Total Expression and Dual Gene-regulatory Mechanisms Maintained in Deletions and Duplications of the Pcdha Cluster*

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The clustered protocadherin-α (Pcdha) genes, which are expressed in the vertebrate brain, encode diverse membrane proteins whose functions are involved in axonal projection and in learning and memory. The Pcdha cluster consists of 14 tandemly arranged genes (Pcdha1–Pcdha12, Pcdhac1, and Pcdhac2, from 5′ to 3′). Each first exon (the variable exons) is transcribed from its own promoter, and spliced to the constant exons, which are common to all the Pcdha genes. Cerebellar Purkinje cells show dual expression patterns for Pcdha. In individual Purkinje cells, different sets of the 5′ genes in the cluster, Pcdha1–12, are randomly expressed, whereas both 3′ genes, Pcdhac1 and Pcdhac2, are expressed constitutively. To elucidate the relationship between the genomic structure of the Pcdha cluster and their expression in Purkinje cells, we deleted or duplicated multiple variable exons and analyzed the expression of Pcdha genes in the mouse brain. In all mutant mice, transcript levels of the constant exons and the dual expression patterns were maintained. In the deletion mutants, the missing genes were flexibly compensated by the remaining variable exons. On the other hand, in duplication mutants, the levels of the duplicated genes were trimmed. These results indicate that the Pcdha genes are independently and randomly expressed from both alleles in individual Purkinje cells of the cerebellum (5). On the other hand, Pcdhac1 and Pcdhac2, located at the 3′ end of the cluster, are constitutively expressed from both alleles in individual Purkinje cells (6). These findings suggest that there are at least two kinds of expression regulations; random and constitutive, in the Pcdha cluster. In addition, the expression levels of Pcdha1–Pcdhac1, but not of Pcdhac2, are enhanced at the neu-

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osomal differentiation stage in embryonic stem (ES) cells, by a cis-regulatory element consisting of five DNase I-hypersensitive sites (HS5-1) (21) (see Fig. 1A). Thus, random gene expression may be regulated by HS5-1. However, almost nothing is known about the dual regulatory mechanisms for the random and constitutive expression of the Pcdha genes.

Various approaches have been taken to elucidate the expression regulation of clustered genes, including searching for a cis-regulatory element (22–24), deleting and duplicating clustered genes (25, 26), and inverting clustered genes (26–29). However, no study to date has reported how the expression of Pcdha genes is influenced by deletion or duplication of the variable exons. Here, we used the targeted meiotic recombination (TAMERE) method (30) to generate mutant mice in which the variable exons, with their promoters, were deleted or duplicated. We compared the expression patterns of the Pcdha genes within the mutant cluster (in the mutant mice) with those of the wild-type (WT) cluster, in WT mice, by quantitative real-time PCR (qRT-PCR), in situ hybridization, and single cell RT-PCR and single nucleotide polymorphism (SNP) analyses of Purkinje cells.

In the mutants, the expression levels and distribution patterns of the CR transcript were essentially unaltered. Interestingly, in the deletion and duplication mutants, the dual regulatory patterns of Pcdha gene expression were maintained. The single cell RT-PCR and SNP analyses, which can distinguish the mRNAs expressed from individual alleles, suggested that the regulators of these dual expression patterns exist outside the variable region. These dual regulatory systems may provide a clue to the role of Pcdha genes in neuronal functions.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animals were maintained in a specific pathogen-free space under a 12-h light/dark regimen. Experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Science Council of Japan and approved by the Animal Experiment Committee of Osaka University.

**Production of Mice Carrying the G1loxP Allele**—The G1loxP mice, in which a loxP site was inserted into the genomic region 3-kb upstream from the CR1 exon, were described previously (14).

**Production of Mice Carrying the G1Neo Allele**—A genomic DNA library made from TT2 ES cells was screened with Pcdha1 cDNA, and a 13-kb genomic DNA fragment containing the a1 and a2 exons was obtained. This fragment was subcloned into the XhoI site of pBluescript II (31), and the Sleeping Beauty cassette (-loxP-IR/DR-L-loxP-PGK-neo-inoxP-IR/DR-R) (32) was inserted into the NheI site between the a1 and a2 exons, to construct the targeting vector. The vector was linearized by NotI and introduced into TT2 ES cells by electroporation. We obtained targeted recombinants and their chimeric offspring using standard methods. The 11R mutant mice were backcrossed with the B6 strain (see supplemental Fig. S2 for details).

**Synaptosomal Complex Protein 1 (Sycp1)-Cre Transgenic Mice**—For efficient trans-allelic recombination (30, 38), we generated Sycp1-Cre transgenic mice, which expressed Cre recombinase specifically in the testis. The transgene vector contained the −737 to +87 promoter region of the Sycp1 gene and the Cre recombinase gene, with a nuclear localization signal and a polyadenylation signal, inserted into pBluescript II. The fragment, digested by SalI and NotI, was microinjected into fertilized eggs derived from B6 mice. Three transgenic lines were generated. They were maintained on the B6 genetic background, and expression of the Cre transgene in the germ cells was confirmed by crossing them with CAG-CAT-EGFP mice (39). For this study, we used one of three lines, in which Cre was expressed specifically in the testis.

