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BEHAVIORAL NEUROSCIENCE

Down-regulation of protocadherin- α A isoforms in mice changes contextual fear conditioning and spatial working memory

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Abstract

Diverse protocadherins (Pcdhs), which are encoded as a large cluster (composed of α , β and γ clusters) in the genome, are localized to axons and synapses. The Pcdhs have been proposed to contribute to the generation of sophisticated neural networks and to regulate brain function. To address the molecular roles of Pcdhs in regulating individual behavior, here we generated knockdown mice of Pcdh-a proteins and examined their behavioral abnormalities. There are two alternative splicing variants of the Pcdh-a constant region, Pcdh- α A and B isoforms, with different cytoplasmic tails. Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, in which the Pcdh- α B splicing variant was absent and the Pcdh-a A isoforms were down-regulated to approximately 20% of the wild-type level, exhibited enhanced contextual fear conditioning and disparities in an eight-arm radial maze. Similar abnormalities were found in Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice, which lacked 57 amino acids of the Pcdh- α A cytoplasmic tail. These learning abnormalities were, however, not seen in Pcdh- $\alpha^{\Delta B / \Delta B}$ mice [in which the neomycin-resistance (neo) gene cassette was removed from the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ alleles], in which the expression level of the Pcdh- α A isoforms was recovered, although the Pcdh- α B isoforms were still completely missing in the brain. In addition, the amount of 5-hydroxytryptamine increased in the hippocampus of the hypomorphic Pcdh-a A mutant mice but not in recovery Pcdh- $\alpha^{\Delta B/\Delta B}$. These results suggested that the level of Pcdh- α A isoforms in the brain has an important role in regulating learning and memory functions and the amount of 5-hydroxytryptamine in the hippocampus.

Introduction

During the development of the nervous system, cell recognition molecules are involved in organizing neuronal networks by regulating neural migration, fasciculation, synaptogenesis and intracellular signaling (Dodd & Jessell, 1988; Edelman & Crossin, 1991; Fields & Itoh, 1996).

The protocadherin (Pcdh)-a family was originally identified as cadherin-related neuronal receptors (CNRs) (Kohmura et al., 1998). The *Pcdh*- α cluster is followed by the *Pcdh*- β and *Pcdh*- γ clusters, which are arranged in tandem on human chromosome 5 and mouse chromosome 18. The mouse $Pcdh-\alpha$ gene cluster is composed of 14

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variable exons and a set of three constant-region exons (Sugino et al., 2000; Wu & Maniatis, 2000). Mature Pcdh-a mRNAs are generated from one of these variable exons, encoding alternative extracellular and transmembrane regions, and a set of constant-region exons, which encode short cytoplasmic domains. For the Pcdh- α third constant exon, two kinds of alternative splicing variants, Pcdha A and B isoforms, are produced (Sugino et al., 2000). Diverse Pcdh proteins appear to be exclusively expressed in the brain, especially in the olfactory bulb, hippocampus and cerebellum. Pcdh proteins, and complexes that include them, are localized to axons and partially to synapses during neuronal development (Kohmura et al., 1998; Phillips et al., 2003; Morishita et al., 2004). Several dozen Pcdh- α and Pcdh- γ mRNAs are differentially expressed in single Purkinje cells with monoallelic chromosome regulation (Esumi et al., 2005; Kaneko et al., 2006). This molecular diversity

of the Pcdhs has led to the speculation that these proteins might underlie the precise formation of the neuronal network (Serafini, 1999; Shapiro & Colman, 1999; Yagi & Takeichi, 2000; Hamada & Yagi, 2001; Yagi, 2008).

Pcdh-y null mice die soon after birth because of a dramatic degeneration of spinal interneurons in the late embryonic stages (Wang et al., 2002; Weiner et al., 2005). Moreover, synaptic density in the intermediate zone of the spinal cord is dramatically decreased. Further investigation has been performed in mutants lacking both Pcdh-y proteins and the proapoptotic protein BAX (a member of proapoptic Bcl-2 family). In these mice, a decrease in synapses, despite the minimal apoptosis and neurodegeneration, was still observed and the activity of the synapses that did form was reduced. These results indicated that Pcdh- γ proteins are essential for the synaptic development of at least some neurons. We recently demonstrated that $Pcdh-\alpha$ mutants lacking the common cytoplasmic tail showed abnormal axonal sorting of olfactory sensory neurons into glomeruli (Hasegawa et al., 2008). However, roles for Pcdh proteins in regulating brain functions that affect behavior have not yet been identified.

In the present study, we generated mutant mice with extensively decreased Pcdh- α proteins, to investigate the physiological role of these proteins *in vivo*. These mutant mice showed enhanced fear-conditioning learning and a moderate disparity from normal in the eight-arm radial maze test. These abnormalities were reversed by the expression of normal levels of Pcdh- α A isoforms. In addition, these hypomorphic Pcdh- α A mutations led to up-regulated levels of 5-hydroxytryptamine (5-HT) in the hippocampus.

Materials and methods

Production of Pcdh-a isoform-specific gene-converted mice

To generate the Pcdh- α Δ Bneo allele, we constructed a targeting vector to insert a termination codon in-frame followed by a loxP site at the first codon of the constant fourth exon, which encodes a subtypespecific region of the Pcdh-a B isoform. To generate the Pcdh-a Δ Aneo allele, we constructed a targeting vector to insert a termination codon just after the A and B common coding region in the constant third exon. The 5' homology arms were NotI/SalI-digested 3.0-kb fragments generated by polymerase chain reaction (PCR) with two primers for the Pcdh-a B allele; CP3-F (5'-ATAAGAATGCGGC-CGCGGCTACAATGTGGGAGAT-3') and CNR-B-R (5'-ACGCG-TCGACTCATTCTGAAAACAAGCCAGAGTG-3') for the Pcdh-α A allele; and CP3-F and CNR-A-R (5'-ACGCGTCGACTCAGATCCT-GGGATAATGAATTTG-3') for the genomic DNA of mouse embryonic stem cells (TT-2). A floxed PGK-neomycin-resistance (neo) cassette was placed down-stream of the 5' homology arms as the positive selection marker. The 3' homology arm was made from Sall/ApaI-digested 9.0- and 9.4-kb fragments generated by longrange PCR with two primers for the Pcdh-a B allele, CNR-B-F (5'-ACGCGTCGACAAAAAGCAGACCCAGGTTTC-3') and CP3-R (5'-CGGGGGCCCTATTAGCACAGCCTCCCT-3'), and two for the Pcdh-a A allele, CNR-A-F (5'-ACGCGTCGACTGCAATCATCTC-CATCCG-3') and CP3-R. The coding regions in the homology arms were completely sequenced to confirm their integrity. For negative selection, an MC1-DT-A cassette was inserted at the end of the 3' arm.

