Analysis of protocadherin-α in mouse behavioral regulation

マウス行動制御における多様化膜分子群 protocadherin-αの機能解析

Fukuda Emi

KOKORO-Biology Group, Laboratories for Integrated Biology,

Graduate School of Frontier Bioscience, Osaka University

2008. 3

要旨

脳はきわめて多数の神経細胞から構成され、複雑であるが組織化された神経回路を形成している。脳の重要な構成要素であるニューロンは多数の樹状突起を伸ばし、周囲のニューロンと結合して情報を受け取っている。そしてそれらの情報を神経回路が正確に処理することにより、脳が正常に機能する。組織化した神経回路形成には、個々の神経細胞を認識し、結合する分子が重要な役割を担っていることが知られている。私は、細胞認識分子が、行動を制御する分子機構の解明を目指し、多様化膜分子群 protocadherin (Pcdh)-αファミリーに注目して、マウス個体レベルにおける学習・記憶を中心とした行動制御での役割について解析を行った。

多様化膜分子群である Pcdh-αファミリーは、神経細胞で発現するカドヘリン様膜タンパク質であり、神経軸索、シナプス領域に局在していることが示唆されている。この Pcdh-αファミリーは、ゲノム染色体上で遺伝子クラスター構造をもち、多様性をもつ細胞外領域は複数の可変領域エクソンにコードされ、共通の細胞内領域は定常領域エクソンに由来している。Pcdh-αファミリーは、個々の神経細胞ごとに異なった発現パターンをもつことが明らかとなっており、神経細胞の認識に関わる分子群であることが示唆されている。Pcdh-αファミリーの行動制御に関わる分子的役割を明らかにするため、私は Pcdh-α遺伝子改変マウスを用いて実験を行った。

マウス Pcdh-αファミリーの全てのメンバーに共通する定常領域には選択的スプライシングにより細胞内領域の異なる Pcdh-αA 型、B 型アイソフォームが存在する。まず私は本研究室で作製された Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスを用いた。この Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスは、Pcdh-αB 型アイソフォームが欠損し、同時に Pcdh-αA 型アイソフォームが野生型に比べ 20%まで減少していた。このマウスは外見の異常は認められず、繁殖も可能であった。しかし、Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスに対して、運動、感覚、情動、学習・記憶などについての網羅的行動解析を行ったところ、運動、感覚、情動のテストにおいては有意な差が認められなかったものの、恐怖条件付け学習テストにおいて、Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスでは空間学習能力の有意な亢進が認められた。また空間作業能力の指標となる 8 方向放射状迷路においても異常が認められた。これら空間学習の異常は、Pcdh-αB 型のみが欠損し Pcdh-αA 型の発現レベルが正常な Pcdh-$\alpha^{\Delta B/\Delta B}$ マウスでは有意な差は認められなかった。これらの結果から、Pcdh-αA 型が空間学習を制御する重要な役割を担っていることが明らかになった。更に、Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスは、感覚運動ゲーティング制御の障害を調べるプレパルス抑制テストで、抑制がかかりにくい有意な異常が認められた。

次に、Pcdh-αタンパク質の減少と行動異常との関連性を探る為に、行動の制御に関連することが知られているモノアミン量について高速液体クロマトグラフィーを用いた解析を行った。その結果、Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスの海馬体では、野生型に比べセロトニン量が有意に増加していた。一方、Pcdh-$\alpha^{\Delta B/\Delta B}$ マウスの海馬体では有意な差は見られなかった。また、Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ マウスの海馬体 CA1 領域において長期増強（LTP）が顕著に減少していることが判明した。

これらの結果から、Pcdh-αファミリーが学習・記憶の過程、感覚運動ゲーティング制御に重要な役割を持つことが明らかになった。またこれらの行動異常には、海馬体でのセロトニン量の異常な増加が関与している可能性が示唆された。一方、Pcdh-αファミリーの減少は海馬体のシナプス伝達効率を変化させることから、Pcdh-αファミリーがシナプス機能、神経回路網に直接的な役割を持っていることが示唆された。

Table of Contents

General introduction

The brain is composed enormous number of neurons, and organized complex neural networks systematically. The normal behavior is highly associated with a network of neural connections. During development, neurons extend dendrites and axons, and they generated complex arborizations. These branch patterns are at least assembled from developmental programs underling genetic-code that give rise to number of neurons, location of projection, the morphology of dendrites and axons, and the synaptic connections. It is thought to be that neurons are labeled with cell-surface recognition proteins so that they project to target sites and make synaptic connections. I have a lively interest in questions that how neurons distinguish the enormous number of different cells, and how the neural connections affect the behavior.

Neuronal interaction and recognition are thought to be mediate, in part, by cell surface proteins. The cadherin superfamily is a group of cell surface proteins that is composed more than 100 members in vertebrates. This family suggested from their molecular structures and diversity that they regulate the cell-cell interactions or transmit signaling from outside to inside of the cell. Extracellular domain contains cadherin repeats, which contain sequences that are involved in calcium binding (Overdiun *et al.*, 1995; Shapiro *et al.*, 1995). Several subgroup of cadherin can be defined based on shared propertied and sequence, classic cadherins, demosomal cadherins, protocadherins (Pcdhs), Flamingo/CELSRs and FAT. Among of these subgroups, Pcdhs are the largest subgroup, more than sixty members, in the cadherin superfamily (Nollet *et al.*, 2000).

The Pcdhs family has been identified more than 70 different Pcdhs genes. The Pcdhs family can be divided into two groups based on their genomic structure; clustered Pcdhs and nonclustered Pcdhs (Radies *et al.*, 2005). Clustered Pcdhs are consist of the *Pcdh*-α, β and γ family, which give rise to over 50 members of proteins by diverse splicing in a small genomic locus (Hirayama & Yagi, 2006). Most of Pcdhs are predominantly expressed in neurons, and some of them are localized in synapses and have led to combinatorial expression patterns. (Kohmura *et al.*, 1998; Phillips *et al.*, 2003; Morishita *et al.*, 2004). The features of the Pcdh-α have suggested the hypothesis that the diversity of the Pcdh-α potentially contributes to the neuronal recognition (Serafani, 1999; Shapiro & Colman, 1999; Yagi & Takeichi, 2000; Hamada & Yagi,

2001). However, the molecular functions of Pcdhs are still poorly understood. To elucidate the molecular function of Pcdhs *in vivo*, my laboratory generated the Pcdhs gene-targeting mice. In this study, to address the roles of Pcdhs in regulating behavior, we subjected these mutant mice to behavioral analysis. Furthermore, we approached to reveal the mechanisms underlying behavioral abnormalities through physiological, anatomical, and neurochemical phenotypes.

CHAPTER 1

Summary

The brain is composed of enormous numbers of neuron, and complex neuronal networks are organized systematically. During the neural development, axons choose specific dendrites for synapse formation by the cell recognize molecules. It is important to understand that how the molecular mechanisms organized the neuronal networks for perceiving the behavior function such as learning and memory. The clustered protocadherins (Pcdhs) family is the subgroup of the diverse cadherin superfamily and they are encoded as a large cluster (composed of α, β and γ clusters) in the genome. Pcdhs are predominantly localized in the brain and Pcdhs are up-regulate during the neuronal development. Then it is possible that they contribute to generate 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-α proteins and examined their behavioral abnormalities. In Pcdh-α proteins, there are two alternative splicing variants of the constant region, Pcdh-α A and B isoforms, with different cytoplasmic tails. Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, which lacked a splicing variant of Pcdh-α B and were down-regulated in Pcdh-α A isoforms to approximately 20%, displayed enhancement of contextual fear conditioning and disparities in the eight-arm radial maze. These learning abnormatities were, however, normalized in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice (removing the neomycin-resistant gene cassette from Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ alleles), which recovered the expression level of Pcdh-α A isoforms but completely lacked the Pcdh-α B isoforms in their brains. Furthermore, Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed a reduced of prepulse inhibition of the acoustic startle response. In addition, the amount of 5-hydroxytryptamine (5-HT) specifically increased in the hippocampus of Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mutant mice. These results suggested that the Pcdh-α family had significant roles for regulating learning and memory function, sensorimoter gating and the amount of 5-HT in the hippocampus.

Introduction

The formation of neural connection with their intended targets is controlled by complex cell-cell interactions. During the development of the nervous system, cell recognition molecules play roles in organizing neuronal networks by regulating neural migration, fasciculation, synaptogenesis and intracellular signaling (Dodd & Jessell, 1988; Edelman & Crossin, 1991; Fields & Itoh, 1996). A large number of the cadherin superfamily has been identified in the CNS and contributes to neural development, including the compartmentalization of the brain during development (Inoue *et al.*, 2001), the formation of neuronal connectivity, synapse formation, stabilities (Togashi *et al.*, 2002; Abe *et al.*, 2004) and synaptic plasticity (Tang *et al.*, 1998; Bozdagi *et al.*, 2000; Manabe *et al.*, 2000).

The protocadherin-α (Pcdh-α) 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-α* gene cluster is composed of 14 variable exons and a set of three constant region exons (Sugino *et al.*, 2000; Wu &

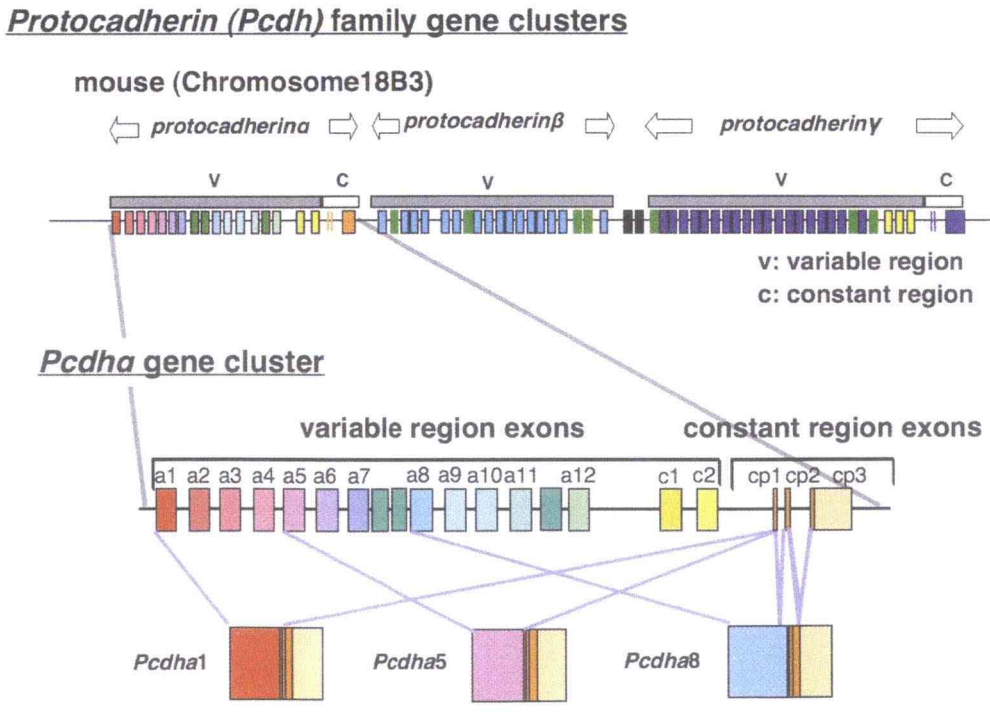


Figure 1. Structure of *protocadherin* gene cluster.

Maniatis, 2000). Mature *Pcdh-α* mRNAs are generated from one of these variable exons encoding alternate extracellular, transmembrane, and a set of constant region exons, which encode short cytoplasmic domains (Fig. 1). In the *Pcdh-α* 3rd constant exon, two kinds of alternative splicing variants, Pcdh-α A and B isoforms, are produced in all *Pcdh-α* transcripts (Sugino *et al.*, 2000) (Fig. 2). Diverse Pcdh proteins appear to be exclusively expressed in the brain, especially in the olfactory, hippocampus and cerebellum. Pcdh proteins, coupled with the complex formation of the proteins, located in axons and partially at the synapses during neuronal development (Kohmura et al., 1998; Phillips et al., 2003; Morishita et al., 2004), and have led to combinatorial expression patterns. This molecular diversity of Pcdhs has led to the speculation that Pcdhs might underlie the precise formation of the neuronal network (Serafani, 1999; Shapiro & Colman, 1999; Yagi & Takeichi, 2000; Hamada & Yagi, 2001).

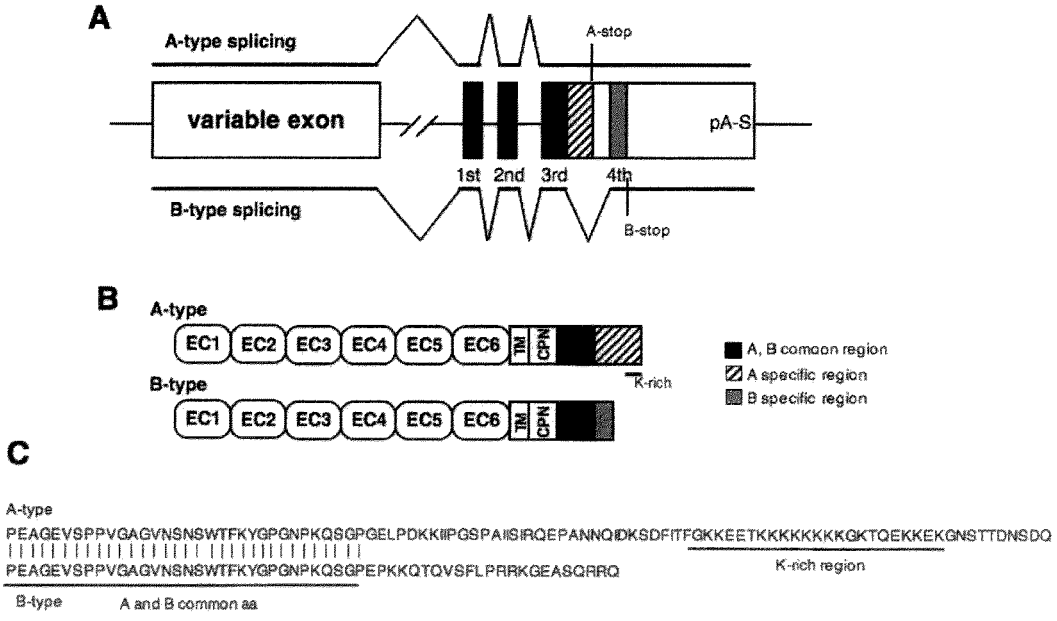


Figure 2. (A) Alternative splicing of *Pcdh-α* A and B isoforms in constant regions. Pcdh-α A- and B-type sequences are derived from different exons in the constant region. Black boxes represent *Pcdh-α* A- and B-type common regions, stripe boxes represent the specific region of A-type and gray boxes represent the specific region of B-type. A-type splicing had three, and B-type splicing had four exons. Termination of both transcripts is a common poly A signal (pA-S). (B) Diagram of the protein structure of Pcdh-α A and B isoforms. Each protein 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) Comparison of amino acid sequences of Pcdh-α A and B.

Recent study of the *Pcdh-γ* cluster using gene-targeting mice showed that Pcdh-γ null mice die soon after birth because of the dramatic neurodegeneration 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-γ proteins and the proapoptotic protein BAX (a member of proapoptic Bcl-2 family). In these mice, the decrease of synapses by minimal apoptosis and neurodegeneration were still observed and the activity of the formed synapses was reduced. These results indicated that Pcdh-γ proteins are essential for synaptic development at least in some neurons; however, the roles of Pcdh proteins in the regulation of brain function have not been identified yet.

In the present study, we generated mutant mice, which extensively decreased Pcdh-α proteins, to investegate their physiological role *in vivo*. These mutant mice showed enhancement of fear conditioning learning and moderate disparity in the eight-arm radial maze test. These abnormalities were recovered by rescuing protein levels of Pcdh-α A isoforms. The mutant mice also showed irregularities in the prepulse inhibition test. In addition, these hypomorphic Pcdh-α A mutants up-regulate the amount of 5-HT levels in the hippocampus.

Materials and Methods

Antibodies

A monoclonal antibody for specific Pcdh-α B proteins (1F4, rat IgG) was produced to immunize the KLH (keyhole limpet hemocyanin) conjugated with the synthesized polypeptide (Cys-RRKGEASQPRQ), which is a specific Pcdh-α B cytoplasmic tail. In the present study, we used anti-CNR/Pcdhα (rabbit polyclonal antibody) as a Pcdh-α A isoform-specific antibody (Murata *et al.*, 2004). Anti-CNRN (rabbit polyclonal antibody), anti-4E11 (rat monoclonal antibody) (Mutoh *et al.*, 2004) against the Pcdh-α4 N-terminus (QIHYSIPEEAKHGT-Cys) and an anti-CNR-A antibody (Takei *et al.*, 2001) against the Pcdh-α A-specific region were used for immunoprecipitation. Anti-synaptophysin (Sigma), anti-synaptotagmin (Chemicon), anti-GAP-43 (Sigma), anti-GAD-67 (Chemicon) and anti-PSD-95 (Affinity Bioreagents, USA) were purchased.

Plasmids and Transfection

Plasmid DNA was prepared as previously described (Murata *et al.*, 2004). Briefly, full-length mouse *Pcdhα-v4-A* or *Pcdhα-v4-B* was tagged with a c-myc epitope, and then cloned into pcDNA3.1 expression vector (Myc-CNR/Pcdhα-v4-A or Myc-CNR/Pcdhα-v4-B). HEK293T cells were maintained in DME medium supplemented with 10% fetal bovine serum. Plasmid DNA was transfected into HEK293T cells using LipofectAMINE 2000 (Invitrogen).

