the basal nucleus of the Meynert, and the somatosensory cortex [anterior(+) to posterior(−) distance (mm) from bregma according to Paxinos et al. (6)]. The sections were stained with cresyl violet or by utilizing endogenous acetylcholine esterase activity (7) for histological examination.

Immunostaining was performed as previously described (8). The primary antibodies were rabbit mouse anti-choline acetyltransferase (ChAT) polyclonal antibody

(Chemicon, Temecula, CA, USA), mouse monoclonal anti-parvalbumin (PV) (Sigma, St. Louis, MO, USA), and rabbit anti-pan-Zic antibodies (9). We confirmed by immunoblot analysis that the rabbit anti-pan-Zic antibody recognizes Zic1, Zic2, and Zic3 proteins (data not shown). Digital images were obtained using either the NanoZoomer Digital Pathology C9600 (Hamamatsu Photonics, Shizuoka, Japan) image scanner or with an Axioskop2 plus microscope equipped with an AxioCam color CCD camera (Zeiss, München, Germany).

Resequencing analysis of *ZIC2* in human subjects

We performed resequencing analysis of *ZIC2* in 278 patients with schizophrenia who were of Japanese descent. The diagnosis of schizophrenia including the samples below was made on the basis of Diagnostic and Statistical Manual of Mental Disorders criteria (DSM-IV), by at least two expert psychiatrists. We then determined the allele frequencies of detected mutations using an expanded sample panel of schizophrenia patients (967 subjects: 457 men, 510 women; mean age 47.3 ± 13.8 [SD] years) and 1060 controls (502 men, 558 women; mean age 47.7 ± 13.6 years) who were documented as being free of mental disorders following brief interviews by expert psychiatrists. Our recruitment of schizophrenia and control subjects did not involve

structured or semi-structured instruments. This study was approved by the ethics committees of RIKEN.

Genomic DNA was isolated by the standard chloroform-phenol extraction method. Protein-coding regions and exon/intron boundaries within the *ZIC2* gene were screened for polymorphisms by direct sequencing of PCR products using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). The primers used for amplification and PCR conditions are available upon request.

Molecular and functional analysis

Plasmid construction

Mouse *Zic2* variants that have the same missense mutations as the human *ZIC2* nonsynonymous mutations (*Zic2*^{A95T} for *ZIC2*^{A95T}, *Zic2*^{R408P} for *ZIC2*^{R409P}, and *Zic2*^{S443R} for *ZIC2*^{S444R}) were generated by PCR (10) using pEFBOS-*Zic2* (11) or pcDNA3-HA-*Zic2* as templates. Hereinafter, we refer to them as *Zic2*-A95T (*Zic2*^{A95T}), *Zic2*-R409P (*Zic2*^{R408P}) and *Zic2*-S444R (*Zic2*^{S443R}), respectively. Expression plasmids for these wild-type *Zic2* and *Zic2* variants were constructed by inserting the open reading frame of wild-type *Zic2*, and variants, in-frame into pcDNA3.1 (Invitrogen,

Carlsband, CA, USA), modified to encode three HA epitope tags, and pCMVtag2 (Stratagene, La Jolla, CA**,** USA), modified to encode one FLAG epitope tag. The N-termini of wild-type Zic2 and the variants were tagged with HA or FLAG. pGL4-ZBS was constructed by inserting a mouse genomic DNA clone containing Zic2-binding sequences (Ishiguro et al., unpublished) into pGL4 (Promega, Madison, WI, USA).

Cell culture, transfection, and immunoblot analysis

NIH3T3, 293T, and C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Lipofectamine and Plus reagent (Invitrogen) or Trans-IT LT1 (Mirus Corp, Madison, WI, USA) were used for transfections. Immunoblot analysis was performed as previously described (12). Briefly, anti-FLAG rabbit polyclonal antibody (Sigma) and horse radish peroxidase (HRP)-conjugated anti rabbit IgG antibody were used as the primary and secondary antibodies, respectively. The signals were detected with ECL (GE healthcare, Tokyo, Japan) and images were captured with a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). The signal intensities were quantified with ImageJ software (http://rsbweb.nih.gov/ij/).