We obtained homologous recombinants using TT-2 cells from F1 male embryos crossing between C57BL/6 and CBA mice. Mice possessing the Δ Bneo and Δ Aneo alleles were produced using standard procedures for chimeric mouse production (Yagi *et al.*, 1993). Successful gene targeting was confirmed by PCR analysis

using the primer pair NA-F (5'-GGTGAGTTGCCAGACAAATTC-3') and mh1-2R (5'-GCCCTGCAAATATCATCAAG-3') and Southern blotting analysis, as shown in Figs 1 and 6. Male chimeras were crossed with C57BL/6 females and the resulting heterozygous mice were backcrossed with C57BL/6 mice for more than eight generations. All of the experiments were performed with littermates from crosses between C57BL/6 backcrossed heterozygotes.

To remove the PGK-neo cassette, we crossed Pcdh- $\alpha^{+/\Delta Bneo}$ mice with the transgenic mice that express Cre recombinase ubiquitously (Kawamoto *et al.*, 2000) (a generous gift from Dr J. Miyazaki, Osaka University, Japan).

Antibodies

A monoclonal antibody for specific Pcdh- α B proteins (1F4, rat IgG) was produced by immunizing rats with keyhole limpet hemocyanin conjugated with a synthesized polypeptide (Cys-RRKGEASQPRQ), which is a specific sequence in the Pcdh- α B cytoplasmic tail. In the present study, we used anti-CNR/Pcdh- α (rabbit polyclonal antibody) as a Pcdh- α A isoform-specific antibody (Murata *et al.*, 2004). Anti-CNRN (rabbit polyclonal antibody) and 4E11 (rat monoclonal antibody) (Mutoh *et al.*, 2004) against the Pcdh- α 4 N-terminus (QIHYSIPEEAKHGT-Cys), and anti-CNR-A antibody (Takei *et al.*, 2001) against the Pcdh- α A-specific region were used for immuno-precipitation. Anti-synaptophysin (Sigma), anti-synaptotagmin (Chemicon), anti-growth-associated protein 43 (Sigma), anti-glutamic acid decarboxylase 67 (Chemicon) and anti-postsynaptic density 95 (Affinity Bioreagents, USA) were purchased.

Plasmids and transfection

Plasmid DNA was prepared as previously described (Murata *et al.*, 2004). Briefly, full-length mouse $Pcdh-\alpha 4-A$ or $Pcdh-\alpha 4-B$ was tagged with a c-myc epitope and then cloned into a pcDNA3.1 expression vector (Myc-CNR/ $Pcdh-\alpha 4-A$ or Myc-CNR/ $Pcdh-\alpha 4-B$). HEK293T cells were maintained in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum. Plasmid DNA was transfected into HEK293T cells using LipofectAMINE 2000 (Invitrogen).

Reverse transcription-PCR

Total RNA from the mouse brain was extracted with TRIzol Reagent (Invitrogen) and cDNA was synthesized after priming with 40 pmol per reaction with oligo-dT primers in a total volume of 20 μ L, using 15 U of Superscript III (Invitrogen) in accordance with the manufacturer's protocol. To detect spliced transcripts in wild-type and mutant mice, the primer pair North-F (5'-GGCAGCCCAACCCTGACT-3') and mh1-2R was used. To define each transcript, Southern blot analysis using an internal probe for the products was performed after reverse transcription-PCR.

Immunoblotting and immunoprecipitation

Mice were deeply anesthetized with diethyl ether. The harvested brains were immediately frozen and tissues were homogenized in $2\times$ sodium dodecyl sulfate sample buffer (100 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue). The lysates were spun at 20 000 g for 20 min and the supernatant was used for immunoblot analysis. For immunoprecipi-



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tation, tissues were homogenized in radioimmunoprotein assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors (5 µg/mL aprotinin, 3 µg/mL leupeptin, 3 µg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and incubated at 4°C for 10 min. The lysates were spun at 9100 g at 4°C for 20 min and the supernatants were precleared with protein G-Sepharose beads (Amersham, Bioscience) at 4°C for 1 h. The samples were then incubated with 1 µg of anti-CNRN or anti-CNR-A at 4°C for 1 h. Protein G-Sepharose beads were added and the samples were incubated at 4°C for more than 2 h. The beads were washed extensively with RIPA buffer and phosphate-buffered saline, and the proteins were dissociated from them by boiling the beads in sample buffer. The proteins were subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis followed by western blot analysis (Murata et al., 2004).

Animal and behavioral experiments

All tests were performed with male mice that were 10 weeks old at the start of testing. The mice were housed four per cage in a room with a 12 h light/dark cycle and *ad libitum* access to food and water. Experimental procedures were in accordance with Guide for the Care and Use of Laboratory Animals in Science Council of Japan and were approved by the Animal Experiment Committee of Osaka University and Kyoto University.

Motor function tests

Motor function tests were conducted as described previously (Miyakawa *et al.*, 2001). The rotarod test was performed using an accelerating rotarod (UGO Basile Accelerating Rotarod). The time that each mouse was able to stay on the rod was measured. In the wire hang test, each animal was placed on the lid of a wire cage and then inverted gently so that the mouse gripped the wire. The latency to fall was recorded with a 60 s cutoff time.

Open field test

Locomotor activity was measured by the open field test. Mice were placed in the center of an open field apparatus ($27.4 \times 27.4 \times 20$ cm; MED Associates, Alban, VT, USA). Data were collected for 2 h and the total distance and time spent in the center were recorded.

Light/dark transition test

Light/dark transition tests were conducted in a two-chamber apparatus as described previously (Miyakawa *et al.*, 2001). One chamber was irradiated brightly, whereas the other chamber was dark. Mice were placed into the light side and allowed to move freely between the two chambers for 10 min. The total number of transitions and total time in the light chamber were recorded.

Home-cage activity

During the home-cage activity test, each subject was housed individually in a cage $(31 \times 21 \times 13 \text{ cm})$ in a room with a 12 h light/dark cycle and *ad libitum* access to food and water. Mice were observed for 3 days and the total distance traveled during the night and day was recorded.

Elevated plus maze

The elevated plus maze formed two open arms $(25 \times 5 \text{ cm})$ and two enclosed arms that had 15-cm-high transparent walls. The two sets of arms were placed diagonally and the apparatus was elevated to 50 cm above the floor. To prevent mice from falling from the apparatus, 3-mm-high Plexiglas ledges were attached to the open arms. The mice were placed in the central square $(5 \times 5 \text{ cm})$ facing one of the closed arms. The test was conducted for 10 min and the time spent in the open arms was recorded.

Porsolt forced swim test

The Porsolt forced swim test was conducted as described previously (Miyakawa *et al.*, 2001). Briefly, mice were placed into a cylinder filled with super hypochlorous water and their behavior was recorded for 10 min. Data acquisition was performed automatically and the time of immobility was recorded.