RT-PCR

Total RNA from mouse brain was extracted with TRIzol Reagent (Invitrogen) and cDNA was synthesized after priming with 40 pmol per reaction of oligo-dT primer in a total volume of 20 μl, using 15 units of Superscript3 (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 internal probe of the products was performed after RT-PCR.

Immunoblotting and Immunoprecipitation

Mice were deeply anesthetized with diethylether. The brains were immediately frozen after dissecting, and tissues or transfected cells were homogenized in 2 x SDS sample buffer (100 mM Tris-HCl, pH6.8, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue). The lysates were centrifuged at 20,000 x g for 20 min, and the supernatant was used for immunoblot analysis. For immunoprecipitation, tissues were homogenized in RIPA buffer (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors (5 μg/ml aprotininm, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, 1 mM PMSF) and incubated at 4°C for 10 min. The lysates were centrifuged at 9,100 x 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 sample was then incubated with 1 μg of anti-CNRN or anti-CNR-A at 4°C for 1 h. Protein G-Sepharose beads were added to the sample, and incubated at 4°C for more than 2 h. After the beads were washed extensively with RIPA buffer and phosphate buffered saline (PBS), the proteins were dissociated by boiling the beads in sample buffer. The proteins were subjected to SDS-PAGE followed by Western blot analysis (Murata *et al.*, 2004).

Histochemistry

For Nissl staining of brain sections with cresylviolet, deeply anesthetized mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). The 40 μm fixed sections were cut on a microtome (Leica SM2000R), and mounted on glass slides.

Animal and behavioral experiments

All tests were performed with male mice that were 10 weeks old at the start of testing. Mice were housed four per cage in a room with a 12 h light/dark cycle with *ad libitum* access to food and water.

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 each mouse was able to stay on the rod was measured. In the wire hang test, each animal was placed on a lid of a wire cage and then inverted gently, so the mouse gripped the wire. Latency to fall was recorded, with a 60 sec

cutoff time.

Open field test

Locomotor activity was measured by the open field test. Mice were placed in the center of the open field apparatus (27.4 x 27.4 x 20 cm; MED-associates, Alban, VT). 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 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 x 21 x 13 cm) in a room with a 12 h light/dark cycle with *ad libitum* access to food and water. Mice were observed for 3 days and the total distance travelled during the night and day was recorded.

Elevated plus maze

The elevated plus maze formed two open arms (25 x 5 cm) and two closed arms with 15 cm-high transparent walls. The two 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 subjects were placed in the central square (5 x 5 cm) facing one of the closed arms. The test was conducted for 10 min and the time in 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 the cylinder filled with super hypochlorous water, and their behavior was recorded for 10 min. Data acquision was performed automatically, and time of immobility was recorded.

Pain test

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

Contextual and cued fear conditioning

On the training day, each mouse was placed in a conditioning chamber (10.5 x 10.5 x

10.5 cm, O'HARA and Co., Tokyo), and allowed to explore freely for 2 min. A tone (75 dB) was sounded as the conditioning stimulus (CS) for 30 sec followed by a 2-sec mild foot shock (0.35 mA) as the unconditioning stimulus (US). One or two more tone-shock pairs were given at 2-min intervals. The animal was returned to its home cage 30 sec after the last pair. 24 h after the conditioning session, the mice were placed back into the conditioning chamber for 5 min, and freezing behavior was measured in context. An hour after context testing, the mice were put into another white Plexiglas chamber for 3 min, and then the tone was turned on for 3 min. Freezing during the first and following 3 min was recorded respectively.

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 x 95 cm diameter) was filled to a depth of 30 cm with 21 ± 1°C water 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 surface, or for a maximum of 60 sec. Four trials per day were conducted for 9 days. Latency to reach the platform, the distance traveled to reach the platform, average swim speed, and the percentage of time spent at the perimeter of the pool were recorded. On the 10^{th} day, the platform was removed, and a probe test was conducted. The percentage of time spent in each quadrant, number of platform crossings, 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 as described previously (Miyakawa *et al.*, 2001). Briefly, mice were deprived of food and maintained at 80-85% of their initial body weight. After the pretraining session, maze acquition trials were performed for 15 consecutive days. For each trial, the choice of arms, latency to get all pellets, distance traveled, the number of different arms chosen within the first eight choices, and the number of working memory and omission errors were scored.

Acoustic startle response/Prepulse inhibition

A startle reflex measurement system was used (O'HARA & Co., Ltd). The background noise level was 70 dB in each chamber. Each mouse placed in a Plexiglas tube for 5 min as an initial period of acclimation. The responses were recorded for 160 msec starting with the onset of the prepulse stimulis. The maximum startle amplitude scored

during the 160 msec was used. Mice received a series of six trial type, two types for startle trials and three types for prepulse inhibition trials. The startle stimulus was 110- or 120-dB sound. The prepulse sound was 74-, 78- or 82-dB sound, which presented 100 ms before startle stimulus. These six trial types were presented in pseudorandom order 10-20 sec intertribal interval. The following formula was used to calculate percentage prepulse inhibition of ASR: 100-{[(startle response on prepulse + startle stimulus trials)/ startle response alone trials] x 100}.

Image analysis

All applications used for behavioral studies were performed on a Macintosh computer, using Image OF, Image WM, and Image FZ (O'Hara & Co., Tokyo, Japan). 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 were modified by Tsuyoshi Miyakawa (available through O'Hara & Co., Tokyo, Japan).

Measurement of monoamines by HPLC

The contents of monoamines and their metabolites were measured using an HPLC with electrochemical detection. Mice were killed by decapitation at 10 weeks old, and the brain regions rapidly dissected out and quickly frozen at –80°C until assayed. Each frozen brain tissue was homogenized with 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 centrifuged at 20,000 x g for 15 min at 4°C. The supernatants were filtered through a syringe filter unit (DISMIC-3; ADVANTEC, Japan) and the pH was adjusted to 3.0 by adding 1 M sodium acetate, and then injected into an HPLC system equipped with an ODS column (Eicompak SC3-ODS; 3 x 100 mm; Eicom, Japan) and an electrochemical detector (EDC-100; Eicom, Japan) 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 a Bradford protein assay solution (Bio-Rad, Richmond, CA, USA) after the precipitates had been solubilized with 0.1 M NaOH.

Statistical analysis

Statistical analysis was conducted using StatView (SAS Institute, Cary, NC). Data were analysed by *t*-test, one-way ANOVA, or repeated measures ANOVA. Values in Tables and graphs are expressed as the mean ± SEM.

Electrophysiology

To compare Pcdh-$\alpha^{\Delta Bnoe/\Delta Bneo}$ with wild-type mice, we performed all experiments using littermates and in a blind fashion. 9- to 11-week-old Pcdh-$\alpha^{\Delta Bnoe/\Delta Bneo}$ and wild-type mice were decapitated under deep halothane anesthesia. Hippocampal slices (400 μm thick) were cut with a Vibratome tissue slicer and placed in a humidified holding chamber for at least 1 h. A single slice was transferred to the recording chamber maintained at 25°C and submerged beneath a continuously perfusing medium that had been saturated with 95% O_2 /5% CO_2. The medium comprised 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$ and 11 mM glucose. All perfusing solution contained picrotoxin (100 μM) to block $GABA_A$ receptor-mediated inhibitory synaptic response. Field potential recordings were made using a glass electrode filled with 3M NaCl placed in the stratum radiatum in CA1 area of the hippocampus. An Axopatch-1D amplifier was used and the signal was filtered at 1 kHz and digitized at 10 kHz. To evoke synaptic responses, a bipolar tungsten electrode was placed stratum radiatum, and Schaffer collateral/commissural fibers were stimulated at 0.1 Hz (= test pulse). A single high-frequency stimulus train (100 Hz, 1 s) was applied at the test pulse intensity to induce LTP. The amount of LTP was expressed as percentages in the slope of EPSPs relative to the baseline. The average size of EPSPs recorded between 50-60 min after high-frequency stimulation was used for statistical comparison. For measurements of paired-pulse facilitation (PPF), afferent fibers were stimulated twice at intervals of 50, 100 and 200 ms in the presence of 25 μM D-APV. For measurements of post-tetanic potentiation (PTP), a train of high-frequency stimuli (100 Hz, 1 s) was delivered at Schaffer collateral/commissural fibers in the presence of 50 μM D-APV. All value was reported as means ± SEM. Student's *t*-test (two tailed, unpaired) was used to determine whether or not there was a significant difference in the mean between two sets of data.

Results

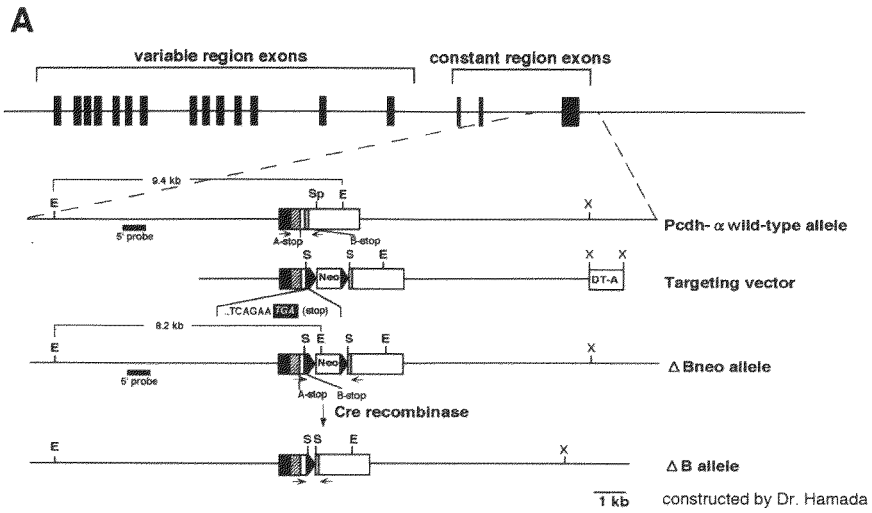
Generation of Pcdh-α isoform-specific gene-converting mice

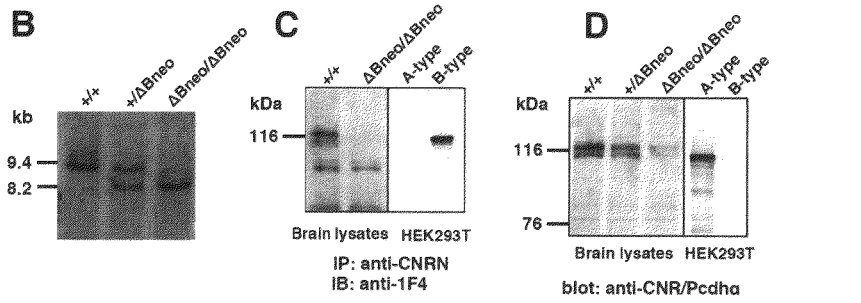
To examine the roles of Pcdh-α proteins *in vivo*, we generated Pcdh-α isoform-specific gene-converting mice. Mice bearing ΔBneo and ΔB alleles were originally produced to assess the roles of *Pcdh-α* B-type transcripts. In the ΔBneo allele, no amino acids (aa) were changed in Pcdh-α A isoforms and Pcdh-α B isoforms were expected not to be expressed. In the ΔBneo allele, a neo gene was inserted into the 4th constant exon for a specific Pcdh-α B region (Fig. 3A). The gene conversion was confirmed by PCR and Southern blot analysis (Fig. 3B, E). To evaluate the expression of Pcdh-α proteins in Pcdh-α$^{+/\Delta Bneo}$ and Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mutant brains, we performed Western blot analysis using a monoclonal antibody (anti-1F4) against Pcdh-α B proteins that specifically detected the Pcdh-α B isoform (specificity showed in Fig. 3C using HEK293T transfectants) and anti-CNR/Pcdhα, which mainly recognized the Pcdh-α A isoform (specificity shown in Fig. 3D using HEK293T transfectants). Using the anti-1F4 antibody, we could not detect Pcdh-α B proteins in the brain extracts of Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice, even in immunoprecipitants with anti-CNRN antibody-recognized Pcdh-α4 proteins (Fig. 3C). At the same time, the amounts of Pcdh-α A isoform proteins were largely decreased to approximately 20% in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mutants and 60% in Pcdh-α$^{+/\Delta Bneo}$ (Fig. 3D). We confirmed by RT-PCR that *Pcdh-α* B mRNAs were completely abolished in Pcdh-α$^{\Delta Bnoe/\Delta Bneo}$ mice but Pcdh-α A was not (Fig. 3F). In the ΔBneo allele, the coding sequences for Pcdh-α A isoform proteins should not be changed; therefore it was possible that this down-regulation of the Pcdh-α A isoform was caused by the insertion of a neo gene cassette in the ΔBneo-targeted allele.

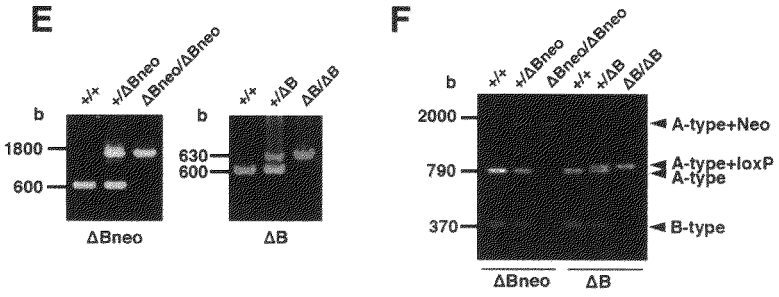
To examine this possibility, we produced a ΔB allele by removing the neo gene cassette from the ΔBneo allele. Two loxP sites were located on both sides of the neo gene cassette; therefore, we could easily remove it from the ΔBneo allele using Cre-recombinase. Crossing the Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice with CAG-Cre mice, we produced mutants (Pcdh-α$^{+/\Delta B}$) containing the ΔB allele (Fig. 3A). Using PCR analysis of genomic DNA with two primers on both sides of the neo gene in the ΔBneo allele, we confirmed that the neo gene was correctly removed at the loxP sites in Pcdh-α$^{+/\Delta B}$ and Pcdh-α$^{\Delta B/\Delta B}$ mice (Fig. 3E). *Pcdh-α* B mRNAs and proteins were also completely

abolished in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice (Fig. 3F, G). On the other hand, interestingly, in Pcdh-$\alpha^{\Delta B/\Delta B}$ mutants, Pcdh-α A isoforms were recovered to a similar level as those of wild-type mice by removing the neo gene cassette in the ΔBneo allele (Fig. 3H). Thus, we obtained two different Pcdh-α mutant strains. Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mutants had about 5-fold fewer Pcdh-α A isoforms and lacked the Pcdh-α B isoforms, and Pcdh-$\alpha^{\Delta B/\Delta B}$ mutants lacked the Pcdh-α B isoforms but had the normal protein level of the Pcdh-α A isoforms. Using these Pcdh-α hypomorphic mutants, we addressed the roles of Pcdh-α proteins to regulate brain function.

Figure 3. Generation of Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice and Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (A) Schematic diagram of the genomic organization of wild-type mouse *Pcdh-α*, the targeting vector, ΔBneo allele targeted a floxed neomycin-resistant gene into B-specific 4th exons in the constant region, and the ΔB allele removed the neomycin-resistant gene by Cre/loxP recombination. The restriction map around the locus used for targeting and Southern blot analysis is shown (E, *EcoRI*; S, *SalI*; X, *XhoI*, Sp, *SpeI)*. The probe for Southern blot analysis and primer for genomic PCR (arrows) are also indicated. Neo, neomycin-resistant gene; DT-A, diphtheria toxin A-fragment. (B) Southern blot analysis of targeted *Pcdh-α* constant region. DNA from the tails of wild-type (+/+), heterozygous (+/ΔBneo) and homozygous (ΔBneo/ΔBneo) target mice was digested with *EcoRI*. Blotted DNA was hybridized with 5' probe, which was placed on a 5' Pcdh-α 3rd exon genomic flanking region. (C) Pcdh-α B isoforms in wild-type (+/+) and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice were detected by Western blotting with anti-1F4 antibody for immunoprecipitants with anti-CNRN antibody. The antigen specificity of anti-1F4 antibody for Pcdh-α B isoforms is shown by using HEK293T cell transfectants of Myc-CNR/Pcdhα-v4-A (A-type) and Myc-CNR/Pcdhα-v4-B (B-type). (D) Expression of Pcdh-α A isoform proteins in wild-type (+/+), Pcdh-$\alpha^{+/\Delta Bneo}$ and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ brains at 4 weeks old. Antigen specificity of anti-CNR/Pcdhα antibody for Pcdh-α A isoform is shown by using HEK293T cells transfectants. (E) Genomic PCR of Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice and Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (F) In Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ and Pcdh-$\alpha^{\Delta B/\Delta B}$ mice, *Pcdh-α* B-type mRNA could not be detected by RT-PCR. (G) Pcdh-α B isoforms in wild-type mice (+/+) were detected by Western blotting for immunoprecipitants with anti-CNRN antibody, but not in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (H) Protein level of Pcdh-α A isoform in wild-type (+/+), Pcdh-$\alpha^{+/\Delta Bneo}$ and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ brains.


