The b1 isoform of protocadherin-gamma (Pcdhγ) interacts with the microtubule-destabilizing protein SCG10

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Abstract Due to their structural characteristics and their diversity, the 22 members of the protocadherin-gamma (Pcdhγ) family have been suggested to contribute to the establishment of specific connections in the nervous system. Here, we focus on a single isoform, Pcdhγ-b1. Its expression is found in different brain regions and in developing spinal cord it is restricted to scattered cells, whereas all cells are labeled using an antibody that recognizes all Pcdhγ isoforms. As a first step to understanding the signaling mechanisms downstream of Pcdhγ, we identify the microtubule-destabilizing protein SCG10 as a cytoplasmic interactor for Pcdhγ-b1 and other isoforms of the Pcdhγ-b subfamily, and show that SCG10 and Pcdhγ-b1 are found together in certain neuronal growth cones.

Keywords: Protocadherin-gamma; Protocadherin; SCG10; Growth cone; Two-hybrid

1. Introduction

The large number of protocadherins (Pcdhs) and their diversity and high expression levels in neural tissue suggest that they might provide a molecular code involved in establishing complex networks of neuronal connections [1–3]. Analysis of the expression of the entire Protocadherin-gamma (Pcdhγ) family shows that Pcdhγ proteins in neurons are localized both in growth cones [4] and at synapses [5]. Furthermore, mice homozygous for deletion of the *Pcdhγ* locus die at birth and show extensive apoptotic death of interneurons in the spinal cord, possibly resulting from erroneous synapse formation [6].

Pcdhs are transmembrane proteins: their extracellular domain comprises a variable number of cadherin ectodomains but their cytoplasmic domain has no similarity with the cadherin intracellular domain (for review, see [7]). The major part of each of the 22 Pcdhγ isoforms is encoded by a single large "variable" exon, which is spliced to three short downstream "constant" exons [8,9]. The resulting proteins differ in their extracellular and transmembrane domains and in first part of their cytoplasmic domains, but share an identical C-terminal cytoplasmic domain (Fig. 1A). On the basis of their sequences, Pcdhγ isoforms may be further subdivided into three subclasses – a, b and c – comprising 12, 7 and 3 members, respectively [3]. The variable portion of the cytoplasmic domains of Pcdhγ isoforms differs between subclasses, suggesting that each subclass might interact with characteristic downstream effectors. This might provide a means by which different subclasses regulate different cellular functions. However, nothing is currently known of the signaling capacities of the Pcdhγ isoforms.

The b subfamily is of particular interest because the cytoplasmic variable domains of these isoforms contain a short conserved sequence including two tyrosine residues, which represent potential sites of post-translational regulation. We have performed a two-hybrid screen to identify putative downstream effectors. We show here that the cytoplasmic domain of Pcdhγ-b1 interacts with SCG10, a protein present in growth cones that is involved in the control of microtubule polymerization during neuronal differentiation.

2. Materials and methods

2.1. Antibodies

To raise antibodies specific to the mouse Pcdhγ-b1 isoform, the variable intracellular region was used as an antigen. It was produced in *Escherichia coli* BL21-RIL as a GST fusion protein expressed from pGEX-6P vector, purified and separated from the GST moiety by endoprotease cleavage. Rabbits were immunized with the purified protein at Eurogentec and specific antibodies were purified from the serum by affinity purification with a His-tag fusion of the variable intracellular region of Pcdhγ-b1 cross-linked to a Sepharose column (Pharmacia). Pan-Pcdhγ antibodies were described previously [4], serum against SCG10 [10] was a gift of Dr. A. Sobel. α-Tubulin was detected using mouse monoclonal antibody DM1A (Sigma).

Western blot analysis was performed as described previously [11] using chemiluminescence with ECL or ECL Plus kit (Amersham), depending on the sensitivity required.

2.2. Two-hybrid screening

Yeast two-hybrid analysis was carried out using the Matchmaker Gal4 Two-Hybrid System3 (Clontech) according to the manufacturer's instructions. Selection of interactors used only histidine and adenine auxotrophy selection. The bait vector was constructed by PCR amplification and in-frame cloning of the Pcdhγ-b1 cDNA region corresponding to nucleotides 2271–2549 (GenBank BC054741) in pGBKT7-BD vector and verified by sequencing. A pre-existing library

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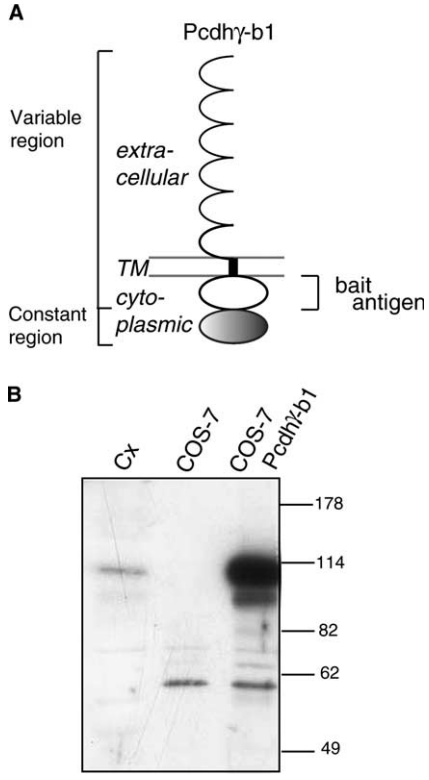

Fig. 1. Pcdhγ-b1 structure and antibodies validation. (A) Schematic representation of Pcdhγ. The extracellular and transmembrane domains, and the first part of the cytoplasmic domains, are different for each Pcdhγ isoform while the C-terminal cytoplasmic tail is identical for all isoforms. The variable part of the cytoplasmic domain was used to generate isoform specific antibodies against Pcdhγ-b1. It also served as bait in the two-hybrid screen and as GST fusion for the in vitro interaction assay. (B) The purified antibodies were tested by Western blotting on extracts of cortex and mock-transfected COS-7 cells or COS-7 cells transfected with Pcdhγ-b1, as negative and positive controls, respectively.

prepared from E12, E14 and E16 mouse ventral spinal cord was subcloned in pGADT7-AD vector. As negative controls, bait vector with lamin and SMN inserts was used.

2.3. