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Molecular and Functional Interaction between Protocadherin-γC5 and $GABA_A$ Receptors

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We have found that the γ2 subunit of the $GABA_A$ receptor (γ2-$GABA_A$R) specifically interacts with protocadherin-γC5 (Pcdh-γC5) in the rat brain. The interaction occurs between the large intracellular loop of the γ2-$GABA_A$R and the cytoplasmic domain of Pcdh-γC5. In brain extracts, Pcdh-γC5 coimmunoprecipitates with $GABA_A$Rs. In cotransfected HEK293 cells, Pcdh-γC5 promotes the transfer of γ2-$GABA_A$R to the cell surface. We have previously shown that, in cultured hippocampal neurons, endogenous Pcdh-γC5 forms clusters, some of which associate with GABAergic synapses. Overexpression of Pcdh-γC5 in hippocampal neurons increases the density of γ2-$GABA_A$R clusters but has no significant effect on the number of GABAergic contacts that these neurons receive, indicating that Pcdh-γC5 is not synaptogenic. Deletion of the cytoplasmic domain of Pcdh-γC5 enhanced its surface expression but decreased the association with both γ2-$GABA_A$R clusters and presynaptic GABAergic contacts. Cultured hippocampal neurons from the Pcdh-γ triple C-type isoform knock-out (TCKO) mouse (*Pcdhg*$^{tcko/tcko}$) showed plenty of GABAergic synaptic contacts, although their density was reduced compared with sister cultures from wild-type and heterozygous mice. Knocking down Pcdh-γC5 expression with shRNA decreased γ2-$GABA_A$R cluster density and GABAergic innervation. The results indicate that, although Pcdh-γC5 is not essential for GABAergic synapse formation or $GABA_A$R clustering, (1) Pcdh-γC5 regulates the surface expression of $GABA_A$Rs via *cis*-cytoplasmic interaction with γ2-$GABA_A$R, and (2) Pcdh-γC5 plays a role in the stabilization and maintenance of some GABAergic synapses.

Introduction

Protocadherin-γC5 (Pcdh-γC5) is 1 of the 22 members of the Pcdh-γ family. This family together with the Pcdh-α and Pcdh-β families, constitute the so-called clustered protocadherins (Pcdhs), which in human, rat, and mouse are composed of >50 members. They are called "clustered" because the genes of the three families are arranged in tandem, in a small locus of a single chromosome (Wu and Maniatis, 1999; Wu et al., 2001; Wu, 2005). Clustered Pcdhs are cell adhesion molecules of the cadherin superfamily that are predominantly expressed in the CNS. They interact *in cis* and *in trans* with other Pcdhs via their cadherin repeat ectodomains (for review, see Brusés, 2000; Redies et al., 2000; Frank and Kemler, 2002; Junghans et al., 2005; Morishita et al., 2006; Morishita and Yagi, 2007; Shapiro et al., 2007; Yagi, 2008).

Because of their cell adhesion properties, large number, and combinatorial expression in neurons, it has been proposed that Pcdhs are involved in the establishment of specific patterns of neuronal connectivity (Kohmura et al., 1998; Shapiro and Colman, 1999; Wang et al., 2002b; Kallenbach et al., 2003; Phillips et al., 2003; Esumi et al., 2005; Frank et al., 2005; Kaneko et al., 2006). Alternatively, it has been proposed that Pcdhs are involved in neurite self-avoidance (Zipursky and Sanes, 2010; Lefebvre et al., 2012).

Pcdh-γC5 is one of the three C-type protocadherins (Pcdh-γC3, Pcdh-γC4, and Pcdh-γC5) that are present in the protocadherin-γ gene cluster (*Pcdhg*). This cluster contains 22 variable exons, which by *cis*-splicing of the mRNA, each combine with three downstream constant exons. Each variable exon (including Pcdh-γC5 variable exon) encodes the ectodomain (containing six cadherin repeats; see Fig. 1*A*), the transmembrane domain and the proximal moiety of the cytoplasmic domain (CD). The three constant exons encode the distal moiety of the CD, which is common to all Pcdh-γs and includes the C terminus (see Fig. 1*A*). Pcdh-γs play a role in both neuronal connectivity and in preventing apoptosis of some neurons (Wang et al., 2002b; Weiner et al., 2005; Prasad et al., 2008; Chen et al., 2012).

In neurons, some Pcdh-γs are synaptically localized but not exclusively (Wang et al., 2002b; Phillips et al., 2003; Blank et al., 2004; Frank et al., 2005; Li et al., 2010). Pcdh-γs are also produced by astrocytes and are involved in both perisynaptic and nonsyn-

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aptic neuron–astrocyte interactions (Garrett and Weiner, 2009; Li et al., 2010). We have previously shown that Pcdh-γC5 is associated with a subset of GABAergic synapses (Li et al., 2010).

In this paper, we show that Pcdh-γC5 interacts with γ2-$GABA_A$ receptor (γ2-$GABA_A$R) via their cytoplasmic domains and that this interaction facilitates the localization of $GABA_A$Rs at the cell surface. The results are also consistent with the hypothesis that, although Pcdh-γC5 is not essential for GABAergic synapse formation, it is involved in the stabilization and maintenance of some GABAergic synapses.

Materials and Methods

Animals. All the animal protocols have been approved by the Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines. Rat brains were used in all experiments except for the studies on the Pcdh-γC deletion mutant *Pcdhg*$^{tcko/tcko}$ mouse cultures. The generation of this triple C-type Pcdh-γ knock-out (TCKO) mouse has been described previously (Chen et al., 2012). This mouse is deficient in the three C-type Pcdh-γs (Pcdh-γC3, Pcdh-γC4, and Pcdh-γC5). For hippocampal neuronal cultures, rat and mouse embryos of either sex were used. For rat brain membrane preparation, female rats were used.

Antibodies. Two rabbit (Rb) antibodies (from two New Zealand female rabbits) to synthetic peptides of the deduced amino acid sequence of the rat Pcdh-γC5 (GenBank accession number GQ131870) were raised in our laboratory as described previously (Li et al., 2010). The Rb antibody to the N terminus amino acids 1–14 (QLRYSVVEESEPGT-C) is specific for Pcdh-γC5 and it does not recognize other Pcdhs. We call this antibody anti-Pcdh-γC5, and it has been characterized previously (Li et al., 2010). The Pcdh-γC5 peptide epitope recognized by this antibody is identical in rat, mouse, and human. In immunoblots of rat brain membranes, the affinity-purified antibody (purified on immobilized antigen) recognizes a 120,000 Mr polypeptide. We have used anti-Pcdh-γC5 to study the regional, cellular, and subcellular localization of Pcdh-γC5 in neuronal cultures and rat brain during development (Li et al., 2010). This antibody precipitated Pcdh-γC5 from brain extracts. Similarly, a Rb antibody to the C terminus amino acids 902–915 (C-GNGNKKKSGKKEKK), which is also common to rat, mouse, and human Pcdh-γC5, was generated. We call this antibody anti-Pcdh-γC5(C). In immunoblots, it recognizes the 120,000 Mr Pcdh-γC5 protein. This is a pan-Pcdh-γ antibody, since the C terminus amino acid sequence recognized by this antibody is common to all members of the Pcdh-γ family. The anti-Pcdh-γC5 and anti-Pcdh-γC5(C) were affinity-purified on their respective immobilized peptide antigen and used in the experiments described below. The guinea pig (GP) anti-α1 (amino acids 1–15), Rb anti-α1 (amino acids 1–15), Rb anti-γ2 (amino acids 1–15), and GP anti-γ2 (amino acids 1–15) of rat $GABA_A$R subunits were raised and affinity-purified (on immobilized antigen peptide) in our laboratory. The mouse monoclonal antibody (Ms mAb) to β2/3 $GABA_A$R subunit was also generated in our laboratory. The generation, affinity purification, specificity, and characterization of these anti-$GABA_A$R antibodies have been described previously (De Blas et al., 1988; Vitorica et al., 1988; Ewert et al., 1992; Miralles et al., 1999; Christie et al., 2002a,b, 2006; Riquelme et al., 2002; Christie and De Blas, 2003; Charych et al., 2004a,b; R. W. Li et al., 2005a; Yu et al., 2007, 2008; Yu and De Blas, 2008; X. Li et al., 2009; Y. Li et al., 2010).

The sheep anti-glutamic acid decarboxylase (GAD) was from Dr. Irwin J. Kopin (NINDS, Bethesda, MD). The GP anti-vesicular GABA transporter (VGAT) (catalog #131004) and the Ms mAb to gephyrin (clone mAb7a; catalog #147021) were from Synaptic Systems. The Ms mAb to postsynaptic density 95 (PSD-95) was from Millipore (clone 6G6-1C9; catalog #MAB1596; used with rat cultures) or from NeuroMab (clone K28/43; catalog #73-028; used with mouse cultures). The GP anti-VGLUT1 was from Millipore Bioscience Research Reagents (catalog #AB5905). The Ms mAb to the 9E10 cMyc epitope (EQKLISEEDL; clone 4A6; catalog #05-724), the Rb anti-enhanced green fluorescent protein (EGFP) (catalog #AB3080P), the Ms mAb to actin (clone C4; catalog #MAB1501), and Rb anti-glutathione *S*-transferase (GST) (catalog #MAB1372) were from Millipore. Ms mAb to TUJ1 (neuron-specific class III β-tubulin) was from Sigma-Aldrich (catalog #T8578). Ms mAb anti-6xHis tag (N114/14; catalog #73-169) was from NeuroMab. Fluorophore-labeled FITC, Texas Red, or AMCA (aminomethylcoumarin) species-specific anti-IgG antibodies were made in donkey (Jackson ImmunoResearch Laboratories).

Yeast two-hybrid. The yeast two-hybrid (Y2H) assay and the screening of a rat brain library has been described previously (Charych et al., 2004a,b). We used as bait the large intracellular loop (IL) of the γ2 short subunit (γ2IL) corresponding to amino acids 318–404 of the rat γ2-$GABA_A$R (GenBank NP_899156). For bait construction, sense and antisense oligonucleotide primers were designed to amplify the IL of γ2-$GABA_A$R. The γ2IL DNA, containing a stop codon at the C-end of the encoded IL peptide, was directionally inserted into the pEG202 polylinker. We confirmed that (1) LexA-γ2IL fusion protein did not activate the *LacZ* reporter and (2) the LexA-γ2IL bait did not activate the genomic LEU2 reporter gene as described previously (Charych et al., 2004a,b). For the positive control, the yeast was transformed with pSH18-34 and pSH17-4, the latter of which contains the LexA DNA binding domain. For a negative control, the yeast was transformed with pSH18-34 and pRHFM1, the latter containing the bicoid protein bait, or with pSH18-34 and pEG202, the empty bait vector. The Y2H procedure for screening the pJG4-5 containing oligo-dT primed rat brain cDNA library (OriGene Technologies) has been described previously (Charych et al., 2004a,b). To test the specificity of the interaction of the CD fragment of Pcdh-γC5 (clone GS113) with the γ2IL, bait pEG202 plasmids containing the IL of other subunits with a stop codon were constructed (β3IL amino acids 303–425, α1IL amino acids 307–393, and γ3IL amino acids 321–427) (Khrestchatisky et al., 1989; Ymer et al., 1989; Khan et al., 1993; Fernando et al., 1995).

To map the binding site of the γ2IL for the cytoplasmic moiety of Pcdh-γC5 (clone GS113), various truncations of the γ2IL cDNA, with a stop codon added at the C terminus, were subcloned into pEG202. To map the Pcdh-γC5 binding site for the γ2IL, various truncations of the Pcdh-γC5 CD were subcloned in pJG4-5. To determine whether γ2IL specifically interacts with Pcdh-γC5 but not with other Pcdh-γs, the CDs of various Pcdhs (Pcdh-α4, Pcdh-γA3, and Pcdh-γC3), including their natural stop codon, were amplified by PCR using as template a Marathon-ready rat brain cDNA library (Clontech) and subcloned in pJG4-5. The quality of all the cloned DNAs was verified by DNA sequencing. We also confirmed that for each of the constructs the protein was expressed in yeast *Saccharomyces cerevisiae* EGY48 by immunoblotting the cell lysate of yeast transformants with mouse anti-LexA mAb or Ms anti-HA mAb.

Cloning the full-length rat Pcdh-γC5. We used the Marathon-ready rat brain cDNA library (Clontech), containing full-length cDNA clones, as template. A 5′-RACE PCR was done using the forward 5′ library adaptor primer 1 and the antisense 5′-CTGTGGGCCGCAGGGTCACCTCCA TG-3′ primer, which was designed from the rat GS113 clone that we isolated by Y2H during the screening. The 5′-RACE product was used as a template in two nested PCRs whose products had overlapping fragments of the rat Pcdh-γC5 cDNAs, covering the whole sequence between both fragments. For the first nested PCR, the sense primer corresponded to the mouse Pcdh-γC5 cDNA sequence (5′-GGCTCTCTCCTCTGTA CTGTGGCTGCC-3′) and the antisense primer corresponded to a rat sequence in GS113 (5′-ACTCCCTGGAGGGCGAGTCCTGG-3′). A 800 bp DNA fragment was obtained, cloned into pCR-XL-TOPO (TOPO XL PCR cloning kit; Invitrogen), and sequenced. This rat sequence was used to design the antisense primer for the second nested PCR. For this PCR, the aforementioned 5′-RACE product was also used as template. The sense primer corresponded to a mouse Pcdh-γC5 sequence localized at the 5′-UTR (5′-CAGCTTCTGCACTCCAGGCTCTGGG-3′) and the antisense primer corresponded to a rat Pcdh-γC5 sequence (5′-GGGTTGACATACACGAAGGAGGAGGCTGGG-3′). A 1500 bp cDNA fragment was subcloned and sequenced. Once the sequences of the two rat Pcdh-γC5 cDNA fragments were determined, rat primers were designed for the cloning of the complete coding region of the rat Pcdh-γC5 cDNA by a nested PCR procedure using the Marathon-ready rat cDNA library as a template. The first PCR included a sense primer correspond-

ing to a cDNA sequence of the rat Pcdh-γC5 located at the 5′-UTR (5′-GCTCTCCAAGAAGGGACTTCTGGG-3′) and an antisense corresponding to a sequence of the rat Pcdh-γC5 located in the 3′-UTR (5′-GGGAGGCTGCCCTGTGGCTCAAGGCC-3′). The PCR product was used as template in a second PCR with two nested primers: a sense primer starting 4 bp upstream from the start codon (5′-GGTCATGGGGCCTATGGCATCACCACAGGTCACTGG-3′) and an antisense primer starting 9 bp downstream from the stop codon (5′-GCCTCCATATTACTTCTTCTCTTTCTTGCCCGACTTCTTCTTGTTGCC-3′). A 2.8 kb DNA fragment was generated, purified, and cloned with the T/A cloning method into pCR-XL-TOPO plasmid and sequenced. The PCR primers were designed such that no mouse sequences from the initial PCR primers were carried into the final cloned rat sequence. We have submitted the rat Pcdh-γC5 cDNA to GenBank (accession number GQ131870).