A
variable region exons
constant region exons
9.4 kb
Sp
E
X
5' probe
A-stop
B-stop
Pcdh- α wild-type allele
S
Neo
DT-A
Targeting vector
TCAGAA TGA (stop)
8.2 kb
ΔBneo allele
Cre recombinase
ΔB allele
1 kb
constructed by Dr. Hamada


B
+/+
+/ΔBneo
ΔBneo/ΔBneo
kb
9.4
8.2
C
kDa
116
A-type
B-type
Brain lysates
HEK293T
IP: anti-CNRN
IB: anti-1F4
D
76
blot: anti-CNR/Pcdhα


E
b
1800
600
ΔBneo
+/ΔB
ΔB/ΔB
630
ΔB
F
2000
790
370
A-type+Neo
A-type+loxP
A-type
B-type

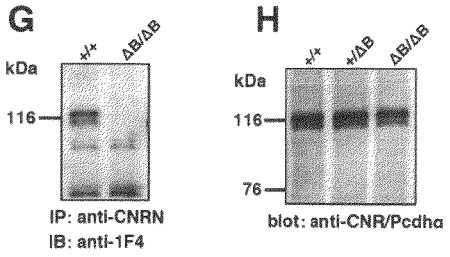

G
H
IP: anti-CNRN
IB: anti-1F4
blot: anti-CNR/Pcdhα

Figure 3

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

No perinatal lethality was observed and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice were born at the expected Mendelian frequency at the time of weaning. Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice were healthy and fertile. We observed no visible abnormalities in 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 in the neocortex, hippocampus, or cerebellum cortex by Nissl staining of the brain sections (Fig. 4). We also could not detect apparent changes of synaptic proteins such as synaptophysin, synaptotagmin, growth-associated protein 43 (GAP-43), glutamic acid decarboxylase 67 (GAD-67) and postsynaptic density 95 (PSD-95) in the hippocampus between genotypes (Fig. 4I).

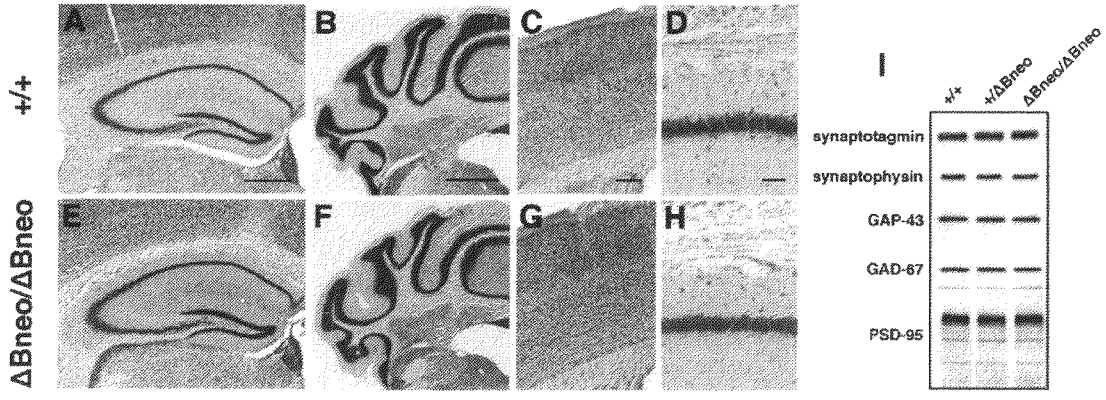


Figure 4. Nissl staining of coronal sections and the expression level of synaptic protein of the brain from wild-type (A, B, C, D) and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice (E, F, G, H). No obviously impairments were detected. (A, E) Nissl staining of the hippocampus at low magnification. (B, F) Cerebellum cortex. (C, G) Layers 1 through 6 in the cerebral cortex. (D, H) Pyramidal cell layer in the hippocampus. (I) Immunoblot analysis of synaptic-marker proteins in wild-type (+/+), heterozygous (+/ΔBneo) and homozygous (ΔBneo/ΔBneo) target mice. Scale bars, (A) 500 μm; (C) 1 mm; (E) 200 μm; (G) 50 μm.

Emotional behavior in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice

We next analysed behavioral function of Pcdh-α using these mutant mice. All behavioral experiments were performed with male mice that were 10 weeks old at the beginning of the behavior tests. Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice had no significant defects of physical characteristics such as body weight, whiskers and fur. No disparity was observed in reaching, reflex, the wire hanging test or rotarod test for motor coordination. As shown in Table 1, they did not show any significant abnormalities in the light/dark transition test, home cage activity; however, we detected a tendency toward anxious or fearful phenotypes with time spent in the center area in the open field test ($P = 0.11$) and the stay time in open arms in the elevated plus maze ($P = 0.13$) in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice, but there was no significant difference from wild-type mice. Pcdh-α B isoform-specific mutants of Pcdh-α$^{\Delta B/\Delta B}$ mice appeared normal in the open field test and the elevated plus maze test. Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice also showed the tendency of changes in the porsolt forced swim test which was used to evaluate depression ($P = 0.05$) and in the hot plate test for pain sensitivity ($P = 0.07$), but not significant.

Table.1 Sensory and motor abilities in Pcdh-α gene-mutated mice.

Pcdh-αΔBneo/ΔBneo mice	+/+	mutants	p-value
Motor Tests			
Wire hang (latency to fall; seconds)	52.3 (±3.0)	52.0 (±3.0)	0.95
Rotarod (latency to fall; seconds)			
Day1	59.8 (±6.4)	55.5 (±6.4)	0.64
Day2	87.0 (±7.2)	89.1 (±7.7)	0.84
Open Field			
Center time (seconds)	11.7 (±2.5)	6.9 (±1.6)	0.11
Distance (cm)	7388 (±562)	6772 (±653)	0.48
Light/dark transition test			
Number of transitions	24.0 (±1.5)	23.6 (±2.1)	0.89
Total time in light (seconds)	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 minutes	29.3 (±3.5)	38.3 (±2.6)	0.05
Immobility (%) 1-10 minutes	42.2 (±4.3)	46.1 (±3.2)	0.46
Pain Test			
Hot Plate (latency; seconds)	11.5 (±1.1)	9.2 (±0.6)	0.07

Data represent the mean (±SEM) (+/+, n=15-17; ΔBneo/ΔBneo, n=16-17).

Pcdh-αΔB/ΔB mice			
Open Field			
Center time (seconds)	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 (±SEM) (+/+, n=16-17; ΔB/ΔB, n=15-16).

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

The cognitive functions of Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice and their sibling were analysed in a contextual and cued fear conditioning test. During the conditioning period, the animals were presented a paired tone and footshock twice as training. In this period, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice showed no difference in freezing (genotype effect, $F_{1,31} = 0.002$, $P = 0.967$) (Fig. 5A). However, when we performed contextual and cued tests 24 h after training, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice showed significant enhancement of freezing in the contextual testing relative to wild-type mice (genotype effect, $F_{1,31} = 5.48$, $P = 0.0258$) (Fig. 5B). On the other hand, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice and their wild-type littermates displayed similar levels of freezing during cued testing (genotype effect, $F_{1,31} = 1.987$, $P = 0.169$) (Fig. 5C). To avoid possible effects of the test sequence in the test battery, we performed the contextual and cued fear conditioning test using another group of mice that had experienced no other behavioral tests. When the littermates received the paired tone and footshock twice, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice also showed greater freezing in the contextual test, but not in the cued test than wild-type mice. These results indicated that Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mutants had enhanced contextual learning.

Next, to confirm whether abnormalities in the contextual fear conditioning test in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice were caused by an decrease of Pcdh-α A isoform proteins or loss of Pcdh-α B isoform proteins, we conducted the contextual and cued fear conditioning test for Pcdh-α$^{\Delta B/\Delta B}$ mice that specifically lacked Pcdh-α B isoform proteins. Pcdh-α$^{\Delta B/\Delta B}$ mice and their wild-type littermates were presented with a pair of tone and footshock twice in the conditioning phase. After 24 h, contextual and cued tests were conducted. Interestingly, Pcdh-α$^{\Delta B/\Delta B}$ mice showed no significant difference in the percentage of freezing in the contextual test compared to wild-type littermates (genotype effect, $F_{1,31} = 0.054$, $P = 0.818$) (Fig. 5E). 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. 5D and F). These findings indicated that enhanced freezing in the altered contextual fear conditioning test displayed in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice was due to the down-regulation of Pcdh-α A isoform proteins.

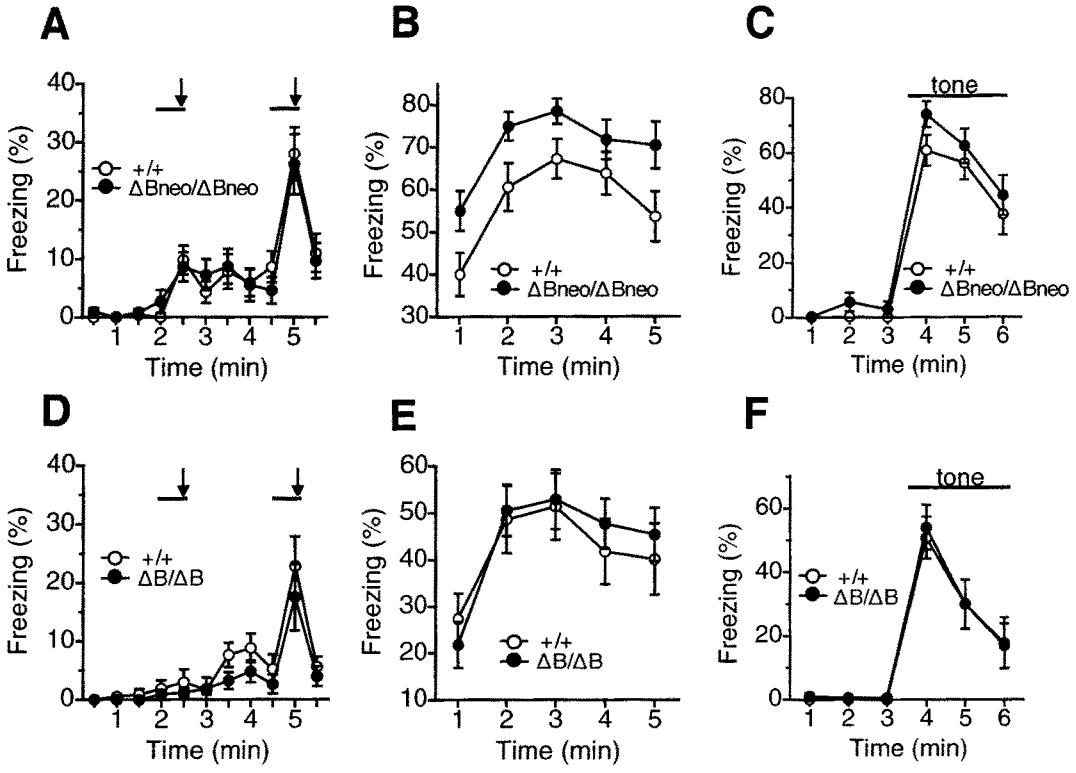


Figure 5. Contextual and cued-fear conditioning in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice (A, B, C) and Pcdh-$\alpha^{\Delta B/\Delta B}$ mice (D, E, F) against wild-type mice (+/+). (A) Percentage of freezing during the conditioning phase. A tone was sounded for 30 sec (bars) followed by a 2-sec footshock (arrows). (B) Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed enhanced freezing during contextual testing conducted 24 h after conditioning (P = 0.026). (C) Freezing during the tone-cued testing 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-$\alpha^{\Delta B/\Delta B}$ mice. (E) Freezing during context testing. No significant difference was observed between 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. There was no significant difference between genotypes. Data are given as the means (±SEM) (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 16; Pcdh-$\alpha^{\Delta B/\Delta B}$ mice, n = 16; wild-type, n = 17 respectively).

Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice in the Morris water maze

To examine spatial learning defects of Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice, we tested littermates of Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ and wild-type mice in the Morris water maze (hidden platform version). Mice were given four trials per day for nine consecutive days of the training and probe tests one day after training. In this test, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice generally showed no significant cognitive impairment. Escape latency, which was the time to reach the platform, was similar between genotypes during training (genotype effect, $F_{1,29} = 0.174$, $P = 0.679$) (Fig. 6A). There was also no abnormality either in swimming speed or in time spent at the perimeter of the pool (Fig. 6B and C) ($P > 0.05$).

During the probe test, in which the learning platform was removed, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice spent more time in the training quadrant compared to other quadrants, similar to wild-type littermates (Fig. 6E). In addition, we found no differences in the number of crossings above the place where the platform was located ($P = 0.541$) (Fig. 6D) and both genotypes spent significantly more time in the training quadrant than in other quadrants in the probe test conducted after reversal training one week after the original training (Fig. 6F). This indicated that Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice had no abnormalities in Morris water maze learning.

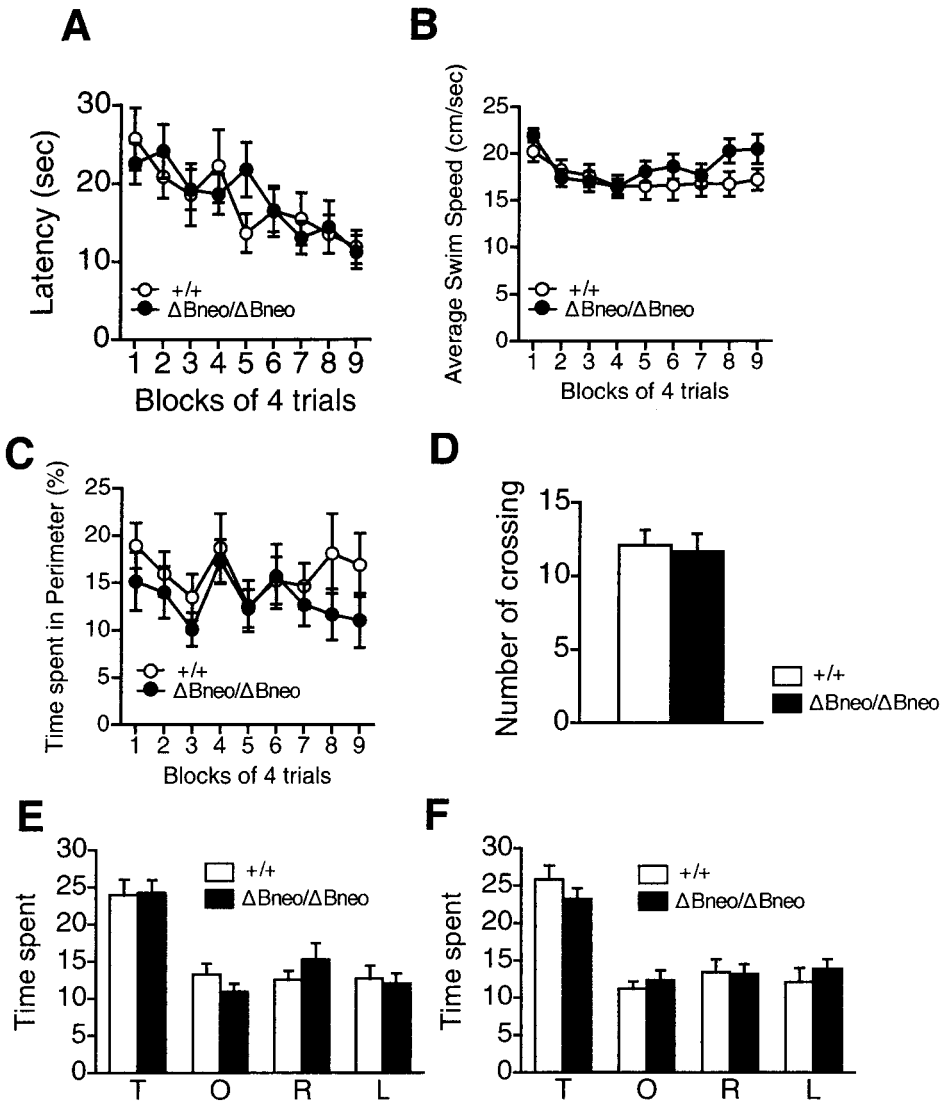


Figure 6. Intact performance of Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice in the Morris water maze. (A) Escape latency, (B) swimming speed, (C) time spent at the perimeter of the pool. There were no significant differences between the two genotypes. (D) Both genotypes also crossed the training site more often than the equivalent sites in the other three quadrants in the probe trials performed after training. (E) In probe trials, Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ 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 1 week after the first probe trials. Both genotypes similarly spent more time in the training quadrant compared with the other quadrants. Data are given as the means (± SEM) (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 16; wild-type, n = 15).

Disparities of Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice in the eight-arm radial maze test

Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice and wild-type littermates were also analysed in the eight-arm radial maze test to examine spatial working memory. In this task, the number of working memory errors of mice revisiting arms that had been visited previously, which is generally interpreted as a measure of working memory performance, was measured (Olton *et al.*, 1979a, b). The number of working memory errors in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice was significantly low during 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 of working memory errors during the delay period, 30 sec in the 15th trial, and 2 min in the 16th to 18th trials, was observed (genotype effect, $F_{1,23} = 2.789$, $P = 0.109$) (Fig. 7A). The amount of total food intake during a trial was significantly less in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice than in 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 delay period (15th to 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. 7B). However, the number of different arms chosen within the first eight choices was not significantly different ($P > 0.05$) (Fig. 7C). There were no significant differences in the distance traveled (Fig. 7D) and the latency to take all 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. 7E), suggesting that the increased number of revisiting errors displayed during trials without delay was unlikely to be caused by reduced motivation to take the pellets. Thus, Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice showed a decreased number of working memory errors in the eight-arm radial maze. Consistent with locomotor activity measured in several tests such as the open field test, light/dark transition and home cage activity (Table 1) there was no distinction in the distance traveled. Taken together, these findings suggested that the hypomorphic Pcdh-α protein level was associated with abnormal memory formation.

