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

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ZIC2 is a causal gene for holoprosencephaly and encodes a zinc-finger-type transcriptional regulator. We characterized Zic2kd+/+ mice with a moderate (40%) reduction in Zic2 expression. Zic2kd+/+ mice showed increased locomotor activity in novel environments, cognitive and sensorimotor gating dysfunctions, and social behavioral abnormalities. Zic2kd+/+ brain involved enlargement of the lateral ventricle, thinning of the cerebral cortex and corpus callosum, and decreased number of cholinergic neurons in the basal forebrain. Because these features are reminiscent of schizophrenia, we examined ZIC2 variant-carrying allele frequencies in schizophrenia patients and in controls in the Japanese population. Among three novel missense mutations in ZIC2, R409P was only found in schizophrenia patients, and was located in a strongly conserved position of the zinc finger domain. Mouse Zic2 with the corresponding mutation showed lowered transcription-activating capacity and had impaired target DNA-binding and cofactor-binding capacities. These results warrant further study of ZIC2 in the pathogenesis of schizophrenia.
Results

Wild-type (Zic2\(^{+/+}\)) and Zic2\(^{ad^+}\) mice are indistinguishable by their body weight and external appearance\(^2\). Both male and female Zic2\(^{ad^+}\) mice are fertile and female Zic2\(^{ad^+}\) mice can foster their progenies without any obvious faults\(^3\). In our previous study, we found that prepulse inhibition (PPI) of acoustic startle response is decreased in Zic2\(^{ad^+}\) mice\(^4\). For a more comprehensive analysis of behavioral phenotypes, we carried out the suite of behavioral tests listed in Table 1. For the light-dark box test, marble burying test, elevated plus maze test, forced swimming test, grip strength test, wire hanging test, footprint test and rotarod test, we found no significant differences in behavior between Zic2\(^{ad^+}\) and wild-type mice (Table 1)\(^2\). For the remaining tests, we found significant differences in behavior between Zic2\(^{ad^+}\) and wild-type mice; as described below.

Locomotor activities were lower or higher in Zic2\(^{ad^+}\) mice than wild-type mice depending on the situation. We first placed the mice in new home cages and then monitored their locomotor activity continuously for 13 days (Figure 1A–C). Our analysis revealed that the locomotor activity was significantly lower in Zic2\(^{ad^+}\) mice than in wild-type mice in the later stationary period (relatively low day-to-day variance, days 6–13) (P = 0.044) (Figure 1A). When we assessed the mean circadian locomotor activities during the stationary period, we found that the activity of Zic2\(^{ad^+}\) mice was significantly lower than that of the wild type in the early dark phase (20:00–24:00) (P = 0.048), but that the circadian rhythm of the Zic2\(^{ad^+}\) mice was normal (Figure 1B).

We also assessed locomotor activity in open field tests with observation times of 15 min. Zic2\(^{ad^+}\) mice showed a significantly higher overall locomotor activity compared to wild-type mice (P = 0.041) (Figure 1C, left), but there were no differences in preference between the two genotypes for the central and peripheral fields (Figure 1C, right). These results suggest that Zic2\(^{ad^+}\) mice have higher locomotor activities than the wild type in a novel environment.

Cognitive function deficits in Zic2\(^{ad^+}\) mice. The Morris water maze test is commonly used to evaluate learning ability and acquisition of spatial memory. In our study, this test consisted of 4 days of training (day 1–4) with a fixed hidden platform, 1 day (day 5) of a probe test without a platform, and 1 day (day 6) of a reverse test in which the hidden platform was placed in the opposite quadrant. To reach the hidden platform, Zic2\(^{ad^+}\) mice needed a significantly longer time in the training session (P = 0.046) and a significantly longer time in the reverse test session (P = 0.037) (Figure 2A, top panel). The moving speed of Zic2\(^{ad^+}\) mice was slightly, and significantly, lower than that of wild-type mice only on day 1 of the training session (Figure 2A, left middle panel, P = 0.036). However, their spatial memory acquisition was not impaired as seen in the results of the probe test (Figure 2A, bottom panel). The motor performance and motivation of Zic2\(^{ad^+}\) mice might not be impaired, given that there were no significant differences between the two genotypes in the moving speed at days 2 to 4 [F(1,18) = 0.117, P = 0.90, RMANOVA, main effect of genotype] (Figure 2A, left middle panel) or in the overall no movement time [F(1,18) = 0.23, P = 0.64, RMANOVA, main effect of genotype] (Figure 2A, right middle panel). Furthermore supporting this notion, the results were similar for Zic2\(^{ad^+}\) and wild-type mice for the other tests related to motor performance and motivation (rotarod, footprint, wire hanging and forced swimming test) (Table 1). Therefore, the water maze test results were considered to reflect an impaired learning ability of Zic2\(^{ad^+}\) mice.