Preparation of the Pcdh-γC5 constructs. The 9E10 cMyc epitope (EQKLISEEDL) was inserted between amino acids 103 and 104 of the mature (after cleavage of the signal peptide) ^{cMyc}Pcdh-γC5 protein (Full), corresponding to the linker region between first and second EC1 and EC2 extracellular cadherin repeats (see Fig. 1*A*, Full, arrow). Two Pcdh-γC5 deletion constructs were made by PCR using as template the full-length rat ^{cMyc}Pcdh-γC5 cDNA clone. The ^{cMyc}Pcdh-γC5-extra construct (see Fig. 1*A*, Extra) contained the signal peptide (29 aa long), the full extracellular domain (660 aa long plus the cMyc-tag), the transmembrane domain (23 aa long), and 10 aa of the intracellular domain (AKCLRRHEDR) adjacent to the transmembrane domain. The ^{cMyc}Pcdh-γC5-intra membrane-bound construct (see Fig. 1*A*, Intra) contained the signal peptide, amino acids 1–12 of the Pcdh-γC5 N terminus (QLRYSVVEESEP) followed by a PKLG linker, the 9 aa of the extracellular domain (amino acids 652–660 of the mature Pcdh-γC5, LTHPPERSD) that are adjacent to the transmembrane domain, the transmembrane domain, and the full cytoplasmic domain of Pcdh-γC5 (232 aa long). The 9E10 cMyc tag was inserted between amino acids 19 and 20 of the short extracellular mature peptide by using the Gene Editor *in vitro* Site-Directed Mutagenesis System (Promega) as described previously (Christie et al., 2006). The cMyc-tagged constructs were directionally cloned into pcDNA3.1(+) vector in the NheI and EcoRI sites.

The Pcdh-γC5-extra-EGFP membrane-bound construct (Extra-EGFP) was prepared in pEGFP-N1. This construct had the whole cytoplasmic domain of Pcdh-γC5 replaced by the EGFP tag (see Fig. 1*A*).

Generation of the small hairpin RNAs of Pcdh-γC5. The procedure has been described previously for other small hairpin RNAs (shRNAs) (R. W. Li et al., 2005b; Yu et al., 2007; X. Li et al., 2009). A shRNA (sh1) targeting a sequence of the extracellular variable region of Pcdh-γC5 mRNA (5′-ATACATCTGAAGCAGTGAAGA-3′, nucleotides 851–871 from start codon) was subcloned into mU6pro vector. The shRNA contains a 21 nt antisense sequence that perfectly matches the target mRNA followed by a loop and the sense sequence, which contains a single mismatch in the middle to facilitate sequencing. Two DNA oligonucleotides encoding each strand of the shRNA were synthesized, annealed, and inserted into BbsI and XbaI sites of mU6pro vector polylinker. A control shRNA (sh1 3m) containing three point mutations was also made. A rescue ^{cMyc}Pcdh-γC5 mRNA was generated by introducing five silent mutations in five consecutive codons (ACATCTGAAGCAGTG → ACGTCCGAGGCTGTT both encoding TSEAV) in the sh1 target region with the GeneTailor Site-Directed Mutagenesis System (Invitrogen).

Preparation of bacterial fusion proteins and in vitro *interaction assay.* Bacterial expression and purification of glutathione *S*-transferase GST-γ2IL (36 kDa) and GST (27 kDa) fusion proteins were done as reported previously (Fernando et al., 1995). The CDs of Pcdhs were subcloned into pET-32a(+) (Novagen), to generate the His-tag bacterial fusion proteins. These have a 18 kDa peptide, containing His-tag, thioredoxin tag, and S-tag added to the N terminus of the Pcdh CD. His-Pcdhs (His-Pcdh-γC5, 45 kDa; His-Pcdh-γC3, 45 kDa; His-Pcdh-γA3, 46 kDa; His-Pcdh-α4, 47 kDa) or His-tag control (18 kDa) proteins were purified from bacterial lysates with His60 Ni Superflow Resin, according to the manufacturer's instructions (Clontech). Equal moles of GST (13 μg) or GST-γ2IL (18 μg), were adsorbed to 50 μl bed volume of glutathione-coated beads, and incubated with 20 μg of purified His-Pcdhs protein or 8.6 μg of purified His-tag control protein in 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5, with a protease inhibitor mixture (Roche; catalog #1697498), overnight at 4°C. After three washes by centrifugation, the bound proteins were eluted from the beads at 4°C with 33 mM glutathione in 50 mM Tris-HCl, pH 8.0. Eluates were analyzed by SDS-PAGE followed by immunoblotting with Ms anti-His and Rb anti-GST antibodies. Image acquisition of protein blots was done with a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences) and analyzed with Odyssey software, version 3.0.

Coimmunoprecipitation of Pcdh-γC5 and $GABA_A$Rs. Rat brain membranes were prepared from female Sprague Dawley rat forebrain as described previously (Li et al., 2010). Detergent extracts were prepared by incubating brain membranes with RIPA buffer (10 mM Tris-HCl, 137 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor mixture (10 μg/ml trypsin inhibitor, type I-S, 10 μg/ml trypsin inhibitor, type II-O, 1 mM benzamidine) for 1.5 h followed by centrifugation at 50,000 × *g* for 1 h. The supernatant was used for immunoprecipitations. The coimmunoprecipitation procedure of [^{3}H]flunitrazepam ([^{3}H]FNZ) binding activity (to $GABA_A$Rs) with the anti-Pcdh-γC5 antiserum has been described previously for another antibody (Charych et al., 2004a). All steps were performed at 4°C. Briefly, a 400 μl aliquot (~600 μg of protein) of the brain membrane RIPA extract was incubated overnight with 50 μl of Rb anti-Pcdh-γC5 antiserum, followed by incubation with 30 μl of protein A-agarose beads for 1 h. After centrifugation, the pellet was washed twice with 500 ml of RIPA buffer in the presence of 1 mM PMSF and protease inhibitors. The binding of 10 nM [^{3}H]FNZ to the pellet (immunoprecipitate) and to the supernatant (100 μl) was performed in a filter assay, as described previously (Charych et al., 2004a). Nonspecific [^{3}H]FNZ binding was determined in the presence of 10 μM clonazepam. Radioactivity was measured with a liquid scintillation analyzer (model Tri-Carb 2900TR; Packard).

For immunoblot analysis of Pcdh-γC5 coprecipitated with anti-$GABA_A$R antibodies, 50 μl of protein A-Sepharose beads (GE Healthcare), suspended in 500 μl of 50 mM Tris-HCl, pH 7.4, was incubated with 50 μl of GP anti-γ2, or GP anti-α1 $GABA_A$R subunit antisera or preimmune (to γ2) GP serum at 4°C overnight. After washing with RIPA buffer, the beads were incubated with 600 μl of the rat brain detergent extract (3 mg of protein) at 4°C overnight and centrifuged, and the pellet was washed with RIPA buffer (containing 1 mM PMSF and the protease inhibitor mixture) four times. The beads were incubated with 70 μl of SDS-PAGE sample dissociation buffer (0.01 M Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 0.005% bromophenol blue) for 20 min at room temperature (RT) followed by centrifugation. The supernatant was collected and placed on boiling water for 8 min and subjected to SDS-PAGE and immunoblotting. In the glycosidase treatment experiments, the boiled supernatant was incubated with either 500 U of endoglycosidase H (Endo H) (New England Biolabs) or 500 U of N-glycosidase F (PNGase F) (New England Biolabs) at 37°C overnight. The digested samples were placed in boiling water for 8 min and subjected to SDS-PAGE and immunoblotting. The immunoreactive protein bands were visualized with a primary Rb anti-Pcdh-γC5 followed by a peroxidase-conjugated secondary antibody and a chemiluminiscence reaction (SuperSignal West Pico Trial Kit; Thermo Fisher Scientific) followed by imaging with ChemiDoc imaging system using Quantity One software (Bio-Rad Laboratories).

Cell cultures and transfections. Hippocampal (HP) neuronal cultures were prepared according to Goslin et al. (1998) as described previously (Christie et al., 2002a,b; Christie and De Blas, 2003). Briefly, dissociated neurons from embryonic day 18 (E18) rat hippocampi (from Sprague Dawley embryos of either sex) were plated at low density (3000–8000 cells per 18 mm diameter coverslip) for immunofluorescence or high density (10,000–20,000 cells per 18 mm diameter coverslip) for transfection, and maintained in rat glial cell conditioned medium up to 21 d. Mouse HP cultures were prepared from E18 embryos of either sex as described above for rat, and maintained in rat glial cell conditioned medium. The human embryonic kidney cell line 293 (HEK293) was cultured in DMEM (Invitrogen) with 10% FBS (Invitrogen) in a 5% CO_2 atmosphere. Cultured rat HP neurons (12 DIV) or HEK293 cells were

transfected with one or a combination of various plasmids as indicated. Two micrograms of each plasmid were used (or 0.5 μg of pEF6-mCherry or 1.5 μg of sh1, sh1 3m, and rescue mRNA plasmid) using the CalPhos Mammalian Transfection Kit (BD Biosciences), according to the instructions provided by the manufacturer. Immunofluorescence was performed 4 d after transfection of HEK293 cells or 6 d after transfection of neurons. For immunoblot experiments, HEK293 cells were cultured in 100 mm plates and transfected with 15 μg of various plasmids.

Protein biotinylation. HEK293 cells in 100 mm culture dishes were transfected with various plasmids as described above. Four days later, the cells were washed twice with PBS, pH 8.0, for 15 min followed by incubation with 1 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) in PBS, pH 8.0, for 30 min. All steps were performed at 4°C. Cells were incubated three times for 10 min each with 100 mM glycine/PBS, pH 8.0, followed by a wash with PBS for 15 min. Cells were incubated with 400 μl of RIPA buffer A (10 mM Tris-HCl, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 1% SDS, pH 8.0) containing the aforementioned protease inhibitor mixture and 1 mM PMSF for 2 h. After centrifugation, the supernatant was incubated with 100 μl of high-capacity Neutravidin-agarose beads (Thermo Fisher Scientific) for 2.5 h. The beads were washed twice with 800 μl of RIPA buffer B (10 mM Tris-HCl, 500 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 1% SDS, pH 8.0) for 15 min each followed by another wash with RIPA buffer A for 15 min. One hundred microliters of SDS-PAGE sample dissociation buffer were then added to the avidin beads and left at room temperature for 20 min. After centrifugation, the supernatant was collected and subjected to SDS-PAGE and immunoblotting.

Immunofluorescence. Immunofluorescence of fixed and permeabilized HP or HEK293 cultures was done as described previously (Christie et al., 2002a,b, 2006; Li et al., 2010). Immunofluorescence of cell surface antigens in neurons or HEK293 cells was done by live-cell incubation with the primary antibody(ies) to the corresponding cell surface antigen(s) at 37°C for 30 min in culture medium in a 5% CO_2 atmosphere. Cultures were washed, fixed, permeabilized, and incubated with 5% normal donkey serum in 0.25% Triton X-100 in PBS at RT followed by incubation for 2 h at RT with a mixture of primary antibodies to the intracellular antigens (in 0.25% Triton X-100 in PBS) followed by incubation for 1 h at RT with a mixture of fluorophore-labeled secondary antibodies in 0.25% Triton X-100 in PBS. Apoptosis in the HP cultures was revealed with a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay using the ApopTag Red *In Situ* Apoptosis Detection Kit from Millipore Bioscience Research Reagents (catalog #S7165) following the manufacturer's recommended procedure.

Image acquisition, analysis, and quantification. Fluorescence images of cultured HP neurons were collected using a Nikon Plan Apo 60×/1.40 objective on a Eclipse T300 microscope (Nikon Instruments) with a CoolSNAP HQ2 CCD camera (Photometrics) driven by IPLab 4.0 (Scanalytics) acquisition software. For total internal reflection fluorescence (TIRF) microscopy, cells were imaged on a Nikon W-TIRF microscope evanescent wave imaging system on a TE2000-E inverted microscope (Nikon Instruments) with an Apo TIRF 100, NA 1.45 objective lens. EGFP fluorescence was excited with an EXCITE 120 (EXFO America) Mercury Halide illuminator. Images were processed with Photoshop 7.0 (Adobe), adjusting brightness and contrast, as described previously (Christie et al., 2002b, 2006; R. W. Li et al., 2005a,b; X. Li et al., 2007).

For the quantification of cMyc clusters in rat neurons transfected with the ^{cMyc}Pcdh-γC5 and derived deletion constructs, 21 transfected neurons per construct (from seven experiments, three cells/experiment) were randomly selected, and the values were calculated as number of clusters per transfected cell. The number of clusters counted was 1495 for ^{cMyc}Pcdh-γC5, 2422 for ^{cMyc}Pcdh-γC5 extra, and 395 for ^{cMyc}Pcdh-γC5-intra. For the quantification of the effect of each cMyc-tagged construct on the density of γ2 clusters in transfected neurons, 80–90 dendritic fields (100 μm^2 per field) from 40 to 45 transfected neurons per construct (two dendritic fields per neuron) were randomly selected from eight to nine transfection experiments (five neurons/experiment/construct). For each cMyc-tagged construct, the number of GAD terminals contacting 15–21 neurons (from six experiments) was also determined. The total number of puncta counted per construct was 3168–5940 γ2 clusters and 590–1036 GAD+ boutons depending on the construct. Cluster density was calculated as number of clusters per 100 μm^2 of dendritic surface. The presynaptic GAD innervation was calculated as the number of GAD+ boutons contacting each transfected cell (20 cells in five transfection experiments). Clusters were counted after the maximum intensities of the fluorophore channel were normalized and the low intensity and diffuse nonclustered background fluorescence signal seen in the dendrites was subtracted. Clusters colocalization in two fluorescence channels was determined by overlaying the images. A cluster in a fluorescence channel was considered to colocalize with a cluster in the other channel when >66% of surface of one of the clusters overlapped with the other cluster. For determining the effect of Pcdh-γC5 shRNAs in HP neurons, 15 transfected neurons were randomly selected from three independent experiments (five cells/experiment) and two dendrites from each neuron. The number of Pcdh-γC5 and γ2 clusters in each 100 μm^2 dendritic field (a dendritic field/dendrite) was determined as well as the number of GAD+ boutons per cell. The total number counted per condition was 652–1641 for cMyc clusters; 614–965 for γ2 clusters, 166–302 for GAD, 768–790 for VGLUT1, and 780–819 for PSD-95.

For quantification of the mouse nontransfected mature HP cultures, data were collected from two culture experiments (each from a different pregnant rat). Individual cultures were prepared from each sister embryo. The tail of each embryo was collected at the time of HP dissection. Genotyping was assessed by PCR analysis after cell plating of all cultures. For quantification of cluster/boutons density in 21 DIV cultures, 84 dendrites from 14 neurons (6 dendrites/neuron) from five coverslips were quantified for each genotype. The number of clusters or boutons present in 100 μm^2 of each dendrite was calculated. The number of puncta counted for each genotype was 1250–1536 for gephyrin clusters, 1176–1502 for γ2 clusters, and 940–1387 for VGAT boutons. Quantification of the percentage of GAD+ neurons in the wild-type (WT) and homozygous (HMZ) cultures was calculated from analyzing 457 and 343 neurons from five and four cultures, respectively. Quantification of the percentage of TUNEL+ neurons in the WT and HMZ cultures was calculated from analyzing 318 and 263 neurons from four and three cultures, respectively. For quantification of clusters in HEK293 cells, for each construct, 10 specifically transfected cells randomly selected from three experiments (three to four cells/experiment) were used. The number of cMyc clusters per construct counted was 200–2175 and 211–913 for γ2. All values are given as mean ± SEM.