Pain test

The mice were placed on a 55° C hot plate and the latency to paw lick or foot shake was recorded.

Contextual and cued fear conditioning

On the training day, each mouse was placed into a conditioning chamber ($10.5 \times 10.5 \times 10.5$ cm; O'Hara & Co., Tokyo, Japan) and

FIG. 1. Generation of Pcdh- $\alpha^{\Delta Bnco/\Delta Bnco}$ and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice. (A) Alternative splicing in the constant regions generates the Pcdh- α A and B isoforms. Black boxes represent Pcdh-a A- and B common regions, striped boxes represent A and gray boxes represent B-specific regions. A-type splicing uses three and B-type splicing uses four exons. The termination region for both transcripts is a common poly A signal (pA-S). (B) Protein structure of the Pcdh-a A and B isoforms. Each isoform family has a common variable region and a different cytoplasmic region. EC1-6, extracellular cadherin domains 1-6; TM, transmembrane domain; CPN, short cytoplasmic domain; K-rich, lysine-rich region. (C) Genomic organization of wild-type mouse Pcdh-a, the targeting vector, the Δ Bneo allele, containing a floxed neo gene in the B-specific fourth exon in the constant region, and the ΔB allele, from which the neo gene was removed by Cre/loxP recombination. The restriction sites around the locus used for targeting and Southern blot analysis (blacklines) are shown (E, EcoRI; S, SalI; X, XhoI, Sp, SpeI). The probe for Southern blot analysis (black lines) and the primers for genomic PCR (arrows) are also indicated. DT-A, diphtheria toxin A fragment. (D) Southern blot analysis of the targeted Pcdh- α constant region. DNA from the tails of wild-type (+/+), heterozygous (+/ Δ Bneo) and homozygous (Δ Bneo/ Δ Bneo) mice was digested with EcoRI. Blotted DNA was hybridized with the 5' probe, which was against a 5' Pcdh- α third exon genomic flanking region. (E) Pcdh- α B isoforms in wild-type (+/+) and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice were detected by western blotting with an anti-1F4 antibody of immunoprecipitants obtained with an anti-CNRN antibody. The antigen specificity of the anti-1F4 antibody for Pcdh- α B isoforms is shown using HEK293T cell transfectants of Myc-CNR/Pcdh- α 4-A (A-isoform) and Myc-CNR/Pcdh- α 4-B (B-isoform). (F) Expression of the Pcdh- α A isoform proteins in the brain of 4-week-old wild-type (+/+), Pcdh- $\alpha^{+/\Delta Bneo}$ and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice. Antigen specificity of the anti-CNR/Pcdha antibody for the Pcdh- α A isoforms is shown using HEK293T cell transfectants. (G) Genomic PCR of Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ and Pcdh- $\alpha^{\Delta B}/\Delta B$ mice. (H) In Pcdh- $\alpha^{\Delta Bneo}/\Delta^{\Delta Bneo}$ and Pcdh- $\alpha^{\Delta B}/\Delta B$ mice, no Pcdh- α B-type mRNA could be detected by reverse transcription-PCR. (I) Pcdh- α B isoforms were detected in wild-type mice (+/+) by western blotting of immunoprecipitants obtained with the anti-CNRN antibody but not in Pcdh- $\alpha^{\Delta B/\Delta B}$ mice. IP, immunoprecipitation. IB, immunoblotting. (J) Protein level of the Pcdh- α A isoforms in the brain of wild-type (+/+), Pcdh- $\alpha^{+/\Delta B}$ and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice.

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allowed to explore freely for 2 min. A tone (75 dB) was sounded as the conditioned stimulus for 30 s followed by a 2 s mild foot-shock (0.35 mA) as the unconditioned stimulus. One or two more tone-shock pairs were given at 2 min intervals and the animal was returned to its home cage 30 s after the last pair. At 24 h after the conditioning session, the mice were placed back into the conditioning chamber for 5 min and their freezing behavior was measured in context. At 1 h after context testing, the mice were placed into a different, white Plexiglas chamber for 3 min and then the tone was turned on for 3 min. Freezing during the first and subsequent 3 min intervals was recorded.

Morris water maze

A hidden-platform version of the Morris water maze test was conducted as described previously (Miyakawa *et al.*, 2001). A plastic circular pool (40 cm high \times 95 cm in diameter) was filled to a depth of 30 cm with water at 21 ± 1 °C made opaque with nontoxic white paint. Each mouse was placed in one of four starting locations facing the pool wall and swam until finding a platform 1 cm below the water's surface or for a maximum of 60 s. Four trials per day were conducted for 9 days. Latency to reach the platform, the distance traveled to reach the platform, the average swim speed and the percentage of time spent at the perimeter of the pool were recorded. On the 10th day, the platform was removed and a probe test was conducted. The percentage of time spent in each quadrant, the number of platform crossings, the average swim speed and the percentage of time spent at the perimeter of the pool were recorded.

Eight-arm radial maze test

The eight-arm radial maze test was conducted as described previously (Miyakawa *et al.*, 2001). Briefly, mice were deprived of food and maintained at 80–85% of their initial body weight. After a pretraining session, maze acquisition trials were performed for 15 consecutive days. For each trial, the choice of arms, latency to obtain all of the pellets, the distance traveled, the number of different arms chosen within the first eight choices and the number of working memory and omission errors were scored.

Image analysis

All of the data analyses for the behavioral studies were performed on a Macintosh computer, using Image OF, Image WM and Image FZ (O'Hara & Co.). Applications were based on the public domain NIH IMAGE program (developed at the U.S. National Institute of Mental Health and available on the Internet at http://rsb.info.noh.gov/nih-image/) and modified by T.M. (available through O'Hara & Co.).

Measurement of monoamines by high performance liquid chromatography

The monoamines and their metabolites were measured using high performance liquid chromatography with electrochemical detection. Mice were killed by decapitation at 10 weeks old and the brain regions were rapidly dissected out and frozen at -80° C until assay. Each sample of frozen brain tissue was homogenized by ultrasonic irradiation in 250 µL of 0.2 M perchloric acid containing isoproterenol as an internal standard. The homogenates were placed on ice for

30 min and spun at 20 000 g for 15 min at 4°C. The supernatants were filtered through a syringe filter unit (DISMIC-3; Advantec, Japan), their pH was adjusted to 3.0 by adding 1 M sodium acetate and then they were injected into a high performance liquid chromatography system equipped with an ODS column (Eicompak SC3-ODS; 3×100 mm; Eicom, Japan) and an electrochemical detector (EDC-100; Eicom) with the potential set at +750 mV. The mobile phase was 0.1 M citric acid and 0.1 M sodium acetate, pH 3.5, containing sodium-1-octansulfonate (190 mg/L), EDTA-2Na (5 mg/L) and 17% methanol. The flow rate was set at 0.25 mL/min. The protein content was measured using Bradford protein assay solution (Bio-Rad, Richmond, CA, USA) after the precipitates had been solubilized with 0.1 M NaOH.