Gel shift assay

Gel shift assays were performed as previously described (12). 293T cells were transfected with the FLAG-tagged *Zic2* or *Zic2*-R409P vectors. Cells were harvested 20 h after transfection and then lysed in cold lysis buffer (10% glycerol, 0.5% NP-40, 300 mM NaCl, 40 mM HEPES-NaOH, pH 7.8, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF). The cell lysate was mixed by rotation for 30 min at 4°C, and then centrifuged at 15 000*g* for 10 min at 4°C to eliminate cell debris. Anti-FLAG M2 antibody-conjugated agarose beads (1 µL/mL lysate, Sigma), which were equilibrated with the lysis buffer, were added to the lysate. The lysates were mixed with the beads by rotation for 2 h at 4°C. The beads were then washed 3 times with lysis buffer to purify the bound proteins and then the FLAG-tagged peptides were eluted. The beads were re-suspended in 25 µL lysis buffer and used in the gel shift assay for quantification of the amount of bound protein. We used infrared dye (IRD, LI-COR Biosciences, Lincoln, NE, USA)-labeled DNA probes derived from a mouse genomic region containing high affinity Zic2-binding sites (Ishiguro et al., unpublished). The amount of probe used in each assay ranged from 50 to 400 fmol. The results are presented as the means of ten independent gel-shift experiments. The bound probe signal intensities were measured by using an Odyssey infrared imaging system (LI-COR), and normalized by the protein

amount determined by immunoblot analysis.

Luciferase reporter assay

Luciferase reporter assays were performed as previously described (11). Briefly, NIH3T3 cells or C3H10T1/2 cells seeded into 24-well culture dishes were transfected with the luciferase reporter plasmid pGL4-ZBS (100 ng), the FLAG- or HA-tagged expression vectors (100 ng), which include the empty vector and those containing the wild-type *Zic2* or *Zic2* variants, and pEF-RL (5 ng). Luciferase activity was measured according to the manufacturer's recommendations (Promega) using a luminometer, Minilumat LB 9506 (EG&G Berthold, Wildbad, Germany).

Immunoprecipitation

Immunoprecipitation was performed as previously described (12) using the cell lysates of 293T cells transfected with the FLAG-tagged vectors and the anti-FLAG M2 antibody-conjugated agarose beads. Anti-DNA-dependent protein kinase (DNA-PK) (Stressgen, Ann Arbor, MI, USA), anti-RNA helicase A (RHA) (12), and anti-FLAG rabbit polyclonal antibodies were used for the immunoblot detection of the precipitated proteins.

Statistical analysis

Parametric data were analyzed by using the two-sided Student's *t*-test (t-test) and non-parametric data were analyzed by using the Mann–Whitney's *U*-test (U-test). The *P* values refer to the t-test, unless otherwise specified. We also used the repeated measure two-way analysis of variance (RMANOVA) or the chi-square test for homogeneity. Differences were defined as statistically significant when $P < 0.05$.

Materials and methods for the experiments in supplemental figures (S1-S3)

Social interaction in an open field

Social interaction with a freely moving mouse (encounter method)

Subject mice were individually put into the center of a white-colored open field (40 × 40 × 30 cm [H]). Immediately after the introduction of the subject mouse, a target mouse was also introduced into the same open field. The duration of contact behavior was measured for 6 h. Contact or separation of mice was recorded by the software as 1 or 2, respectively. Data was collected and analyzed using a personal computer and commercially available software (Time HC: O'Hara, Tokyo, Japan).

Social discrimination test

This test was performed in the open-field test apparatus with a luminance of 70 lux. The