Results

Yeast two-hybrid and *in vitro* pull-down assays show a specific and direct interaction between the cytoplasmic domain of Pcdh-γC5 and the large intracellular loop of the γ2 $GABA_A$R subunit

Clone GS113 was isolated from the rat brain cDNA library in a Y2H assay. It showed strong interaction with the large IL of the γ2 short subunit (γ2IL) of the $GABA_A$R. The γ2IL was used as bait in the library screen. Clone GS113 includes the following: (1) a 693 bp open reading frame encoding the 231 aa C-terminal polypeptide of the Pcdh-γC5; (2) the stop codon; and (3) a 222 bp 3′-UTR. In addition, GS113 contains a poly(A) tail. The encoded C terminus protein fragment had 98.3 and 93.1% identity to the analogous polypeptide fragment of the mouse and human Pcdh-γC5, respectively. GS113 encodes 231 of the 232 aa of the cytoplasmic domain of Pcdh-γC5 (Fig. 1*A*, GS113). Only 1 aa adjacent to the putative transmembrane domain of Pcdh-γC5 was missing from the CD. Of the 231 aa, the 107 aa polypeptide proximal to the transmembrane domain (Fig. 1*A*, GS113, orange) plus the missing amino acids correspond to the cytoplasmic variable region, which is specific for Pcdh-γC5. The 124 aa C-terminal polypeptide, distal to the transmembrane domain (Fig. 1*A*, GS113, blue), corresponds to the constant cytoplasmic region, which is common to all Pcdh-γs. In the Y2H assay (Table 1, part A), GS113 also showed strong interaction with γ3IL but

did not interact with the ILs of α1 and β3 GABA$_A$R subunits or the cytoplasmic 50 aa C terminus polypeptide of the GluA3 (GluR3) AMPA receptor subunit (fragment named GluA3C in Table 1, part A).

Additional Y2H experiments showed that γ2IL did not interact with Pcdh-γC3 CD, Pcdh-γA3 CD, or Pcdh-α4 CD (Table 1, part B), indicating that γ2IL specifically interacts with Pcdh-γC5 but not with other protocadherins. Moreover, *in vitro* pull-down assays with bacterial fusion proteins, confirmed that the γ2IL interacts with the Pcdh-γC5 CD but not with the CD of other Pcdhs tested. Thus, His-Pcdh-γC5 CD was pulled down by immobilized GST-γ2IL (Fig. 1*B*, red asterisk; *C*, lane 1). In contrast, His-Pcdh-γA3 CD, His-Pcdh-γC3 CD, and His-Pcdh-α4 CD were not pulled down by GST-γ2IL (Fig. 1*B*). However, His-Pcdh-γC5 CD was not pulled down by the GST control protein (Fig. 1*C*, lane 2), even when more GST than GST-γ2IL was immobilized on the beads. The Y2H and pull-down experiments strongly indicate that γ2IL specifically interacts with the Pcdh-γC5 CD but not with that of other Pcdhs, including the closely related Pcdh-γC3. Moreover, the pull-down experiments showed that γ2IL and Pcdh-γC5 CD directly interact with each other, not needing additional proteins.

Further analysis showed that both the variable and constant regions of the Pcdh-γC5 CD are essential for the interaction with γ2IL. Complete or partial deletion of the variable or constant regions of Pcdh-γC5 CD eliminated the interaction with γ2IL (Table 1, part C). These results are consistent with the absence of interaction of the CD of Pcdh-γC3, Pcdh-γA3, or Pcdh-α4 with γ2IL as shown above.

We have also identified the domain of the γ2IL-GABA$_A$R that interacts with Pcdh-γC5 CD. This region, corresponding to amino acids 362–404 located at the C-end of γ2IL (Table 1, part D), is highly conserved among the ILs of the GABA$_A$R gamma subunits (Khan et al., 1993; Fernando et al., 1995). However, it differs from the corresponding region in the non-gamma GABA$_A$R subunits. This is consistent with the observed strong interaction of GS113 with both γ2IL and γ3IL and the lack of interaction with α1IL and β3IL (Table 1, part A).

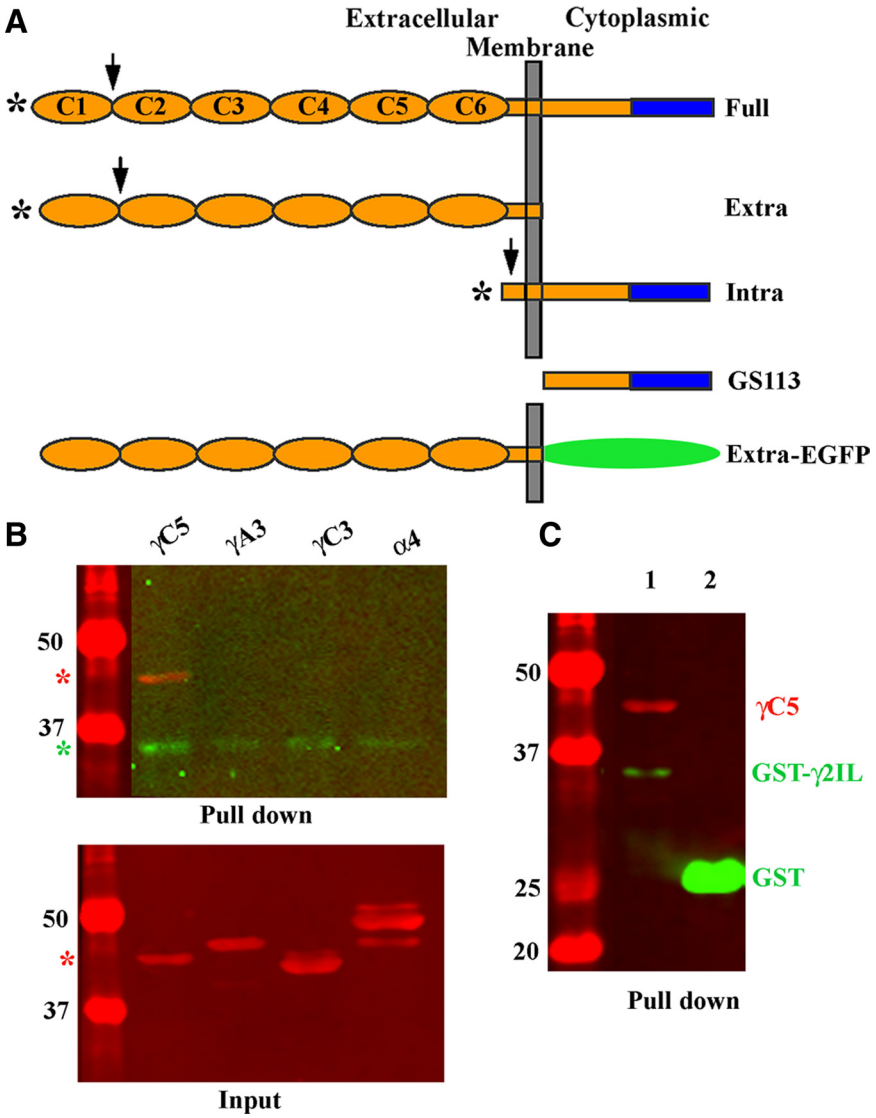

Figure 1. Diagram of the Pcdh-γC5 constructs and *in vitro* interaction of Pcdh-γC5 CD with γ2IL-GABA$_A$R subunit. ***A***, ^{cMyc}Pcdh-γC5 (Full) has six cadherin repeats (C1–C6) in the ectodomain. An exon encodes the variable region (orange), which has an amino acid sequence specific for Pcdh-γC5. The distal part of the cytoplasmic domain (blue) is common to all Pcdh-γs and is encoded by three constant exons. The membrane-bound ^{cMyc}Pcdh-γC5-extra (Extra) has the six cadherin repeats, but the cytoplasmic domain is missing. The membrane-bound ^{cMyc}Pcdh-γC5-intra (Intra) has the cytoplasmic domain and a small piece of the ectodomain, but the six cadherin repeats are missing. GS113 was isolated by Y2H from a rat brain library using the γ2IL as bait. It corresponds to the cytoplasmic Pcdh-γC5. The arrows show the location of the cMyc epitope. The asterisk shows the position of the epitope (N terminus) recognized by the Rb anti-Pcdh-γC5. The cMyc and N terminus epitopes are present in the three ^{cMyc}Pcdh-γC5 membrane-bound constructs. The membrane-bound Extra-EGFP has the whole cytoplasmic domain of Pcdh-γC5 replaced by the EGFP tag (green). The diagrams depict the mature membrane-bound Pcdh-γC5 constructs after removal of the signal peptide. ***B***, *In vitro* pull down of bacterial fusion proteins. The upper double immunofluorescence blot shows that His-Pcdh-γC5 CD (γC5, red asterisk) was pulled down by GST-γ2IL. However, His-Pcdh-γA3 CD (γA3), His-Pcdh-γC3 CD (γC3), or His-Pcdh-α4 CD (α4) was not pulled down by GST-γ2IL. All lanes show that GST-γ2IL (green asterisk) was eluted by glutathione. The bottom immunoblot shows the input of the corresponding Pcdh CDs. ***C***, Double immunofluorescence blot. His-Pcdh-γC5 CD (red band, lane 1) was pulled down by GST-γ2IL (green band, lane 1) but not by GST (green band, lane 2) even when more GST was bound to the glutathione beads. His-Pcdh CD fusion proteins were detected with a Ms anti-His mAb, while GST or γ2-GST were detected with a Rb anti-GST Ab. In each blot, the left lane has protein markers (red protein bands) with the molecular weight shown in kilodaltons (black numbers at the left).

Cloning and sequencing the full-length rat Pcdh-γC5

We cloned and sequenced the full-length rat Pcdh-γC5 cDNA from a rat brain cDNA library, as described in Materials and Methods. We have submitted the sequence to GenBank (accession number GQ131870). The Pcdh-γC5 cDNA includes 3138 bp of the rat Pcdh-γC5 mRNA (81 bp of the 5′-UTR, the full 2832 bp coding region, a stop codon, and the full 222 bp 3′-UTR) plus a poly(A) tail. The open reading frame encodes a 944-aa-long polypeptide with 96.7 and 92.4% identity with the mouse (GenBank NM033583) and human transcript variant 1 (GenBank NM018929) Pcdh-γC5 protein, respectively, which are also 944 aa

Table 1. The cytoplasmic domain of Pcdh-γC5 interacts with the large intracellular loop of the γ2 GABA$_A$R subunit

A

Bait	Interaction with Pcdh-γC5 CD (LacZ Reporter)
γ2IL	+++
γ3IL	+++
α1IL	–
β3IL	–
GluA3C	–
PEG202	–

B

Prey	Interaction with γ2IL bait
Pcdh-α4	–
Pcdh-γA3	–
Pcdh-γC3	–

C

Prey	Amino acids	Interaction with γ2IL bait
Pcdh-γC5 CD	686 - 915	+++
Pcdh-γC5 CD - variable	686 - 791	–
Pcdh-γC5 CD - constant	792 - 915	–
Pcdh-γC5 CD	739 - 844	–
Pcdh-γC5 CD	686 - 844	–
Pcdh-γC5 CD	739 - 915	–

D

Bait	Amino acids	Interaction with Pcdh-γC5 CD
γ2IL	318 - 404	+++
γ2IL	318 - 355	–
γ2IL	337 - 377	–
γ2IL	362 - 404	+++
γ2IL	378 - 404	++

long. Of the 944 aa, 29 correspond to the signal peptide. The amino acids 1–660 of the mature protein correspond to the extracellular domain, amino acids 661–683 to the putative transmembrane domain, and amino acids 684–915 to the cytoplasmic domain. The extracellular domain, the putative transmembrane domain, and the 108 aa of the cytoplasmic domain proximal to the transmembrane domain are encoded by the same exon and have an amino acid sequence unique to Pcdh-γC5 (Fig. 1*A*, Full, orange). The cytoplasmic 124 aa C-end peptide is encoded by three exons and has a sequence common to all Pcdh-γs (Fig. 1*A*, Full, blue).

The amino acid sequence of the translated open reading frame of the mature (after cleavage of the signal peptide) and nonglycosylated rat Pcdh-γC5 corresponded to a molecular weight of 99,018 Da. Nevertheless, the Mr of the glycosylated protein in the brain is ~120,000 as we have previously shown (Li et al., 2010). When the complete sequence of the rat Pcdh-γC5 cDNA was compared with the rat genome sequence, we found that it aligned with four exons at region P11 of rat chromosome 18. One exon corresponded to the variable region unique to Pcdh-γC5, while three exons encoded the constant region. The last of these three exons also encoded the 3′-UTR.

Pcdh-γC5 and GABA$_A$Rs coimmunoprecipitate in rat brain extracts

The interaction of Pcdh-γC5 and GABA$_A$R in rat brain extracts was demonstrated by coimmunoprecipitation of GABA$_A$Rs with Pcdh-γC5. Figure 2*A* shows that the anti-Pcdh-γC5 antiserum coimmunoprecipitated GABA$_A$Rs, as shown by the presence in the immunoprecipitate of specific [^{3}H]FNZ binding (total minus nonspecific binding). Thus, 23% of the solubilized GABA$_A$Rs were precipitated by the anti-Pcdh-γC5 antiserum. The presence of specific [^{3}H]FNZ binding activity indicated that (1) the coprecipitated GABA$_A$Rs had the γ2 subunit and (2) that this subunit was incorporated into fully assembled GABA$_A$Rs, since only fully assembled pentamers containing the γ2 subunit (plus α and β subunits) show [^{3}H]FNZ binding. [^{3}H]FNZ binds to the interface between the α and the γ2 subunits and amino acids from both subunits contribute to [^{3}H]FNZ binding. The γ2 subunit without α and β subunits show no [^{3}H]FNZ binding, and the GABA$_A$R pentamers having α and β subunits, but not γ2, neither show [^{3}H]FNZ binding. The specificity of Pcdh-γC5 interaction with γ2 observed in Y2H was also observed in the brain extracts, since a specific anti-Pcdh-γC4 Rb antibody to an extracellular epitope of Pcdh-γC4, made in our laboratory, did not precipitate [^{3}H]FNZ binding (data not shown).

The interaction between GABA$_A$Rs and Pcdh-γC5 was also demonstrated by the reciprocal coimmunoprecipitation experiment. An anti-γ2 and an anti-α1 GABA$_A$R subunit antiserum each coprecipitated the 120 kDa Pcdh-γC5 protein from brain extracts (Fig. 2*B*, asterisk and arrow). Moreover, the γ2 antiserum precipitated a stronger Pcdh-γC5 protein band of ~140 kDa (Fig. 2*B*, arrowhead). The anti-γ2 GP preimmune serum (PIS) did not precipitate any of the two Pcdh-γC5 proteins. The immunoreactivity of Pcdh-γC5 with the coprecipitated 120 and 140 kDa proteins was blocked by 50 μg/ml antigenic peptide (Fig. 2*C*). Further evidence that the coprecipitated 120 and 140 kDa proteins corresponded to Pcdh-γC5 was obtained by showing that another Pcdh-γC5(C) antibody to a different epitope recognized both the 120 kDa (Fig. 2*D*, arrow and asterisk) and the 140 kDa protein (Fig. 2*D*, arrowhead). For the experiment in Figure 2*D*, the electrophoresis conditions were aimed to increase the separation between the two protein bands.

To determine whether the 120 and 140 kDa peptides represent different glycosylation forms of the same Pcdh-γC5 polypeptide, the immunoprecipitates obtained with anti-γ2 or anti-α1 were digested with Endo H or PNGase F followed by SDS-PAGE and immunoblotting with anti-Pcdh-γC5. Endo H did not affect the mobility of the 120 or 140 kDa proteins, while PNGase F increased the mobility of both proteins, maintaining their relative mobility to each other (Fig. 2*E*). These results indicate that (1) the different mobility of the

two proteins is not due to differential glycosylation of the same polypeptide, (2) both protein are glycosylated, and (3) both glycosylated forms correspond to mature post-endoplasmic reticulum Pcdh-γC5. The 140 kDa protein was also present in the input (Fig. 2*E*, arrowhead), although in a much lower concentration than the 120 kDa protein (Fig. 2*E*, arrow). These experiments and the ones presented in Figure 2, *B* and *D*, show that the anti-γ2 antiserum preferentially coprecipitates the less abundant 140 kDa protein over the more abundant 120 kDa protein. In contrast, the anti-α1 antiserum preferentially coprecipitates the 120 kDa over the 140 kDa protein. Nevertheless, anti-α1 also precipitates the 140 kDa protein and anti-γ2 also precipitates the 120 kDa protein. Both anti-α1 and anti-γ2 precipitate the 120 kDa protein to a similar extent.