**Generation of Pcdha11R/G1loxP and Pcdha11R/G16Neo Mice**—By mating 11R mice and Sycp1-Cre transgenic mice, 11R mice carrying the Sycp1-Cre transgene were generated. We crossed these mice and mice bearing the G1loxP allele, and then selected male offspring bearing the 11R allele, G1loxP allele, and the Sycp1-Cre transgene (Pcdha11R/G1loxP, Sycp1-Cre). We then crossed Pcdha11R/G1loxP, Sycp1-Cre male and B6 female mice, and genotyped the pups using genomic DNA extracted from the tail. Some of these pups carried the Pcdha11R/G1loxP allele, or Pcdha11R/G1loxP or Pcdha11R/G1loxP alleles as a result of TAMERE in the testis. To identify the Pcdha11R/G1loxP or Pcdha11R/G1loxP mice, we performed Southern blotting and PCR analyses (supplemental Fig. S3). The Pcdha11R/G1loxP mice, in which the targeted mutation was inserted into the CBA allele, were backcrossed with the CBA strain for two generations, and their pups were analyzed. The Pcdha11R/G1loxP mice were obtained by crossing Pcdha11R/G1loxP parents, which were backcrossed with B6 for more than two generations.

**Generation of Pcdha11R/CR11-12/del(11-c2)/del(11-c2) and Pcdha11R/CR11-12/del(11-c2)/dup(12-c2) Mice**—By crossing 11R mice bearing the Sycp1-Cre transgene and mice bearing the G16Neo allele, we generated male mice bearing the 11R allele, G16Neo allele, and Sycp1-Cre transgene (Pcdha11R/G16Neo, Sycp1-Cre). We obtained pups from crosses and their chimeric offspring. The G16Neo mutant mice were backcrossed with the C57BL/6 (B6) strain (see supplemental Fig. S1 for details).

**Production of Mice Carrying the 11R Allele**—We constructed a targeting vector to insert, in-frame, a GAP43-HcRed gene cassette (encoding the N-terminal peptide, MLCCMRRTK, of GAP43 (34), the HcRed protein, which is a far-red fluorescent protein (35), and a stop codon), a floxed PGK-neo, an internal ribosome entry site (36), a Kozak sequence (37), and then the a11 coding sequence, between the 7133 bp of the 5’ homologous arm (amplified with the 11R5’F and 11R5’R primers (sequences available under supplemental “Experimental Procedures”) and 2542 bp of the 3’ homologous arm (amplified with the 11R3’F and 11R3’R primers (sequences available in Supplemental Table S1)). An MC1DT-A cassette (31) was inserted at the end of the 5’ arm to allow selection. The 11R-targeting vector was linearized by NotI and introduced into TT2 ES cells by electroporation. We obtained targeted recombinants and their chimeric offspring using standard methods. The 11R mutant mice were backcrossed with the B6 strain (see supplemental Fig. S2 for details).
Deletions and Duplications in the Pcdha Cluster

FIGURE 1. loxP-insertion, deletion, and duplication alleles in the variable region of the Pcdha cluster. A, a genomic structure of the Pcdha wild-type (WT) allele. It consists of 14 first exons (white boxes) in the variable region and three CR exons (CR1–CR3), black boxes) in the constant region. The first exons are termed a1–a12, ac1, and ac2 from 5′ to 3′. Pcdha genes are produced by splicing each first exon to the CR exons, and termed Pcdha1–Pcdha12, Pcdhac1, and Pcdhac2. Dnase I-hypersensitive sites (HS5-1 and HS7) are shown as ovals. Arrows indicate the direction of transcription. B, G1loxP allele: a loxP site was inserted between ac2 and CR1. C, G1Neo allele: loxP sites were inserted between a1 and a2 (see supplemental Fig. S1 for details). D, 11R allele: an HcRed gene (red box), a loxP site, and an internal ribosome entry site (ires, yellow box) were inserted between the promoter and coding region of a11 (see supplemental Fig. S2 for details). E, the del(11–c2) allele: deletion of a11–ac2. F, the del(2–11) allele: deletion of a2–a11. G, the dup(2–10) allele: duplication of a2–a10. H, the dup(12–c2) allele: duplication of a12–ac2. The del(11–c2) and dup(12–c2) alleles were produced by Cre-loxP-mediated trans-allelic meiotic recombination between the 11R and G1loxP alleles. The del(2–11) and dup(2–10) alleles were produced from the G1Neo and 11R alleles. The deleted DNA segments are indicated by dashed lines, and the duplicated segments are shown under the position of the original segment. The loxP sites are shown as blue triangles.

of Pcdha11R/G1Neo, Sycp1-Cre males and B6 females, and genotyped them using genomic DNA extracted from the tail. To detect the deletion and duplication events caused by TAMERE, we performed Southern blotting and PCR analyses (supplemental Fig. S3). Some Pcdha+/-del(2–11) or Pcdha+/-dup(2–10) mice were generated from TAMERE in the testis. The Pcdha+/-del(2–11) mice, in which the CBA allele was mutated, were backcrossed with the CBA strain for two generations. Pcdha+/-dup(2–10)/dup(2–10) mice were obtained by crossing Pcdha+/-dup(2–10) parents, and were backcrossed with B6 for more than two generations.