Statistical analysis

Statistical analyses were conducted using StatView (SAS Institute, Cary, NC, USA). Data were analysed by Student's *t*-test (two-tailed, unpaired), two-way ANOVA or two-way repeated measures ANOVA. Values in tables and graphs are expressed as the mean \pm SEM.

Results

Generation of Pcdh-a isoform-specific gene-converted mice

To examine the roles of Pcdh-a proteins in vivo, we generated Pcdh-a isoform-specific gene-converted mice. Mice bearing $\Delta Bneo$ and ΔB alleles were produced to assess the roles of $Pcdh-\alpha$ B-type transcripts. In the Δ Bneo allele, no amino acids (aa) were changed in the Pcdh- α A isoforms but the Pcdh-a B isoforms were not expected to be expressed (Fig. 1A-C) (aa sequence shown in Supporting information, Fig. S1). In the Δ Bneo allele, a neo gene was inserted into the fourth constant exon, which encodes the specific Pcdh- α B region (Fig. 1C). The gene conversion was confirmed by PCR and Southern blot analyses (Fig. 1D and G). To evaluate the expression of Pcdh- α proteins in Pcdh- $\alpha^{+/\Delta Bneo}$ and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ brains, we performed western blot analysis using a monoclonal antibody (anti-1F4) against Pcdh-a B proteins that specifically detected the Pcdh- α B isoform (specificity shown in Fig. 1E using HEK293T transfectants) and anti-CNR/Pcdh-a, which mainly recognized the Pcdh-a A isoforms (specificity shown in Fig. 1F using HEK293T transfectants). Using the anti-1F4 antibody, we could not detect any Pcdh- α B proteins in the brain extracts of Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, even in immunoprecipitates obtained with an anti-CNRN antibody that recognizes Pcdh-a4 proteins (Fig. 1E). At the same time, the amount of Pcdh-a A isoform proteins decreased greatly, to approximately 20% of the wild-type level, in Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mutants and to 60% in Pcdh- $\alpha^{+/\Delta Bneo}$ (Fig. 1F). We confirmed by reverse transcription-PCR that the Pcdh-a B mRNAs were abolished in Pcdh- $\alpha^{\Delta Bnoe/\Delta \hat{B}neo}$ mice but Pcdh- α A mRNAs were not (Fig. 1H). In the Δ Bneo allele, the coding sequences for the Pcdh- α A isoform proteins should not have been changed; therefore, it was possible that this down-regulation of the Pcdh- α A isoform was caused by the inserted neo gene cassette in the Δ Bneo-targeted allele.

To examine this possibility, we produced a ΔB allele by removing the neo gene cassette from the ΔB neo allele. A loxP site was located on both sides of the neo gene cassette; therefore, we could easily remove it from the ΔB neo allele using Cre recombinase. By crossing the Pcdh- $\alpha^{\Delta B$ neo/ ΔB neo mice with CAG-Cre mice, we obtained mutants (Pcdh- $\alpha^{+/\Delta B}$) containing the ΔB allele (Fig. 1C). PCR analysis of the genomic DNA using primers against both sides of the neo gene in the ΔB neo allele confirmed that the neo gene was correctly removed at the loxP sites in the Pcdh- $\alpha^{+/\Delta B}$ and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice (Fig. 1G). The *Pcdh*- α B mRNAs and proteins were also completely abolished in the Pcdh- $\alpha^{AB/\Delta B}$ mice (Fig. 1H and I); however, the Pcdh- α A isoforms recovered to a level similar to those of wild-type mice when the neo gene cassette was removed (Fig. 1J). Thus, we obtained two different Pcdh- α mutant strains, i.e. Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mutants, which had about 20% of the wild-type level of Pcdh- α A isoforms and lacked the Pcdh- α B isoforms, and Pcdh- $\alpha^{\Delta B/\Delta B}$ mutants, which lacked the Pcdh- α B isoforms but had the normal protein level of the Pcdh- α A isoforms. Using mice bearing these hypomorphic *Pcdh*- α mutations, we addressed the roles of Pcdh- α proteins in regulating brain function.

Phenotypes of the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mutant

The Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice exhibited no perinatal lethality, were healthy and fertile, and were found at the expected Mendelian frequency at the time of weaning. We observed no visible abnormalities in the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice during development or adulthood. The brains of Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice were similar in size and shape to those of wild-type mice. No obvious impairments were detected in the organization of the neocortex, hippocampus or cerebellar cortex by Nissl staining of brain sections (Fig. S2). We also did not detect any apparent differences in the levels of synaptic proteins, such as synaptophysin, synaptotagmin, growth-associated protein 43, glutamic acid decarboxylase 67 or postsynaptic density 95, in the hippocampus of the mutant vs. wild-type mice (Fig. S2).

Emotional behavior of the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice

We next analysed the function of Pcdh- α in the behavior of these mutant mice. All of the behavioral experiments were performed with male mice that were 10 weeks old at the beginning of the behavior tests. The Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice had no significant defects in their physical characteristics, such as in their body weight, whiskers and fur. No disparities were observed for the mutant mice in the wire hanging test or in the rotarod test for motor coordination. As shown in Table 1, their responses in the light/dark transition test and their home-cage activity scores were similar to wild-type. However, the scores of time spent in the center area in the open field test showed a tendency of the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice to exhibit anxious or fearful phenotypes (P = 0.11), as did their results for the stay time in the open arms in the elevated plus maze (P = 0.13). However, the difference from wild-type mice was not significant. In contrast, the Pcdh- $\alpha^{\Delta B/\Delta B}$ mice, which were Pcdh- α B isoform-specific mutants, appeared normal in the open field and elevated plus maze tests. The $Pcdh\text{-}\alpha^{\Delta Bneo/\Delta Bneo}$ mice also showed a tendency to different behavior than wild-type mice in the Porsolt forced swim test, which is used to evaluate depression (P = 0.05), and in the hot-plate test for pain sensitivity (P = 0.07) but the differences were not significant.