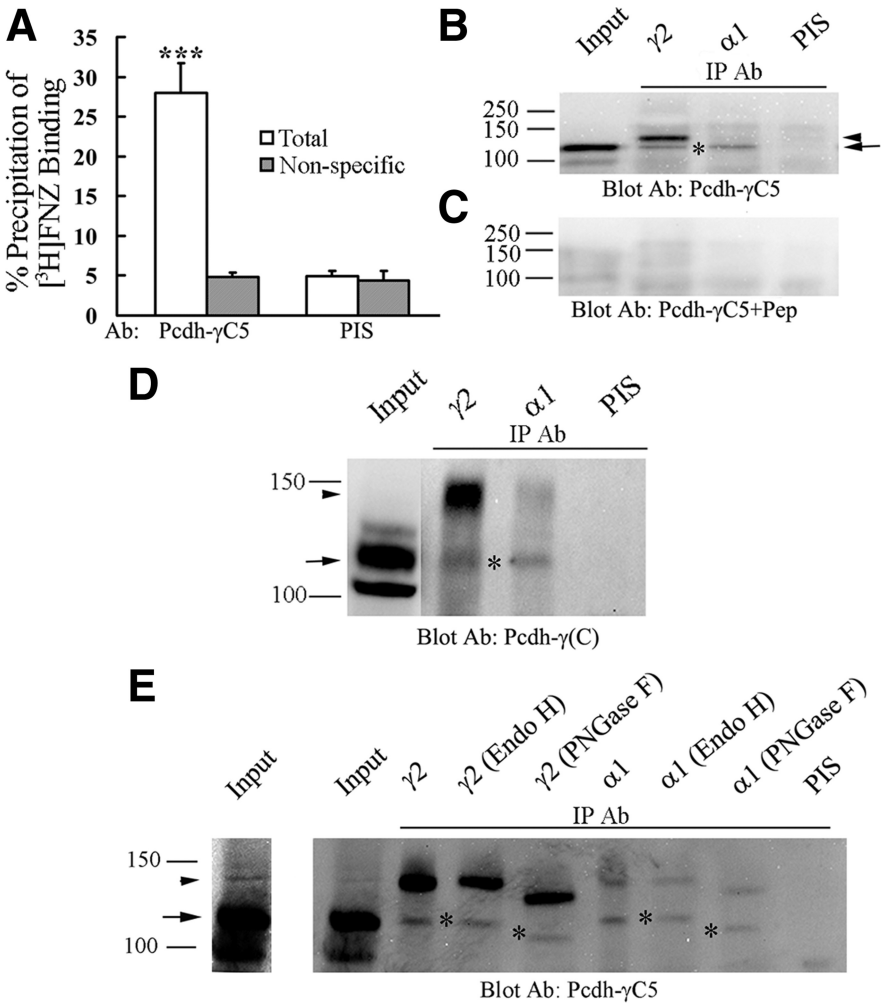

Figure 2. Pcdh-γC5 and $GABA_A$Rs coprecipitate in rat brain extracts. ***A***, The anti-Pcdh-γC5 antiserum precipitates [^{3}H]FNZ binding activity of solubilized $GABA_A$Rs from a rat forebrain membrane fraction. The total [^{3}H]FNZ binding activity precipitated by 50 μl of anti-Pcdh-γC5 antiserum (27.9 ± 3.8%) is significantly higher ($n = 4$; ***$p < 0.001$ in two-tailed Student's *t* test) than both the nonspecific binding activity (not displaceable by excess of clonazepam) that was precipitated by this antiserum (4.9 ± 0.5%) and the binding activity precipitated by 50 μl of the PIS (4.9 ± 0.6%) from the same rabbit that produced the anti-Pcdh-γC5 antiserum. Thus, the specific binding (total binding minus nonspecific binding) is 23%. Error bars indicate SEM. ***B***, Anti-γ2 and anti-α1 $GABA_A$R subunit antisera (made in GP) but not the γ2 GP PIS precipitate the 120 kDa Pcdh-γC5 protein (asterisk and arrow), as shown by immunoblotting with Rb anti-Pcdh-γC5. In addition, the γ2 antiserum precipitates a stronger protein band of 140 kDa (arrowhead). The α1 antiserum also precipitates the 140 kDa protein to a lesser extent. The PIS in ***B–E*** corresponds to preimmune serum from the same rabbit that produced the anti-γ2 antiserum. ***C***, The immunoreactivity of anti-Pcdh-γC5 with the immunoprecipitated 120 and 140 kDa proteins was blocked by 50 μg/ml antigenic peptide. ***D***, Antibody Pcdh-γC5(C) to the C terminus, which recognizes all members of the Pcdh-γ family, reacts with both the 120 kDa (arrow and asterisk) and 140 kDa (arrowhead) proteins coprecipitated with γ2 and α1 antisera, but not with the γ2 PIS. ***E***, The anti-γ2 or anti-α1 precipitates were digested with Endo H or PNGase F followed by SDS-PAGE and immunoblotting with anti-Pcdh-γC5. Endo H did not affect the mobility of the 120 kDa (arrow and asterisks) or 140 kDa (arrowhead) Pcdh-γC5 proteins, while PNGase F increased the mobility of both proteins. The 140 kDa protein (arrowhead) was also present in the input, although to a much lower concentration than the 120 kDa protein. The input image in the first two left lanes is the same, but the first lane was exposed for a longer time to better visualize the 140 kDa protein band. The numbers at the left of the immunoblots show the position of the molecular weight protein markers in kilodaltons.

Pcdh-γC5 expressed in HEK293 cells is transported to the cell surface

HEK293 cells, which have been transfected with ^{cMyc}Pcdh-γC5 (Full) and incubated with anti-Pcdh-γC5 and/or anti-cMyc under live-cell condition to label surface Pcdh-γC5, showed the presence of ^{cMyc}Pcdh-γC5 clusters at the cell surface, as shown by immunofluorescence labeling with both Rb anti-Pcdh-γC5 and Ms anti-cMyc antibodies (Fig. 3*A–C*, arrowheads). Note that both antibodies gave identical immunolabeling. Surface clusters were also obtained after HEK293 cells were transfected with the membrane-bound constructs ^{cMyc}Pcdh-γC5-intra (Fig. 3*D–F*, Intra, arrowheads) or ^{cMyc}Pcdh-γC5-extra (Fig. 3*G–I*, Extra, arrowheads) after live-cell incubation with both antibodies. Note that the two antibodies recognize epitopes localized at the extracellular domain of each of the three ^{cMyc}Pcdh-γC5 constructs (see Materials and Methods) (Fig. 1*A*).

The cluster density was considerably higher in cells transfected with ^{cMyc}Pcdh-γC5-extra (19.5 ± 1.5 clusters/100 μm^2) compared with that of cells transported with ^{cMyc}Pcdh-γC5 (6.1 ± 0.4 clusters/100 μm^2; $p < 0.001$) or ^{cMyc}Pcdh-γC5-intra (4.8 ± 0.4 clusters/100 μm^2; $p < 0.001$), as shown in Figure 3*J*. The results are consistent with the notion that the cytoplasmic domain is involved in the intracellular retention of Pcdh-γC5, decreasing the translocation of Pcdh-γC5 to the cell surface. In these live-cell Ab incubation experiments, the labeled clusters correspond to proteins that have been translocated from intracellular compartments and exposed to the cell surface.

TIRF microscopy of EGFP fluorescence in live HEK293 transfected cells shows that Pcdh-γC5-extra-EGFP (Extra-EGFP) accumulates at cell–cell contacts (Fig. 3*K*, arrowheads), indicating that the extracellular domain of Pcdh-γC5 has homophilic *trans*-adhesive properties.

In the absence of α and β subunits, Pcdh-γC5 promotes the transfer of the γ2 subunit to the cell surface, and this transfer depends on the cytoplasmic domain of Pcdh-γC5

It has been shown that, when HEK293 cells are transfected with the γ2 subunit alone (in the absence of α and β subunits), some of the γ2 subunit goes to the surface but no-functional $GABA_A$Rs are made (Connolly et al., 1996, 1999). When we transfected

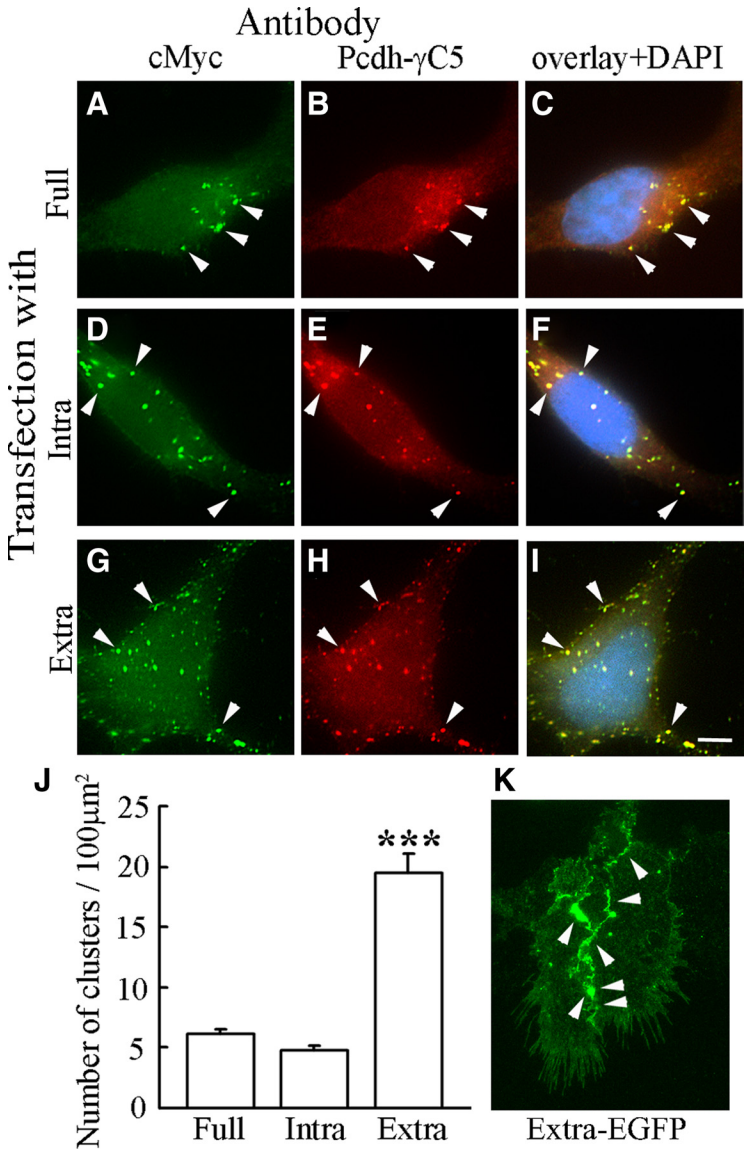

Figure 3. Various Pcdh-γC5 constructs are expressed at the surface of transfected HEK293 cells. Double-label immunofluorescence of transfected HEK293 cells after live-cell incubation with a mixture of Ms anti-cMyc and Rb anti-Pcdh-γC5 antibodies. ***A–I***, HEK293 cells were transfected with ^{cMyc}Pcdh-γC5 (***A–C***, full), or ^{cMyc}Pcdh-γC5-intra (***D–F***, intra), or ^{cMyc}Pcdh-γC5-extra (***G–I***, extra). Immunofluorescence was done with Ms anti-cMyc (***A***, ***D***, ***G***, green) and Rb anti-Pcdh-γC5 (***B***, ***E***, ***H***, red). After fixation and permeabilization, cells were incubated with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) (***C***, ***F***, ***I***, blue). The cMyc and Pcdh-γC5 antibodies show identical labeling of clusters (arrowheads). There is no immunofluorescence labeling in nontransfected cells (data not shown). ***J***, Quantification of cluster density at the cell surface of HEK293 cells transfected with various ^{cMyc}Pcdh-γC5 DNA constructs ($n = 10$ transfected cells per construct from 3 experiments; ***$p < 0.001$, one-way ANOVA Tukey–Kramer multiple-comparison test). Error bars indicate SEM. ***K***, TIRF microscopy of EGFP fluorescence in live HEK293 cells that have been transfected with Pcdh-γC5 extra-EGFP (Extra-EGFP). Note that Extra-EGFP accumulates at cell– cell contacts (arrowheads). Scale bar, 5 μm (for all image panels).

HEK293 cells with the γ2 GABA$_A$R subunit only, and the live cells were incubated with anti-γ2, the cells showed very low and diffuse immunofluorescence surface labeling with the anti-γ2 antibody (Fig. 4*J*). The anti-γ2 fluorescence intensity at the cell surface was considerably lower than when the cells were cotransfected with γ2 together with α1 and β3, as shown below. Moreover, no γ2 surface microclusters were formed, which were present when γ2 was cotransfected with α1 and β3 (compare Fig. 4*J* with Fig. 5*A*). In the absence of α1 and β3, most of the γ2 subunit was retained in the endoplasmic reticulum, which was observed by immunolabeling with the anti-γ2 antibody after fixation and permeabilization (Fig. 4*K*). However, when HEK293 cells were cotransfected with γ2 (in the absence of α and β subunits) together with ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra, the γ2 subunit formed clusters at the cell surface that frequently colocalized with ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra surface clusters (Fig. 4*A–F*, arrowheads). Nevertheless, when the γ2 subunit was coexpressed with ^{cMyc}Pcdh-γC5-extra under the same labeling conditions, no γ2 clusters were detected at the surface, although ^{cMyc}Pcdh-γC5-extra clusters were formed at the cell surface (Fig. 4*G–I*).

Similar experiments cotransfecting with γ2 and either ^{cMyc}Pcdh-α4 or Pcdh-γC3-EGFP showed very low γ2 fluorescence and no γ2 clusters at the cell surface (data not shown). These results are consistent with the aforementioned Y2H, *in vitro* pull-down, and coimmunoprecipitation experiments, supporting the notion that γ2 specifically interacts with Pcdh-γC5 but not with other Pcdhs.

Quantification revealed that, in HEK293 cells cotransfected with γ2 and either ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra, the percentages of γ2 clusters colocalizing at the cell surface with cMyc (85.0 ± 4.9 vs 82.5 ± 4.7%; $p > 0.05$; $n = 10$; two-tailed Student's *t* test), respectively, or the percentages cMyc clusters colocalizing with γ2 clusters (44.6 ± 7.7 vs 51.0 ± 6.5%; $p > 0.05$; $n = 10$), respectively, were similar. However, no γ2 clusters were detected at the surface of cells cotransfected with γ2 and ^{cMyc}Pcd-γC5-extra.