Determination of the Targeted Allele in Mutant Mice—The TT2 ES cell line was established from fertilized F1 eggs from a cross between B6 and CBA mice. To identify which of these alleles was targeted, we used SNPs of Pcdha1 (rs24884904) and/or Pcdha10 (rs13498878) between the B6 and CBA strains. For Pcdha1, the PCR was performed as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 7 min. The primers were Pcdha1SNF and Pcdha1SNPR (sequences available in supplemental Table S1). The PCR product was digested with BmgTI20I (Taken) yielding 202- and 139-bp bands from the B6 allele and a 341-bp band from the CBA allele (see supplemental Fig. S3J). For Pcdha10, the PCR was performed as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and then 72 °C for 7 min. The primers were Pcdha10SNF and Pcdha10SNPR of H2O. The product was analyzed using the 7900HT Sequence Detection System (Applied Biosystems). To quantify the number of molecules, five serial 10-fold dilutions of linearized plasmid vector of each gene were constructed and analyzed simultaneously to obtain standard curves. The plasmid vector inserts were full-length cDNAs of Pcdha1 to Pcdhac2, and partial cDNAs of the β-actin (Actb) genes. The mean quantity of each amplified Pcdha gene was normalized to the quantity of Actb. The value was divided by the mean amount of the WT transcript, and statistical analysis was performed. These qRT-PCR results for the WT, heterozygous, and homozygous mice were analyzed using an one-factor analysis of variance with Sheffe’s F test.

In Situ Hybridization—The in situ hybridization was performed essentially as described previously with small modifications (40). The details are provided under supplemental "Experimental Procedures".

Purkinje Cell Counts—To analyze the proportion of Purkinje cells that expressed Pcdha genes, we used the brain from P21 Pcdha<del(11–c2)/del(11–c2)> mutant mice (n = 4) and their WT littermates (n = 4), or from P21 Pcdha<del(2–11)/del(2–11)> mutant mice (n = 4) and their WT littermates (n = 4). We cut 12–14 10-μm thick sagittal sections at 200-μm intervals from the midline, and performed in situ hybridization with probes specific for Pcdha1, Pcdha10, Pcdha12, Pcdhac1, Pcdhac2, and Purkinje cell protein 2 (Pcp2) (41). The sections were then photographed (sequences available in supplemental Table S1). Digestion with Sau3AI yielded 202- and 139-bp bands from the B6 allele and a 341-bp band from the CBA allele (see supplemental Fig. S3G).
Deletions and Duplications in the Pcdha Cluster

Before using the TAMERE system, we individually inserted three loxP sites into the variable region of the Pcdha cluster (Fig. 1, B–D). First, the “G1loxP” allele (14) was generated by inserting a loxP site between the ac2 exon and the first exon of the constant region (CR1) in the Pcdha cluster (Fig. 1B). Second, a loxP site was inserted into the sequence between the a1 and a2 exons, to generate the “G16Neo” allele (Fig. 1C and supplemental Fig. S1). Finally, a loxP site was inserted between the promoter and coding region of a11, to generate the “1IR” allele (Fig. 1D and supplemental Fig. S2).

To delete or duplicate the sequence between the loxP site of the 1IR allele and that of the G1loxP allele by the TAMERE system, we obtained male mice that possessed the 1IR and G1loxP alleles (1IR/G1loxP), and the Sycp1-Cre transgene, which elicits Cre recombinase expression specifically in the testis. The male mice were crossed with WT female mice, and the genotypes of the F1 pups were analyzed by PCR. The minority of F1 pups carried the del(11-c2) allele, in which exons a11 to ac2 were deleted (Fig. 1E), or the dup(12-c2) allele, in which exons a12 to ac2 were duplicated (Fig. 1H). F1 pups carrying these deletion or duplication alleles were obtained at 12.7% (17 of 134 pups). All of these heterozygous and homozygous mutant mice survived to adulthood and were fertile.

To delete or duplicate the sequence between the loxP site of the G16Neo allele and that of the 1IR allele by the TAMERE system, we crossed male mice carrying the G16Neo and 1IR alleles (G16Neo/1IR), and Sycp1-Cre with WT female mice, and genotyped their F1 pups by PCR. The minority of F1 pups carried one of two mutant alleles: the del(2–11) allele, in which the variable exons from a2 to a11 were deleted (Fig. 1F), or the dup(2–10) allele, in which the variable exons from a2 to a11 were duplicated (Fig. 1G). F1 pups carrying these deletion or duplication alleles were obtained at 24.8% (31 of 125 pups). The genotypes of these mice were confirmed by PCR and Southern blotting analyses (supplemental Fig. S3). All of these heterozygous and homozygous mutant mice survived to adulthood and were fertile.