Fear conditioning is a test for associative learning that depends partly on hippocampal function, as is the Morris water maze test. The association of conditioned stimuli (CS, tone) and unconditioned noxious stimuli (US, electric foot shock) was learned in the conditioning on day 1. The results were quantitatively evaluated by the freezing response of the subjects. Zic2\(^{ad^+}\) mice showed a significantly reduced freezing response in the context test on day 2 (P = 0.037, U-test, Figure 2B). These mice also showed a significantly reduced freezing response in the cue test on day 3 (P = 0.049, U-test, Figure 2C).

We also observed abnormal behavioral traits in Zic2\(^{ad^+}\) mice in the Y-maze spontaneous alternation test. Zic2\(^{ad^+}\) mice showed a significantly lower alteration percentage (P = 0.046, U-test) and a significantly higher number of arm entries (P = 0.0040) than the wild-type mice (Figure 2C), suggesting that Zic2\(^{ad^+}\) mice have working memory impairment and a higher level of locomotor activity in a novel environment. Together with the absence of behavioral traits related to mood disturbances or anxiety-like behaviors, our results from the water maze, fear-conditioning and Y-maze tests are consistent with the impaired cognitive function in Zic2\(^{ad^+}\) mice.

Abnormalities in social behavior in Zic2\(^{ad^+}\) mice. We next assessed the social behaviors of the Zic2\(^{ad^+}\) mice by the resident-intruder assay. Juvenile wild-type mice were placed into the home cages of

Table 1 | Summary of Zic2\(^{ad^+}\) behavioral analysis.

| Test                          | Response | Implication to schizophrenia |
|-------------------------------|----------|-----------------------------|
| Home cage activity            | decreased* | Negative?                   |
| Open field                    |          |                             |
| Locomotor                     | increased* | Positive (psychomotor agitation) |
| % center                      | no change |                             |
| Morris Water Maze             |          |                             |
| Latency-training              | increased* | Cognitive (learning deficits) |
| Latency-reverse               | increased* | Cognitive (learning deficits) |
| Probe test                    | no change |                             |
| Speed-training                | initially slow* |                             |
| No move-training              | no change |                             |
| Fear conditioning             |          |                             |
| Conditioning                  | no change | Cognitive (fear memory deficits) |
| Contextual                    | decreased* | Cognitive (fear memory deficits) |
| Y-maze                        |          |                             |
| No. of entries                | increased* | Positive (psychomotor agitation) |
| % alteration                  | decreased* | Cognitive (working memory deficits) |
| Social interaction            |          |                             |
| Novel environment             | no change |                             |
| Resident intruder attack      | decreased* | Negative (social withdrawal) |
| Social dominance              |          | Negative (social withdrawal) |
| Social recognition            | no change |                             |
| Acoustic startle response     | increased* | Cognitive (sensorimotor gating) |
| PPI of acoustic startle response | decreased* | Cognitive (sensorimotor gating) |
| Light-Dark box                | no change |                             |
| Marble burying                | no change |                             |
| Burrowing                     | no change |                             |
| Elevated Plus maze            | no change^3 |                             |
| Forced swimming               | no change^3 |                             |
| Tail suspension               | no change |                             |
| Grip strength                 | no change^3 |                             |
| Wire hanging                  | no change^3 |                             |
| Footprint                     | no change^3 |                             |
| Rotarod                       | no change^3 |                             |

*Zic2\(^{ad^+}\) compared to Zic2\(^{+/+}\); *Possible relevance to the three classes of schizophrenia symptoms (positive, negative, and cognitive dysfunction); ^Ogura et al. (2001); *P < 0.05 in statistical tests between Zic2\(^{+/+}\) and Zic2\(^{ad^+}\).
the resident Zic2<sup>kd+</sup> mice and the behavior of the test mice (Zic2<sup>kd+</sup> mice or control wild-type mice) were analyzed for 15 min (Figure 3A, 3B, supplemental video). The time spent attacking (P = 0.049; Figure 3A, left panel) and the frequency of attacks were significantly lower in Zic2<sup>kd+</sup> mice than in control wild-type mice (P = 0.049; Figure 3A, right panel). The frequency of body contact also tended to be higher in Zic2<sup>kd+</sup> mice than in wild-type mice (P = 0.12). We also observed abnormal social behavior in Zic2<sup>kd+</sup> mice in the social dominance tube test (Figure 3C). In this test, Zic2<sup>kd+</sup> mice and control wild-type mice were placed at opposite ends of a transparent plastic tube in a head-to-head direction, and the first mouse to escape was judged the loser (Figure 3B). In general, mice of both genotypes moved forward and pushed each other within the tube. Zic2<sup>kd+</sup> mice became losers more frequently than the wild-type mice (P = 0.024, chi-square test). We also performed a social interaction test in a novel environment (open field) with caged (Figure S1B) and non-caged partners (Figure S1A), but found no clear differences in the number and duration of the contacts between Zic2<sup>kd+</sup> and wild-type mice.