We also performed the eight-arm radial maze test against Pcdh-α$^{\Delta B/\Delta B}$ mice and their wild-type littermates. There were no significant irregularities between genotypes in the number of working memory errors, the amount of total food intake, the number of different arms chosen within the first eight choices, the distance traveled and the

number of omission error trials ($P > 0.05$) (Fig. 8). These results indicated that the down-regulation of Pcdh-α A isoform proteins also cause for disparities of spatial working memory.

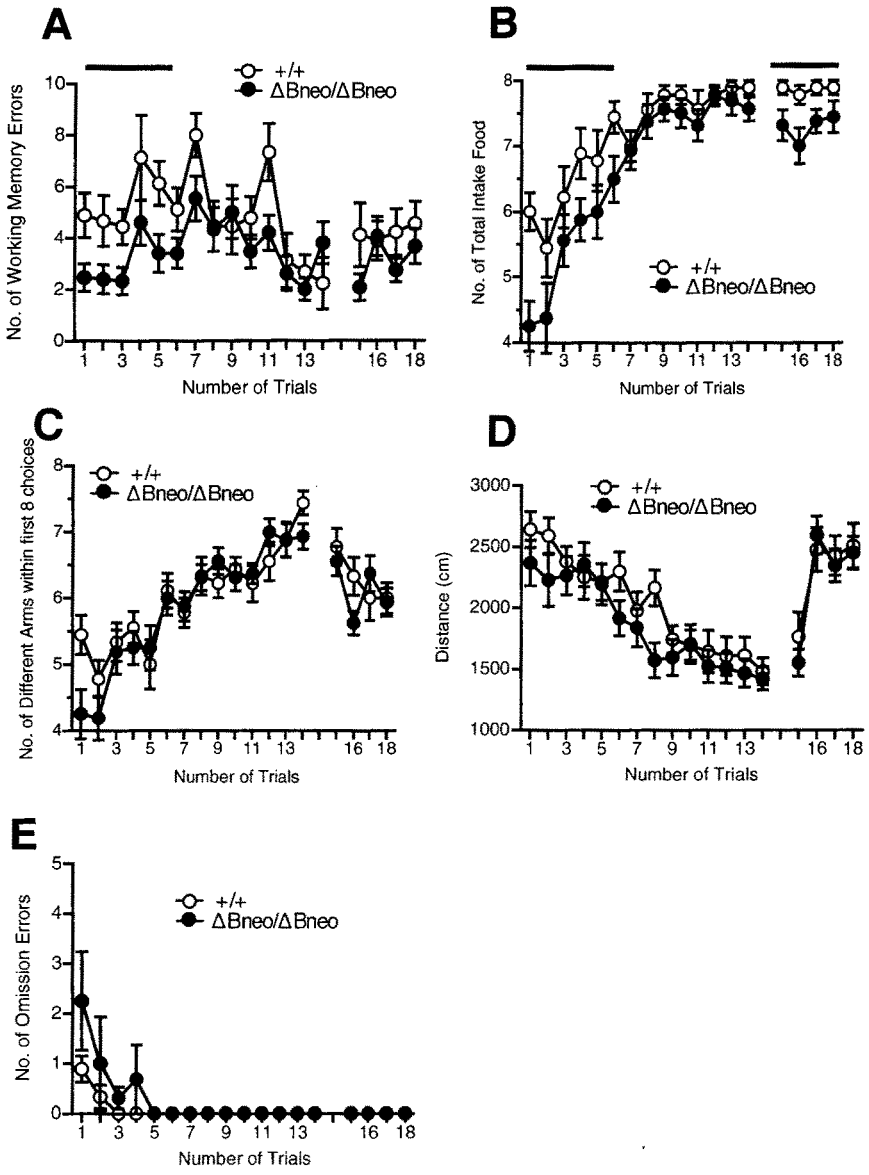


Figure 7. Eight-arm radial maze test of wild-type (+/+) and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice. Mice were given 14 trials and 4 delay trials (30 sec for the 15th trial and 120 sec for the 16th to 18th trials). In delay trials, mice were confined to the center platform for each delay time after taking the fourth pellet. (A) The number of working memory errors. A significant genotype effect was observed during the 1st to 6^{h} days (P = 0.035). (B) Amount of total food intake. A significant genotype effect was observed during the first 6 trials (P = 0.044) and delay trials (15th to 18th trials) (P = 0.048). (C) The number of different arms chosen within the first eight choices. No significant genotype effects were observed (P > 0.05). (D) Total distance during the trials. There were no significant differences between genotypes. (E) Number of omission errors. Data are given as the means (±SEM) (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 15; wild-type, n = 9).

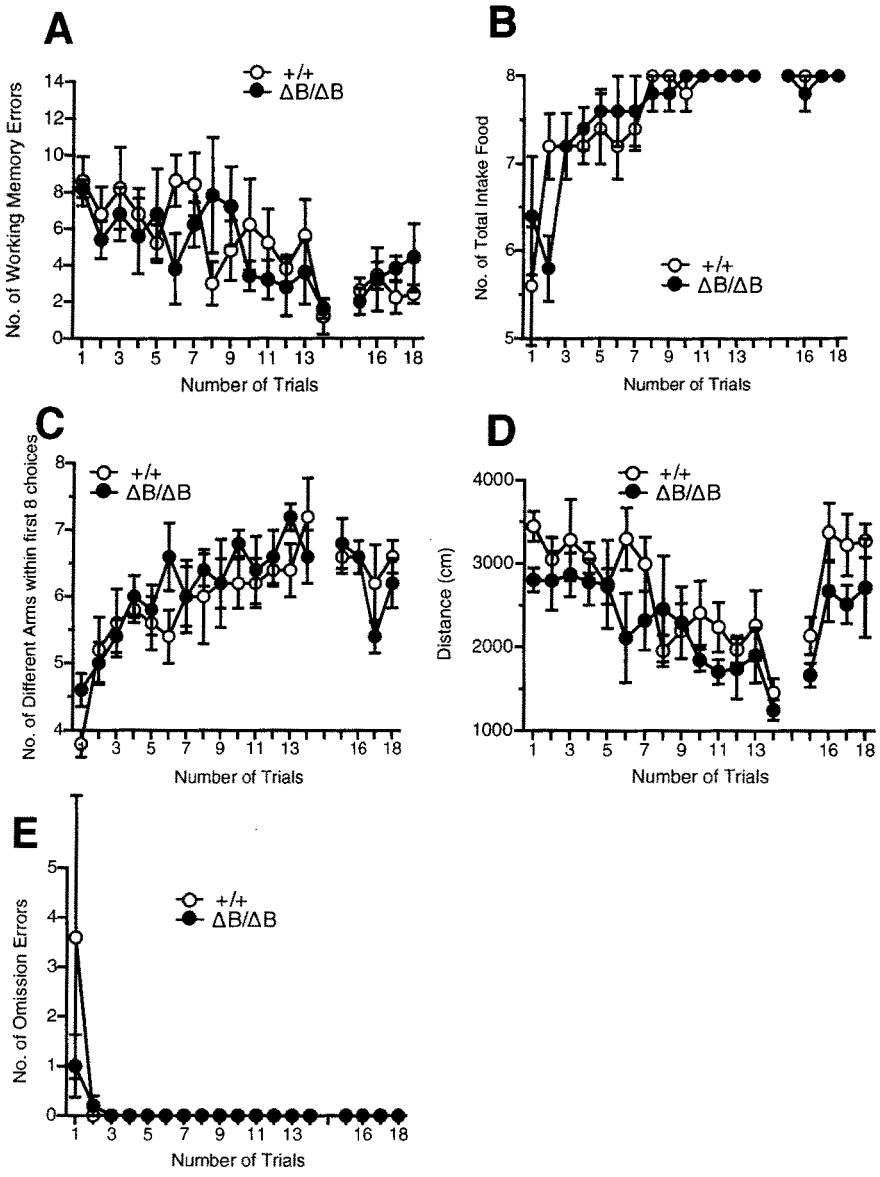


Figure 8. Eight-arm radial maze test of wild-type (+/+) and Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (A) The number of working memory errors in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (B) The amount of total food intake in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (C) The number of different arms chosen within the first eight choices was not significantly different. (D) Total distance during the trials. There were no significant differences between genotypes. (E) The number of omission errors was not different. Data are given as the means (±SEM). (Pcdh-$\alpha^{\Delta Bo/\Delta B}$ mice, n = 5; wild-type, n = 5).

Prepulse inhibition (PPI) impaired in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice

Prepulse inhibition (PPI) of the acoustic startle response (ASR) is a measure of sensorimotor gating and concerns an attenuation of the startle response by a week stimulus presented a short time before the startle stimulus. ASR is an unconditioned reflexive response to a sudden acoustic stimulus and the primary circuits mediating PPI is a relatively simple pathway located in the lower brainstem (Davis *et al.*, 1982; Fendt *et al.*, 2001). To investigate a possible role of sensorimotor gating, PPI was preformed in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice and their wild-type littermates. The ASR induced by 110-, 120- dB acoustic stimuli were indistinguishable between mutants and wild-type mice (110-dB, P = 0.39; 120-dB, P = 0.29) (Fig. 9A). Next, PPI of ASR was analysed. Non-startling week stimuli were presented 100 ms before the 110- or 120-dB startle stimuli. The percentage of PPI, represented the degree of suppression of ASR by 74-, 78- and 82-dB prepulse stimuli, were significant decreased in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice than wild-type littermates (Fig. 9B). The difference of the percentage of PPI between genotypes was greater when the startle amplitude was 110-dB than 120-dB. This distinction of degree of response between 110-dB and 120-dB probably because of a ceiling effect was induced by massive intensity of the startle stimulus. These results indicated decrease of the sensorimoter gating in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice.

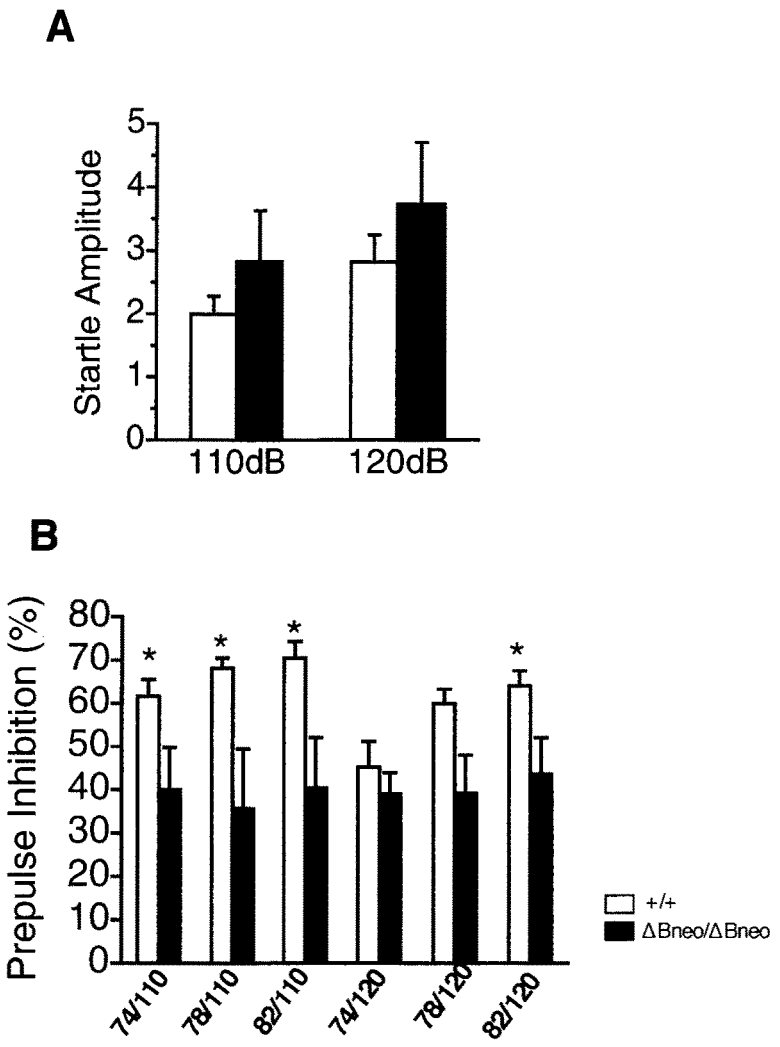


Figure 9. Prepulse inhibition of acoustic startle response of wild-type (+/+) and Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice. (A) Startle responses to stimuli 110- and 120-dB sound. There was no significant difference between genotypes. (B) Prepulse inhibition of acoustic startle response. Three different prepulse stimuli (74-, 78-, 82-dB) were presented 100 ms before the 110- or 120-dB startle stimuli. Data are given as the means (±SEM) (*, $P < 0.05$, Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 16; wild-type, n = 17).

5-HT level in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice

Anxiety or behavioral performance is probably associated with alternate monoaminergic transmission in the forebrain such as the frontal cortex or hippocampus. Previous studies have shown that abnormal freezing in the contextual fear conditioning test was related to the amount of monoaminergic transmission (Inoue *et al.*, 1994; Kim *et al.*, 1997). To evaluate abnormal monoaminergic transmission in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice, detailed neurochemical analysis was performed. In the frontal cortex, no obvious differences were found in noradrenaline (NA), 5-hydroxyindoleacetic acid (5-HIAA, serotonergic metabolites), 5-HT, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC, dopamine metabolites), and homovanillic acid (HVA, dopamine metabolites) amounts in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice (Fig. 10B). In the hippocampus, however, 5-HT level was significantly increased in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice ($P = 0.0001$) (Fig. 10A).

On the other hand, significant increase of 5-HT levels could not be found in Pcdh-α B-specific mutants of Pcdh-α$^{\Delta B/\Delta B}$ mice in the hippocampus (Fig. 10C) and in the frontal cortex (Fig. 10D). NA and 5-HIAA levels were also not different in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice.These results indicated that the 5-HT level in the hippocampus was associated with the hypomorphic Pcdh-α A isoform.

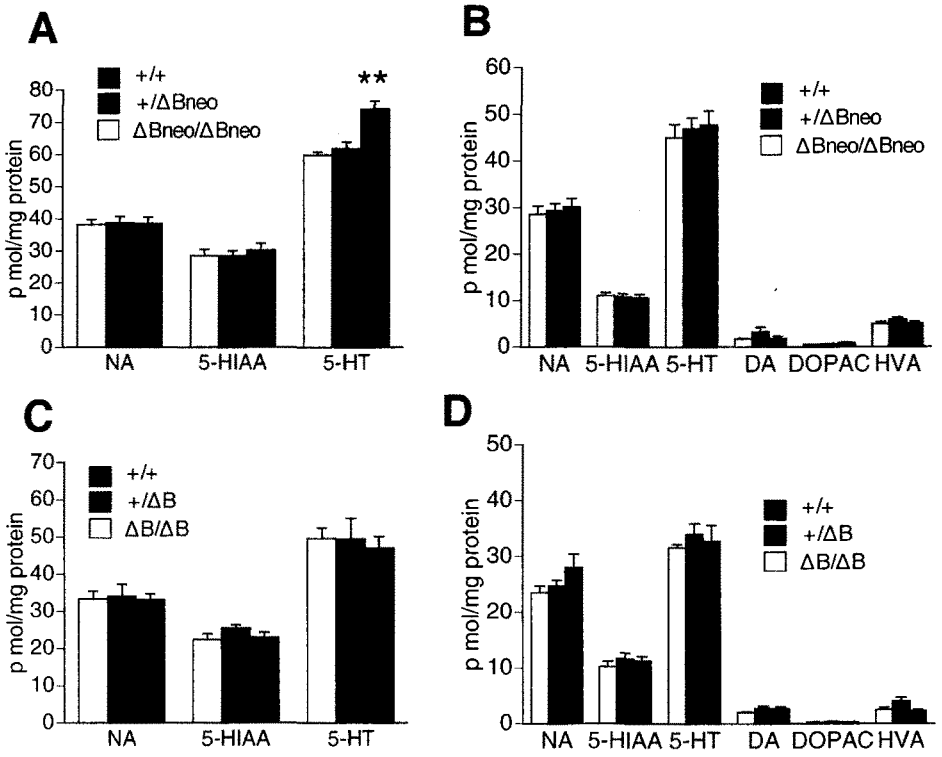


Figure 10. Monoamines in wild-type (+/+, white bar), heterozygous (+/ΔBneo, +/ΔB, gray bar) and homozygous (ΔBneo/ΔBneo, ΔB/ΔB black bar) mice. The amount of monoamines was measured by HPLC assay. (A) Monoamine level in the hippocampus. 5-HT was significantly increased in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice (**, P = 0.0001). (B) There were no significant differences in the monoamine level in the frontal cortex for genotypes. (C) Monoamine level in the hippocampus in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. (D) In the frontal cortex in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice. NA, noradrenaline; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.

Reduced hippocampal synaptic plasticity in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice

Because alternations in the strength of synaptic connection between hippocampal neurons are assumed to underlie contextual and spatial memories, we investigate synaptic plasticity in the hippocampal Schaffer collateral synapses (SC-CA1). There was no significant difference on paired-pulse facilitation (PPF), which is sensitive to presynaptic release probability, between the wild-type and mutant mice ($P > 0.05$) (Fig. 11A). Post-tetanic potentiation (PTP) was also indistinguishable in magnitude between genotypes ($P > 0.05$) (Fig. 11B).