Expression of Pcdha Genes in the del(11-c2) Allele—We used the Pcdha(del(11-c2)/del(11-c2)) and Pcdha+/del(11-c2) mice to examine the effects of deleting exons a11 to ac2. First, we performed qRT-PCR of the whole brain at P21. The expression level of

RESULTS

Deletions and Duplications of the Pcdha Cluster—Little is known about the relationship between the genomic structure of the clustered Pcdha genes and their gene expression. Therefore we deleted or duplicated the clustered genes in mice using the TAMERE system.

with a BIOREVO BZ-9000 microscope (Keyence). Purkinje cells that were stained around the nucleus in the Purkinje cell layer were considered to be signal positive, and were counted. Damaged or uniformly stained sections were excluded. The ratio obtained by dividing the number of Pcp2-negative cells by the number of Pcp2-positive cells was subjected to statistical analysis. The results for WT and homozygous mice were analyzed using Student’s t test or Welch’s t test with Microsoft Excel and Statcel2 software (OMS Publishing Inc.).

Split Single Cell RT-PCR and SNP Analysis—The single cell RT-PCR was performed essentially as described previously, with small modifications (42). The details are provided under supplemental “Experimental Procedures”.

FIGURE 2. Expression of Pcdha genes in Pcdha(del(11-c2)) and Pcdha(del(11-c2)) mice. A, qRT-PCR analysis of Pcdha transcripts in the brain of WT (n = 4), Pcdha(del(11-c2)) (n = 4), and Pcdha(del(11-c2)/del(11-c2)) mice (n = 4) on postnatal day 21 (P21). Expression levels are shown as the ratio to WT. The CR transcript level was unchanged in the mutants. The Pcdha1, Pcdha2, and Pcdha10 transcripts were increased, and those of Pcdha5 to Pcdha7 were decreased in the deletion mutants. The Pcdha6, Pcdha7, and Pcdha10 levels of the Pcdha(del(11-c2)/del(11-c2)) mice were significantly changed compared with Pcdha(del(11-c2)) mice. *, p < 0.05; **, p < 0.01, versus WT; #, p < 0.05, versus Pcdha(del(11-c2)) mice. Data are shown as the mean ± S.D. B, distribution of the CR and Pcdha10 transcripts in sagittal sections of the WT and Pcdha(del(11-c2)/del(11-c2)) brain at P21, examined by in situ hybridization. Anterior is to the left, posterior to the right. Scale bar, 1 mm. The frames correspond to the fields displayed in C and D. C, expression patterns of the CR and Pcdha10 transcripts in the P21 cerebral cortex of WT and Pcdha(del(11-c2)/del(11-c2)) mice, examined by in situ hybridization. Scale bar, 100 μm. Frames show the high-magnification insets. Scale bar, 5 μm. D, expression pattern of the CR and Pcdha10 transcripts in the CA3 region of the hippocampus in WT and Pcdha(del(11-c2)/del(11-c2)) mice at P21, examined by in situ hybridization. Scale bar, 100 μm. B–D, the expression pattern of the CR transcripts was not significantly altered, but the number of cells expressing Pcdha10 dramatically increased in the Pcdha(del(11-c2)) brain. Arrows show Pcdha10-positive cells in the insets of WT mice.

were obtained at 12.7% (17 of 134 pups). All of these heterozygous and homozygous mutant mice survived to adulthood and were fertile.

To delete or duplicate the sequence between the loxP site of the G16Neo allele and that of the 1IR allele by the TAMERE system, we obtained male mice that possessed the 1IR and G1loxP alleles (1IR/G1loxP), and the Sycp1-Cre transgene, which elicits Cre recombinase expression specifically in the testis. The male mice were crossed with WT female mice, and the genotypes of the F1 pups were analyzed by PCR. The minority of F1 pups carried the del(11-c2) allele, in which exons a11 to ac2 were deleted (Fig. 1E), or the dup(12-c2) allele, in which exons a12 to ac2 were duplicated (Fig. 1H). F1 pups carrying these deletion or duplication alleles

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To delete or duplicate the sequence between the loxP site of the 1IR allele and that of the G1loxP allele by the TAMERE system, we obtained male mice that possessed the 1IR and G1loxP alleles (1IR/G1loxP), and the Sycp1-Cre transgene, which elicits Cre recombinase expression specifically in the testis. The male mice were crossed with WT female mice, and the genotypes of the F1 pups were analyzed by PCR. The minority of F1 pups carried the del(11-c2) allele, in which exons a11 to ac2 were deleted (Fig. 1E), or the dup(12-c2) allele, in which exons a12 to ac2 were duplicated (Fig. 1H). F1 pups carrying these deletion or duplication alleles

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the spliced CR transcripts (CR1–CR3 exons), which are common to all the Pcdha genes, was unchanged in the Pcdha\(^{del(11-c2)/del(11-c2)}\) mice, whereas the expression levels of some of the remaining genes had changed (Fig. 2A). The expression levels of Pcdha1, Pcdha2, and Pcdha10 in the Pcdha\(^{del(11-c2)/del(11-c2)}\) mice exhibited 2.9-, 2.5-, and 8.24-fold increases, respectively, relative to the levels in WT mice (Fig. 2A). The expression levels of Pcdha5, Pcdha6, and Pcdha7 decreased by 0.6-, 0.32-, and 0.46-fold, respectively (Fig. 2A). There were no significant changes in the expression levels of Pcdha3, Pcdha4, or Pcdha9 (Fig. 2A). The expression levels of the Pcdha genes in the Pcdha\(^{del(11-c2)/del(11-c2)}\) mice were intermediate between those of the WT and Pcdha\(^{del(11-c2)/del(11-c2)}\) mice (Fig. 2A).