Enhanced freezing of Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice in the contextual fear-conditioning test

The cognitive functions of Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice and their siblings were analysed in a contextual and cued fear-conditioning test. During the conditioning period, the animals were twice presented with a paired tone and foot-shock, as training. During this period, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed no difference from wild-type in their freezing response (genotype effect, $F_{1,31} = 0.002$, P = 0.967) (Fig. 2A). When the contextual and cued tests were performed at

TABLE 1. Sensory and motor abilities in Pcdh-a gene-mutated mice

	+/+	Mutants	P-value
Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice (+/+, $n = 15-17$; $\Delta Bneo/\Delta Bneo$, $m = 16-17$)			
Motor tests			
Wire hang (latency to fall) (s)	52.3 ± 3.0	52.0 ± 3.0	0.95
Rotarod (latency to fall) (s)			
Day 1	59.8 ± 6.4	55.5 ± 6.4	0.64
Day 2	87.0 ± 7.2	89.1 ± 7.7	0.84
Open field			
Center time (s)	11.7 ± 2.5	6.9 ± 1.6	0.11
Distance (cm)	7388 ± 562	6772 ± 653	0.48
Light/dark transition test			
No. transitions	24.0 ± 1.5	23.6 ± 2.1	0.89
Total time in light (s)	172.2 ± 11.4	160.8 ± 11.0	0.48
Home-cage activity			
Distance night (cm)	1003 ± 180	824 ± 177	0.49
Distance day (cm)	204 ± 21	174 ± 17	0.28
Elevated plus maze			
Time on open arms (%)	14.8 ± 4.5	7.4 ± 1.6	0.13
Porsolt forced swim			
Immobility (%) 1-4 min	29.3 ± 3.5	38.3 ± 2.6	0.05
Immobility (%) 1–10 min	42.2 ± 4.3	46.1 ± 3.2	0.46
Pain test			
Hot plate (latency) (s)	11.5 ± 1.1	9.2 ± 0.6	0.07
Pcdh- $\alpha^{\Delta B/\Delta B}$ mice (+/+, $n = 16-17$; $\Delta B/\Delta B$, $n = 15-16$) Open field			
Center time (s)	23.6 ± 2.8	24.3 ± 2.6	0.86
Distance (cm)	3028 ± 154	2983 ± 98	0.81
Elevated plus maze			
Time on open arms (%)	7.1 ± 1.7	6.6 ± 1.2	0.81

Data represent the mean \pm SEM.

24 h after training, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed a significant enhancement of freezing in the contextual test relative to wild-type mice (genotype effect, $F_{1,31} = 5.48$, P = 0.0258) (Fig. 2B). However, Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice and their wild-type littermates displayed similar levels of freezing during the cued testing (genotype effect, $F_{1,31} = 1.987$, P = 0.169) (Fig. 2C). To avoid possible effects of the test sequence in the test battery, we performed the contextual and cued fear-conditioning tests using another group of mice that had experienced no other behavioral tests. After the littermates had received the paired tone and foot-shock twice, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice again showed greater freezing than wild-type mice in the contextual test but not in the cued test. These results indicated that the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mutants exhibited enhanced contextual learning.

Next, to confirm whether the abnormalities in the fear-conditioning test shown by the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice were caused by the decrease in Pcdh-a A or the loss of Pcdh-a B isoform proteins, we conducted the fear-conditioning test using the Pcdh- $\alpha^{\bar{A}B/\Delta B}$ mice, which lacked the Pcdh- α B proteins but had normal levels of Pcdh- α A proteins. Pcdh- $\alpha^{\Delta B/\Delta B}$ mice and their wild-type littermates were presented twice with a paired tone and foot-shock in the conditioning phase. After 24 h, contextual and cued tests were conducted. Interestingly, the Pcdh- $\alpha^{\Delta B/\Delta B}$ mice showed no significant difference in their freezing behavior in the contextual test compared with their wild-type littermates (genotype effect, $F_{1,31} = 0.054$, P = 0.818) (Fig. 2E). Also, no differences were observed in the training (genotype effect, $F_{1,31} = 1.511$, P = 0.228) and cued tests (genotype effect, $F_{1,31} = 0.027$, P = 0.870) (Fig. 2D and F). These findings indicated that the enhanced freezing in the altered contextual fear-conditioning test displayed by the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice was due to the downregulation of Pcdh-a A isoform proteins.



FIG. 2. Contextual and cued fear conditioning in Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ (A–C) and Pcdh- $\alpha^{\Delta B/\Delta B}$ (D–F) mice compared with wild-type mice (+/+). (A) Percentage of time showing freezing during the conditioning phase. A tone was sounded for 30 s (bars) followed by a 2 s foot-shock (arrows). (B) Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed enhanced freezing during the contextual test conducted at 24 h after conditioning (P < 0.03). (C) Freezing during the tone-cued test in wild-type (+/+) and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice. There was no significant difference between wild-type (+/+) and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice. (D) Percentage of freezing during the conditioning phase in wild-type (+/+) and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice. (F) Freezing during the tone-cued testing in wild-type (+/+) and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice. (F) Freezing during the tone-cued testing in wild-type (+/+) and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice. There was no significant difference between genotypes. Data are given as the mean \pm SEM (Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 16; Pcdh- $\alpha^{\Delta B/\Delta B}$ mice, n = 17, respectively).

Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ behavior in the Morris water maze

To examine the spatial learning defects of the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, we subjected them and their wild-type littermates to the Morris water maze (hidden platform version). The mice were subjected to four trials per day for nine consecutive days in the training phase and the probe test was performed at 1 day after the training. In this test, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice generally showed no significant cognitive impairment. The escape latency, which was the time that they took to reach the platform, was similar between genotypes during training (genotype effect, $F_{1,29} = 0.174$, P = 0.679) (Fig. 3A). The mutant also showed no abnormality in swimming speed or the time spent at the perimeter of the pool (Fig. 3B and C) (P > 0.05).

During the probe test, in which the learning platform was removed, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice spent more time in the training quadrant than in the other quadrants, similar to their wild-type littermates (Fig. 3E). In addition, we found no differences in the number of crossings above the place where the platform had been located (P = 0.541) (Fig. 3D). Both genotypes also spent significantly more time in the training quadrant than in the other quadrants in the probe test conducted after reversal training at 1 week after the original training (Fig. 3F). Thus, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed no abnormalities in Morris water maze learning.