However, LTP induced by a strong tetanic stimulation (100Hz, 1s) was significantly smaller in Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice (143.8±1.8 % of baseline at 50-60 min after application of tetanus, $n = 9$) than in wild-type mice (163.4±3.3 % of baseline, $n = 10$, t-test, $P < 0.01$) (Fig. 11C). Initial EPSP slopes were normalized in each experiment to the averaged slope value during the control period (-30 to 0 min). Representative traces (average of 10 consecutive responses) in the inset were EPSPs obtained at the times indicated by the numbers in the graph. Thus, down-regulation of Pcdh-α proteins significantly altered the synaptic plasticity in the SC-CA1 pathway.

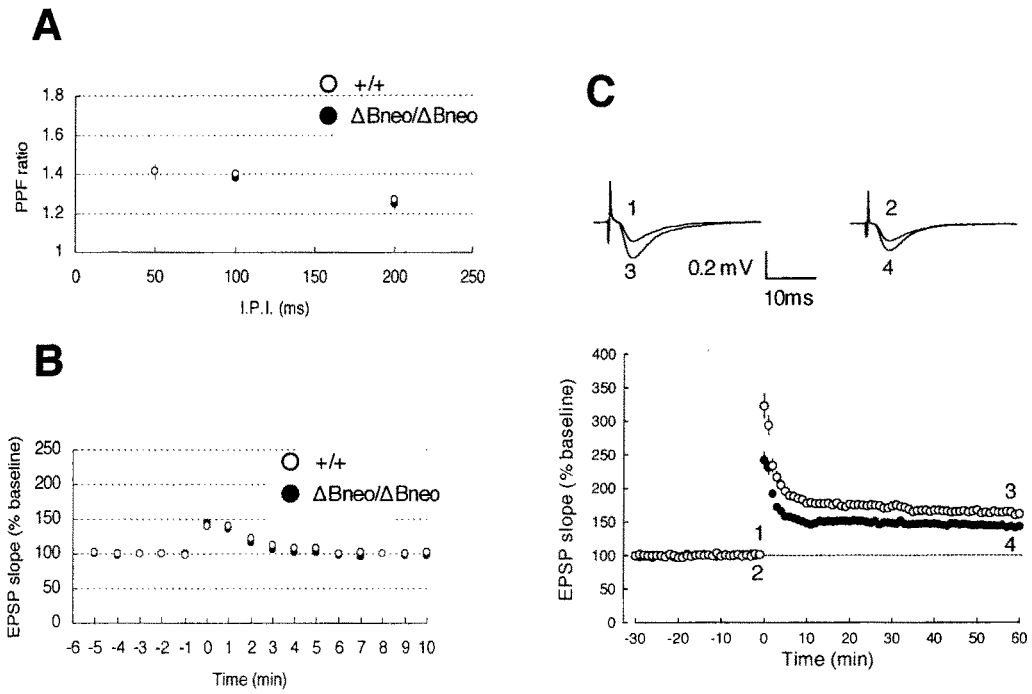


Figure 11. Functional properties of hippocampal CA1 synapses. (A) Paired-pulse facilitation induced by stimulating afferent fibers twice at intervals of 50, 100, 200 ms was not indistinguishable between wild-type and mutants mice (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 9; wild-type, n = 10). (B) Post-tetanic potentiation was not different between genotypes (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 13; wild-type, n = 11). (C) The averaged time course of LTP for 60 min after tetanic stimulation in wild-type and mutant mice (Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, n = 9; wild-type, n = 10). At time 0, tetanic stimulation (100Hz, 1s) was delivered to the Schaffer collateral-commissural pathway. LTP in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice is significantly smaller than that in wild-type mice (P < 0.01).

Discussion

In this study, we reported abnormal phenotypes, which were fear-related learning, working memory, sensorimotor gating and 5-HT level in the hippocampus in Pcdh-α gene-converting mice. However, Pcdh-$\alpha^{\Delta B/\Delta B}$ mutants, which recovered the protein level of Pcdh-α A, but still lacked Pcdh-α B isoforms, normalized these learning abnormalities and the hippocampal 5-HT level. These results suggested that Pcdh-α A isoform proteins could regulate normal contextual learning, working memory and the hippocampal 5-HT level.

In fear conditioning learning tests, hypomorphic Pcdh-α A mutants had significant enhanced abnormalities only in contextual fear learning but not in tone cued fear learning. Pavlovian fear conditioning of contextual cues requires the normal functioning of both the hippocampus and the amygdala, whereas conditioning to tones requires normal functioning of the amygdala, but not the hippocampus (Kim & Fanselow, 1992). These results suggested that down-regulation of Pcdh-α A proteins to approximately 20% influenced hippocampus-dependent normal functions. Moreover, it is known that working memory in the radial maze task and spatial learning of the Morris water maze task are also largely dependent on hippocampal function. Here we also found abnormalities in the working memory of Pcdh-α A hypomorphic mutants, suggesting that their differences in working memory are derived from their hippocampal defects. However, Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice showed normal in spatial learning in the Morris water maze task, which is also dependent on hippocampal function. It is possible that enhanced learning ability might hardly be detectable in the Morris water maze task compared to the radial maze task and fear conditioning test. Indeed, a previous study presented that Telencephalin-deficient mice showed enhanced performance in the radial maze task but not the Morris water maze task (Nakamura *et al.*, 2001).

The Pcdh-$\alpha^{\Delta Bneo/\Delta ABeo}$ mice also showed significantly reduced PPI of ASR, which is an operational sensorimotor gating. Abnormal sensory inhibition may reflect a deficit in processing incoming sensory information. The prosposed primary circuit of PPI is the pathway through inferior colliculus, superior colliculus and pedunculopontine tegmental nucleus (Fendt *et al.*, 2001). Thus, Pcdh-α proteins would affect the regulation of sensorimotor gating through these neuronal processes. Impairment of

sensorimotor gating is characteristic of some neurophychiatric disorders such as schizophrenia with attentional abnormalities (Braff & Geyer *et al.*, 1990). PPI deficits reversed by antipsychotic drugs, such as olanzapine, clozapine or haloperidol like in schizophrenia patients. Our preliminary data from pharmacological study, Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice pretreated with haloperidol, a D2 receptor antagonist, did not display significant deficits in PPI compared to wild-type mice. Studies of serotonin gene-targeting mice suggested the 5-HT release or 5-HT-dopamine balance were reasoned for abnormal sensorimotor gating (Dulawa *et al.*, 2000; Ojima *et al.*, 2004; Tanaka *et al.*, 2006). It is possible that the abnormal PPI in Pcdh-$\alpha^{\Delta Bneo/\Delta ABeo}$ mice could be caused by 5-HT release.

In the hippocampus, the level of 5-HT was enhanced in Pcdh-α A hypomorphic mutants, whereas NA was not changed. Interestingly, in the frontal cortex, NA and 5-HT tended to enhance, but not significantly in Pcdh-α A hypomorphic Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice. On the other hand, 5-HT level was not changed in Pcdh-$\alpha^{\Delta B/\Delta B}$ mice, lacked only Pcdh-α B isoforms both in the hippocampus and the frontal cortex. We found that *Pcdh-α* mRNAs were extensively expressed in the raphe nucleus where 5-HT neurons are concentrated. The mechanism by which Pcdh-α A proteins could increase the 5-HT level in the hippocampus remains to be determined. It is possible that Pcdh-α A proteins play roles in normally generating serotonergic projections to the hippocampus from the raphe nucleus. 5-HT is involved in many physiological functions in the brain, including learning and memory formation in the hippocampus (Buhot *et al.*, 2003). Here the hypomorphic Pcdh-α A mutants had an increased level of 5-HT in the hippocampus together with abnormalities of contextual fear learning and working memory. These results suggested that increasing the 5-HT level of the hippocampus in mutants might be caused their learning abnormalities.

A previous study showed that Pcdh-γ null mice died soon after birth because of increasing neuronal cell death and decreasing synaptic formation (Wang *et al.*, 2002). The deletion of the Pcdh-γ locus in mice induced neuronal degeneration in various areas of the brain and spinal cord; therefore, the roles of Pcdh molecules in regulating brain function *in vivo* have not been examined. Here, two types of Pcdh-α hypomorphic mice, with down-regulated and truncated Pcdh-α proteins, were born normally. Survival of the mutant mice provided significant information about the molecular function to regulate behavior. Pcdh-α A isoforms and full-length Pcdh-γ

proteins can make a protein complex on the membrane surface, and also colocalized partially in the molecular layers of the hippocampus where synapses are enriched (Murata *et al.*, 2004). Therefore, diverse Pcdh molecules, including Pcdh-γ, which makes a protein complex and regulates membrane translocation with Pcdh-α proteins, possibly play significant roles in generating synapses, and learning and memory formation in the brain.

Pcdh-α proteins can bind to integrins via their RGD motif conserved in the first cadherin domain among Pcdh-α proteins (Mutoh *et al*, 2004; Morishita *et al*, 2006). Integrins play significant roles in regulating synaptic plasticity in the CA1 synapse of the hippocampus (Kramar *et al*, 2006; Huang *et al*, 2006). We also found that down-regulate of Pcdh-α proteins resulted in reduce of LTP at the hippocampal CA1 synapses. Reduce of LTP was not associated with any irregularities in the depolarization envelope during titanic stimulation. The extents of PPF and PTP were not comparable between the wild-type and mutant mice. There is a possibility that interaction between Pcdh-α proteins and intergins might regulate synaptic plasticity in the hippocampus. It was also previously reported that specific cytoplasmic tails of Pcdh-α A and Pcdh-α B associate with neurofilament M (NFM) and fascin, respectively (Triana-Baltzer & Blank, 2006). NFM could be associated with Pcdh-α A isoforms, playing a structural and scaffolding role in neurons. Moreover, because NFM is likely to make a protein complex with N-methyl-D-aspartate (NMDA) receptors, which play crucial roles in regulating synaptic plasticity, and learning and memory in hippocampus, and dopamine receptors (Ehlers *et al.*, 1998; Kim *et al.*, 2002). Taken together, it is possible that Pcdh-α A isoforms specifically regulating synaptic plasticity in the hippocampus associate with neurofilaments and NMDA receptors (Fig. 17B); however, further analysis will be required to assess the role of Pcdh-α A proteins in the regulation of the brain.

In the human *Pcdh* gene cluster, many single-nucleotide polymorphisms (SNPs), several haplotypes, and deletion alleles are detected (Noonan *et al.*, 2003; Kirov *et al.*, 2003; Miki *et al.*, 2005). Here we showed that hypomorphs of Pcdh-α proteins induced defects of learning and memory formation in mice. Human germline polymorphisms in *Pcdh* family genes might provide genetic variation of brain functions and neurological disorders.

In this study we report for the first time that Pcdh-α gene-converting mice showed

behavioral abnormalities of fear-related contextual learning and working memory and sensorimotor gating. Moreover, the cause of fear related learning and working memory was depended on Pcdh-α A isoform proteins but not Pcdh-α B isoforms. It will be important to reveal the molecular function of diverse Pcdh molecules, including splicing variants and Pcdh-β and -γ, to regulate synapse function and generate functional neural networks in the brain.

CHAPTER 2

Summary

The clustered protocadherins (Pcdhs) family is expressed in the nervous system and constitutes the largest subgroup of the cadherin superfamily. Pcdhs proteins are located neurons during the neurodevelopment and partially at the synapses. The localization patterns of the Pcdhs have suggested the hypothesis that the diversity of the Pcdhs potentially contributes to the neuronal recognition. Little is known, however, about the roles of the Pcdhs. To address the molecular roles of Pcdhs in regulating behavior more in detail, we generated mutant mice, deleted the constant region exons, and examined their behavioral abnormalities. These mutant mice, in which Pcdh-α proteins were deficiency or substantial decrease, displayed enhancement of freezing of contextual learning. Moreover, these mice also showed significant enhanced freezing of cued learning. We also found that these mice showed a reduced of Prepulse inhibition of the acoustic startle response and increased the amount of 5-hydroxytryptamine (5-HT) in the hippocampus. Furthermore, the number of c-Fos-positive cells had tendency to increase in the septum after contextual learning. These results suggested that Pcdh-α family played roles for learning and memory, sensorimotor gating, the amount of 5-HT in the hippocampus and the neural activity involved with behavior.

Introduction

In CHAPTER 1, we showed abnormal phenotypes, which were contextual learning, working memory, sensorimotor gating and 5-HT level in the hippocampus in Pcdh-α gene-converting mice. These abnormalities, except for sensorimotor gating were found in hypomorphic Pcdh-α A mice, which were down-regulated to approximately 20% Pcdh-α A proteins. However, Pcdh-$\alpha^{\Delta B/\Delta B}$ mutants, in which recovered the protein level of Pcdh-α A, but still lacked Pcdh-α B isoforms, normalized these abnormalities and the hippocampal 5-HT level. Thus, we concluded that the Pcdh-α A isoforms had significant roles for regulating learning and memory function, sensorimoter gating and the amount of 5-HT in the hippocampus; however, the neural circuits involved with abnormal behavior have not been cleared.

In CHAPTER 2, we approached the roles of Pcdh-α proteins underlying abnormal behavior more in detail. To prevent the residual 20% Pcdh-α A isoforms in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice mask the subtle abnormal phenotype, we used the mutant mice, in which deleted the constant region exons. These mutant mice showed also enhanced freezing in the contextual test. In addition, they were displayed enhancement freezing in the cued test. These mice also showed reduced Prepulse inhibition (PPI), and increased 5-HT level in the hippocampus. Moreover, the number of c-Fos-positive cells in the septum increased after contextual test in the fear conditioning test.

Materials and Methods

Antibodies

In the present study, we used anti-CNR/Pcdhα (rabbit polyclonal antibody) as a Pcdh-α A isoform-specific antibody (Murata *et al.*, 2004). Anti-CNRN (rabbit polyclonal antibody) and anti-EC1 (rabbit polyclonal antibody) against 104 amino acids (from G27 to N131) of the Pcdha-a4 protein were used for immunoprecipitation. Anti-c-fos rabbit polyclonal antibody (SC-52; Santa Cruz Biotechnology) were perchased.

Immunoblotting and Immunoprecipitation

The procedures were described in CHAPTER 1.

Animal and behavioral experiments

All tests were performed with male mice that were 10 weeks old at the start of testing. Mice were housed four per cage in a room with a 12 h light/dark cycle with *ad libitum* access to food and water.

Contextual and cued fear conditioning

The procedures were described in CHAPTER 1.

Morris water maze

The procedures were described in CHAPTER 1.

Acoustic startle response/Prepulse inhibition

The procedures were described in CHAPTER 1.

Image analysis

All applications used for behavioral studies were performed on a Macintosh computer, using Image WM, and Image FZ (O'Hara & Co., Tokyo, Japan). 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 were modified by Tsuyoshi Miyakawa (available through O'Hara & Co., Tokyo, Japan).

Measurement of monoamines by HPLC

The procedures were described in CHAPTER 1.

Immunohistochemistry

Mouse brains were rapidly removed and immersed in OCT compound (Miles) just 1 h after training, contextual and cued test in the fear conditioning test. The tissues were quickly frozen in isopentane cooled with dry ice. Fresh-frozen sections were then cut on a cryostat (Leica CM3050, Germany) and thaw-mounted on slides. The 10 μm sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) for 10 min. The fixed sections were processed for immunohistochemistry with anti-c-Fos antybody and biotin-labeled anti-rabbit IgG secondary antibody. c-Fos positive nuclei were counted manually blinded to the mouse genotypes.

Statistical analysis

Statistical analysis was conducted using StatView (SAS Institute, Cary, NC). Data were analysed by *t*-test, one-way ANOVA, or repeated measures ANOVA. Values in graphs are expressed as the mean ± SEM.

Results

Performance in the contextual and cued fear conditioning test

To confirm and investigate more detail roles of Pcdh-α proteins *in vivo*, we used Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice in which deleted the constant region exons (CR1, CR2 and CR3a). Because of the lack of constant region exons, Pcdh-α proteins were deficiency or substantial decrease in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice were born normally and healthy, and they were fertile. No apparent abnormalities were detected during development or adulthood. Then we performed behavioral tests against Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice and their wild-type littermates.

We performed the contextual and cued fear conditioning test to Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. Pcdh-$\alpha^{\Delta CR/\Delta CR}$ and their wild-type littermates were presented with two pairs of tone and footshock as training. During the conditioning period, Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice were presented no difference in freezing compared with wild-type mice (Fig. 12A). The contextual test was conducted 24 h after training. In this period, Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice displayed tendency to enhance freezing during 1 to 2 minutes (P = 0.09) but not significant (Fig. 12B). However, unlike Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ or Pcdh-$\alpha^{\Delta Aneo/\Delta Aneo}$ mice, Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice showed significant impairment in the cued test (P = 0.009) relative to wild-type mice (Fig. 12C). These results indicated that Pcdh-α proteins effect on enhanced freezing both contextual and cued test.