Next, we examined the distribution of transcripts in the Pcdha\(^{del(11-c2)/del(11-c2)}\) brain at P21 by in situ hybridization, using variable exon-specific probes for Pcdha1 to Pcdha12 and the CR probe common to all the Pcdha genes. CR probe-positive cells were observed throughout the WT brain (Fig. 2, B–D). A similar CR staining pattern was seen in the Pcdha\(^{del(11-c2)/del(11-c2)}\) brain (Fig. 2, B–D). Pcdha1–Pcdha12 transcripts are randomly expressed (see supplemental Note 2), and Pcdha1 and Pcdha2 transcripts are constitutively expressed in Purkinje cells of the WT cerebellum (5, 6). This expression pattern was also observed in the cells of the cerebral cortex and in the pyramidal cells of the hippocampal CA3 region of P21 WT mice (data not shown). In these regions, in Pcdha\(^{del(11-c2)/del(11-c2)}\) mice, the Pcdha1–Pcdha9 transcripts

![Image of in situ hybridization analysis of Pcdha genes in the Purkinje cells of Pcdha\(^{del(11-c2)/del(11-c2)}\) mice.](image-url)
FIGURE 5. Single cell RT-PCR and SNP analysis of the Pcdha genes in individual Purkinje cells of Pcdha<sup>+/del(11-c2)</sup> mice. A, by mating Pcdha<sup>del(11-c2)/del(11-c2)</sup> (CBA) and WT (JF1) mice, the first filial generation (F1) mice, namely Pcdha<sup>+/del(11-c2)</sup>, were generated. After reverse transcription of the RNAs of a single Purkinje cell isolated from the cerebellum neurons, the cDNA was split into three tubes. In each tube, PCR was performed using primers for the specific genes. B, electrophoresis results of the second-round PCR products by the split single cell RT-PCR for the Pcdha and Pcp2 genes in individual Purkinje cells. #1–17 numbers designate individual cells. 1–3, tubes into which the cDNA from an individual Purkinje cell was divided; independent PCRs were performed for each tube. C, after sequencing the PCR products, SNP analysis was used to distinguish between Pcdha transcripts from the del(11-c2) allele and those from the WT allele. Transcripts from the WT and the del(11-c2) alleles are shown as blue and red circles, respectively. The Pcdha6 gene has no SNP between the B6 and JF1 strains, and is undistinguishable. Transcripts that were undistinguishable or not determined are shown as plus signs. Nonspecific bands are shown as minus signs. Pcdha10 was clearly expressed from the del(11-c2) allele in all the cells examined.
Deletions and Duplications in the Pcdha Cluster

![Image of graphs and diagrams related to Pcdha expression patterns in different mice genotypes](image)

**Figure 6. Expression of Pcdha genes in the del(11-c2) allele.** A. qRT-PCR analysis of Pcdha transcripts in the brain of WT (n = 4), Pcdha<sup>+/del(11-c2)</sup> (n = 4), and Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice (n = 4) at P21. The levels of Pcdha1, Pcdha12, and CR transcripts in the Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice increased significantly compared with WT. The Pcdha1 and Pcdha12 transcript levels were unchanged. The Pcdha1 expression level of Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice was significantly different from that of the Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice. Expression levels are shown as the ratio to WT. **, p < 0.05; **, p < 0.01, versus WT; #, p < 0.01, versus Pcdha<sup>+/del(11-c2)</sup> mice. Data are shown as the mean ± S.D. B. Expression of the CR, Pcdha1, and Pcdha12 transcripts in sagittal sections of WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> brains at P21, examined by *in situ* hybridization. Anterior is to the left, posterior to the right. Scale bar, 1 mm. Frames correspond to the fields displayed in C and D. C, the expression patterns of CR, Pcdha1, and Pcdha12 transcripts in the cerebral cortex of WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> brains at P21, examined by *in situ* hybridization. Scale bar, 100 μm. D, the expression patterns of CR, Pcdha1, and Pcdha12 transcripts in the CA3 region of the hippocampus of the WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> brain at P21 by *in situ* hybridization. Scale bar, 100 μm. B–D, there was no obvious difference in the distribution of CR transcripts between the WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> brains, whereas the level of the Pcdha1 transcript clearly increased. The level of Pcdha12 was also slightly increased in the Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice. Arrows show Pcdha1-positive cells in the insets of WT mice.

were also randomly expressed. However, the number of cells expressing Pcdha10 and its expression level had dramatically increased in the cerebral cortex and hippocampus (Fig. 2, B–D). This result was consistent with the qRT-PCR analysis.