**Zic2<sup>kd+</sup> mouse brain shows an altered morphology and reduction of forebrain cholinergic neurons and amygdalar Zic-positive cells.** To elucidate the molecular basis of the behavioral abnormalities observed in Zic2<sup>kd+</sup> mice, we performed a morphometric analysis of the Zic2<sup>kd+</sup> mouse brain by MRI (Figure 4). We showed that Zic2<sup>kd+</sup> mouse brains had enlarged lateral ventricles compared with the brains of wild-type mice (Figure 4A and B). The ratio of the volume of lateral ventricles to brain was 35% higher in the brains of Zic2<sup>kd+</sup> mice than in those of wild-type mice. The 3D superimposition of lateral ventricles in the brain indicated that the enlargement was most notable in the anterior horn region (Figure 4B and C). Enlargement of the lateral ventricles in Zic2<sup>kd+</sup> mice might partly reflect the reduction in the mass of the septum (Figure 4A, right panel), which we also observed in MRI 2D coronal images through the anterior commissure (data not shown). The hippocampal size did not show any clear differences between the two genotypes. Morphometric analysis of histological sections revealed that compared to the wild type the thickness of the cerebral cortex and the corpus callosum was slightly but significantly thinner in Zic2<sup>kd+</sup> mice (cerebral cortex, P = 0.0038; corpus callosum, P = 0.0028; Figure 4D) and that the position and shape of the medial structure rostral to the hippocampus (fimbria including septofimbrial nucleus or septal triangular nucleus) were significantly narrower (P < 0.001 for both, Figure 4D).

We also found the amygdala in Zic2<sup>kd+</sup> mice to be morphologically different to that in wild-type mice. In wild-type mice, Zic-positive cells were abundant in the amygdalohippocampal area (AHA) and sparse in the medial and cortical nuclei (Figure 4E). In Zic2<sup>kd+</sup> mice, the Zic-positive cells were less abundant than in wild-type mice in the equivalent rostrocaudal positions (Figure 4E, 8/8). Furthermore, the high cell density in the AHA of wild type animals shown in toluidine blue staining seemed reduced and the intense signals detected by acetylcholine esterase staining in the AHA was debilitated (Figure 4E), in Zic2<sup>kd+</sup> mice. As shown by acetylcholine esterase stained sections (Figure 4E), in some cases (6/8), the medial protrusion of the amygdala in the coronal sections tended to be blunted in Zic2<sup>kd+</sup> mice compared to the wild type.

The reduction of the septal mass and expression of Zic2 in the basal forebrain structures<sup>40</sup> led us to investigate the number of cholinergic neurons that are densely distributed in the septum. We counted the choline acetyl transferase (ChAT)-positive cholinergic neurons in comparable coronal sections from the brains of Zic2<sup>kd+</sup> and wild-type mice. The results indicated that the numbers of cholinergic neurons were decreased in the medial septum, diagonal band and substantia innominata regions, but not in other regions including cerebral cortex, striatum and caudoputamen, (Figure 5A–C). In

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**Figure 1** | Spontaneous motor performance abnormalities in Zic2<sup>kd+</sup> mice. (A) Home cage activity was measured for 13 days. On day 1 the mice were put into a new home cage. Mean activities per day are indicated. Activity counts represent the number of time bins (approximately 0.20–0.25 s each) in which spontaneous activity including locomotor activity, rearing, and other activities such as stereotypic movements, were detected. *P < 0.05 in t-test. (B) Circadian activities. The values indicate the summation of the activities corresponding time bins (bin = 1 h) of the last 8 days (days 6–13) when the daily change in the total activity level (A) was minimal. *P < 0.05 in t-test. (C) Open field test. (left) Total distance traveled in the open box for 15 min observation period. (right) Percentage of the total time in the central area of the field (30% of the total field area). *P < 0.05 in t-test. Data is presented as means ± SEM. The number of mice in each group is given in parentheses.
addition, the numbers of PV-positive neurons were not different in the medial septum or diagonal band (Figure 5D). Therefore, the number of basal forebrain cholinergic neurons is selectively reduced by the reduction of Zic2 expression. We also examined the number of ChAT-positive cells with or without Zic-like immunoreactivities in the affected regions (medial septum, diagonal band and substantia innominata) at early postnatal stages (P5–7, Figure 5E). In Zic2kd−/− mice, we observed a significant reduction in the number of Zic− ChAT+ cells in the diagonal band region compared to the number in wild-type mice (P < 0.001, Figure 5E, left panel). The number of Zic− ChAT+ cells in the medial septum and Zic− ChAT+ cells in the diagonal band and medial septum region also tended to be reduced in Zic2kd−/− mice compared to the wild type (Zic− ChAT+ cells in the medial septum, P = 0.062; Zic− ChAT+ cells in the diagonal band and medial septum region, P = 0.12 and P = 0.056 respectively). These results were consistent with those obtained in adult mice, suggesting that the reduction in the number of ChAT-positive neurons in the basal forebrain primarily stemmed from the reduction in Zic2 gene expression during embryonic or prenatal development.