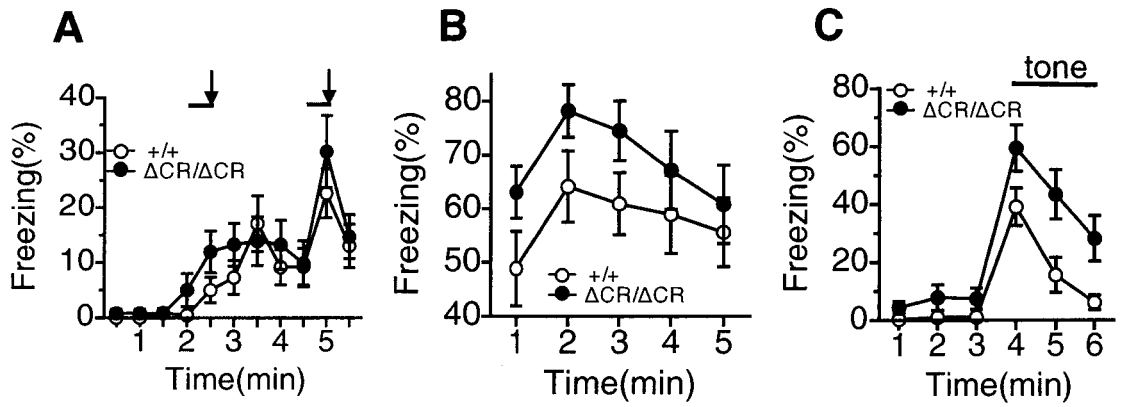


Figure 12. Contextual and cued-fear conditioning in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. (A) Percentage of freezing during training phase in wild-type (+/+) and Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. A tone was sounded for 30 sec (bars) followed by a 2-sec footshock (arrows). (B) Percentage of freezing during contextual testing conducted 24 h after conditioning. (C) Freezing during the tone-cued testing was significantly enhanced in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice (P = 0.009). Data are given as the means (± SEM). (Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, n = 16; wild-type mice; n = 17).

Pcdh-α$^{\Delta CR/\Delta CR}$ mice in the Morris water maze

We tested Pcdh-α$^{\Delta CR/\Delta CR}$ and wild-type mice in the Morris water maze. Mice were given four trials per day for nine consecutive days of the training and probe tests one day after training. Pcdh-α$^{\Delta CR/\Delta CR}$ mice displayed no significant difference similar to Pcdh-α$^{\Delta Bneo/\Delta Bneo}$ mice in the Morris water maze. Escape latency, which was the time to reach the platform, was no difference between two genotypes during training ($P > 0.05$) (Fig. 13A). There was also no abnormality either in swimming speed or in time spent at the perimeter of the pool (Fig. 13B and C) ($P > 0.05$). During the probe test, in which the platform was removed, Pcdh-α$^{\Delta CR/\Delta CR}$ mice spent more time in the training quadrant compared to other quadrants (Fig. 13E). We found tendency to increase the number of crossings above the place where the platform was located, but not significant ($P = 0.24$) (Fig. 13D). Pcdh-α$^{\Delta CR/\Delta CR}$ mice also spent significantly more time in the training quadrant than in other quadrants in the probe test conducted after reversal training one week after the original training (Fig. 13F). These results indicated that Pcdh-α$^{\Delta CR/\Delta CR}$ mice had no abnormalities in Morris water maze.

Prepulse inhibition (PPI) impaired in Pcdh-α$^{\Delta CR/\Delta CR}$ mice

To evaluate abnormal sensorimoter gating in Pcdh-α$^{\Delta CR/\Delta CR}$ mice, PPI was also measured in Pcdh-α$^{\Delta CR/\Delta CR}$ and their wild-type littermate controls. Mice were received the ASR induced by 110-, 120-dB acoustic stimuli. Pcdh-α$^{\Delta CR/\Delta CR}$ mice tended to enhance the startle amplitude compared to wild-type mice at 120-dB acoustic stimuli ($P = 0.1$) (Fig. 14A). The percentage of PPI, represented the degree of suppression of ASR by 74-, 78- and 82-dB prepulse stimuli, were significant decreased in Pcdh-α$^{\Delta CR/\Delta CR}$ mice than wild-type littermates (Fig. 14B). To validate the possible roles of Pcdh-α$^{\Delta CR/\Delta CR}$ mice in PPI, we measured PPI using another group of mice. In this group, Pcdh-α$^{\Delta CR/\Delta CR}$ mice displayed higher levels of ASR than wild-type mice ($P = 0.003$, 110-dB; $P = 0.0264$, 120-dB, data not shown). Pcdh-α$^{\Delta CR/\Delta CR}$ mice also showed greater diminished PPI than wild-type mice. These results indicated that Pcdh-α proteins were relevant to the sensorimoter gating.

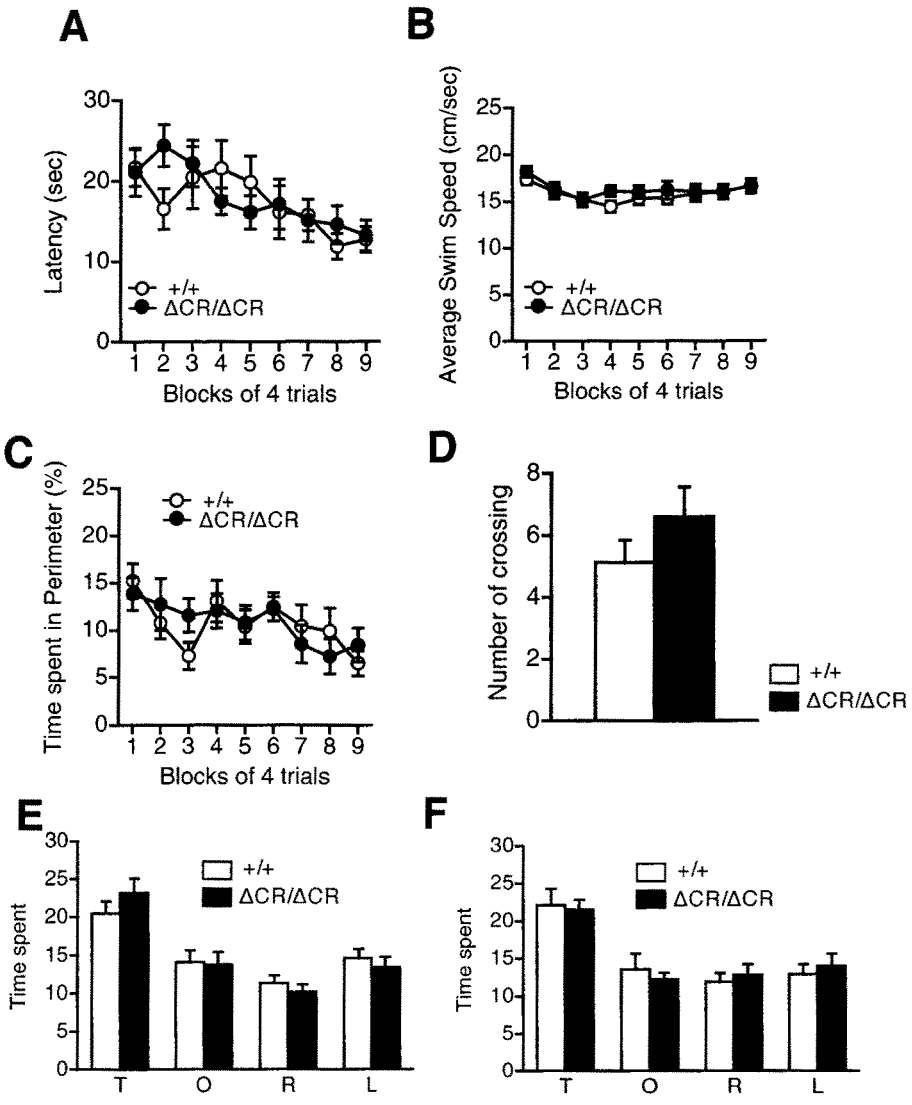


Figure 13. Performance of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice in the Morris water maze. (A) Escape latency, (B) swimming speed, (C) time spent at the perimeter of the pool. There were no significant differences between genotypes. (D) The number of crossing the training site. Both genotypes also crossed more often than the equivalent sites in the other three quadrants in the probe trials. (E) Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice and their wild-type (+/+) littermates searched for where the platform had been located in probe trials (O, opposite quadrant; R, right quadrant; L, light quadrant). (F) Probe trials were conducted again 1 week after the first probe trials. Data are given as the means (± SEM) (Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, n = 17; wild-type, n = 16).

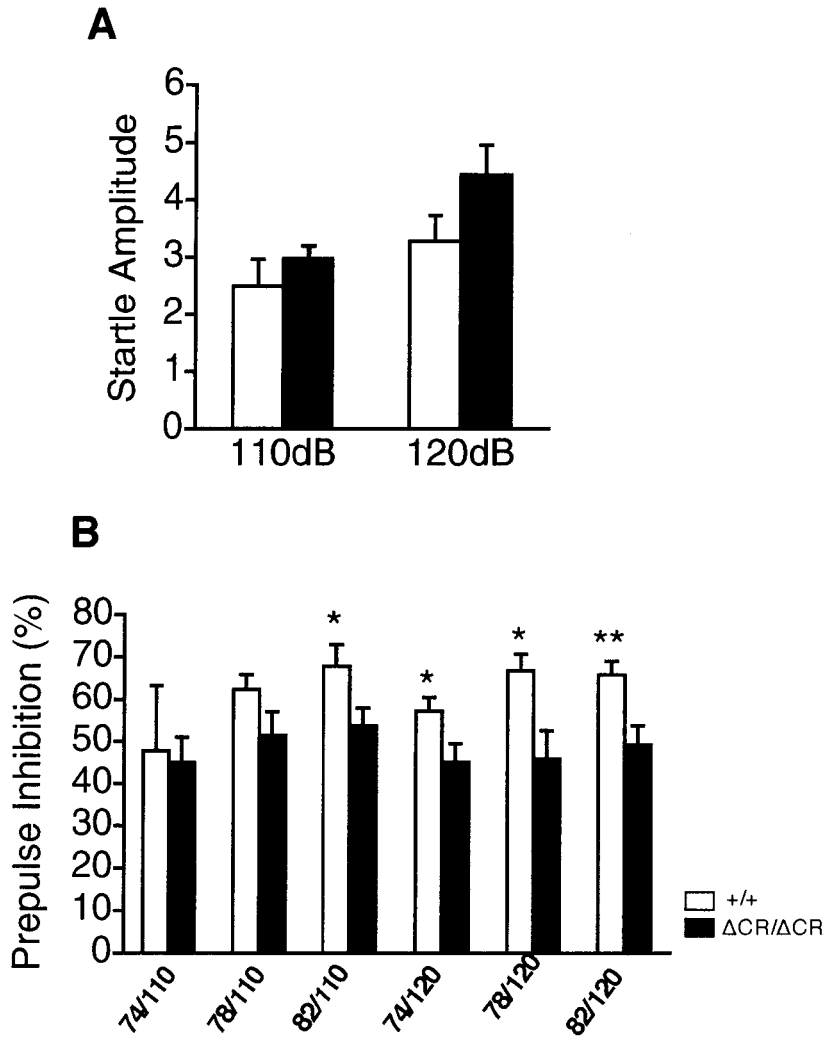


Figure 14. Prepulse inhibition of acoustic startle response of wild-type (+/+) and Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. (A) Acoustic startle responses to stimuli 110- and 120-dB sound. (B) PPI of acoustic startle response. PPI was lower in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice than in wild-type mice. Data are given as the means (±SEM) (*, $P < 0.05$, **, $P < 0.01$, Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, $n = 19$; wild-type, $n = 18$).

Analysis of monoamine level in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice

The effect of Pcdh-α deficiency on monoaminergic transmission was determined by HPLC. In the hippocampus, 5-HT level was significantly increased in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice compared with wild-type mice ($P < 0.001$) (Fig. 15). No statistically differences in levels of NA, 5-HIAA was found between genotypes. Thus, the hippocampal 5-HIAA/5-HT ratio was not change in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice and wild-type mice. These results indicated that the 5-HT level in the hippocampus was associated with Pcdh-α peroteins.

c-Fos-positive neurons in the septum in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice

We explored possible alternations of neuronal activity involved with the enhanced freezing in the fear conditioning test. Pcdh-$\alpha^{\Delta CR/\Delta CR}$ and wild-type mice were examined c-Fos expression as a marker for postsynaptic activity to define the pattern of neurons excited by fear conditioning test. There were no different of the number of c-Fos-positive neurons after training and cued test between two genotypes.

Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice also showed no impairments the number of c-Fos-positive neurons in the hippocampus. However, the number of c-Fos-positive neurons tended to increase in the septum after contextual testing in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice compared to wild-type mice, suggested elevated activation of septum neurons in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice (Fig. 16).

Figure 16. c-Fos-positive neurons in the septum. (A) Photomicrographs showing representative c-Fos labeling in the hippocampus (top panels) and septum (bottom panels) in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ (right panels) and wild-type (left panels) mice. Insets, High magnifications of c-Fos staining. Scale bars, hippocampus, 200μm; septum, 100μm; inset, 50μm. (B) Number of c-Fos-positive neurons in the septum. (C) Freezing during the contextual testing. Data are given as the means (±SEM) (Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, $n = 4$; wild-type, $n = 4$).

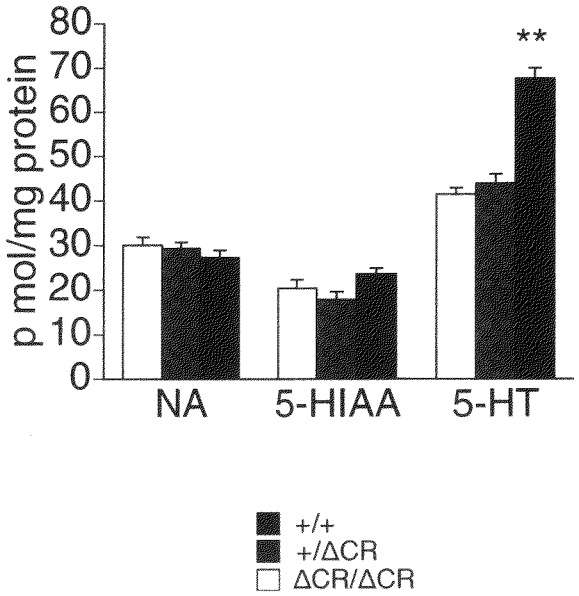
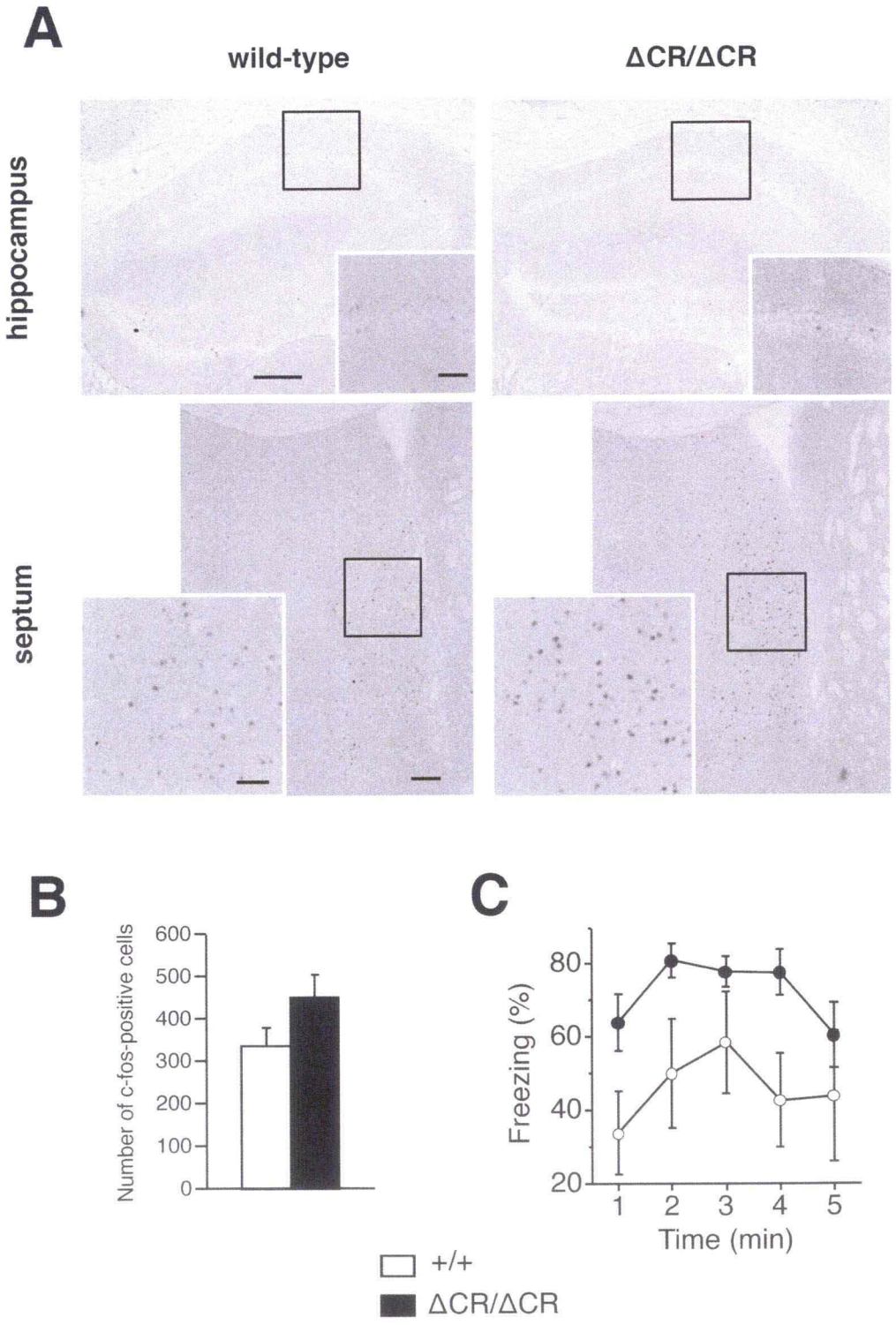


Figure 15. Monoamines in wild-type (+/+, white bar), heterozygous (+/ΔCR, gray bar) and homozygous (ΔCR/ΔCR, black bar) mice. 5-HT level was significantly increased in the hippocampus of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice (**, $P < 0.001$). NA, noradrenaline; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine.