To quantify the expression frequency of Pcdha10, we examined Purkinje cells of the cerebellum. In the Purkinje cells, the expression frequency of Pcdha10 was higher in Pcdha<sup>del(11-c2)/del(11-c2)</sup> than in WT mice (middle of Fig. 3A), whereas the expression frequency of the CR was similar between WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice (top of Fig. 3A). We examined the ratio of Pcdha10-positive cells to total Purkinje cells, using a specific marker for Purkinje cells, Pcp2. Only 12% of the WT Purkinje cells were Pcdha10-positive, whereas 84% of the Pcdha<sup>del(11-c2)/del(11-c2)</sup> Purkinje cells were Pcdha10-positive (Fig. 3B). In addition, the expression frequency of Pcdha10 was close to that of Pcdha1 (77 ± 21%) and Pcdha2 (100 ± 10%). The expression patterns of the other Pcdha genes did not dramatically change in the cerebellum (Fig. 4). No significant change was detected in the number of Pcp2-positive Purkinje cells between the WT and Pcdha<sup>del(11-c2)/del(11-c2)</sup> mice (WT, 434 ± 33 cells per section; 475 ± 43 cells per section; p = 0.18).

Finally, to examine expression of Pcdha genes from the single del(11-c2) allele, we performed single cell RT-PCR and SNP analysis of the Purkinje cells of Pcdha<sup>+/del(11-c2)</sup> mice at P21. We were able to distinguish the del(11-c2) allele (CBA) from the WT allele (JF1) by SNP analysis (Fig. 5A). All of the Purkinje cells analyzed expressed Pcdha10 from the del(11-c2) allele (Fig. 5, B and C). Cells expressing Pcdha1 to Pcdha9 from the del(11-c2) allele were rare (Fig. 5, B and C). In this experiment, we could not detect Pcdha10 from the WT allele, and found Pcdha6 at high frequency. These results indicated that from the del(11-c2) allele, the expression pattern of Pcdha10 changed from random to constitutive, but the expression pattern of the Pcdha1–Pcdha9 genes remained random (only Pcdha10 from the del(11-c2) allele in the 9 of 17 cells). In addition, deletion of the a11–ac2 exons altered the expression from the del(11-c2) allele but not from the WT allele. Therefore, these results suggest that the original expression regulator for the Pcdha1 and Pcdha2 genes in the WT allele regulated Pcdha10 in the del(11-c2) allele. In addition, the dual expression pattern of random and constitutive expression was reallocated among the Pcdha genes of the del(11-c2) allele, suggesting that regulators of the dual expression were conserved for the del(11-c2) allele.

Expression of Pcdha Genes in the del(2–11) Allele—We next examined the effects of deleting exons a2 to a11 in the Pcdha<sup>del(2–11)/del(2–11)</sup> and Pcdha<sup>+/del(2–11)</sup> mice. First, we performed qRT-PCR analysis for the whole brain at P21. In the
Pcdha<sup>del(2–11)/del(2–11)</sup> mice, the expression levels of CR transcripts showed a 1.43-fold increase compared with WT (Fig. 6A). The expression levels of Pcdha1 and Pcdha12 in the Pcdha<sup>del(2–11)/del(2–11)</sup> mice showed 49- and 1.97-fold increases, respectively, whereas those of Pcdha1c1 and Pcdha2c2 showed no significant differences from WT mice (Fig. 6A). In the Pcdha<sup>−/−del(2–11)</sup> mice, the expression levels of the Pcdha1, Pcdha12, and CR transcripts were intermediate between the levels in the WT and Pcdha<sup>del(2–11)/del(2–11)</sup> mice (Fig. 6A).

For the in situ analysis, we used cRNA probes for the Pcdha1, Pcdha12, Pcdha1c1, Pcdha2c2, and CR transcripts in the Pcdha<sup>del(2–11)/del(2–11)</sup> brain at P21. There was no obvious difference in the distribution of the CR (CR in Fig. 6, B–D), Pcdha1c1, or Pcdha2c2 (supplemental Fig. S4) transcripts between the WT and Pcdha<sup>del(2–11)/del(2–11)</sup> brains. However, in the Pcdha<sup>del(2–11)/del(2–11)</sup> brain the Pcdha1-positive cells were dramatically increased, and the Pcdha12-positive cells were slightly increased in the cerebral cortex and hippocampus, compared with the WT brain (Pcdha1 and Pcdha12 in Fig. 6, B–D). These results were consistent with the qRT-PCR analysis.

To quantitate the expression frequency of Pcdha1 and Pcdha12, we examined Purkinje cells. In the Pcdha<sup>del(2–11)/del(2–11)</sup> Purkinje cells, Pcdha1 and Pcdha12 were expressed more frequently than in WT Purkinje cells (Pcdha1 and Pcdha12 in Fig. 7A), but the expression pattern of the CR was not markedly changed (CR in Fig. 7A). We counted the number of Pcdha1- or Pcdha12-positive Purkinje cells and calculated their ratio to the number of Pcp2-positive cells. The proportion of Purkinje cells that were Pcdha1-positive was higher in the Pcdha<sup>del(2–11)/del(2–11)</sup> mice than in WT mice (WT, 4.6%; Pcdha<sup>del(2–11)/del(2–11)</sup>, 61.6%), as was the proportion of Pcdha12-positive cells (WT, 41%; Pcdha<sup>del(2–11)/del(2–11)</sup> 67.7%) (Fig. 7B). No significant changes were observed in the number of Pcp2-positive Purkinje cells between the WT and Pcdha<sup>del(2–11)/del(2–11)</sup> mice (WT, 500 ± 72 per section; Pcdha<sup>del(2–11)/del(2–11)</sup>, 489 ± 19 cells per section; p = 0.79).