Figure 2 | Cognitive function deficits in Zic2kd−/− mice. (A) Morris water maze test. (top) Mean latency to reach the platform during the training session (days 1–4) and reverse test session (day 6). Values indicate the mean of all six trials on the day. (middle left) Moving speed in the training session. (middle right) No movement time in the training session. (bottom) The results of the probe test (day 5) as indicated by the period of time (s) in the indicated quadrant within the 60 s testing period. *P < 0.05 in t-test. (B) Fear conditioning test. Mean percentage freezing are indicated for the conditioning test (day 1) before and after the electrical foot shock (preUS [mean of the 2 min before unconditioned-stimulus, US] and US [1 min after US] respectively), context test (day 2, mean of the total testing period [5 min]), and cue test (day 3) before and after pre tone (preCS [mean of the 2 min before conditioned stimulus, CS] and CS [mean of the 2 min with CS], respectively). *P < 0.05 in Mann-Whitney U-test. (C) Y-maze test. (left) Percentage altered selection of the entered arm. (right) Total number of arm entries. *P < 0.05 in Mann-Whitney U-test; **P < 0.01 in t-test. Data is presented as means ± SEM. The number of mice in each group is given in parentheses.
latencies to win were as follows: tube test. (left) Won rate in the total of 66 matches. The means simultaneously recording from opposite directions. (C) Social dominance away from the intruder mouse. The left and right images indicate the humans. We therefore set out to examine whether contribute to the onset of schizophrenia, in at least a subset of mouse, whereas the SCIENTIFIC REPORTS of the above behavioral and histological abnormalities in Zic2kd/1 mice were reminiscent of schizophrenia symptoms in patients. As a first step to address this possibility, we searched ZIC2 for nonsynonymous mutations in patients with schizophrenia. Many nonsynonymous mutations are reported in patients with HPE2,23; however, there are no reports of an association of ZIC2 mutations with psychiatric illnesses.

Sequence analysis of the entire ZIC2 protein coding regions and adjacent introns in 278 patients revealed four nonsynonymous mutations in the coding regions (Table 2). We then examined the allele frequencies of these mutations in 967 patients with schizophrenia and in 1060 control subjects (Table 2). Ins239H was the most commonly detected mutation, but its frequency (~9%) was similar in patient and control groups. This finding is consistent with the results of previous studies24,25. The three remaining mutations, A95T, R409P, and S444R, were novel. A95T and R409P were single-nucleotide mutations not found in normal subjects. The frequency of S444R was not significantly different between the patient group (0.39%) and normal subjects (0.18%; P = 0.80 by Fisher’s exact test). Patients with this mutation showed no obvious psychotic symptoms; however, we could not perform detailed physical examinations on these patients, nor examine the genotypes of their relatives because we could not obtain their consent on these issues.

The Zic2-R409P shows impaired transcriptional activation. Zic orthologues are widely distributed among the eumetazoans and show evolutionary conserved domains in their protein-coding regions28. Multi-species alignment of the three Zic2 mutations revealed that R409P was located within the highly conserved regions of the published Zic sequences, including those of the protostomians and cnidarians (Figure 6A and data not shown). We also showed that R409 position has conserved in a zinc-finger-type transcription factor, GLI1, in which the side chain of the encoded amino acid residue is responsible for side-chain-base interactions (Figure S2)29. Both A95T and S444R were conserved in most of the vertebrate Zic2 sequences examined, but these sequences did not align with those in invertebrates (Figure 6A and data not shown). We used the computer algorithm PolyPhen Polyphen to predict the effect of the mutations on protein structure and function. PolyPhen analysis predicted that the amino acid change in R409P most likely caused abnormal protein structure and function, whereas it was only a possibility for S444R and even less likely for A95T (Table 2).