A
wild-type
ΔCR/ΔCR
hippocampus
septum
B
Number of c-fos-positive cells
600
500
400
300
200
100
0
C
Freezing (%)
80
60
40
20
1
2
3
4
5
Time (min)
+/+
ΔCR/ΔCR

Discussion

Bahavioral responses of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice were markedly different from wild-type mice in contextual and cued fear conditioning test assessing fear-related learning, and sensorimoter gating. The 5-HT level was increased in the hippocampus of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice brain. Furthermore, the number of c-Fos, as a marker for postsynaptic activity, in the septum had tendency to increase.

Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice displayed enhanced freezing both contextual and cued learning compared to their wild-type littermates, whereas hypomorphic Pcdh-α A mice, which were 5-fold down-regulated Pcdh-α A proteins in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ and truncated Pcdh-α A proteins in Pcdh-$\alpha^{\Delta Aneo/\Delta Aneo}$ mice showed enhanced freezing only contextual learning. This difference could be account for the amount of residual Pcdh-α proteins. The residual Pcdh-α A isoforms in Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice might be enough to represent similar level in cued learning of wild-type mice. Based on results from Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, Pcdh-α proteins were essential for freezing not only contextual learning but also cued learning. Pavlovian fear conditioning of contextual cues requires the function both the hippocampus and the amygdala, whereas conditioning to tones requires the amygdala, but not the hippocampus (Kim & Fanselow, 1992). Thus, these results from Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice suggested that Pcdh-α proteins were important for normal function of both the hippocampus and the amygdala. Similar to Pcdh-$\alpha^{\Delta Bneo/\Delta Bneo}$ mice, we also did not detect the difference in the Morris water maze, other hippocampal-dependent task, between Pcdh-$\alpha^{\Delta CR/\Delta CR}$ and wild-type mice, suggesting Pcdh-α proteins would not affect the Morris water maze.

The neuronal activity measured by c-Fos staining after contextual test in the fear conditioning test had tendency to increase in the septum of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice brain compared to wild-type mice. Previous study using the fear conditioning paradigm have provided evidence that the processing of contextual learning by the hippocampus would be controlled by the amygdala through a modulation of septal-hippocampal excitability (Gray *et al.*, 1987). Furthermore, the median raphe nucleus is the origin of the serotonergic pathway that projects to the septum and hippocampus. This pathway is hypothesized to be the substrate of the serotonergic behavioral system (Bobillier *et al.*, 1975). Our results produced the possibility that the enhancement of freezing in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice expressed in the contextual test might be caused by the

hyperactivation of septal-hippocampal neurons. However, we could not detect obvious differences of c-Fos-positive cells in the amygdala and the hippocampus. Therefore we need the other analysis to define the neural activity in these areas clearly. Additional studies to define and characterize cell populations responsible for the effects of fear learning will help to investigate the neuronal mechamisms for Pcdh-α proteins.

We confirmed that Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice also showed significantly reduced levels of PPI of ASR, which measured sensorimotor gating. In addition, Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice displayed increased ASR. Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, it was unlikely that reduced PPI was simply related to the increasedstartle reactivity. In an assessment of 13 stains of mice with widely varying startle response magnitudes and amount of PPI, there was no clear relationship between ASR and PPI (Paylor and Crawley, 1997). As Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice, pharmacological study would be necessary such as clozapine or haloperidol for reversing of PPI deficit. We found that Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice slightly altered the locomotor activity in the open-arm test, or emotional behavior in the light/dark test compared to wild-type mice (data not shown). They may therefore be useful as an animal model of neurophychiatric disorders such as schizophrenia or depression.

In conclusion, our finding suggests that Pcdh-α proteins is strongly relevant to fear conditioning learning, sensorimoter gating in behavior, and the 5-HT levels in the hippocampus. Moreover, Pcdh-α proteins may alter the neuronal activity even if it is not directly. Revealing how the diversity of Pcdhs family contributes to the formation of brain connectivity and regulates the behavior is an attractive challenge for future.

Acknowledgments

I am very grateful to Prof. T. Yagi for the guidance and advice in the entire course of my Ph. D. study. I thank Mr. M. Sanbo, Ms. C. Sanbo, Ms. N. Yamauchi, Ms. H. Masuda, and Ms. E. Naruto for technical support. Gene-targeting mice was generated by Dr. S. Hamada (Fukuoka Women's University) and Dr. R. Yasuda. I also thank Prof. T. Miyakawa (Fujita Health University) for behavioral technical support, Dr. T. Yamamoto (Yokohama City University), Dr. H. Yamamoto (Tokyo Institute of Psychiatry) for HPLC measurements and Dr. S. Kobayashi and Prof. T. Manabe (University of Tokyo) for Electrophysiological support, critical reading and discussion. I am grateful to Prof. J. Miyazaki (Osaka University, Japan) for the CAG-Cre transgenic mice. I thank the members of the KOKORO Biology Laboratory for assistance and discussions.

Abbreviations

aa, amino acids; CNR, cadherin-related neuronal receptor; PPI, Prepulse inhibition; ASR, acoustic startle response; NA, noradrenaline; neo, neomycin-resistant; Pcdh, protocadherin; 5-HT, 5-hydroxytryptamine, serotonin.

References

Abe, K., Chisaka, O., Van, Roy, F., Takeichi, M. (2004) Stability of dendritic spines and synaptic contacts is controlled by alpha N-catenin. *Nat Neurosci,*. **7**, 357-363.

Bobillier, P., Pettijean, F., Salvert, D., Ligier, M., Seguin, S. (1975) Differential projections of the nucleus raphe dorsalis and nucleus raphe centralis as revealed by autoradiography. *Brain Res.*, **85**, 205-210.

Buhot, M. C., Wolff, M., Benhassine, N., Costet, P., Hen, R., Segu, L. (2003) Spatial learning in the 5-HT1B receptor knockout mouse: selective facilitation/impairment depending on the cognitive demand. *Learn Mem.*, **10**, 466-477.

Bozdagi, O., Shan, W., Tanaka, H., Benson, D. L., Huntley, G. W. (2000) Increasing numbers of synaptic puncta during late-phase LTP: N-cadherin is synthesized, recruited to synaptic sites, and required for potentiation. *Neuron*, **28**, 245-259.

Davis, M., Gendelman, D. S., Tischler, M. D., Gendelman, P. M. (1982) A primary acoustic startle circuit: lesion and stimulation studies. *J. Neurosci.*, **2**, 791-805.

Dodd, J. & Jessell, T. M. (1988) Axon guidance and the patterning of neuronal projections in vertebrates. *Science,* **242**, 692-699.

Dulawa, S. C., Scearce-Levie, K.A., Hen, R., Geyer, M.A. (2000) Serotonin releasers increase prepulse inhibition in serotonin 1B knockout mice. *Psychopharmacology*, **149**, 306-312.

Edelman, G. M. & Crossin, K. L. (1991) Cell adhesion molecules: implications for a molecular histology. *Annu. Rev. Biochem.*, **60**, 155-190.

Ehlers, M. D., Fung, E. T., O'Brien, R. J., Huganir, R. L. (1998) Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments. *J. Neurosci.*, **18**, 720-730.

Fendt, M., Li, L., Yeomans, J. S. (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. *Psychopharmacology*, **156**, 216-224.

Fields, R. D. & Itoh, K. (1996) Neural cell adhesion molecules in activity-dependent development and synaptic plastisity. *Trends. Neurosci.*, **19**, 473-480.

Hamada, S. & Yagi, T. (2001) The cadherin-related neuronal receptor family: a novel diversified cadherin family at the synapse. *Neurosci Res.*, **41**, 207-215.

Hirayama T, Yagi T. (2006) The role and expression of the protocadherin-alpha clusters in the CNS. *Curr Opin Neurobiol.*, **16**, 336-342.

Huang, Z., Shimazu, K., Woo, N. H., Zang, K., Muller, U., Lu, B., Reichardt, L. F. (2006) Distinct roles of the beta 1-class integrins at the developing and the mature hippocampal excitatory synapse. *J. Neurosci.*, **26**, 11208-11219.

Inoue, T., Tsuchiya, K., Koyama, T. (1994) Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. *Pharmacol. Biochem. Behav.*, **49**, 911-920.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S., Osumi, N. (2001) Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. *Development*, **128**, 561-569.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., and Miyazaki, J. (2000) A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett.*, **470**, 263-268.

Kim, J. J. & Fanselow, M. S. (1992) Modality-specific retrograde amnesia of fear. *Science*, **256**, 675-677.

Kim, J. J., Shih, J. C, Chen, K., Chen, L., Bao, S., Maren, S., Anagnostaras, S. G., Fanselow, M. S., De, Maeyer, E., Seif, I., Thompson, R. F. (1997) Selective enhancement of emotional, but

not motor, learning in monoamine oxidase A-deficient mice. *Proc. Natl Acad. Sci. USA.*, **94**, 5929-5933.

Kim, O. J., Ariano, M. A., Lazzarini, R. A., Levine, M. S., Sibley, D. R. (2002) Neurofilament-M interacts with the D1 dopamine receptor to regulate cell surface expression and desensitization. *J. Neurosci.*, **22**, 5920-5930.

Kirov, G., Georgieva, L., Williams, N., Nikolov, I., Norton, N., Toncheva, D., O'Donovan, M., Owen, M. J. (2003) Variation in the protocadherin gamma A gene cluster. *Genomics*, **82**, 433-440.

Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., Yagi, T. (1998) Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. *Neuron*, **20**, 1137-1151.

Kramar, E. A., Lin, B., Rex, C. S., Gall, C. M., Lynch, G. (2006) Integrin-driven actin polymerization consolidates long-term potentiation. *Proc. Natl Acad. Sci. USA.*, **103**, 5579-5584.

Manabe, T., Togashi, H., Uchida, N., Suzuki, S. C., Hayakawa, Y., Yamamoto, M., Yoda, H., Miyakawa, T., Takeichi, M., Chisaka, O. (2000) Loss of cadherin-11 adhesion receptor enhances plastic changes in hippocampal synapses and modifies behavioral responses. *Mol Cell Neurosci.*, **15**, 534-546.

Miki, R., Hattori, K., Taguchi, Y., Tada, M. N., Isosaka, T., Hidaka, Y., Hirabayashi, T., Hashimoto, R., Fukuzako, H., Yagi, T. (2005) Identification and characterization of coding single-nucleotide polymorphisms within human protocadherin-alpha and -beta gene clusters. *Gene*, **349**, 1-14.

Miyakawa, T., Yamada, M., Duttaroy, A., Wess, J. (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. *J. Neurosci.*, **21**, 5239-5250.

Morishita, H., Murata, Y., Esumi, S., Hamada, S., Yagi, T. (2004) CNR/Pcdhalpha family in subplate neurons, and developing cortical connectivity. *Neuroreport*, **15**, 2595-2599.

Morishita, H., Umitsu, M., Murata, Y., Shibata, N., Udaka, K., Higuchi, Y., Akutsu, H., Yamaguchi, T., Yagi, T., Ikegami, T. (2006) Structure of the cadherin-related neuronal receptor/protocadherin-alpha first extracellular cadherin domain reveals diversity across cadherin families. *J. Biol. Chem.*, **281**, 33650-33663.

Murata, Y., Hamada, S., Morishita, H., Mutoh, T., Yagi, T. (2004) Interaction with protocadherin-gamma regulates the cell surface expression of protocadherin-alpha. *J. Biol. Chem.*, **279**, 49508-49516.

Mutoh, T., Hamada, S., Senzaki, K., Murata, Y., Yagi, T. (2004) Cadherin-related neuronal receptor 1 (CNR1) has cell adhesion activity with beta1 integrin mediated through the RGD site of CNR1. *Exp. Cell Res.*, **294**, 494-508.

Nakamura, K., Manabe, T., Watanabe, M., Mamiya, T., Ichikawa, R., Kiyama, Y., Sanbo, M., Yagi, T., Inoue, Y., Nabeshima, T., Mori, H., Mishina, M. (2001) Enhancement of hippocampal LTP, reference memory and sensorimotor gating in mutant mice lacking a telencephalon-specific cell adhesion molecule. *Eur. J. Neurosci.*, **13**, 179-189.

Nollet, F., Kools, P., van, Roy, F. (2000) Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol.*, **299**, 551-572.

Noonan, J. P., Li, J., Nguyen, L., Caoile, C., Dickson, M., Grimwood, J., Schmutz, J., Feldman, M. W., Myers, R. M. (2003) Extensive linkage disequilibrium, a common 16.7-kilobase deletion, and evidence of balancing selection in the human protocadherin alpha cluster. *Am. J. Hum. Genet.*, **72**, 621-635.

Ojima, T., Ito, C., Sakurai, E., Sakurai, E., Watanabe, T., Yanai, K. (2004) Effects of serotonin-dopamine antagonists on prepulse inhibition and neurotransmitter contents in the rat cortex. *Neurosci Lett.*, **366**, 130-134.

Olton, D. S., Becker, J. T., & Handelmann, G. E. (1979a) Hippocampus, space, and memory. *Behav. Brain Sci.*, **2**, 313-365.

Olton, D.S. & Papas, B.C. (1979b) Spatial memory and hippocampal function. *Neuropsychologia*, **17**, 669-682.

Overduin, M., Harvey, T. S., Bagby, S., Tong, K. I., Yau, P., Takeichi, M., Ikura, M. (1995) Solution structure of the epithelial cadherin domain responsible for selective cell adhesion. *Science*, **267**, 386-389.

Paylor, R., Crawley, J. N. (1997) Inbred strain differences in prepulse inhibition of the mouse startle response. *Psychopharmacology*. **132**. 169-180.

Phillips, G. R., Tanaka, H., Frank, M., Elste, A., Fidler, L., Benson, D. L., Colman, D. R. (2003) Gamma-protocadherins are targeted to subsets of synapses and intracellular organelles in neurons. *J. Neurosci.*, **23**, 5096-5104.

Redies, C., Vanhalst, K., Roy, F. (2005) delta-Protocadherins: unique structures and functions. *Cell Mol Life Sci.*, **62**, 2840-2852.

Serafini, T. (1999) Finding a partner in a crowd: neuronal diversity and synaptogenesis. *Cell*, **98**, 133-136.

Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M.S., Grübel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R., Hendrickson, W. A. (1995) Structural basis of cell-cell adhesion by cadherins. *Nature*, **374**, 327-337.

Shapiro, L., Colman, D. R. (1999) The diversity of cadherins and implications for a synaptic adhesive code in the CNS. *Neuron*, **23**, 427-430.

Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., Yagi, T. (2000) Genomic organization of the family of CNR cadherin genes in mice and humans. *Genomics*, **63**,

75-87.

Takei, Y., Hamada, S., Senzaki, K., Mutoh, T., Sugino, H., Yagi, T. (2001) Two novel CNRs from the CNR gene cluster have molecular features distinct from those of CNR1 to 8. *Genomics*, **72**, 321-330.

Tanaka, K., Shintani, N., Hashimoto, H., Kawagishi, N., Ago, Y., Matsuda, T., Hashimoto, R., Kunugi, H., Yamamoto, A., Kawaguchi, C., Shimada, T., Baba, A. (2006) Psychostimulant-induced attenuation of hyperactivity and prepulse inhibition deficits in Adcyap1-deficient mice. *J Neurosci.*, **26**, 5091-5097.

Tang, L., Hung, C. P., Schuman, E. M. (1998) A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation. *Neuron*, **20**, 1165-1175.

Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., Takeichi, M. (2002) Cadherin regulates dendritic spine morphogenesis. *Neuron*, **35**, 77-89.

Triana-Baltzer, G. B., Blank, M. (2006) Cytoplasmic domain of protocadherin-alpha enhances homophilic interactions and recognizes cytoskeletal elements. *J. Neurobiol.*, **66**, 393-407.

Wang, X., Weiner, J. A., Levi, S., Craig, A. M., Bradley, A., Sanes, J. R. (2002) Gamma protocadherins are required for survival of spinal interneurons. *Neuron*, **36**, 843-854.

Weiner, J. A., Wang, X., Tapia, J. C., Sanes, J. R. (2005) Gamma protocadherins are required for synaptic development in the spinal cord. *Proc. Natl Acad. Sci. USA.*, **102**, 8-14.

Wu, Q. & Maniatis, T. (2000) Large exons encoding multiple ectodomains are a characteristic feature of protocadherin genes. *Proc. Natl Acad. Sci. USA.*, **97**, 3124-3129.

Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y., Aizawa, S. (1993) A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. *Anal. Biochem.*, **214**, 77-86.

Yagi, T. & Takeichi, M. (2000) Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes. Dev.*, **14**, 1169-1180.

Appendix

Production of Pcdh-α isoform-specific gene-converting mice

To generate the Pcdh-α ΔBneo 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 4th exon encoding a subtype-specific region of the Pcdh-α B isoform. The 5' homology arms were a *NotI/SalI*-digested 3.0-kb fragment generated by PCR with two primers for the Pcdh-α B allele; CP3-F, (5'-ATAAGAATGCGGCCGCGGCTACAATGTGGGAGAT-3') and CNRB-R, (5'-ACGCGTCGACTCATTCTGAAAACAAGCCAGAGTG-3') for TT-2 genomic DNA. A floxed PGK-neomycin-resistant (neo) cassette was placed downstream of the 5' homology arms as the positive selection marker. The 3' homology arm was made from *SalI/Apa*I-digested 9.0-kb and 9.4-kb fragments generated by long-range PCR with two primers for the Pcdh-α B allele; CNRB-F, (5'-ACGCGTCGACAAAAAGCAGACCCAGGTTTC-3'); CP3-R, (5'-CGGGGGCCCTATTAGCACAGCCTCCCT-3'). The coding regions in the homology arms were completely sequenced to confirm their integrity. For negative selection, a MC1-DT-A cassette was inserted at the end of the 3' arm.