These experiments show that ^{cMyc}Pcdh-γC5 increases the surface clustering of the γ2 GABA$_A$R subunit and strongly suggest that also increases the amount of the γ2 GABA$_A$R subunit at the cell surface. To determine that indeed this was the case, we did surface biotinylation experiments. Figure 4*M* shows that cotransfection of HEK293 with ^{cMyc}Pcdh-γC5 and EGFP-γ2 significantly increased the amount of EGFP-γ2 present at the cell surface (196.6 ± 8.8%; $p < 0.01$), over HEK293 cells transfected only with EGFP-γ2 (100%). The EGFP-γ2 subunit is functionally similar to γ2 (Kittler et al., 2000; Arancibia-Cárcamo et al., 2009). A smaller increase in the cell surface expression of EGFP-γ2 was observed in cells cotransfected with ^{cMyc}Pcdh-γC5-intra (152.8 ± 8.9%; $p < 0.05$) but not with ^{cMyc}Pcdh-γC5-extra (116.3 ± 12.9; $p = 0.17$), when compared with cells transfected only with EGFP-γ2. Note that ^{cMyc}Pcdh-γC5 facilitated the surface expression of EGFP-γ2 more efficiently than ^{cMyc}Pcdh-γC5-intra and ^{cMyc}Pcdh-γC5-extra, even when the expression of ^{cMyc}Pcdh-γC5 protein in the cotransfected cells was lower than the other two cotransfected constructs (Fig. 4*M*, black dots). Also note that the absence of γ2 GABA$_A$R subunit clusters in Figure 4, *G* and *J*, does not preclude the existence of diffuse γ2 GABA$_A$R subunit at the cell surface. This

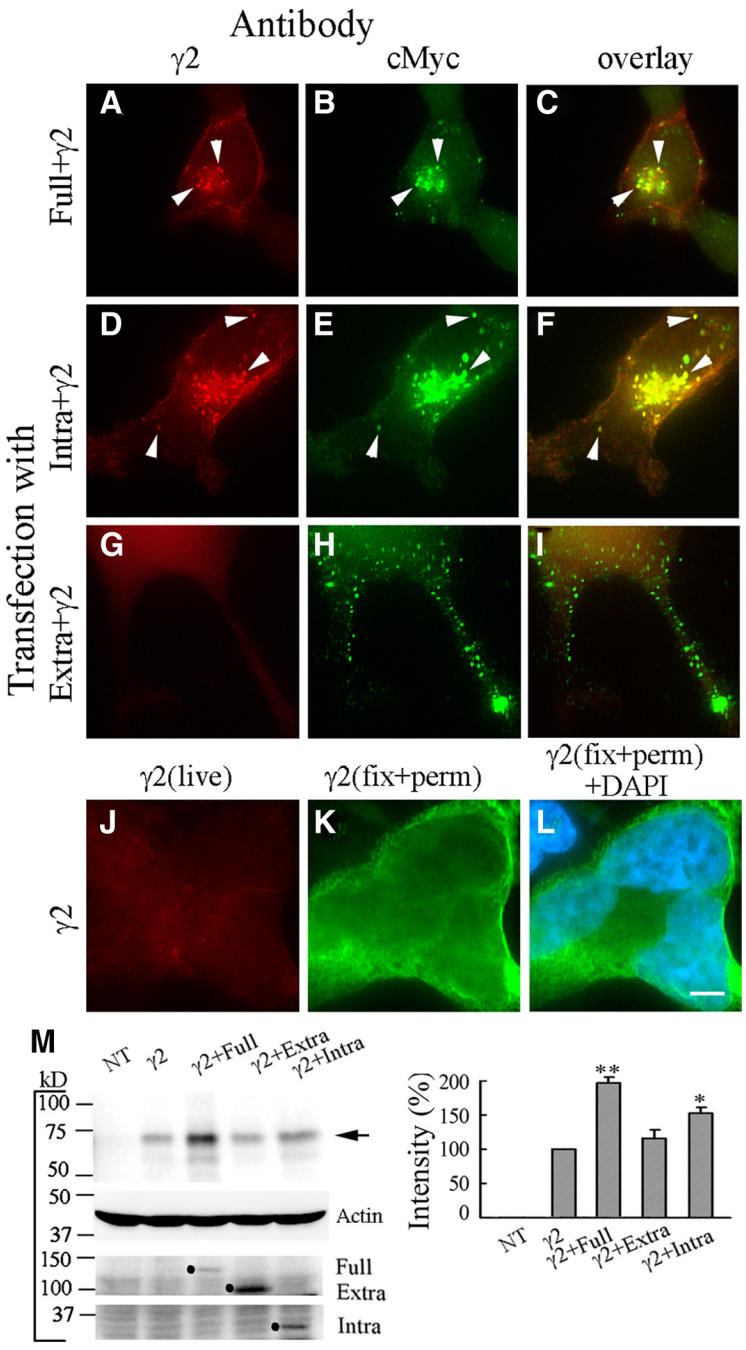

Figure 4. Pcdh-γC5 helps the γ2 $GABA_A$R subunit translocate to the surface of HEK293 cells and form clusters that colocalize with Pcdh-γC5 clusters. ***A–I***, Immunofluorescence of HEK293 cells cotransfected with nontagged γ2 and one of the ^{cMyc}Pcdh-γC5 constructs followed by live-cell incubation with a mixture of GP anti-γ2, Ms anti-cMyc, and Rb anti-Pcdh-γC5. The γ2 subunit, when coexpressed with either ^{cMyc}Pcdh-γC5 (***A–C***, Full + γ2) or ^{cMyc}Pcdh-γC5-intra (***D–F***, Intra + γ2), formed surface γ2 clusters that frequently colocalized with those of ^{cMyc}Pcdh-γC5 (***A–C***, arrowheads) and ^{cMyc}Pcdh-γC5-intra (***D–F***, arrowheads). However, the γ2 subunit, when was coexpressed with ^{cMyc}Pcdh-γC5-extra (***G–I***, Extra + γ2), formed no γ2 clusters at the cell surface (***G***), while ^{cMyc}Pcdh-γC5-extra formed surface clusters (***H***). The labeling with anti-Pcdh-γC5 (data not shown) was identical with that of anti-cMyc antibody. ***J–L***, HEK293 cells were transfected with the nontagged γ2 subunit alone followed by live-cell incubation with GP anti-γ2 antibody followed by fixation, permeabilization, and incubation with Rb anti-γ2 antibody. The γ2 subunit was expressed in HEK293 cells (***K***) but not on the surface of the same cells (***J***). The nuclear DAPI staining is shown in the overlay (***L***). Scale bar, 5 μm. ***M***, Immunoblots of HEK293 cells, nontransfected (NT), or transfected with EGFP-γ2 only (γ2), or cotransfected with EGFP-γ2 and one of the ^{cMyc}Pcdh-γC5 constructs (γ2 + Full; γ2 + Extra; γ2 + Intra). Cell surface proteins were biotinylated, affinity-purified on Neutravidin-agarose, and subjected to SDS-PAGE and immunoblotting with Rb anti-EGFP antibody (top blot). The 70 kDa EGFP-γ2 protein band is indicated by an arrow. The actin loading control (total cell lysate) shows that the amount of protein in the cultures, used for the purification of the biotinylated proteins, was similar under the various transfection conditions. The bottom blots are two regions of the same immunoblot, with anti-Pcdh-γC5, of transfected HEK293 total cell lysates showing the expression of the three ^{cMyc}Pcdh-γC5 constructs of the expected mobility: Full (120 kDa), Extra (98 kDa), and Intra (32 kDa). The protein bands of the three expressed constructs are indicated by a black dot on the left side of the protein band. The graph on the right side shows the quantification of the EGFP-γ2 protein band of the top immunoblot blot normalized for actin ($n = 2$ experiments; $^*p < 0.05$; $^{**}p < 0.01$ in one-way ANOVA Tukey–Kramer multiple-comparison test). Error bars indicate SEM.

notion is supported by the presence of the γ2 $GABA_A$R subunit polypeptide in the corresponding biotinylated fractions (Fig. 4*M*). The biotinylated γ2 $GABA_A$R subunit protein band corresponds to surface γ2 $GABA_A$R subunit and not to cytoplasmic contamination, since immunoblots with an anti-tubulin antibody showed that tubulin, a cytoplasmic protein used as control, was absent from the biotinylated fraction, although it was present in the total cell lysate (data not shown).

Next, we addressed whether Pcdh-γC5 exerts similar effects on $GABA_A$Rs containing γ2 in combination with α and β subunits.

In cotransfected HEK293 cells, Pcdh-γC5 and $GABA_A$R show high degree of colocalization at the cell surface, and this colocalization depends on both the cytoplasmic domain of Pcdh-γC5 and the presence of the γ2 subunit in the $GABA_A$Rs

It has been shown that when HEK293 cells are cotransfected with γ2 in combination with α1 and β3 $GABA_A$R subunits, functional $GABA_A$R heteropentamers are formed and γ2-containing $GABA_A$Rs are translocated to the surface (Connolly et al., 1996, 1999). We have cotransfected HEK293 cells with α1, β3, and γ2 $GABA_A$R subunits, and the live cells were incubated with a mixture of anti-γ2, anti-β3, and anti-α1 antibodies recognizing extracellular epitopes of these subunits. The HEK293 cells showed a combination of diffuse labeling and microclusters on the cell surface (Fig. 5*A–C*) with identical localization of the immunofluorescence signal for the three antibodies (Fig. 5 shown for γ2 and β3). However, when the HEK293 cells were cotransfected with the three $GABA_A$R subunits and either ^{cMyc}Pcdh-γC5 (Fig. 5*D–F*) or ^{cMyc}Pcdh-γC5-intra (Fig. 5*G–I*), and the live cells were incubated with a mixture of anti-γ2, anti-cMyc, and anti-Pcdh-γC5 antibodies, the $GABA_A$Rs formed larger clusters at the cell surface. Moreover, a significant number of the clusters, visualized with anti-γ2 as in Figure 5, *D* and *G*, or with anti-α1 or anti-β3 (data not shown), colocalized with the surface ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra clusters, as revealed by immunoflu-

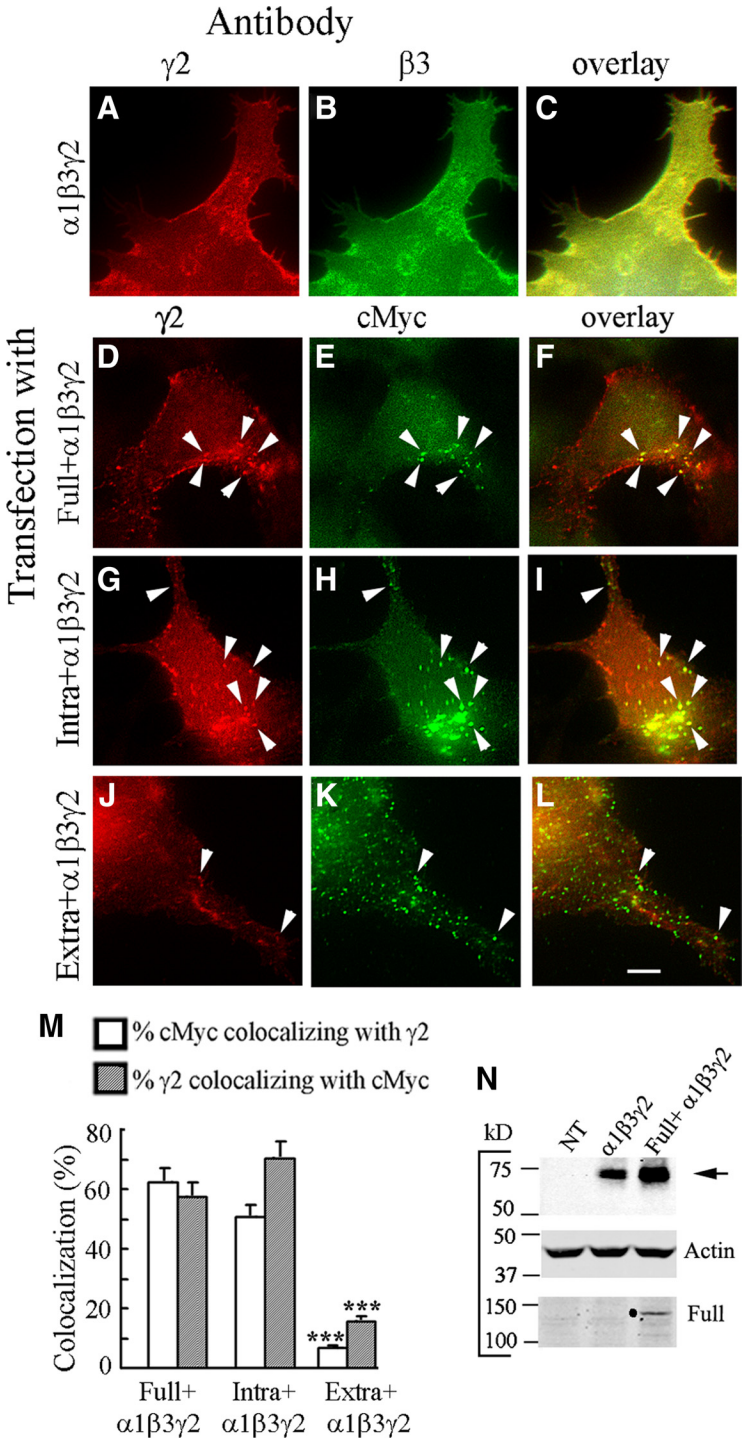

Figure 5. In cotransfected HEK293 cells, Pcdh-γC5 induces the formation of large GABA$_A$R clusters at the cell surface, many of which colocalize with Pcdh-γC5 clusters. ***A–C***, Immunofluorescence of HEK293 cells cotransfected with nontagged GABA$_A$Rs α1, β3, and γ2 subunits and incubated with γ2, β3, and α1 antibodies under live-cell conditions. The three antibodies GP anti-γ2 (***A***), Ms anti-β2/3 (***B***), and Rb anti-α1 (data not shown) subunits showed identical immunofluorescence pattern characterized by the presence of microclusters on the surface of HEK293 cells. ***D–L***, HEK293 cells cotransfected with the three GABA$_A$R subunits and one of the ^{cMyc}Pcdh-γC5 constructs were incubated with GP anti-γ2 (***D***, ***G***, ***J***, red), Ms anti-cMyc (***E***, ***H***, ***K***, green), and Rb anti-Pcdh-γC5 (data not shown) under live-cell incubation condition. Cotransfection of GABA$_A$Rs with ^{cMyc}Pcdh-γC5 (***D–F***, Full+α1β3γ2) and ^{cMyc}Pcdh-γC5-intra (***G–I***, Intra+α1β3γ2) induced the formation of large γ2 clusters on HEK293 cell surface, many of them colocalizing with the Pcdh-γC5 clusters. The arrowheads point to colocalization of γ2 and ^{cMyc}Pcdh-γC5 construct clusters. In contrast, cotransfection of HEK293 cells with GABA$_A$Rs and ^{cMyc}Pcdh-γC5-extra (***J–L***, Extra+α1β3γ2) shows few γ2 clusters on cell surface, and only a few ^{cMyc}Pcdh-γC5-extra clusters colocalize with γ2 clusters (arrowheads). In all of the experiments, the labeling with anti-Pcdh-γC5 (data not shown) was identical with that of anti-cMyc. Scale bar, 5 μm. ***M***, Quantification of the colocalization between γ2 and ^{cMyc}Pcdh-γC5 construct clusters ($n = 10$ transfected cells per construct from 3 experiments; ***$p < 0.001$, one-way ANOVA Tukey–Kramer multiple-comparison test). Error bars indicate SEM. ***N***, Immunoblots of HEK293 cells, nontransfected (NT) or cotransfected with α1, β3, and EGFP-γ2 (α1β3γ2), or cotransfected with ^{cMyc}Pcdh-γC5, α1, β3, and EGFP-γ2 (Full+α1β3γ2) after purification of biotinylated surface proteins. Immunoblot with Rb anti-EGFP antibody (top blot) shows the 70 kDa EGFP-γ2 protein band (arrow). Underneath are the total lysate loading actin control and the immunoblot with anti-Pcdh-γC5, which shows the presence of the 120 kDa ^{cMyc}Pcdh-γC5 (Full) protein band (marked by a dot on the left side) in the total lysates of cells cotransfected with Full+α1β3γ2, but not in the nontransfected cells or cell transfected with α1β3γ2.

orescence with anti-cMyc (Fig. 5*D–I*, arrowheads) or anti-Pcdh-γC5 (data not shown).