We characterized the function of these mutations by generating the equivalent mutations in mouse Zic2 proteins, and assessing their activities in vitro. The size of the mutant Zic2 proteins was mostly comparable to that of the wild type protein, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 6B). However, the band corresponding to Zic2-KD was not significantly different between the patient group (0.39%) and normal subjects (0.18%; P = 0.80 by Fisher’s exact test). Patients with this mutation showed no obvious psychotic symptoms; however, we could not perform detailed physical examinations on these patients, nor examine the genotypes of their relatives because we could not obtain their consent on these issues.

Figure 3 | Social behavior abnormalities in Zic2+/- mice. (A) Resident-intruder test. (left) Total time spent in the indicated behaviors. (right) The number of attacking events. *P < 0.05 in t-test. Data is presented as means ± SEM. (B) Captured video image of the resident-intruder test. In this case, the Zic2+/+ mouse (+/+ , top, black) was attacking the white intruder mouse, whereas the Zic2-/- mouse (kd/+, bottom black) was moving away from the intruder mouse. The left and right images indicate the simultaneous recording from opposite directions. (C) Social dominance tube test. (left) Won rate in the total of 66 matches. The means ± SEM latencies to win were as follows: Zic2+/+, 36.5 ± 5.2 s; Zic2-/-, 38.3 ± 6.4 s. (right) Captured video images from a representative match. From top to bottom, the beginning to the end of the match is sequentially indicated. In this case, the Zic2+/+ mouse was pushed out from the plexiglass tube (30 cm) and the Zic2-/- mouse became the winner. *P < 0.05 in chi-square test. The number of mice in each group is given in parentheses.

Screening of ZIC2 mutations in patients with schizophrenia. Some of the above behavioral and histological abnormalities in Zic2+/- mice are reminiscent of schizophrenia symptoms in humans. We therefore set out to examine whether ZIC2 mutations contribute to the onset of schizophrenia, in at least a subset of patients. As a first step to address this possibility, we searched ZIC2 for nonsynonymous mutations in patients with schizophrenia. Many nonsynonymous mutations are reported in patients with HPE2,23; however, there are no reports of an association of ZIC2 mutations with psychiatric illnesses.

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Figure 4 | Morphological features of the brains from Zic2kd/1 mice. (A) Volumetric analysis of the entire brain, lateral ventricle (LV), and hippocampus (HPC). The values for tissue volumes in Zic2kd/1 mice are indicated as percentages of the corresponding wild-type values. The values for ratio of volumes in LV/whole brain and HPC/whole brain are also indicated as percentages of the corresponding wild-type values. A total of 15 pairs of Zic2+/+ and Zic2kd/1 mice were subjected to in vivo MRI imaging. *P < 0.05, **P < 0.01, ***P < 0.001 in t-test. Data is presented as means ± SEM. (B) 3D reconstruction of the outer surface of the brains of Zic2+/+ (+/+) and Zic2kd/1 (kd/+ ) mice (gray) with lateral ventricle (green). Dorsal (left) and anterior-lateral (right) views are indicated. Note the enlarged lateral ventricles and the narrowed interspace between the left and right lateral ventricles containing septum (asterisk). (C) Morphometric analysis. Sections were subjected to acetylcholine esterase staining. (a)–(d) Lines denote the distances measured in each section (a, cerebral cortex thickness; b, corpus callosum thickness; c, medial structure rostral to the hippocampus [limb including septofimbrial nucleus or septal triangular nucleus]; d, subformical organ width). The measurements were done on 15 pairs of the most comparable from the serial sections of adult male Zic2+/+ and Zic2kd/1 mice brains. (D) Morphometric analysis. The lengths are presented as a percentage relative to the corresponding wild-type values. *P < 0.05, **P < 0.01, ***P < 0.001 in t-test. Data is presented as means ± SEM. (E) Morphological abnormalities in the amygdala. The coronal sections from Zic2+/+ (+/+) and Zic2kd/1 (kd/+ ) mice were subjected to immunostaining with the anti-Zic antibody, toluidine blue (TB), and acetylcholine esterase staining (AchE). The black arrowhead and arrow indicate the Zic-positive cells in the amygdalohippocampal area (AHA) and medial nucleus, respectively. The white arrowheads indicate the enhanced AchE signals in the AHA. AMY, amygdalar complex; BLA, basolateral nucleus of amygdala; COA, cortical nucleus of amygdala; DT, dorsal thalamic nuclei; LA, lateral nucleus of amygdala; MEA, medial nucleus of amygdala; MH, medial habenular nucleus; MN, meningeal membrane. Scale bars, 0.5 mm.
Zic2. Taken together, these results suggested that the R409P mutation dampens the transcriptional activation capacity of Zic2 by altering the properties of the Zic2-containing molecular complex.