Disparities between Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ and wild-type mice in the eight-arm radial maze test

The Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice and their wild-type littermates were analysed using the eight-arm radial maze test to examine their spatial working memory. In this task, the number of working memory errors

made by mice revisiting arms that had been visited previously, which is generally interpreted as a measure of working memory performance, was determined (Olton & Papas, 1979; Olton et al., 1979). The number of working memory errors made by the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice was significantly lower than that made by wild-type mice during the first six trials (genotype effect, $F_{1,23} = 5.015$, P = 0.035; interaction between trials and genotype, $F_{5,115} = 0.045$, P = 0.999). No significant genotype effect on working memory errors during the delay period (30 s in the 15th trial and 2 min in the 16th-18th trials) was observed (genotype effect, $F_{1,23} = 2.789$, P = 0.109) (Fig. 4A). The amount of total food intake during a trial was significantly lower for the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice than for wild-type mice during the first six trials (genotype effect, $F_{1,23} = 4.547$, P = 0.044; interaction between trials and genotype, $F_{5,115} = 0.837$, P = 0.526) and the delay period (15th-18th trials) (genotype effect, $F_{1,23} = 4.347$, P = 0.048; interaction between trials and genotype, $F_{3,69} = 0.486$, P = 0.693) (Fig. 4B). However, the number of different arms chosen within the first eight choices was not significantly different (P > 0.05) (Fig. 4C). There were no significant differences in the distance traveled (Fig. 4D) or in the latency to take all of the pellets during the trials, including the delay period (data not shown). In addition, there was no significant genotype effect on the number of omission error trials (genotype effect, $F_{1,23} = 0.713$, P = 0.407; interaction between trials and genotype, $F_{17,391} = 0.648$, P = 0.853) (Fig. 4E), suggesting that the increased number of revisiting errors displayed during the trials without delay was unlikely to be caused by a reduced motivation to take the pellets. Thus, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed a decreased number of working memory errors in the eight-arm radial maze. Consistent with the locomotor activity measured in several tests, such as the open field test, light/dark transition and home-cage activity (Table 1), there was



FIG. 3. Intact performance of Pcdh- $\alpha^{ABneo/ABneo}$ mice in the Morris water maze. (A) Escape latency, (B) swimming speed and (C) time spent at the perimeter of the pool. There were no significant differences between the two genotypes. (D) Both genotypes crossed the training site more often than the equivalent sites in the other three quadrants in the probe trials performed after training. (E) In the probe trials, Pcdh- $\alpha^{ABneo/ABneo}$ mice and their wild-type (+/+) littermates searched for where the platform had been located. Both genotypes spent more time in the training quadrant T compared with the other quadrants (O, opposite quadrant; R, right quadrant; L, light quadrant) in the probe trials performed after training. (F) Probe trials were conducted again at 1 week after the first probe trials. Both genotypes spent similarly more time in the training quadrant compared with the other quadrants. Data are presented as the mean \pm SEM (Pcdh- $\alpha^{ABneo/ABneo}$ mice, n = 16; wild-type, n = 15).

no difference in the distance traveled. Taken together, these findings suggest that the reduction in Pcdh- α protein level in these mutants was associated with abnormal memory formation.

We also performed the eight-arm radial maze test with Pcdh- $\alpha^{AB/\Delta B}$ mice and their wild-type littermates. There were no significant differences between the genotypes in the number of working memory errors, the amount of total food eaten, the number of different arms chosen within the first eight choices, the distance traveled or the number of omission error trials (P > 0.05) (Fig. S3). These results indicated that the down-regulation of the Pcdh- α A isoform proteins also caused disparities in spatial working memory.

Generation of a mouse bearing another hypomorphic mutation of the Pcdh- α A isoforms

To confirm the roles of Pcdh- α A isoform proteins in regulating brain function, we generated another hypomorphic Pcdh-a A isoform geneconverted mouse. By conventional gene targeting, we obtained heterozygous and homozygous mutant mice bearing the Δ Aneo allele and the gene conversion was confirmed by Southern blot analysis (Fig. 5C). In the \triangle Aneo allele, the Pcdh- α A isoform proteins were truncated at the 57 aa cytoplasmic tail. Also, a neo gene was inserted into the third constant exon (Fig. 5A and B) (aa sequence shown in Fig. S1). We performed Western blot analysis to examine the expression of Pcdh- α proteins in the Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mutant brains. Because of the 56 aa cytoplasmic tail deletion, we could not use the anti-CNR/Pcdh- α antibody, which recognizes the Pcdh- α cytoplasmic region. We therefore used the 4E11 antibody, which recognizes the EC1 region, to detect immunoprecipitates obtained with anti-CNRN (which recognizes the N-terminal region of Pcdh-a4 proteins) and anti-CNR-A (which recognizes Pcdh-a A isoform proteins). In immunoprecipitates obtained with anti-CNRN, we found truncated Pcdh-a proteins (Fig. 5D). In addition, full-length Pcdh-a A

isoform proteins were not found in the Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mutants by immunoprecipitation or western blotting with the anti-CNR-A antibody (Fig. 5D). Thus, we confirmed that the Pcdh- α A isoform proteins were truncated in Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice.

In the behavioral experiments, the Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ mutants showed abnormalities in contextual learning and working memory. These abnormal phenotypes were due to the down-regulated Pcdh- α A isoforms because the defects in contextual learning were not found in the Pcdh- $\alpha^{\Delta B}/\Delta B$ mice, which are Pcdh- α B isoform-specific mutants; therefore, we performed learning and memory tests for the Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice bearing the other Pcdh- α A hypomorphic mutation.

In the fear-conditioning test, the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice were presented with three pairs of tone and foot-shock, as training. More Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice displayed freezing behavior during the first 2 min (P = 0.018) in the contextual test but not in the training and cued tests (Fig. 6A–C). Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice and their wild-type littermates were also presented with three pairs of tone and foot-shock, as training (Fig. 6D). In the contextual test conducted at 24 h after training, significantly more Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice showed freezing behavior during the second and third minutes (P = 0.032) (Fig. 6E); however, no significant differences from wild-type were observed in the training and cued tests (Fig. 6D and F). These phenotypes were similar to those of the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, strongly suggesting that the enhanced freezing in the contextual fear-conditioning test was due to the low levels of Pcdh- α A isoform proteins.

In the eight-arm radial maze test, the number of working memory errors made by the Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice was significantly lower during the first five trials than that of wild-type mice (genotype effect, $F_{1,35} = 4.609$, P = 0.039; interaction between trials and genotype, $F_{4,140} = 0.601$, P = 0.662). However, no significant genotype effect on working memory errors during the delay period was observed (genotype effect, $F_{1,30} = 0.007$, P = 0.932) (Fig. 7A). The amount of total food taken by the Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice showed a greater



FIG. 4. Eight-arm radial maze test of wild-type (+/+) and Pcdh- $\alpha^{ABnco/\Delta Bnco}$ mice. Mice were subjected to 14 trials and four delay trials (30 s for the 15th trial and 120 s for the 16th–18th trials). In the delay trials, the mice were confined to the center platform for the delay time after taking the fourth pellet. (A) Number of working memory errors. A significant genotype effect was observed during the first to sixth days (P < 0.05). (B) Amount of total food intake. A significant genotype effect was observed during the first to sixth drials) (P < 0.05). (C) Number of different arms chosen within the first eight choices. No significant genotype effects were observed (P > 0.05). (D) Total distance traveled during the trials. There were no significant differences between genotypes. (E) Number of omission errors. Data are presented as the mean \pm SEM (Pcdh- $\alpha^{ABnco/ABnco}$ mice, n = 15; wild-type, n = 9).

decrease than by wild-type mice during the first five trials but the difference was not significant (P = 0.075) (Fig. 7B). The number of different arms chosen within the first eight choices was not significantly different (Fig. 7C). There were also no significant differences in the distance traveled (Fig. 7D), omission errors (Fig. 7E) and latency to take all of the pellets during the trials, including the delay period (data not shown). Thus, Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice showed abnormalities in working memory in the eight-arm radial maze that were similar to those of the Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ mice. These results indicated that reduced Pcdh- α A isoforms induced abnormal working memory formation. Taken together, these findings indicate that Pcdh- α A isoforms in the brain play important roles in regulating the learning and memory abilities of mice.