In the absence of the γ2 subunit, cotransfection with α1, β3, and ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra led to GABA$_A$R microclusters that did not colocalized with Pcd-γC5 clusters, indicating that the γ2 subunit was essential for the colocalization of the GABA$_A$Rs and Pcd-γC5 clusters (data not shown).

When HEK293 cells were cotransfected with the three GABA$_A$R subunits and ^{cMyc}Pcdh-γC5-extra, followed by live-cell incubation condition, the formation of GABA$_A$R clusters at the cell surface was highly reduced. The ^{cMyc}Pcdh-γC5-extra clusters seldom had colocalizing GABA$_A$R clusters (Fig. 5*J–L*, arrowheads).

Quantification (Fig. 5*M*) revealed that the percentages of the cMyc clusters colocalizing with γ2-GABA$_A$R clusters in HEK293 cells cotransfected with the three GABA$_A$R subunits and ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra or ^{cMyc}Pcdh-γC5-extra were 62.2 ± 5.1, 50.7 ± 4, or 6.6 ± 1.1%, respectively (mean ± SEM). The percentages of γ2-GABA$_A$R clusters colocalizing with cMyc clusters were 57.3 ± 5.1, 70.5 ± 5.9, and 15.8 ± 1.5%, respectively. Thus, colocalization with GABA$_A$R clusters was significantly less for ^{cMyc}Pcdh-γC5-extra ($p < 0.001$; $n = 10$) than for ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra. The results showed that the cytoplasmic domain of Pcdh-γC5 is necessary and sufficient for the colocalization of Pcdh-γC5 and GABA$_A$Rs.

Although, under our live-cell labeling conditions, we expect most of the labeled antigen to reside at the cell surface, we also expect some labeling to be localized internally due to endocytosis. To determine whether Pcdh-γC5 and GABA$_A$R cocluster at the cell surface before endocytosis or after endocytosis, we cotransfected HEK293 cells with ^{cMyc}Pcdh-γC5 and γ2, α1, and β3. The live-cell Ab incubation conditions were for 15 min at 4°C or in the presence of 400 mM sucrose at 37°C,

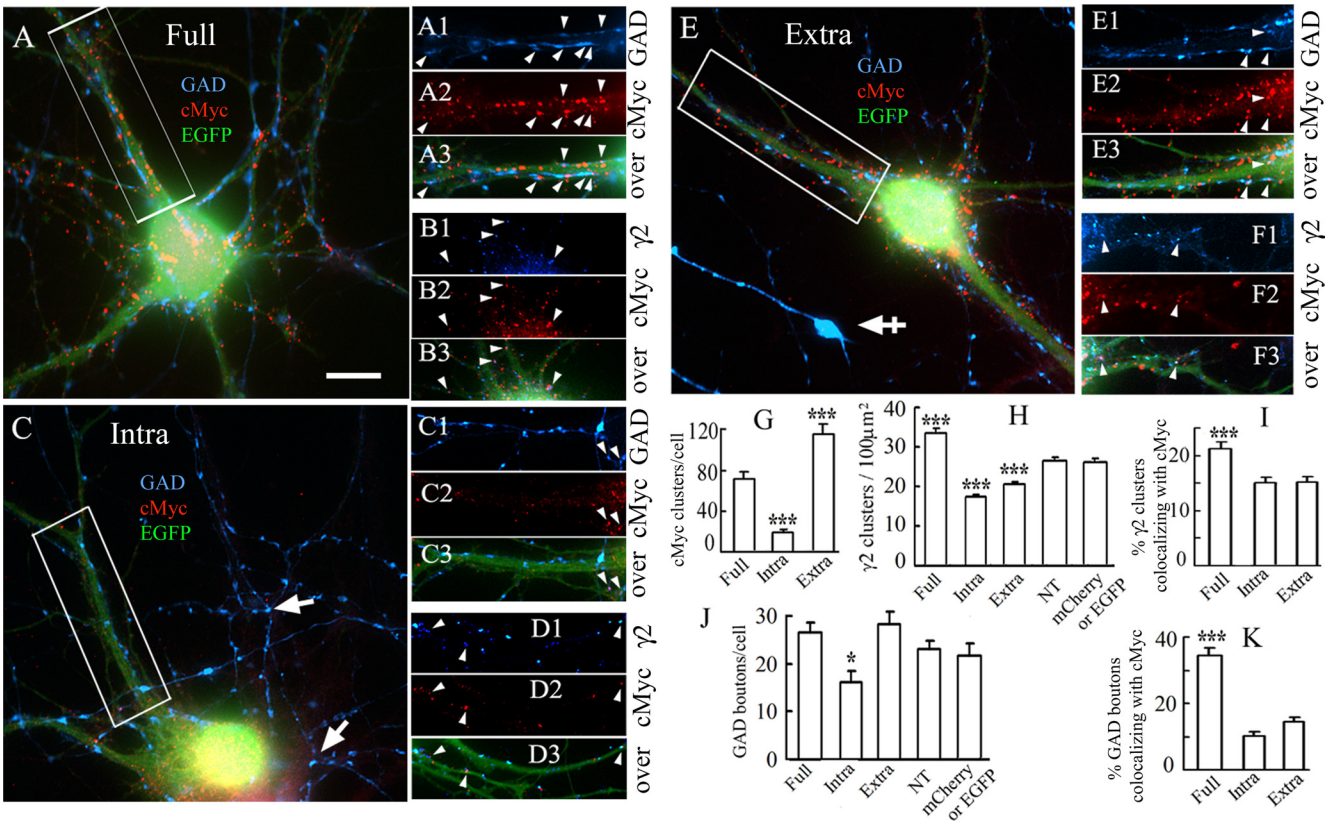

Figure 6. Transfection of hippocampal pyramidal cells with ^{cMyc}Pcdh-γC5 constructs. Effect on endogenous GABA$_A$R clusters and GAD+ synaptic contacts. Triple-label immunofluorescence of transfected HP neurons. ***A***, ***A1–A3***, ***B1–B3***, Neurons were transfected with ^{cMyc}Pcdh-γC5 and EGFP. ***C***, ***C1–C3***, ***D1–D3***, Neurons were transfected with ^{cMyc}Pcdh-γC5-intra and EGFP. ***E***, ***E1–E3***, ***F1–F3***, Neurons were transfected with ^{cMyc}Pcdh-γC5-extra and EGFP. The transfected neurons show both green fluorescence and surface cMyc labeling (red). ***A1–A3***, ***C1–C3***, and ***E1–E3*** show the fluorescence corresponding to the boxed area in ***A***, ***C***, and ***E***, respectively. ***B1–B3***, ***D1–D3***, and ***F1–F3*** are from sister cultures transfected with ^{cMyc}Pcdh-γC5, ^{cMyc}Pcdh-γC5-intra, and ^{cMyc}Pcdh-γC5-extra, respectively. The cultures were incubated with Ms anti-cMyc (red in all panels) under live-cell condition followed by fixation, permeabilization, and incubation with sheep anti-GAD (***A***, ***A1***, ***C***, ***C1***, ***E***, ***E1***, blue) or GP anti-γ2 (***B1***, ***D1***, ***F1***, blue). The arrowheads show surface clusters of the ^{cMyc}Pcdh-γC5 constructs (red) that colocalize with GAD+ boutons (blue) or γ2-GABA$_A$R clusters (blue). ***C*** shows a transfected and a nontransfected neuron next to each other. The arrows point to GAD+ boutons contacting dendrites of the nontransfected neuron. In ***E***, the crossed arrow shows the growth cone of a GAD-containing axon. Scale bar, 5 μm. ***G***, Quantification of the density of the surface cMyc clusters on HP neurons transfected with the ^{cMyc}Pcdh-γC5 constructs ($n = 21$ neurons per construct from 7 transfection experiments). ***H***, Quantification of the density of γ2-GABA$_A$R clusters on HP neurons transfected with the ^{cMyc}Pcdh-γC5 constructs ($n = 40$ or 45 neurons per construct from 8 or 9 transfection experiments). ***I***, Quantification of the percentage of the γ2 clusters that colocalize with clusters of the ^{cMyc}Pcdh-γC5 constructs ($n = 25$ or 26 neurons per construct from 5 transfection experiments). ***J***, Quantification of the number of GAD+ boutons contacting neurons transfected with the ^{cMyc}Pcdh-γC5 constructs ($n = 15–21$ neurons per construct from 6 transfection experiments). ***K***, Quantification of the percentage of GAD+ boutons colocalized with clusters of the ^{cMyc}Pcdh-γC5 constructs ($n = 20$ neurons per construct from 5 transfection experiments). For ***G–K***, ***$p < 0.001$; *$p < 0.001$, one-way ANOVA Tukey–Kramer multiple-comparison test. Error bars indicate SEM.

conditions known to prevent endocytosis and internalization. We found extensive surface coclustering of Pcdh-γC5 and GABA$_A$Rs, indicating that the association between Pcd-γC5 and GABA$_A$R in these clusters occurred before endocytosis (data not shown).

Surface biotinylation experiments (Fig. 5*N*) also showed that ^{cMyc}Pcdh-γC5 facilitated the surface expression of γ2-GABA$_A$Rs (α1, β3, and EGFP-γ2) compared with HEK293 cells transfected with the same combination of GABA$_A$R subunits in the absence of ^{cMyc}Pcdh-γC5 (233 ± 13 vs 100%, respectively; $p < 0.01$; $n = 5$ in two-tailed paired *t* test)

The experiments with cotransfected HEK293 cells show that the interaction of Pcdh-γC5 with γ2-GABA$_A$R facilitates both the presence of GABA$_A$Rs at the cell surface and the clustering of GABA$_A$Rs. They also show that the cytoplasmic domain of Pcdh-γC5 plays a central role in both and that the effects are specific for Pcdh-γC5.

Cultured hippocampal pyramidal cells transfected with ^{cMyc}Pcdh-γC5 have increased density of endogenous GABA$_A$R clusters

Transfection of cultured HP neurons with ^{cMyc}Pcdh-γC5 or ^{cMyc}Pcdh-γC5-intra or ^{cMyc}Pcdh-γC5-extra led to the formation of clusters of the various constructs on the neuronal surface, as shown by immunofluorescence with mouse anti-cMyc under live-cell incubation condition (Fig. 6*A*,*A2*,*B2*,*C*,*C2*,*D2*,*E*,*E2*,*F2*, red color). Quantitative analysis (Fig. 6*G*) showed that ^{cMyc}Pcdh-γC5-extra led to the formation of significantly more surface clusters of this construct (115.4 ± 10.0 clusters/neuron; $p < 0.001$) than ^{cMyc}Pcdh-γC5 (71.2 ± 6.9 clusters/neuron), being ^{cMyc}Pcdh-γC5-intra the construct that led to the fewest number of surface clusters (18.8 ± 3.2 clusters/neuron; $p < 0.001$ compared with the other two values). Since permeabilized neurons show plenty of expression of the three constructs in transfected neurons, the results indicate that, as in the HEK293 transfection experiments described above, the removal of the intracellular domain highly increases ^{cMyc}Pcdh-γC5 at the cell surface. In contrast, the removal of the extracellular domain significantly decreases the amount of ^{cMyc}Pcdh-γC5 at the cell surface.

Figure 6*H* shows that pyramidal cells transfected with ^{cMyc}Pcdh-γC5 and EGFP exhibited increased density of endogenous γ2-GABA$_A$R clusters (33.4 ± 1.2 γ2 clusters/100 μm^2; $p < 0.001$) compared with that of cells transfected only with EGFP (or

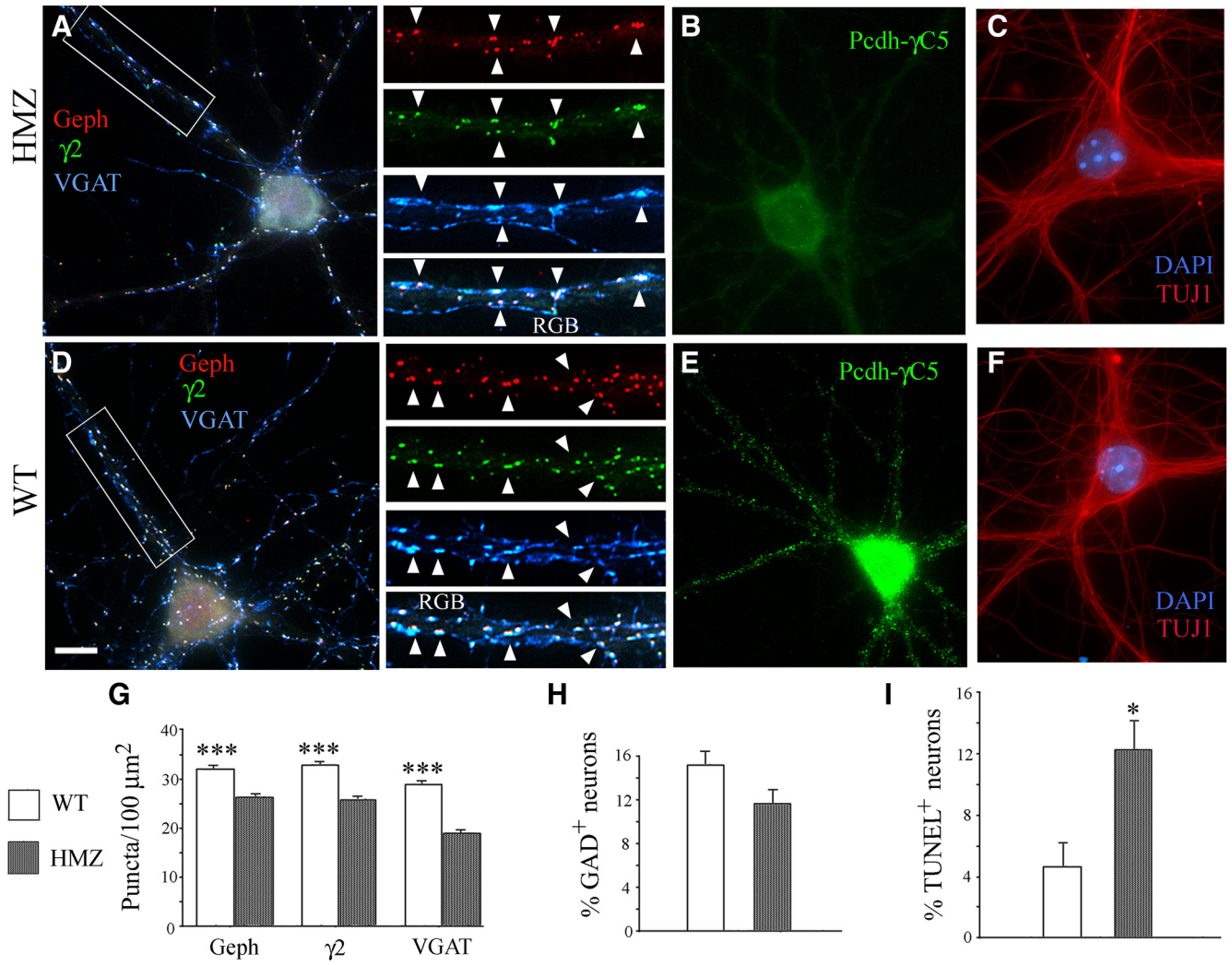

Figure 7. Hippocampal cultures of the TCKO mouse show reduced GABAergic synaptic contacts. ***A–C***, Neuronal cultures from the homozygous TCKO mouse. These neurons show abundant GABAergic synapses (***A***), lack of Pcdh-γC5 expression (***B***), and normal and abundant neuronal processes as shown by the anti-TUJ1 antibody (***C***). ***D–F***, Neuronal cultures from sister wild-type mice. They show GABAergic synapses (***D***), expression of Pcdh-γC5 (***E***), and abundant neuronal processes (***F***). The boxed areas in ***A*** and ***D*** are shown at higher magnification in the smaller panels. Presynaptic VGAT boutons (blue) are apposed to gephyrin (red) and γ2-GABA$_A$R clusters (green) as indicated by arrowheads. Gephyrin and γ2-GABA$_A$R clusters colocalize with each other. Scale bar, 10 μm (for all panels except for the enlarged boxed area, which correspond to 5.5 μm). ***G***, Quantification of the density of GABAergic marker puncta in the WT and HMZ mice ($n = 14$ neurons per genotype from 5 coverslips). ***H***, Quantification of the percentage of GAD+ neurons in the WT and HMZ cultures ($n = 5$ and 4 coverslips, respectively). ***I***, Quantification of the percentage of TUNEL+ neurons in the WT and HMZ cultures ($n = 4$ and 3 coverslips, respectively). For ***G–I***, ***$p < 0.001$; *$p < 0.001$, two-tailed Student's t test. Error bars indicate SEM.