**Discussion**

Our behavioral analysis in mice uncovered some novel roles of Zic2 related to higher brain function. A summary of the results from this behavioral analysis and that of previous studies is provided in Table 1.

The locomotor activity differences between wild-type and Zic2kd/+ mice were context-dependent. In home cages, the mutant mice showed reduced locomotor activity in the early dark phase compared to wild-type mice, but in the open field test the mutant mice showed higher locomotor activity than wild-type mice. The tendency for Zic2kd/+ mice to display higher activity in a novel environment, compared to wild-type mice was also observed in the Y-maze test and the light-dark box test (Figure S1C). Therefore, Zic2kd/+ mice appear to be generally hyperactive upon exposure to a novel environment. This hyperactivity is possibly consistent with symptoms of schizophrenia in humans; hyperactivity in response to a novel environment has been suggested as a useful animal correlate of schizophrenia symptoms and has been noted in some genetically engineered mouse models of schizophrenia.

We demonstrated cognitive dysfunction in Zic2kd/+ mice by the water maze test, the fear-conditioning test, and the Y-maze test. In addition, abnormal PPI, which is deemed to reflect impaired

**Figure 5** | Decreased number of cholinergic neurons in the brains of Zic2kd/+ mice. (A, B) Immunostaining of the brains of Zic2+/+ (+/+) (A) and Zic2kd/+ (kd/+) (B) mice with anti-ChAT antibody. Coronal sections through the septum and diagonal bands derived from adult male mouse were subjected to immunoperoxidase staining. Scale bar, 1 mm. (C) Number of the ChAT+ neurons in the sections. Mean numbers of ChAT+ neurons in 20 sections from the Zic2+/+ and Zic2kd/+ mice brains are indicated. (D) PV-positive cell numbers. The measurements were taken in comparable regions to those subjected to ChAT-immunostaining. (E) The numbers of ChAT- and Zic-immunoreactive neurons in early postnatal (P5-7) Zic2+/+ and Zic2kd/+ brains. Double labeling was performed with the anti-ChAT antibody and anti-pan Zic antibody. CC, cerebral cortex; CP, caudoputamen; DB, diagonal band; LV, lateral ventricle; LS, lateral septum; MS, medial septum; MY, Mynert nucleus; SI, substantia innominata; STR, striatum. (C–E) Data is presented as means ± SEM. The number of mice in each group is given in parentheses. *P < 0.05, ***P < 0.001 in t-test.
sensormotor-gating function seen in schizophrenia, is reported in Zic2
d mice. These results corroborate that the cognitive function deficits in Zic2
d mice are not simple, but multimodal ones including sensormotor gating function.

Social behavioral abnormalities in Zic2
d mice were characterized by a reduction in aggressive behavior compared to the wild-type controls in the absence of clear deficits in the affiliative behaviors. The aggressivity assessed in the resident-intruder and social dominance tube tests may be related to their territory protecting behavior. The absence of depression-like behavior in these mice excludes the possibility that their reduced aggressivity was the result of a general loss in motivation.

Collectively, the behavioral phenotypes of Zic2
d mice seem to be implicated in the three classes of schizophrenia symptoms (positive/negative symptoms and cognitive dysfunction). When we compare the Zic2
d mice phenotype with those of other typical schizophrenia model mice (Table 3), novelty-induced hyperactivity and prepulse inhibition reduction were commonly found in the dominant negative DISC1 transgenic and NRG1 transmembrane KO and conditional KO of ErbB4 in PV-positive interneuron. In addition, the enlargement of lateral ventricle and decrement of working memory were shared with Zic2
d and some of them (Table 3).

The morphological abnormalities in the brain of Zic2
d include a reduction in the septal mass, thinning of the cerebral cortex and corpus callosum, narrowing of the limbia hippocampi, and a regional reduction of amygdalar nuclei. These abnormalities have a pathophysiological resemblance to neuropsychiatric disorders in humans. In particular, enlarged lateral ventricles and decrease in whole brain volume are a symptom of the first episode of schizophrenia and have been observed in some genetically-engineered mouse models of schizophrenia. These finding add further support for the genetic involvement of ZIC2 in the pathogenesis of schizophrenia.