Finally, to examine the mutants in more detail, we performed single cell RT–PCR and SNP analysis of the Purkinje cells of the Pcdha<sup>−/−del(2–11)</sup> mice at P21. We distinguished between the del(2–11) allele (CBA) and WT allele (F1) by SNP analysis (Fig. 8A). From the del(2–11) allele, either Pcdha1 or Pcdha12 were expressed in cells 1–4, 6–9, 12–16, 18, and 19, and both Pcdha1 and Pcdha12 were expressed in cells 5, 10, 11, 17, 20, and 21; meanwhile, from the WT allele, Pcdha12 was expressed only in cells 2 and 21 (Fig. 8, B and C). Pcdha1 was not detected from the WT allele, perhaps explained by low frequency of Pcdha1 in the WT allele (Fig. 7B). These data indicated that every del(2–11) allele of the individual Purkinje cells expressed either Pcdha1 or Pcdha12, and that deletion of the a2–a11 exons did not change the expression from the WT allele. The expression of Pcdha1 and Pcdha12 from the del(2–11) allele appeared to be random, although their expression frequencies were high. On the other hand, the expression patterns of Pcdha1c1 and Pcdha2c2 from the del(2–11) allele were unchanged, i.e. constitutive, and they were expressed at almost the same level (see Figs. 6A, 8, B and C, and supplemental Fig. S4). These results suggested that the dual expression regulation was conserved in
The deletion of the randomly expressed variable exons increased the expression frequency of exons a1 and a12 in individual Purkinje cells.

Expression Levels of Pcdha Genes in the dup(2–10) Allele—To examine the effects of duplicating exons a2 to a10, we used the whole brain of Pcdhadup(2–10)/dup(2–10) mice, and examined the expression levels by qRT-PCR analysis. The expression level of the CR transcript in the Pcdhadup(2–10)/dup(2–10) brain was similar to that in the WT brain (Fig. 9A). In the Pcdhadup(2–10)/dup(2–10) brain, the expression levels of duplicated genes Pcdha2, Pcdha7, Pcdha8, and Pcdha10 were significantly decreased, but those of duplicated genes Pcdha3, Pcdha4, Pcdha5, Pcdha6, and Pcdha9 were unchanged compared with those of the WT brain (Fig. 9A). Thus, the expression levels of the duplicated genes were unchanged or lowered, and never doubled, suggesting that the expression of the duplicated genes was reallocated. Although the expression levels of Pcdha1, Pcdha2, Pcdha7, Pcdha8, and Pcdha10 were decreased, that of CR was not significantly changed, suggesting that these decreases might have been too slight to influence the expression levels of CR, or that the statistical power for detecting changes in CR was insuffi-
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The possibility that the Pcdha$^{\text{dup}(2-10)/\text{dup}(2-10)}$ brain exhibited a slight decrease in CR expression does not conflict with the finding that the Pcdha$^{\text{del}(2-11)/\text{del}(2-11)}$ brain exhibited a slight increase in CR expression. These results raise the possibility that the region between the a2 and a11 exons contains a suppressive element for transcription.

Next, we examined the Pcdha$^{\text{dup}(2-10)/\text{dup}(2-10)}$ brain by in situ hybridization. The distribution of the CR transcript in the Pcdha$^{\text{dup}(2-10)/\text{dup}(2-10)}$ brain was similar to that in the WT brain (Fig. 9B). In the Pcdha$^{\text{dup}(2-10)/\text{dup}(2-10)}$ Purkinje cells, the expression patterns of Pcdha1 to Pcdha12 were random, but those of Pcdha1 and Pcdha4 were constitutive, similar to WT Purkinje cells (data not shown). These results suggested that the dual expression regulation was conserved in the Pcdha$^{\text{dup}(2-10)/\text{dup}(2-10)}$ brain.

Expression Levels of Pcdha Genes from the dup(12-c2) Allele—We examined the effects of duplicating the a12 to ac2 exons using the Pcdha$^{a12}$ and Pcdha$^{\text{dup}(12-c2)/\text{dup}(12-c2)}$ mice, and qRT-PCR analysis. The expression level of the CR transcript in the Pcdha$^{a12}$ and Pcdha$^{\text{dup}(12-c2)/\text{dup}(12-c2)}$ brains was essentially the same as in the WT brain (Fig. 10A). Among the duplicated genes, the expression level of Pcdha2 was significantly increased, but those of Pcdha12 and Pcdha11 were not changed (Fig. 10A). Among the single genes, the expression levels of Pcdha6, Pcdha9, Pcdha10, and Pcdha11 were significantly decreased, but those of Pcdha1, Pcdha5, Pcdha7, and Pcdha8 showed no significant change. Although the expression levels of some genes were changed, that of the CR transcript showed no significant change, suggesting that expression of the Pcdha genes from the dup(12-c2) allele was reallocated.