mCherry) (26.2 ± 0.9 γ2 clusters/100 μm^2) or nontransfected neurons (26.6 ± 0.9 γ2 clusters/100 μm^2). However, and as shown in Figure 6*J*, the ^{cMyc}Pcdh-γC5-transfected neurons showed no significant difference in the density of GAD+ boutons (26.5 ± 2.0 boutons/cell; $p = 0.266$, one-way ANOVA) compared with nontransfected controls (23.0 ± 1.8 boutons/cell) or neurons transfected with EGFP or mCherry (21.7 ± 2.5 boutons/cell). These results show that ^{cMyc}Pcdh-γC5 significantly increases the number of γ2-GABA$_A$R clusters in the transfected cells, but it does not promote the GABAergic contacts in these cells.

In contrast, neurons transfected with ^{cMyc}Pcdh-γC5-intra or ^{cMyc}Pcdh-γC5-extra had significantly lower density of endogenous γ2-GABA$_A$R clusters (17.3 ± 0.7 clusters/100 μm^2, $p < 0.001$; 20.4 ± 0.8 clusters/100 μm^2, $p < 0.001$, respectively) compared with the aforementioned controls and the neurons transfected with ^{cMyc}Pcdh-γC5 (Fig. 6*H*). The neurons transfected with ^{cMyc}Pcdh-γC5-intra also showed no significant difference in the density of GAD+ boutons/cell (16.0 ± 2.4 boutons/cell) from that of the controls ($p > 0.05$), as shown in Figure 6*J*. Nevertheless, the difference was significant when compared with that of ^{cMyc}Pcdh-γC5 ($p < 0.05$) or ^{cMyc}Pcdh-γC5-extra ($p < 0.01$) in one-way ANOVA Tukey–Kramer multiple-comparison test (Fig. 6*J*). There was also significantly lower colocalization of γ2-GABA$_A$R clusters or GAD+ boutons with ^{cMyc}Pcdh-γC5-intra or ^{cMyc}Pcdh-γC5-extra than with ^{cMyc}Pcdh-γC5 (Fig. 6*I*,*K*). Illustrative examples of the effect of the three ^{cMyc}Pcdh-γC5 constructs (1) on γ2-GABA$_A$R clustering (Fig. 6*B1*,*D1*,*F1*) and (2) on GABAergic innervation (Fig. 6*A*,*A1*,*C*,*C1*,*E*,*E1*) are shown. Colocalization examples between the transfected constructs (cMyc) and GAD or γ2 is indicated by arrowheads.

The results indicate that (1) Pcdh-γC5 promotes endogenous GABA$_A$R clustering in Pcdh-γC5 transfected neurons, (2) Pcdh-γC5-intra and Pcdh-γC5-extra have a dominant-negative effect on endogenous GABA$_A$R clustering, and (3) deletion of the extracellular or the cytoplasmic domain significantly reduces the association of these Pcdh-γC5 constructs with GABAergic synapses (association with GAD+ boutons and γ2 clusters).

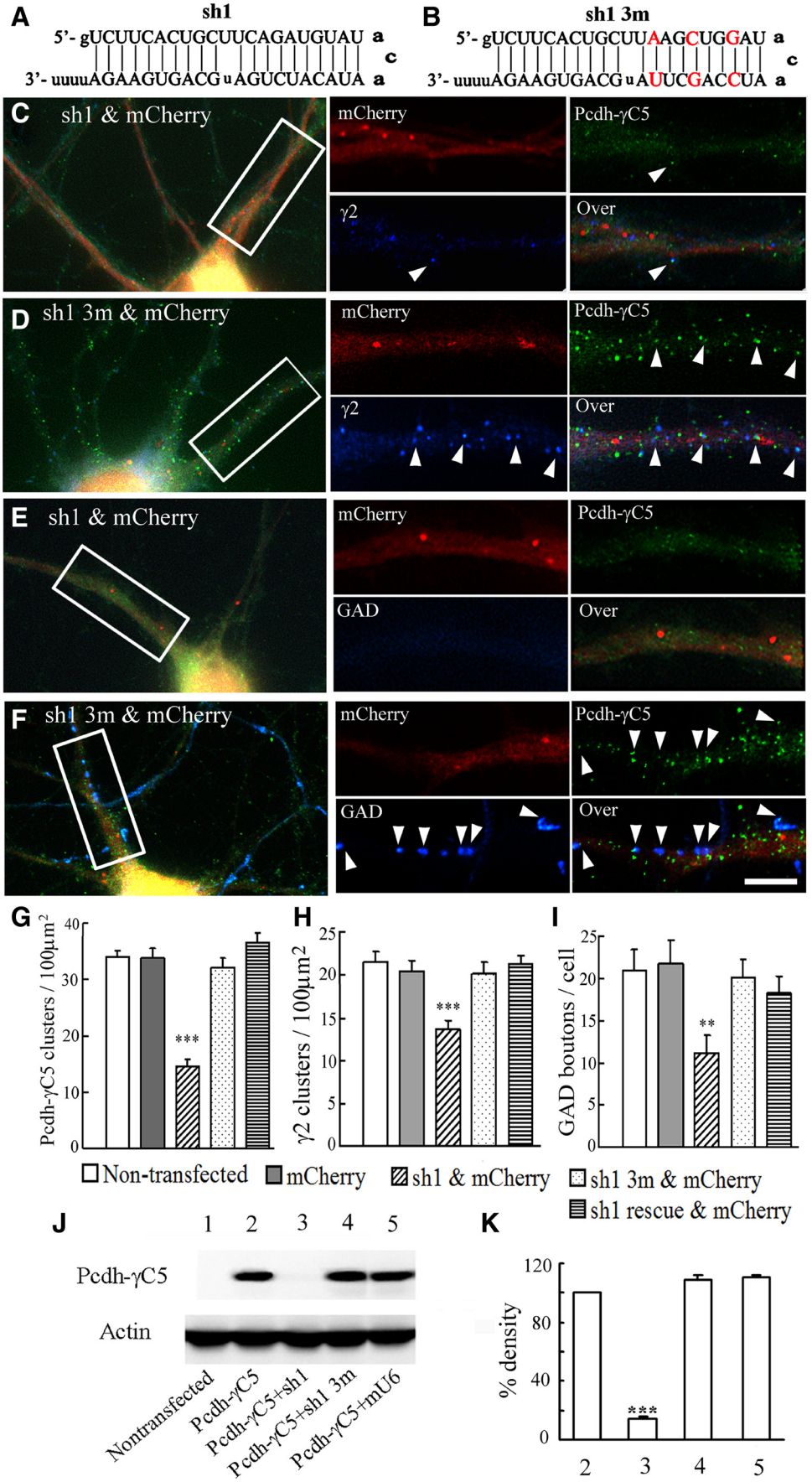

Figure 8. Knocking down Pcdh-γC5 in hippocampal neurons leads to a decrease in both γ2-GABA$_A$R cluster density and GABAergic innervation. ***A***, ***B***, The sh1 and sh1 3m Pcdh-γC5 shRNAs used in this study. The sh1 3m carries three point mutations shown in red. ***C–F***, HP neurons cotransfected with sh1 and mCherry (***C***, ***E***) have lower density of (*Figure legend continues.*)

Hippocampal cultures from a protocadherin-γ TCKO mouse mutant show decreased density of GABAergic synapses

The *Pcdhg*$^{tcko/tcko}$ mutant mice show severe motor defects and perinatal lethality (Chen et al., 2012) displaying similar levels and patterns of neuronal apoptosis and eventual loss of several spinal cord and retinal neuronal populations as in the mice lacking the entire Pcdh-γ cluster (Wang et al., 2002b; Weiner et al., 2005; Lefebvre et al., 2008; Prasad et al., 2008). Nevertheless, cultured HP neurons from the *Pcdhg*$^{tcko/tcko}$ HMZ mutants looked healthy and survived for >3 weeks in culture, for as long as sister cultures from heterozygous (HTZ) and WT mice. The neurons from the HMZ mouse showed no Pcdh-γC5 immunoreactivity, while WT neurons did (Fig. 7, compare *B*, *E*). Neurons from the HMZ mouse had normal and extensive neuronal processes similar to that of the WT, as shown by the TUJ1 antibody (Fig. 7, compare *C*, *F*). The dendritic branching of the HMZ neurons (Fig. 7*A*,*B*) was similar to that of the WT (Fig. 7*D*,*E*) and HTZ neurons (data not shown).

The mature HP cultures from the HMZ mice displayed numerous GABAergic synaptic contacts, as revealed by the presence of postsynaptic gephyrin and γ2-$GABA_A$R clusters apposed to VGAT-containing presynaptic terminals (Fig. 7*A* and enlarged insets, arrowheads), with an appearance similar to that of the GABAergic synaptic contacts of the WT cultures (Fig. 7*D*, enlarged insets, arrowheads). These results indicate that Pcdh-γC5, as well as the other two Pcdh-γCs (Pcdh-γC3 and Pcdh-γC4) codeleted in the TCKO mutant, is not essential for the formation or maintenance of GABAergic synapses.

Nevertheless, quantification (Fig. 7*G*) showed that the mature HMZ HP cultures had a significant decrease in GABAergic synaptic contacts, as shown by the decreased density (clusters or boutons per square micrometer) of gephyrin clusters (26.1 ± 0.7 vs 32.0 ± 0.9; $p < 0.001$), γ2 clusters (25.7 ± 0.7 vs 32.7 ± 0.8; $p < 0.001$), and VGAT+ boutons (19.0 ± 0.7 vs 28.9 ± 0.7; $p < 0.001$) when compared with that of littermate WT mice, respectively. The corresponding values from HTZ HP cultures were not significantly different from that of the WT cultures.

It has been shown that deletion of the entire *Pcdhg* gene cluster leads to apoptosis and loss of a neuronal subpopulation in the spinal cord and retina (Wang et al., 2002b; Weiner et al., 2005; Lefebvre et al., 2008; Prasad et al., 2008). More recently, it has been shown with the *Pcdhg*$^{tcko/tcko}$ mutants that the C-type Pcdh-γs are specifically required for the survival of the affected neurons (Chen et al., 2012). Therefore, the decreased GABAergic synaptic density that we have observed in the HMZ HP cultures could have been confounded by the death of some GABAergic interneurons. Consistent with this possibility, we found that the HMZ cultures had higher percentage of apoptotic neurons than the WT as revealed by a TUNEL assay (12.3 ± 3.9 vs 4.6 ± 1.6; $p = 0.028$; Fig. 7*I*). There was a strong trend for a reduction in the percentage of GABAergic neurons in the HMZ cultures compared with the WT (Fig. 7*H*), although the difference was not statistically significant (11.7 ± 1.3 vs 15.2 ± 1.3; $p = 0.064$).

Thus, the reduced density in GABAergic synaptic contacts in the HMZ cultures could be in part due to increased apoptosis and consequently a reduced number of presynaptic GABAergic interneurons. Nevertheless, the results do not rule out a negative effect of the TCKO deletion on the GABAergic synapses themselves, since deletion of the Pcdh-γ cluster results in synaptic defects even when apoptosis is genetically prevented (Weiner et al., 2005). Moreover, since all three C-type Pcdh-γs are deleted in the TCKO mutants, we cannot ascertain that any observed phenotype is due to the specific loss of Pcdh-γC5 but not to the loss of Pcdh-γC3 and/or Pcdh-γC4. Furthermore, the deep sequencing (RNA-Seq) data of the TCKO mice shows upregulation of several Pcdh mRNAs from the α, β, and γ families, which could compensate for the loss of Pcdh-γC5, thus potentially reducing a synaptic phenotype (Chen et al., 2012). Moreover, we have shown above that the main effect of Pcdh-γC5 is on the postsynaptic γ2-$GABA_A$R. To overcome these problems, we specifically knocked down Pcdh-γC5 in postsynaptic neurons and studied the effect on GABAergic synapses. Nevertheless, the results with the TCKO mutant mice clearly show that Pcdh-γC5 as well as Pcdh-γC3 and Pcdh-γC4 are not essential for the formation and maintenance of GABAergic synapses.

Knocking down endogenous Pcdh-γC5 expression in postsynaptic hippocampal neurons leads to decreased $GABA_A$R cluster density and GABAergic innervation

We made a shRNA (sh1) that specifically targets a mRNA sequence located at the extracellular variable region of Pcdh-γC5 together with a control shRNA of the same sequence containing three point mutations (sh1 3m), as shown in Figure 8, *A* and *B*. We also made a rescue Pcdh-γC5 mRNA that contains five silent mutations at the sh1 targeting region (see Materials and Methods). Figure 8*C–G* shows that neurons cotransfected with sh1 and mCherry had a significant reduction in the density of endogenous Pcdh-γC5 clusters (14.5 ± 1.3 clusters/100 μm^2; $p < 0.001$) when compared neurons transfected with sh1 3m (32.1 ± 1.7 clusters/100 μm^2), or neurons transfected with mCherry (33.8 ± 1.7 clusters/100 μm^2) or sister nontransfected neurons (34.0 ± 1.1 clusters/100 μm^2). Neurons cotransfected with sh1 and the rescue mRNA restored Pcdh-γC5 cluster density (36.5 ± 1.7 clusters/100 μm^2). Comparison between multiple groups using one-way ANOVA Tukey–Kramer multiple-comparison test showed that there is no significant difference in the Pcdh-γC5 cluster density between nontransfected HP neurons and neurons transfected with sh1 3m, mCherry, or with sh1 plus rescue mRNA ($p > 0.05$).

The effect of sh1 on the protein expression of ^{cMyc}Pcdh-γC5 was also tested by immunoblotting of HEK 293 cells after cotransfection with sh1 and ^{cMyc}Pcdh-γC5 (Fig. 8*J*,*K*). The expression of the 120 kDa cMyc-Pcdh-γC5 protein band was knocked down (to 14.5 ± 1.3%; $p < 0.001$) compared with HEK293 cells transfected with ^{cMyc}Pcdh-γC5 only (100%). In contrast, sh1 3m or the mU6 vector, did not significantly affect the expression of cotransfected ^{cMyc}Pcdh-γC5 (109.2 ± 3.2%, $p = 0.21$; 110.5 ± 2.0%, $p = 0.12$, respectively). In these experiments, actin was used as loading control (Fig. 8*J*). The knockdown of Pcdh-γC5 by sh1 had no effect on actin expression.