Using mouse embryonic stem cells (TT2 cells) from F1 male embryos between C57BL/6 and CBA, we obtained homologous recombinants. Mice having ΔBneo alleles were produced by standard procedures for chimeric mice production (Yagi *et al.*, 1993). The gene-targeting event was confirmed by PCR analyses using the primer pair NA-F (5'-GGTGAGTTGCCAGACAAATTC-3') and mh1-2R (5'-GCCCTGCAAATATCATCAAG-3'), and Southern blotting analysis as shown in Fig. 3. Male chimera were crossed with C57BL/6 females, and then the obtained heterozygous mice were backcrossed with C57BL/6 mice for more than eight generations. All experiments were performed with littermates crossed with their C57BL/6 backcrossed heterozygotes.

To remove a PGK-neo cassette, we crossed Pcdh-α$^{+/\Delta Bneo}$ mice with Cre transgenic mice which express Cre recombinase ubiquitously (Kawamoto *et al.*, 2000), a generous gift from Dr. Miyazaki. All experiments were preformed with littermates crossed with their Pcdh-α$^{+/\Delta B}$ heterozygous parents.

Production of Pcdh-α constant region deleted mice

Two strains of mice with targeted mutations were generated: a *loxP* site inserted 3 kb upstream of CR1 (G1loxP allele), and in CR3b for the B-type splicing variant (ΔB allele). To generate the G1loxP allele, a targeting vector was constructed to insert a floxed *PGK-neo* gene cassette 3.0 kb upstream of CR1 (Supplementary Fig. 1A). Clones containing the CR1 exon of *Pcdha* were obtained from the TT-2 genomic phage DNA library (Yagi et al., 1993a). A 14.6-kb genomic DNA fragment was subcloned into pBluescript II SK(-). The 5' homology arm was a *Not*I/*Xho*I-digested 6.0-kb fragment of the 14.6-kb genomic clone. A floxed *PGK-neo* positive selection marker was placed downstream of the 5' homology arm. The 3' homology arm was an *Xho*I/*Kpn*I-digested 5-kb fragment from the 14.6-kb genomic clone. Downstream of the 3' arm is the diphtheria toxin A (DT-A)(Yagi et al., 1993b) gene, containing a poly-A signal, as a negative selection marker. Fifteen targeted events were identified by PCR using primers G1-F and G1-R, and confirmed by Southern blotting. To remove the *MC1-neo* cassette and generate the G1loxP allele, *Cre-pac* (Taniguchi et al., 1998) was transiently expressed in the targeted embryonic stem cells prior to generating chimeras. As for the ΔB allele, the procedure was described in CHAPTER 1 (Fig. 3 and Supplementary Fig. 1A). To delete the constant region exons (CR1, CR2 and CR3a), male mice were generated by breeding that carry the CAG-Cre transgene and two targeted mutations: one G1loxP allele, and one ΔB allele (Supplementary Fig. 2). The *loxP* sites in G1loxP and ΔB are in the same orientation. These males were crossed with C57BL/6 females, and the genotypes of their offspring were determined by PCR analyses using primers G1-F and mh1-2R to detect *trans*-allelic recombination events (Herault et al. 1998; Wu et al. 2007). Two of 14 offspring carried the ΔCR allele, and were used to establish the strain. The amplified PCR products with primers G1-F and mh1-2R were sequenced, and Southern blotting confirmed the trans-allelic recombination event.

Proteins in Pcdh-α mutant mice that lack constant region exons

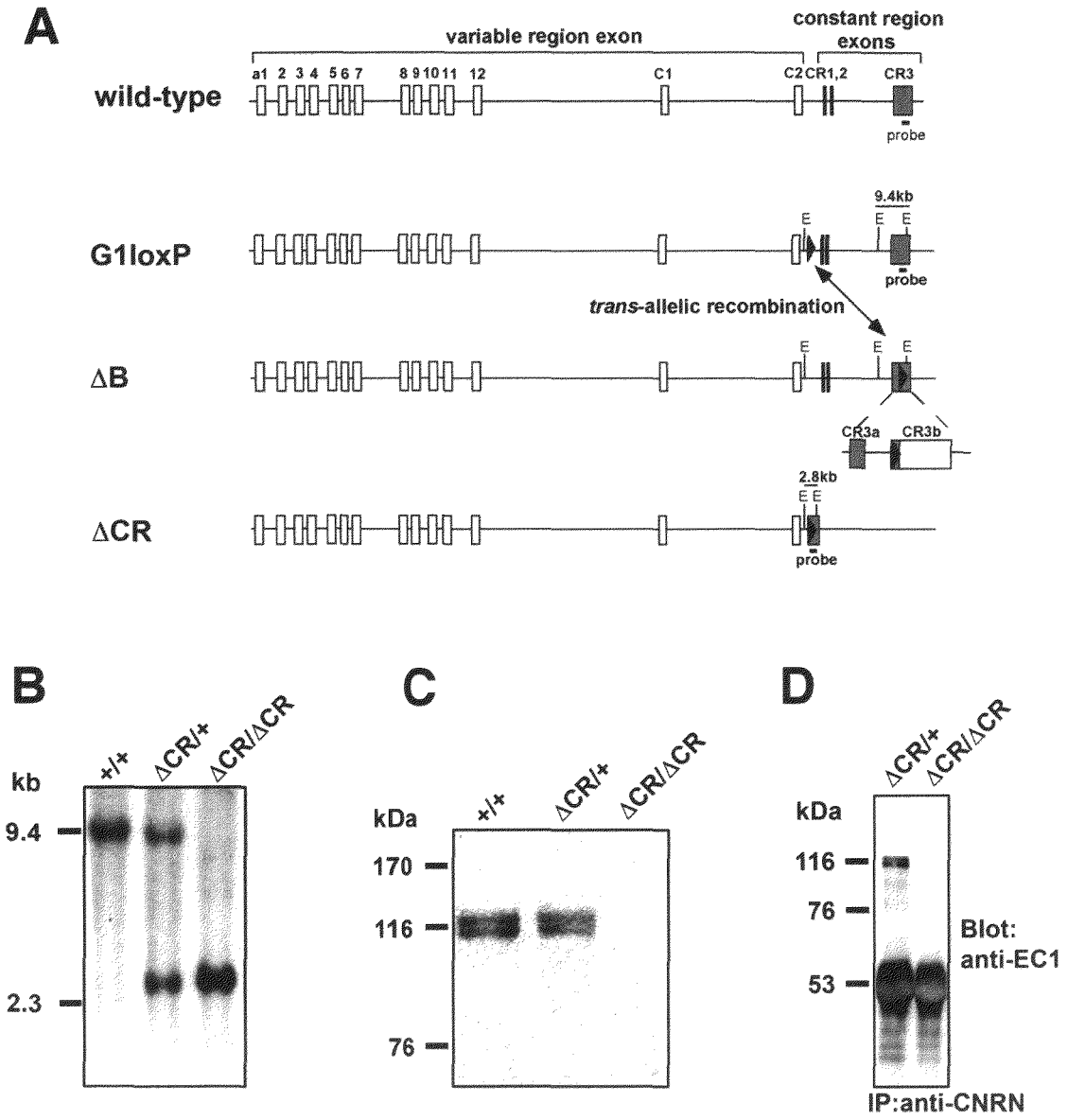
Mice bearing ΔCR allele were produced by *trans*-allelic recombination between G1loxP and ΔB allele (Supplementary Fig. 1A). The gene conversion was detected by Southern blot analysis (Supplementary Fig. 1B). Deletion of the constant region exons was also confirmed from the result that no proteins were detected in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice

brain using anti-CNR/Pcdhα, which recognized the Pcdh-α cytoplasmic region by Western blot analysis (Supplementary Fig. 1C). To evaluate the effect on proteins that depends on missing the constant region exons, we performed Western blot analysis. Because of the deletion of constant region, we used the anti-EC1 antibody, which recognized the EC1 region of Pcdh-α proteins for immunoprecipitants with anti-CNRN antibody, which recognized the N-terminal region of Pcdh-α4 proteins. We could not detect not only full-length but also truncated Pcdh-α proteins in Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice (Supplementary Fig. 1D). Therefore, we obtained Pcdh-α mutant mice in which Pcdh-α proteins were deficiency or substantial decrease.

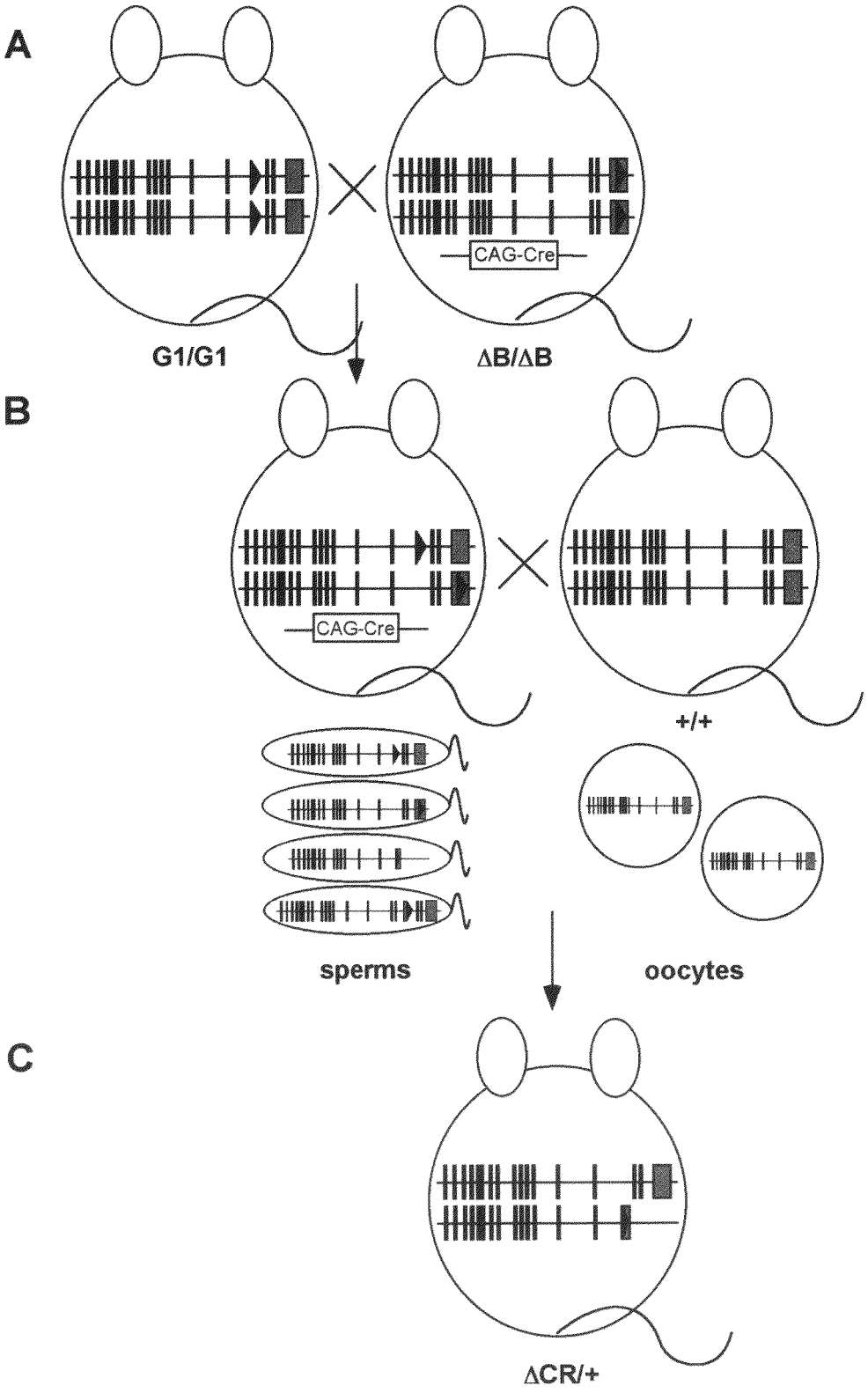
Supplementary Figure Legends

Supplementary Figure 1. Generation of Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice. (A) Schematic diagram of the genomic organization of wild-type mouse *Pcdh-α*, GaloxP, ΔB allele and ΔCR allele deleted the constant region exons. The restriction map around the locus used for targeting and Southern blot analysis is shown (E, *EcoRI).* The probes for Southern blot analysis are also indicated. (B) Southern blot analysis of deleted *Pcdh-α* constant region. DNA from the tails of wild-type (+/+), heterozygous (+/ΔCR) and homozygous (ΔCR/ΔCR) mice was digested with *EcoRI.* Blotted DNA was hybridized with probe, which was placed on a 5' Pcdh-α 3rd exon genomic flanking region. (C) Western blot analysis using anti-CNR/Pcdhα antibody in wild-type (+/+), Pcdh-$\alpha^{+/\Delta CR}$ and Pcdh-$\alpha^{\Delta CR/\Delta CR}$ brains. (D) Pcdh-α proteins in wild-type (+/+) and Pcdh-$\alpha^{\Delta CR/\Delta CR}$ mice were detected by Western blotting with anti-EC1 antibody for immunoprecipitants with anti-CNRN antibody.

Supplementary Figure 2. Breeding procedure to generate the ΔCR allele. (A) Mice carrying the G1loxP allele were crossed with mice carrying the ΔB allele and CAG-Cre transgene. (B) Male F1 progeny were screened for the CAG-Cre transgene, the G1loxP and ΔB allele by genotyping. These F1 progeny were crossed with wild-type mice. (C) F2 progeny were screened for the mutation produced by *trans*-allelic recombination.



Supplementary Figure 1



Supplementary Figure 2

研究業績

(1) 学術論文

Emi Fukuda, Shun Hamada, Sonoko Hasegawa, Shota Katori, Makoto Sanbo, Tsuyoshi Miyakawa, Toshifumi Yamamoto, Hideko Yamamoto, Takahiro Hirabayashi, Takeshi Yagi: Down-regulation of protocadherin-α A isoforms in mice changes contextual fear conditioning and spatial working memory.: European Jornal of Neuroscience.(投稿中)

Sonoko Hasegawa, Shun Hamada, You Kumode, Shota Katori, Emi Fukuda, Yasuo Uchiyama, Takahiro Hirabayashi, Peter Mombaerts, Takeshi Yagi: The protocadherin-α family is required for axonal coalescence into glomeruli of the olfactory bulb in mouse.: Molecular and Cellular Neuroscience. (投稿中)

(2) 国際会議における発表

Emi Fukuda, Shun Hamada, Tsuyoshi Miyakawa, Takahiro Hirabayashi, Takeshi Yagi
"Phenotypic analysis of CNR/Protocadherin α gene-targeting mice."
Neuroscience 2005, Washington, D.C., 2005 年 11 月 12—16 日

(3) 国内学会における発表

福田絵美、濱田俊、平林敬浩、八木健
CNR/Pcdh α スプライシングバリアント特異的遺伝子変換マウスの表現型解析
第 27 回日本神経科学大会・第 47 回日本神経化学会大会 合同大会、 2004 年 9 月 21—23 日

福田絵美、濱田俊、平林敬浩、八木健
CNR/Pcdh α splicing variant 遺伝子変換マウスの表現型解析
第 27 回日本分子生物学会、2004 年 12 月 8—11 日

福田絵美、八木健
Down regulation of CNR/Protocadherin α family enhanced fear-related spatial learning in mice.
第 28 回日本神経科学大会サテライトシンポジウム、2005 年 7 日 24—25 日

福田絵美、濱田俊、宮川剛、平林敬浩、八木健
CNR/Protocadherin α 遺伝子改変マウスの表現型解析
第 28 回日本神経科学大会、2005 年 7 日 26—28 日

濱田俊、福田絵美、香取将太、長谷川園子、江角重行、山本秀子、山本敏文、八木健
CNR/Protocadherin α 蛋白質カルボキシル末端領域の機能解析

第28回日本神経科学大会、2005年7日26—28日

Emi Fukuda, Shun Hamada, Shizuka Kobayashi, Ayako Watabe, Tsuyoshi Miyakawa, Koichi Tanda, Takahiro Hirabayashi, Toshiya Manabe, Takeshi Yagi
Down regulation of CNR/Protocadherin-a family enhanced fear-related spatial learning in mice
第27回日本分子生物学会、2005年12月7—10日

濱田俊、福田絵美、香取将太、八木健
CNR/Protocadherinαタンパク質の細胞内局在制御
第29回日本神経科学大会、2006年7月19—21日

福田絵美、濱田俊、平林敬浩、八木健
Protocadherin-αファミリーは感覚運動ゲーティングと恐怖条件付け学習を制御する
第30回日本神経科学大会、第50回日本神経化学会大会、第17回日本神経回路学会大会 合同大会、2007年9月10—12日

香取将太、濱田俊、福田絵美、江角重行、野口由紀子、山本秀子、山本敏文、八木健
プロトカドヘリンαの細胞内領域は、セロトニン神経の正常な投射の形成に必須である
第30回日本神経科学大会、第50回日本神経化学会大会、第17回日本神経回路学会大会 合同大会、2007年9月10—12日

(4) その他
特許の名称：プロトカドヘリンαを用いた精神神経疾患モデル動物およびその利用
特許の申請者：八木健、濱田俊、福田絵美
特許の申請日：2006年7月18日
出願番号：特願2006-195401
出願人：国立大学法人大阪大学