Analysis of monoamine levels in mice bearing $Pcdh-\alpha$ hypomorphic mutations

Neurochemical analyses were performed to examine the effect of the down-regulation of Pcdh- α proteins on monoaminergic neurotransmission in Pcdh- $\alpha^{\Delta Bneo}$ mice. In the frontal cortex of Pcdh- $\alpha^{\Delta Bneo}$ mice, no obvious differences were found in the levels of noradrenalin, 5-hydroxyindoleacetic acid (5-HIAA) (serotonergic metabolites), 5-HT, dopamine, 3,4-dihydroxyphenylacetic acid (dopamine metabolites) or homovanillic acid (dopamine metabolites) (Fig. 8B). In the hippocampus, however, the 5-HT level was significantly increased in these mice (P < 0.001), although the noradrenalin and 5-HIAA levels were not different



FIG. 5. Generation of Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice, another Pcdh- α A mutant. (A) Genomic organization of the wild-type *Pcdh*- α genome, the targeting vector, and the Δ Aneo targeted allele. A floxed neo gene was inserted at the beginning of the *Pcdh*- α A-type-specific region. Restriction enzyme sites around the locus in each allele are shown (E, *EcoRI*; S, *SaII*; X, *XhoI*, Sp, *SpeI*). Probes for Southern blot analysis and primers for genomic PCR (arrows) are indicated. Neo, neomycin-resistance transferase; DT-A, diphtheria toxin A fragment. (B) Protein structure of Pcdh- α A and the truncated Δ A proteins. Pcdh- α A isoforms lacked the 57-aa cytoplasmic tail and had an additional Glu-Ser-Thr (EST) (Fig. S1). EC1-6, extracellular cadherin domains 1–6; TM, transmembrane domain; CPN, short cytoplasmic domain; K-rich, lysine-rich region. (C) Southern blot analysis of the mutated Pcdh- α third constant exon locus in the constant region. DNA from the tails of wild-type (+/+), heterozygous (+/ Δ Aneo) and homozygous (Δ Aneo/ Δ Aneo) mice was digested with *EcoRI*. Blotted DNA was hybridized with a 5' probe, against a 5' flanking region of the *Pcdh*- α third constant exon. (D) Protein expression in wild-type (+/+) and Pcdh- $\alpha^{\Delta Aneo/\DeltaAneo}$ (-/-) mice. Immunoprecipitates (IP) obtained using an anti-CNRN or anti-CNR-A antibody from the brain extracts of each mouse were immunoblotted with the anti-4E11 or anti-CNR-A antibody, respectively.

from wild-type (Fig. 8A). Furthermore, no significant increase in 5-HT levels was found in the hippocampus or frontal cortex of the Pcdh- $\alpha^{\Delta B/\Delta B}$ mice (Fig. 8C and D). In the Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$

mice, however, the level of 5-HT in the hippocampus was significantly higher than in wild-type mice (P < 0.001) (Fig. 8E and F). These results suggested that the 5-HT level in the



FIG. 6. Contextual and cued fear conditioning in Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ (A–C) and Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ (D–F) mice. (A) Percentage of time showing freezing during the training phase. A tone was sounded for 30 s (bars) followed by a 2 s foot-shock (arrows). (B) Enhanced freezing was observed in the Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ mice during the contextual test conducted at 24 h after conditioning. (C) Freezing during the tone-cued test in Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ mice. (D) Percentage of time showing freezing during the conditioning phase in wild-type (+/+) and Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice. (E) Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice also showed enhanced freezing during contextual testing. (F) There was no significant difference between wild-type (+/+) and Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice during the tone-cued test. **P* < 0.05 vs. control group. Data are given as the mean \pm SEM (Pcdh- $\alpha^{\Delta Bneo}/\Delta Bneo}$ mice, *n* = 19; wild-type mice, *n* = 9: Pcdh- $\alpha^{\Delta Aneo}/\Delta Aneo}$ mice, *n* = 14; wild-type mice, *n* = 22 respectively).

hippocampus was associated with the hypomorphic Pcdh- α A isoforms.

Discussion

In this study, we report abnormal phenotypes in Pcdh- α geneconverted mice; specifically, enhanced fear-related learning, disparities in working memory and an enhanced 5-HT level in the hippocampus. These abnormalities were found in both Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice, carrying a hypomorphic Pcdh- α A allele that expressed Pcdh- α A proteins at about 20% of the wild-type levels, and Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice, in which the Pcdh- α A proteins were truncated. However, Pcdh- $\alpha^{\Delta B/\Delta B}$, in which the protein level of Pcdh- α A was normal but the Pcdh- α B isoforms were absent, did not exhibit these learning abnormalities or the increased hippocampal 5-HT level. These results suggest that the Pcdh- α A isoform proteins might regulate normal contextual learning, working memory and the hippocampal 5-HT level.

In the fear-conditioning learning tests, mice carrying either of two different hypomorphic Pcdh- α A alleles showed significant abnormalities in contextual fear learning but not in tone-cued fear learning. Pavlovian contextual fear conditioning requires normal functioning of both the hippocampus and amygdala, whereas conditioning to tones requires the amygdala but not the hippocampus (Kim & Fanselow, 1992). Thus, our results suggested that Pcdh- α A proteins play important roles in the hippocampus. Moreover, it is known that working memory in the radial maze task and spatial learning in the Morris water maze task are largely dependent on hippocampal function. Here we also found abnormalities in the working memory of the mice producing little to no Pcdh- α A, which could have been derived from hippocampal defects. However, Pcdh- $\alpha^{ABneo/ABneo}$ mice showed no spatial learning deficit in the Morris water maze task,

which is also dependent on hippocampal function. It is possible that the enhanced learning ability may be hardly detectable in the Morris water maze task compared with the radial maze task and the fearconditioning test. Indeed, a previous study showed that mice mutant in Telencephalin, a cell-adhesion molecule exclusively distributed in the telencephalon of the mammalian brain, showed enhanced performance in the radial maze task but not in the Morris water maze task (Nakamura *et al.*, 2001).