test consisted of a habituation session, a first test session, and a second test session. Each session was continued for 15 min and took place in the following order. In the habituation session, two empty cylindrical wire cages (inner size, 7 cm ϕ × 15 cm [H]; outer size, 9 cm ϕ × 16.5 cm [H]; 21 of 3-mm-ϕ stainless wires vertically and longitudinally placed between top and the bottom gray polyvinyl discs, manufactured by the RIKEN Rapid Engineering Team) were placed in two adjacent corners. In the first test session, a mouse (7-wk-old male DBA/2, purchased from Nihon SLC, Shizuoka, Japan) that was new to the test mouse was put into one of the two cylindrical cages. In the second test session, another mouse that was also new to the test mouse was put in the remaining cylindrical cage. Between the three sessions there were 4-min intervals, during which the test mouse was returned to its home cage. The three sessions were video-recorded from above, and the times spent in the two corner squares containing the cylinders within the 3 × 3-square subdivision (17.7 × 17.7 cm square) were measured with Image J OF4 program (O'Hara). For the two test sessions, video recording was also done from an obliquely upward position to observe contact between the test mouse and the in-cage mouse. Contact with the in-cage mouse was defined as a forward movement to the mouse in the cage with subsequent direct contact using the head. The position and posture of the in-cage mouse were observable through the slits

of the wires. The contacts were counted on the video images by an observer who was blind to the genotypes. Each in-cage mouse was used once a day; when the habituation session began, it was simultaneously placed in its cylindrical cage on the corners of an open-field box that was not being used for the tests. These rules were thought to minimize the difference between the two in-cage mice in the second test sessions in regard to their acclimation to the cylindrical cage and the open-field-box environment. After each use, the cylindrical cage was extensively washed with water and rinsed with 90% ethanol, which was then evaporated off, to minimize the effects of remnant ethanol.

Light-dark box test

A four-channel light-dark box system was equipped in the same sound-proof room as the open field. Each light box was made of white plastic (20 × 20 × 20 [H] cm) and illuminated by LEDs (250 Lux at the center of the box) and a CCD camera was placed on the ceiling. Each dark box was made of black plastic (20 × 20 × 20 [H] cm) and an infrared camera was placed on the ceiling. There was a tunnel for transition on the center panel between the light box and dark box (3 × 5 cm) with a sliding door. In the light-dark box test, mice were individually introduced into the light box, and the door of

the tunnel automatically opened immediately after the software detected the mouse. Then mice were allowed to move freely in the light-dark box system for 10 min. The total distance traveled, percentage duration of time within the light box, number of transitions between the light and dark boxes and the first latency to enter the dark box were measured as indices. Data was collected and analyzed using Image J LD4 program (O'Hara, Tokyo, Japan).

Quantification of PV-positive cells in the cerebral cortex

The cortical thickness was determined on PV-positive coronal frozen sections. The cells were manually counted on the digitized images at the following positions: +1.98 to +2.68 for medial and dorsolateral prefrontal cortex; +0.74 to +1.10 for motor cortex; +1.98 to +1.74 for hippocampus (anterior [+] to posterior [−] distance [mm] from the bregma according to Paxinos and Franklin (6)).

Subcellular localization

NIH3T3 cells were transfected with pCMVtag2-FLAG-Zic2WT or pCMVtag2-FLAG-Zic2R409P. After 24 h, the cells were subjected to immunofluorescence staining and were then analyzed by laser scanning confocal

microscopy as previously described (13). The nuclear localization index was calculated, and the evaluation method was performed, as described previously (14).

Determination of the ZIC2 zinc finger 5 structure

The R409P mutation was mapped to the fifth zinc finger of ZIC2. To assess the effect of this missense mutation, we performed a computer simulation of the fifth zinc finger structure of wild-type ZIC2 and R409P ZIC2 using Modellar software (http://salilab.org/modeller/). We used the ‘advanced’ modeling option to calculate the structure using multiple zinc finger structure models (2GLI [GLI1 chain A], 1UBD [Yy1 chain C], 1F2I [Zif12 chain G], 2RPC [ZIC3 chain A]). Five models were created by the Modeller software, and these models were superimposed with GLI-DNA complexes (2GLI) complex using the Swiss-PdbViewer (DeepView) software (http://spdbv.vital-it.ch/) with the ‘Magic fit’ option. The software iMol (http://www.pirx.com/iMol/) was used for the PDB viewer.