Regarding the basis of neural circuits underlying the higher brain function abnormalities observed in Zic2
d mice, we consider the following two observations to be significant. Firstly, we observed a reduction in the number of cholinergic neurons in the basal forebrain, which raises the possibility that abnormal cholinergic regulation of higher brain function underlies the behavioral abnormalities seen in Zic2
d mice. Basal forebrain cholinergic neurons are thought to be capable of regulating the cortical processing of sensory stimuli within various domains. In addition, recent studies indicate that the cholinergic system modulates cognitive deficits in schizophrenia and that cholinergic transmission is a potential target of therapeutics for the improvement of cognitive functions. Thus, further evaluation of the cholinergic transmission dynamics in Zic2 mutants would be beneficial for a better understanding of the role of Zic2 in cognitive function. We also examined the distribution of PV-positive cells in medial and dorsolateral prefrontal cortices and in the hippocampus (Figure S3) because the distribution of PV-positive cortical neurons, which represent a subset of GABAergic inhibitory neurons, is altered in some animal models of schizophrenia and is thought to be a key abnormality underlying the pathogenesis of schizophrenia. However, we did not observe any significant alterations in the distribution of PV-positive cells in Zic2
d cortices (Figure S3).

Our second key observation relates to those implying that abnormalities of the amygdala underlie the social behavior abnormalities in Zic2
d mice. The reduced aggressivity of Zic2
d mice was indicative of abnormal social behavior and we hypothesized that abnormalities of the amygdala were involved for a number of reasons. Firstly, it is well known that the amygdala is essential for controlling aggressive behaviors. Also, lesions in the rat medial amygdala cause a reduction in aggressive behavior. Adding further support, a recent study showed that the AHA and medial amygdala project into the hypothalamic aggression area (mediobasal hypothalamus), which plays a central role in the control of aggressive behavior. These facts led us to hypothesize that the reduced aggressivity in Zic2
d mice is related to the altered morphology of AHA. However, there have been limited studies focusing on the role of AHA in aggressive behavior. Therefore, further investigation of the amygdalar abnormalities in Zic2
d mice would contribute to our understanding of the neural circuits controlling aggressive behavior.

The molecular mechanism of developmental disturbances that lead to the cholinergic neuronal loss and amygdalar dysgenesis remains elusive. As one interpretation, these abnormalities may reflect a milder representation of the HPE-like abnormality and cortical dysgenesis partly as a result of the abnormal Zic2-expressing meningeal progenitor cells in Zic2
d mice. In terms of forebrain cholinergic neuron development, we found that the p75-expressing cholinergic progenitor neurons in the prospective medial septum and diagonal band are missing in Zic1/Zic3 compound mutant mice. Since the structure and function of the vertebrate Zic family of proteins is highly conserved, Zic2 might function to expand the medial forebrain cholinergic neural progenitor cells by inhibiting their exit from the proliferating cell cycle in a manner analogous to that in the Zic1/Zic3 compound mutant mice.

Resequencing analysis of Zic2 in Japanese patients with schizophrenia revealed three novel nonsynonymous mutations in ZIC2. Functional analysis of these mutations in the Zic2
d mouse model of schizophrenia indicated that the R409P mutation results in severe loss-of-function. We showed that the transcriptional activation capacity of the Zic2-R409P protein was about 20% that of the wild-type protein; which corresponds to the decreased protein production from the Zic2
d allele shown previously. This finding in turn validates Zic2
d mice as an animal model of the R409P mutation in schizophrenia. The patient with the R409P mutation was diagnosed with residual-type schizophrenia.
Figure 6 | Properties of ZIC2 variants found in schizophrenia patients. (A) Structure of the ZIC2 protein. Gray boxes with numbers indicate C2H2 motifs in the zinc finger domain of ZIC2. The positions of the A95T, R409P, and S444R mutations are indicated. Multiple alignments of the flanking regions of three mutations are indicated along with the reference sequences. Shaded characters show conserved cysteine and histidine residues in the C2H2 zinc fingers. Black box indicates an evolutionary conserved domain (ZOC, Zic-Opa-Conserved) domain. Gray box indicates the mutated residues and the corresponding residues in other species (Hs, Homo sapiens [human]; Mm, Mus musculus [mouse]; Dr, Danio rerio [zebrafish]; Lb, Loligo alerta [squid]; Dm, Drosophila melanogaster [fly]; Nv, Nematostella vectensis [sea anemone]). (B) Immunoblotting of mouse wild-type Zic2 and Zic2 variants. NIH3T3 cells were transfected with the FLAG-tag expression plasmids. The Zic2 proteins were detected by the anti-FLAG antibody. Arrow indicates the fast migrating component in FLAG-Zic2-S444R. (C) NIH3T3 cells were transfected with a Zic2-responsive luciferase reporter vector together with a vector expressing wild-type FLAG-Zic2 (WT), or the FLAG-Zic2-A95T, -R409P, -S444R mutant proteins. All luciferase activities were normalized to the activities of the co-transfected elongation factor 1 promoter-driven Renilla luciferase. The means ± SEM of three independent experiments of three samples each are shown. (D) Gel mobility shift assay. IRD-labeled target DNAs were incubated with partially purified FLAG-Zic2-WT or FLAG-Zic2-R409P proteins expressed in 293T cells. The probes and the amount are indicated at the top. (E) Quantification of the gel shift assay result. Data are presented as means ± SEM. There were statistically significant differences between the FLAG-Zic2-WT and FLAG-Zic2-R409P-bound DNA probes at each dose (50 fmol, \( P < 0.001 \); 100 fmol, \( P = 0.0022 \); 200 fmol, \( P = 0.012 \); and 400 fmol, \( P = 0.0089 \). (F) FLAG-Zic2-WT or FLAG-Zic2-R409P expressed in 293T cells were immunoprecipitated with the anti-FLAG antibody. Proteins in the input cell lysates (input) and immunoprecipitates (IP) were analyzed by immunoblotting using anti-DNA-PK, anti-RHA, and anti-FLAG antibodies. There was a decrease in the amount of co-precipitated DNA-PK in FLAG-Zic2-R409P immunoprecipitates compared to those from FLAG-Zic2-WT, despite comparable amounts of FLAG-Zic2-R409P and FLAG-Zic2-WT.
Many studies have investigated the ZIC2 mutations in patients with HPE. A recent meta-analysis study of previously published results showed that the vast majority of ZIC2 mutations (98%) cause significant loss-of-function. This suggests that HPE is caused by severely impaired function of ZIC2. Interestingly, only the very few cases (three families), in which the function of ZIC2 was shown to be null, included two independent parents with normal brain imaging despite the identification of ZIC2 missense mutations (Q36P or D152F). Together with these results, our findings raise the possibility that mildly impaired ZIC2 function does not result in HPE, but in psychotic illnesses.