By in situ hybridization analysis, no obvious difference in the dis-
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We also examined the CR transcript in these mutants. Because all the spliced Pcdha transcripts contain the CR exons, the level of spliced CR transcript was assumed to be equal to the sum of all the spliced transcripts of Pcdha genes. We found that the expression level of the CR transcript in the engineered mutants was hardly influenced by the number of variable exons. The putative cis-elements may therefore determine the overall expression levels of the Pcdha genes in the brain.

Deletions and Duplications Suggest Mechanisms Involving Positional Effects and Random Promoter Choice in the Pcdha Cluster—In the WT mouse, the variable exons located in the most 3’ position of the variable region (ac1 and ac2) are constitutively expressed (5, 6). Likewise, in the mouse bearing the del(11-c2) allele, the most 3’ variable exon (a10) was constitutively expressed. This result indicates that the constitutive expression of Pcdhac1 and Pcdhac2 requires their location at the most 3’ position of the cluster, and suggests that the specific promoters of these genes may not be essential for their constitutive expression. Our results also indicate the existence of a regulatory cis-element for Pcdhac1 and Pcdhac2 outside of the variable exons, a11–ac2, because the constitutive expression was independently regulated by individual alleles.

In the HoxD cluster, serial deletions and duplications revealed that the Hoxd gene located at the 5’ end of the cluster is preferentially expressed in digit development, and the regulation of expression is reallocated among the genes in the mutant HoxD cluster. The cis long-distance digit enhancer is located 5’ upstream of the HoxD cluster (24). The preferential expression from the 5’ end of the HoxD cluster is regulated by enhancer tropism (25, 43). Similarly, the 3’ end of the Pcdha cluster, namely Pcdhac2, was preferentially expressed among the Pcdha genes (data not shown). The Pcdha cluster might therefore be regulated by enhancer tropism like the HoxD cluster. In fact, DNase I-hypersensitive sites, HS7 and HS5-1, are located downstream of the clustered variable Pcdha exons (21) (see Fig. 11). HS5-1 is an enhancer for the Pcdhac1–Pcdhac12 and Pcdhac1 genes, but no enhancer for the Pcdhac2 gene has yet been found (21), although HS7 is a candidate enhancer.

Single cell RT-PCR analysis of Purkinje cells revealed that one or two genes among Pcdha1–Pcdha12 are selectively expressed from the WT Pcdha gene locus (5, 6). Likewise, in the present study, we showed that one or two genes (between Pcdha1 and Pcdha12 were selectively expressed from a Pcdha del(2–11) gene locus at much higher frequency than that seen from the WT Pcdha gene locus. On the other hand, in mice bearing the dup(2–10) allele, each duplicated Pcdha gene appeared to be expressed at a lower frequency. These results indicate that only one or two of the Pcdha1–Pcdha12 genes is
expressed selectively, independent of the number of a1–a12 exons, although at least two genes among Pcdha1–Pcdha12 are necessary. This phenomenon can be explained by the idea that a putative cis-element selects one or two gene(s) within Pcdha1–Pcdha12. In other words, the cis-element may be competitively shared by all of the randomly regulated variable exons. In the β-globin and HoxD clusters, common enhancers are thought to regulate the differential transcription by promoter competition (25, 44, 45). Likewise, the differential expression of the Pcdha cluster may be controlled by a common enhancer, and our data are consistent with a competition mechanism, resulting in random promoter choice. The putative cis-element is thought to be located outside of the variable region, because the random expressions were maintained in mice bearing the del(2–11) or dup(2–10) allele. The putative cis-element may be HS5-1.

Potential Functional Significance of the Random and Constitutive Pcdha Gene Expression in Neurons—The Pcdha cluster encodes 14 kinds of single pass transmembrane proteins. The a1–a12, ac1, and ac2 exons encode six cadherin-like extracellular domains, a transmembrane domain, and part of a cytoplasmic domain, and the CR1–CR3 exons encode the rest of the cytoplasmic domain (4, 19, 46). In amino acid sequence, the variable regions of Pcdha1–Pcdha12 are similar to each other, and are distinct evolutionarily from those of Pcdhac1 and Pcdhac2 (47, 48). The extracellular 1 domains of Pcdha1–Pcdha12 have an Arg-Gly-Asp (RGD) sequence, which binds to integrin-β1 (49), whereas the extracellular 1 domains of Pcdhac1 and Pcdhac2 have no RGD sequence (47). Thus, Pcdha1–Pcdha12 are functionally different from Pcdhac1 and Pcdhac2. Furthermore, the expression patterns of Pcdha1–Pcdha12 are different from those of Pcdhac1 and Pcdhac2 (5, 6). This expression difference may also reflect distinct functions of the Pcdhas.

In this study, we showed evidence for two independent regulatory mechanisms, one directing random expression and one directing constitutive expression of the variable exons in the Pcdha cluster (see Fig. 11). Thus, in the WT allele, the Pcdha1–Pcdha12 genes, and the Pcdhac1 and Pcdhac2 genes appear to be regulated independently. These dual expression mechanisms may reflect two different functions of the Pcdha genes in neurons. For instance, the randomly expressed Pcdha1–Pcdha12 may functionally contribute to the enormous diversity of neurons, whereas the constitutively expressed Pcdhac1 and Pcdhac2 may be essential genes for all neurons.

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