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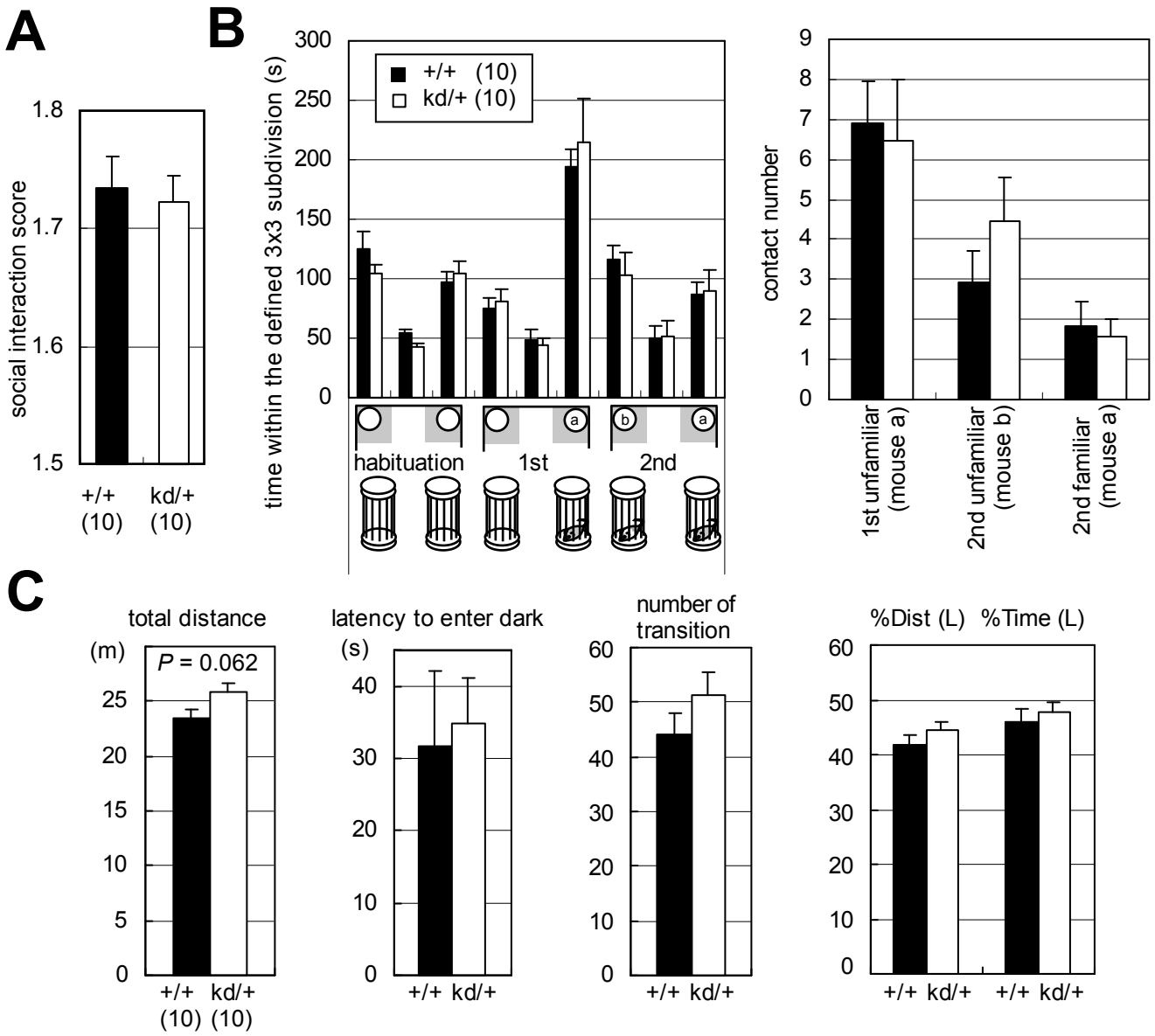
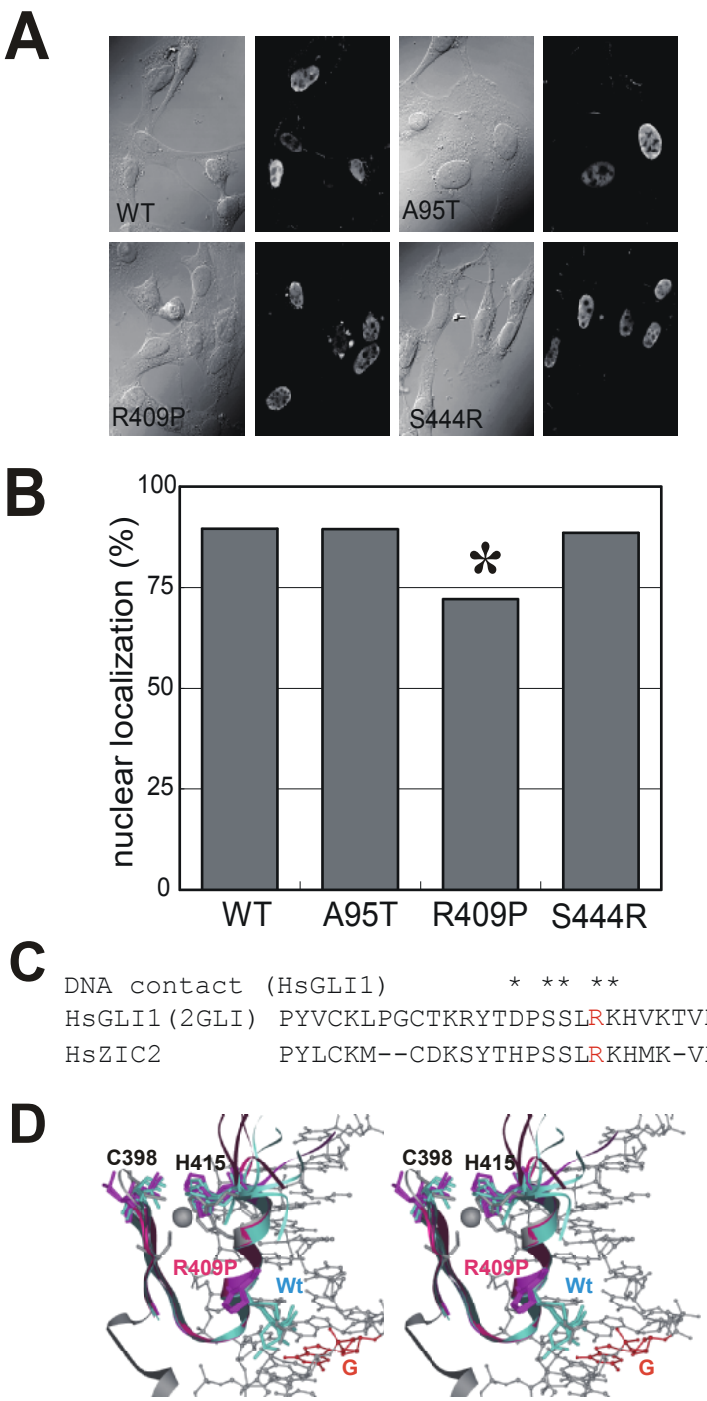