In summary, behavioral and morphological phenotypes in Zic2<sup>+/+</sup> mice were reminiscent of those of schizophrenia. Additionally, the detection of rare, but significantly defective, missense mutations in patients with schizophrenia suggests that further analysis of ZIC2 in neuropsychiatric patients is meaningful. Since this study focused on missense mutations, there still remains the possibility that mutations in introns and/or flanking regions that provoke partial loss of function are associated with schizophrenia.

**Methods**

**Animals.** Animal experiments were approved by the Animal Experiment Committee of the RIKEN Brain Science Institute (approval no. H22-EP068), and the mice were maintained by the institute’s Research Resource Center. Mutant mice heterozygous for the Zic<sup>2<sup>+/−</sup></sup> allele (Zic2<sup>+/−</sup>) were described previously<sup>8,s,11</sup>. Zic2<sup>+/−</sup> 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, and were maintained in C57BL/6J background.

**Behavioral tests.** Home cage activity measurement, open field test, classical fear conditioning, Y-maze test were done as described<sup>50,51</sup>.

**Resident-intruder test.** Group-reared mice were 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. Video recording from two opposite directions 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 Zic<sup>2<sup>+/−</sup></sup>− 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.

**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, Rheinsetten, 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 x 256 x 256, FOV = 25.6 x 25.6 x 25.6 mm. 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<sup>22</sup>. 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.** Histological examination was done as described<sup>23</sup>. 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 innomina, the basal nucleus of the Meynert, and the somatosensory cortex (anterior (+) to posterior (−)) distance (mm) from bregma according to Paxinos et al. Together with these results, our findings raise the possibility that mildly impaired ZIC2 function does not result in HPE, but in psychotic illnesses.

**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.

**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).

**Molecular and functional analysis.** Mouse Zic2 variants that have the same missense mutations as the human ZIC2 nonsynonymous mutations (Zic2<sup>203<sup>E</sup></sup> for ZIC2<sup>203E</sup>, Zic2<sup>210<sup>E</sup></sup> for ZIC2<sup>210E</sup>, and Zic2<sup>240<sup>E</sup></sup> for ZIC2<sup>240E</sup>) were generated by PCR using pEFBOS-Zic2<sup>−/−</sup> or pcDNA3-HA-Zic2 as templates. Hereinafter, we refer to them as Zic2<sup>203E</sup>-Zic2<sup>210E</sup>-Zic2<sup>240E</sup> variants that have the same missense mutations as the human ZIC2 nonsynonymous mutations (Zic2<sup>203<sup>E</sup></sup> for ZIC2<sup>203E</sup>, Zic2<sup>210<sup>E</sup></sup> for ZIC2<sup>210E</sup>, and Zic2<sup>240<sup>E</sup></sup> for ZIC2<sup>240E</sup>) respectively. Expression plasmids for wild-type Zic2 and Zic2 variants were constructed pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pcMVtag2 (Strategene, La Jolla, CA, USA). 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, immunoblot analysis, luciferase reporter assay, gel shift assay, and immunoprecipitation were performed as previously described<sup>24,s,25</sup>.

**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.

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