The level of 5-HT was enhanced in the hippocampus of mice bearing the Pcdh-a A hypomorphic mutations, whereas noradrenalin was not changed. We also found that Pcdh-a mRNAs were extensively expressed in the raphe nuclei, where 5-HT neurons are concentrated (S.K., S.H., S.E. and T.K., unpublished data). The mechanism by which Pcdh-a A proteins might increase the 5-HT level in the hippocampus remains to be determined. It is possible that Pcdh- α A proteins are involved in generating serotonergic projections from the raphe nucleus to the hippocampus. The level of 5-HT in the hippocampus was significantly elevated in both the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ and Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mutants, whereas an enhanced 5-HIAA level was detectable only in the Pcdh- $\alpha^{\Delta Aneo}$ mutants. It is possible that the 5-HT turnover ratio (5-HIAA/5-HT) is different between the Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ and Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mutants. The serotonergic pathway is involved in many physiological functions in the brain, including learning and memory formation in the hippocampus (Buhot et al., 2003). Our present results suggest that the 5-HT level in the hippocampus might be one of the causes of the learning abnormalities in these mice.

A previous study showed that $Pcdh-\gamma$ null mice die soon after birth because of increased neuronal cell death and decreased synapse formation (Wang *et al.*, 2002). Deletion of the $Pcdh-\gamma$ locus in mice induces neuronal degeneration in various areas of the brain and spinal cord; therefore, the roles of Pcdh- γ molecules in regulating brain function *in vivo* have not been examined. Here, mice carrying



FIG. 7. Radial maze test of wild-type (+/+) and Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice. (A) The number of working memory errors made by the Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice was significantly lower during the first five trials (P < 0.05). (B) The amount of total food intake tended to decrease in the Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice during the first five trials but not significantly. (C) The number of different arms chosen within the first eight choices was not significantly different. (D) Total distance during the trials. There was no significant difference between wild-type (+/+) and Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice. (E) The number of omission errors was not different. Data are given as the mean \pm SEM (Pcdh- $\alpha^{\Delta Aneo/\Delta Aneo}$ mice, n = 18; wild-type, n = 19).

either of two Pcdh- α hypomorphic alleles, encoding down-regulated or truncated Pcdh- α proteins, respectively, were observed to develop normally. Therefore, the surviving mutant mice could be analysed for behavioral and molecular differences from wild-type. Pcdh- α A isoforms and full-length Pcdh- γ proteins can form a protein complex on the membrane surface and also partially colocalize in the molecular layers of the hippocampus where synapses are enriched (Murata *et al.*, 2004). Therefore, diverse Pcdh molecules, including Pcdh- γ , which forms a protein complex with and regulates the membrane translocation of Pcdh- α proteins, may play important roles in generating synapses and in learning and memory formation in the brain.

The Pcdh- α proteins can bind to integrins via their conserved RGD(Arg-Gly-Asp) motif, which is located in the first cadherin



FIG. 8. Monoamines in wild-type (+/+, white bars), heterozygous (+/ Δ Bneo, gray bars) and homozygous (Δ Bneo/ Δ Bneo, black bars) mice. The monoamines were measured by high performance liquid chromatography assay. (A) Monoamine level in the hippocampus. 5-HT was significantly increased in Pcdh- α^{Δ Bneo/ Δ Bneo} mice. (B) There were no significant differences in the monoamine level in the frontal cortex between genotypes. (C) Monoamine level in the hippocampus in Pcdh- $\alpha^{\Delta B/\Delta B}$ mice. (D) Monoamine level in the frontal cortex in Pcdh- $\alpha^{\Delta A/\Delta B}$ mice. (E) Monoamine level in the hippocampus in Pcdh- $\alpha^{\Delta A/\Delta neo}$ mice. (F) Monoamine level in the frontal cortex in Pcdh- $\alpha^{\Delta A/\Delta neo}$ mice. **P < 0.001 vs. control group; *P < 0.01 vs. control group. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; NA, noradrenalin.

domain (Mutoh *et al.*, 2004; Morishita *et al.*, 2006). Integrins play important roles in regulating synaptic plasticity in the CA1 synapse of the hippocampus (Huang *et al.*, 2006; Kramar *et al.*, 2006). It is possible that interactions between Pcdh- α proteins and integrins regulate synaptic plasticity in the hippocampus. It was also previously reported that the specific cytoplasmic tails of Pcdh- α A and Pcdh- α B associate with neurofilament M and fascin, respectively (Triana-Baltzer & Blank, 2006). Neurofilament M may therefore associate with Pcdh- α A isoforms, playing a structural and scaffolding role in neurons. Moreover, because neurofilament M appears to form a protein complex with *N*-methyl-D-aspartate receptors, which play crucial roles in regulating synaptic plasticity and learning and memory in the hippocampus, and with dopamine receptors (Ehlers *et al.*, 1998; Kim *et al.*, 2002), it is possible that Pcdh- α A isoforms specifically regulate synaptic plasticity in the hippocampus together with neurofilaments and *N*-methyl-D-aspartate receptors. However, further analysis will be required to assess the role of Pcdh- α A proteins in the regulation of activities in the brain.

In the human *Pcdh* gene cluster, many single-nucleotide polymorphisms, several haplotypes and deletion alleles have been detected (Kirov *et al.*, 2003; Noonan *et al.*, 2003; Miki *et al.*, 2005). Here we showed that hypomorphic alleles for Pcdh- α proteins induced abnormalities in learning and memory formation in mice. Human germline polymorphisms in *Pcdh* family genes might provide genetic variations in brain functions and neurological disorders. In fact, a recent report demonstrated a striking increase in homozygosity of a minor allele of the human *Pcdh*- α cluster locus in patients with bipolar disorder pathogenesis (Pedrosa *et al.*, 2008).

In this study we report for the first time that Pcdh- α gene-converted mice showed behavioral abnormalities in fear-related contextual learning and working memory. Moreover, the cause of these disparities depended on Pcdh- α A but not Pcdh- α B isoforms. It will be important to elucidate the molecular functions of diverse Pcdh molecules, including splicing variants and Pcdh- β and - γ , in regulating synapse function and generating functional neural networks in the brain.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Comparison of the amino acid sequences of Pcdh- α A, Δ A, and B.

Fig. S2. Nissl staining of coronal sections and the expression level of synaptic proteins of the brain from wild-type (A, B, C, D) and Pcdh- $\alpha^{\Delta Bneo/\Delta Bneo}$ mice (E, F, G, H).

Fig. S3. Eight-arm radial maze test of wild-type (+/+) and Pcdh- $\alpha^{\Delta B/\Delta B}$ mice.

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Abbreviations

5-HIAA, 5-hydroxyindoleacetic; 5-HT, 5-hydroxytryptamine, serotonin; aa, amino acid; CNR, cadherin-related neuronal receptor; DT-A, diphtheria Toxin A fragment gene; EDTA, ethylenediametetracetic acid; neo, neomycin resistance; ODS, octadecylsilica; Pcdh, protocadherin; PCR, polymerase chain reaction; RIPA, radioimmunoprotein assay.

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