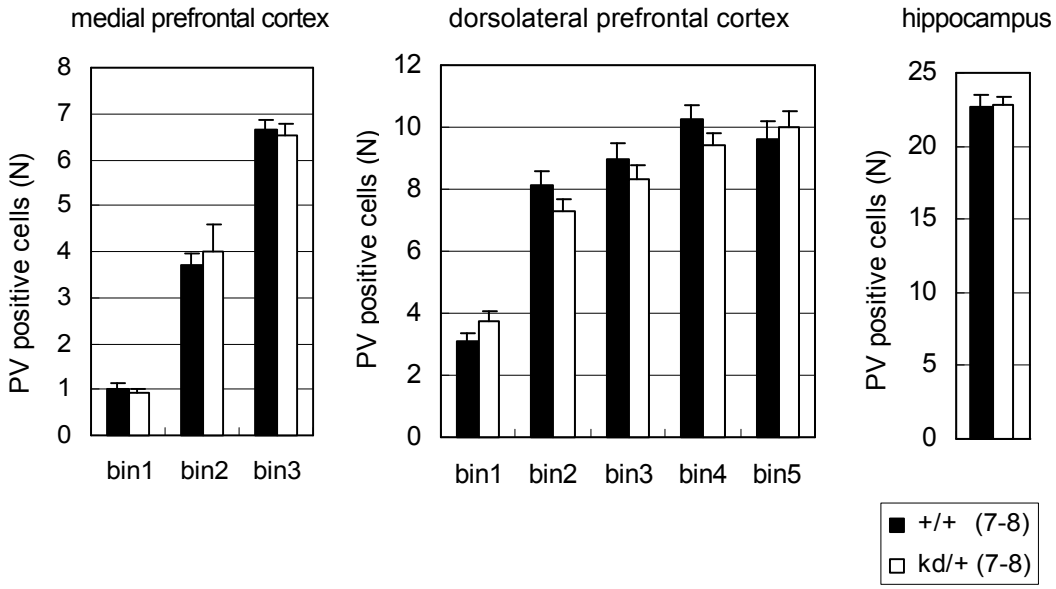
Supplemental Figure 1

Results of some additional behavioral experiments. (A) Social interactions in an open field with an uncaged partner. Contact or separation of mice was recorded by the software as 1 or 2, respectively. Values are presented as mean ± SEM. (B) Social interactions in an open field with a caged partner (social discrimination test). In habituation, the two empty cylinder wire cages (9 cm [diameter] × 16.5 cm) were placed in the two adjacent corners of the open field for 15 min. In the first test session (15 min), an unfamiliar mouse was placed in one of the two cylinder cages. In the second session (15 min), another unfamiliar mouse was placed in the cylinder cage while the mouse that appeared in the first session remained as a familiar mouse. The approaches made by wild-type and Zic2kd/+ mice to the cages were measured by the length of time they stayed in the rectangular region (17.7 cm × 17.7 cm) including the cage (left). The mouse-to-mouse contact (right) was counted by human naked eye observations. Values are presented as mean ± SEM. (C) Light-dark (LD) box test. The mice were individually introduced into the light box of the LD chamber apparatus, and the door of the tunnel opened immediately after the introduction of a mouse. Mice were then allowed to move freely within the LD chamber for 10 min. The total distance traveled, percentage of distance traveled in the light box (%Dist (L)), percentage of time within the light box (%Time (L)), the number of transitions between the light and dark boxes and the first latency to enter the dark box are indicated as mean ± SEM. The number of mice in each group is given in brackets.



Supplemental Figure 2

Additional analysis of the ZIC2 mutant proteins. (A) Nuclear localization of Zic2 FLAG-Zic2-WT, -A95T, -R409P, and -S444R. Transfected NIH3T3 cells were stained with anti-FLAG antibody (right). The differential interference contrast images of the corresponding fields are indicated in the left of each immunostaining. (B) Percentage of cells with nuclear localization. The immunostained FLAG-Zic2-WT, -A95T, -R409P, or -S444R-expressing NIH3T3 cells were subjected to the nuclear localization analysis (56). Percentages of the cells with greater signals in nuclear region than in the cytoplasmic regions among the total immunostained cells are indicated as mean ± SEM. *P < 0.05 in chi-square test, compared with wild-type (WT). (C) Sequence alignment of human (Hs) ZIC2 R409 and its flanking region with the corresponding region of human GLI1, which shares similar zinc finger domains to that of ZIC2. Red letter, R409 in ZIC2 and the corresponding residue in GLI1. (D) Stereoview of the zinc finger 5 region of ZIC2 (blue) and ZIC2-R409P (red). Prediction of the 3D structure is based on the 3D structure of GLI1 (2GLI in protein data bank [PDB, http://www.pdb.org/pdb/home/home.do]).



Supplemental Figure 3

Distribution of PV-positive neurons. Coronal sections (10 μm) through the medial prefrontal cortex, dorsolateral prefrontal cortex, and hippocampus regions were subjected to the cell number counting analysis. Cerebral cortices from the pia surface were divided into 3 bins [for medial prefrontal cortex, 0.5 mm $\times$ 0.2 (thickness) mm/bin], 5 bins [for dorsolateral prefrontal cortex or 0.5 mm (width) $\times$ 0.2 mm/bin]. Values are presented as means $\pm$ SEM. The number of mice in each group is given in brackets. There were no significant differences among the bins (t-test with Bonferroni's correction).