As previously reported (Li et al., 2010) in these HP cultures, a significant amount of GABAergic synapses had associated

←

(*Figure legend continued.*) Pcdh-γC5 clusters (green), γ2-$GABA_A$R clusters (***C***, blue), and GAD+ boutons (***E***, blue) than those cotransfected with sh1 3m and mCherry (***D***, ***F***, respectively). The right-side panels show enlargement of the boxed areas in the left panels. Scale bar: Left-side panels, 9 μm; right-side panels, 5 μm. ***G–I***, Quantification of the effect of knocking down Pcdh-γC5 (sh1 and mCherry) on the density of Pcdh-γC5 clusters, γ2-$GABA_A$R clusters, and GAD+ boutons compared with various controls including a rescue mRNA. ($n = 15$ neurons per condition from 3 experiments; ***$p < 0.001$; **$p < 0.01$, one-way ANOVA Tukey–Kramer multiple-comparison test). ***J***, Immunoblots with antibodies to Pcdh-γC5 and actin of HEK293 cotransfected cells show that sh1 knocks down the protein expression of cotransfected ^{cMyc}Pcdh-γC5; however, sh1 3m or the mU6 plasmid have no effect. ***K***, Quantification of the ^{cMyc}Pcdh-γC5 protein band intensities in the immunoblots of ***J*** ($n = 2$ experiments; ***$p <$ 0.01 in one-way ANOVA Tukey–Kramer multiple-comparison test). Error bars indicate SEM.

Pcdh-γC5, as shown by the colocalization of endogenous Pcdh-γC5 clusters with endogenous γ2-$GABA_A$R clusters (Fig. 8*C*,*D*, insets, arrowheads) and GAD+ boutons (Fig. 8*F*, insets, arrowheads).

Knocking down Pcdh-γC5 in HP neurons with sh1 (plus mCherry) significantly decreased the density of γ2-$GABA_A$R clusters (13.7 ± 1.1 clusters/100 μm^2; $p < 0.001$) compared with neurons transfected with sh1 3m and mCherry (20.3 ± 1.3 clusters/100 μm^2) or nontransfected neurons (21.7 ± 1.2 γ2 clusters/100 μm^2) or neurons transfected only with mCherry (20.6 ± 1.2 γ2 clusters/100 μm^2), as shown in Figure 8, *C*, *D*, and *H*. The γ2 cluster density was restored (21.4 ± 1.0) when neurons were cotransfected with sh1 and the rescue mRNA and mCherry (Fig. 8*H*).

Knocking down Pcdh-γC5 in HP neurons by sh1 also significantly decreased the number of presynaptic GABAergic boutons contacting these neurons (11 ± 2.1 boutons/cell; $p < 0.01$) compared with neurons transfected with sh1 3m (20.1 ± 2.2 boutons/cell), sister nontransfected neurons (20.9 ± 2.5 GAD+ boutons /cell), or neurons transfected only with mCherry (21.7 ± 2.9 GAD+ boutons/cell), as shown in Figure 8, *E*, *F*, and *I*. GABAergic innervation was rescued in neurons cotransfected with sh1 and the rescue mRNA (18.3 ± 2.0 GAD+ boutons/cell) as shown in Figure 8*I*. One-way ANOVA Tukey–Kramer multiple-comparison test showed that there are no significant differences ($p > 0.05$) in γ2 cluster density or GAD innervation between nontransfected neurons, neurons transfected with mCherry, neurons transfected with sh1 3m, or neurons cotransfected with sh1 and the rescue mRNA. Note that transfection with mCherry alone or in combination with other plasmids, produced some mCherry aggregates (Fig. 8*C–F*, red color) that did not interfere with the normal expression of Pcdh-γC5, γ2, or Pcdh-γC5 clusters or GABAergic innervation (Fig. 8*G–I*).

We have also quantified the effect of knocking down Pcdh-γC5 on glutamatergic synapses and we found no significant effect over control neurons. HP neurons transfected with sh1 and mCherry showed similar PSD-95 cluster density (21.1 ± 1.2 clusters/100 μm^2; $p = 0.62$) and VGLUT1 bouton density (19.8 ± 1.4 boutons/100 μm^2; $p = 0.20$) to that of sister nontransfected neurons (20.4 ± 0.9 clusters/100 μm^2 and 20.3 ± 0.9 boutons/100 μm^2, respectively). These results show that knocking down Pcdh-γC5 had no effect on the density of glutamatergic synapses.

Discussion

The γ2 subunit is present in the large majority of synaptic $GABA_A$Rs and this subunit is necessary, although not sufficient, for the postsynaptic localization of the $GABA_A$Rs (Essrich et al., 1998; Schweizer et al., 2003; Li et al., 2005b; Serwanski et al., 2006). Nevertheless, Pcdh-γC5 is present only in a subset of GABAergic synapses. Therefore, the interaction between the cytoplasmic domains of Pcdh-γC5 and γ2-$GABA_A$R cannot explain why only some GABAergic synapses accumulate Pcdh-γC5. In transfected neurons, there was increased colocalization of ^{cMyc}Pcdh-γC5 clusters with GABAergic terminals and endogenous γ2-$GABA_A$R clusters over that of ^{cMyc}Pcdh-γC5-intra and/or ^{cMyc}Pcdh-γC5-extra, suggesting that both the cytoplasmic and extracellular domains of Pcdh-γC5 are involved in the association of Pcdh-γC5 with a subset of GABAergic synapses. An attractive hypothesis is that the ectodomain of postsynaptic Pcdh-γC5 is involved in a homophilic transsynaptic interaction with presynaptic Pcdh-γC5, while the cytoplasmic domain of postsynaptic Pcdh-γC5 is involved in the *cis*-interaction with postsynaptic $GABA_A$Rs. The existence of transsynaptic Pcdh-γC5 homophilic interactions is consistent with our previous studies showing that in GABAergic synapses Pcdh-γC5 is localized both presynaptically and postsynaptically (Li et al., 2010) and with our results of Figure 3*K* showing that the extracellular domain of Pcdh-γC5 has homophilic *trans*-adhesive properties. Moreover, the transcellular interactions of Pcdh-γs are predominantly homophilic (Fernández-Monreal et al., 2009). The association of Pcdh-γC5 with a subset of GABAergic synapses would result from Pcdh-γC5 accumulating only at the GABAergic synapses where strong homophilic transsynaptic interactions of Pcdh-γC5 could be established. Individual neurons express several Pcdh-γs, which through *cis*-heterophilic interactions of the ectodomains form combinatorial Pcdh-γs heterotetramers (Schreiner and Weiner, 2010). The strength of the homophilic transsynaptic interaction of Pcdh-γC5 would depend on the homophilic matching between the presynaptic and postsynaptic Pcdh-γ tetramers (Schreiner and Weiner, 2010).

Contrary to the other Pcdh-γs, which are expressed in the embryo, Pcdh-γC5 is expressed postnatally during the second week, coinciding with the peak of synaptogenesis in the rat brain (Frank et al., 2005; Li et al., 2010) and with the highest developmental expression of the γ2-$GABA_A$R in the rat hippocampus and cerebellum (Laurie et al., 1992). Nevertheless, Pcdh-γC5 is not essential for GABAergic synapse formation or maintenance. Thus, in our rat HP cultures, Pcdh-γC5 is not expressed before 12–14 DIV (Li et al., 2010) while GABAergic synapses appear around 8 DIV (Christie et al., 2002a; Chiou et al., 2011). We have also shown that cultured HP neurons from the *Pcdhg*$^{tcko/tcko}$ deletion mutant mouse, where Pcdh-γC3, Pcdh-γC4, and Pcdh-γC5 genes have been deleted, had plenty of GABAergic synaptic contacts. HP neuronal cultures of a Pcdh-γ KO mouse (*Pcdhg*$^{del/del}$), which lacked the 22 members of the Pcdh-γ family, also had plenty of GABAergic and glutamatergic synaptic contacts (Wang et al., 2002b). Also in a retina conditional Pcdh-γ KO, synaptic connectivity was normal (Lefebvre et al., 2008). All these results indicate that Pcdh-γC5 and other Pcdh-γs are not essential for GABAergic synapse formation and maintenance.

Our results also showed no increase in GABAergic innervation in neurons overexpressing ^{cMyc}Pcdh-γC5 or with ^{cMyc}Pcdh-γC5-extra. Since the latter is highly trafficked to the neuronal surface, the results indicate that Pcdh-γC5 and the membrane-bound extracellular domain are not synaptogenic. In agreement with this hypothesis, Pcdh-γC5 did not promote GABAergic axon contacts in a neuronal-HEK293 mixed-culture assay, in which HEK293 cells were transfected with Pcdh-γC5 (data not shown). In contrast, HEK293 cells transfected with neuroligin 2, as a control, received numerous contacts from GABAergic axons (data not shown).

The hypothesis that Pcdh-γC5 is involved in the stabilization and maintenance of some GABAergic synapses was tested in the TCKO mouse. The HP cultures showed decreased density of γ2-$GABA_A$R clusters and GABAergic innervation. However, we could not exclude the possibility that these effects could result from increased apoptosis and death of some presynaptic interneurons. Moreover, any phenotype of the TCKO mice could not be unambiguously attributed to the absence of Pcdh-γC5, since Pcdh-γC3 and Pcdh-γC4 were also deficient in these mice. Furthermore, the TCKO mice shows upregulation of several Pcdhs, which could compensate for the loss of Pcdh-γC5, thus potentially reducing a synaptic phenotype. A better approach was to do specific perturbations of Pcdh-γC5 expression by (1) knocking down Pcdh-γC5 and (2) studying the dominant-negative effect of ^{cMyc}Pcdh-γC5-intra overexpression. Both

showed decreased density of γ2-$GABA_A$R clusters and GABAergic innervation. These results support the hypothesis that Pcdh-γC5 is involved in the stabilization and maintenance of some GABAergic synapses. The decrease in GABAergic innervation of the neurons in which Pcdh-γC5 has been knocked down is likely the consequence of the reduced density of γ2-$GABA_A$R clusters in these neurons. In support of this notion, we have shown that, in this type of HP culture, disruption of the postsynaptic γ2-$GABA_A$R clusters and gephyrin clusters (both are reduced by knocking down γ2-$GABA_A$R or gephyrin expression with either γ2 shRNA or gephyrin shRNA) is followed by reduction in the GABAergic innervation of these neurons (Li et al., 2005b; Yu et al., 2008).

Pcdh-γC5 facilitates the trafficking of γ2-containing $GABA_A$Rs to the cell surface and to GABAergic synapses. In support of this notion, our data show that (1) in HP neurons, overexpression of ^{cMyc}Pcdh-γC5 leads to increased density of γ2-$GABA_A$R clusters; (2) in HEK293 cells, ^{cMyc}Pcdh-γC5 increases the surface expression of both the γ2 subunit and assembled γ2-containing $GABA_A$Rs to the cell surface; and (3) these effects require the presence of the cytoplasmic domain of Pcdh-γC5. We have shown at the EM level that, in the brain, Pcdh-γC5 can also be localized in intracellular organelles near the GABAergic postsynaptic membrane, consistent with the regulation of postsynaptic $GABA_A$R trafficking by Pcdh-γC5. A synaptic role and a trafficking role of Pcdh-γC5 are nonexclusive and are consistent with the hypothesis proposed by Fernández-Monreal et al. (2009), stating that Pcdh-γs are trafficked and inserted in cell–cell contacts and synaptic membranes, being involved in homophilic transcellular interactions, and that this process is regulated by control of the intracellular trafficking of Pcdh-γs via their cytoplasmic domain. These studies were done with Pcdh-γA3 and Pcdh-γB2. Our results with Pcdh-γC5 support and expand the hypothesis.

Additional support for a role of Pcdh-γC5 on the trafficking of $GABA_A$Rs is derived from our finding that the amino acid sequence (amino acids 378–404) of the γ2IL that interacts with Pcdh-γC5 also interacts with various proteins involved in $GABA_A$R trafficking, such as $GABA_A$R-associated protein (GABARAP), Golgi-specific DHHC zinc finger protein (GODZ), and calcium-modulating cyclophilin ligand (CALM) (for review, see Chen and Olsen, 2007; Luscher et al., 2011). Therefore, Pcdh-γC5 in concert with GABARAP, GODZ, and/or CALM could be involved in the trafficking of γ2-containing $GABA_A$Rs to the cell surface and GABAergic synapses.

Only a few molecules that bind to the cytoplasmic domain of Pcdh-γs had been previously identified. The microtubule-destabilizing protein SCG10 interacts with Pcdh-γBs CD (Gayet et al., 2004). The focal adhesion kinases PYK2 and FAK interact with both Pcdh-γ CD and Pcdh-α CD (Chen et al., 2009), and programmed cell death protein 10 (PDCD10) interacts with Pcdh-γ CD (Lin et al., 2010). The receptor tyrosine kinase Ret instead interacts with the ectodomains of Pcdh-α and Pcdh-γ, but it phosphorylates their cytoplasmic domain (Schalm et al., 2010). While Ret, SCG10, PYK2, FAK, and PDCD10 interact with several or all members of the Pcdh-γ family, γ2-$GABA_A$R specifically interacts with Pcdh-γC5.

It is also worth mentioning that two Pcdh-γC5 molecular species (120 kDa and a 140 kDa) coprecipitate from brain extracts with anti-γ2 and anti-α1 $GABA_A$R antibodies. The 120 kDa is the predominant form of Pcdh-γC5 in the brain. The low abundant 140 kDa form is preferentially coimmunoprecipitated by anti-γ2. Both anti-α1 and anti-γ2 coprecipitate the 120 kDa protein to a similar extent, which supports the notion that the 120 kDa Pcdh-γC5 interacts with the assembled $GABA_A$R pentamers (containing α, β, and γ subunits) since Pcdh-γC5 directly interacts with γ2 but not with α1, nevertheless is precipitated by anti-α1. The coprecipitation of [^{3}H]FNZ binding by anti-Pcdh-γC5 also supports the notion that the assembled $GABA_A$R pentamer interacts with Pcdh-γC5 since [^{3}H]FNZ binds only to assembled $GABA_A$Rs. Regarding the 140 kDa Pcdh-γC5 form, we do not know yet whether the preferential coimmunoprecipitation of this form with anti-γ2 over anti-α1 is because the 140 kDa Pcdh-γC5 preferentially interacts with nonassembled γ2. It is also possible that the 140 kDa protein is not Pcdh-γC5, but this is an unlikely possibility since both the 140 kDa and the 120 kDa proteins are recognized by two anti-Pcdh-γC5 antibodies to different epitopes (the N and C terminus, respectively). Thus, both proteins have the same N terminus and C terminus. We have shown that the 120 and 140 kDa forms are mature post-ER glycosylated proteins. Their difference in Mr is not due to differential glycosylation of the same polypeptide. A possibility is that the 120 and 140 kDa Pcdh-γC5 isoforms result from differential Pcdh-γ *cis*- and *trans*-splicing (Wu and Maniatis, 1999; Tasic et al., 2002; Wang et al., 2002a). Other possibilities are differential posttranslational modification (other than glycosylation) or a tight association of a subpopulation of the 120 kDa Pcdh-γC5 with another protein of ~20 kDa, which increases the affinity of Pcdh-γC5 for γ2 binding. These possibilities are currently under investigation.

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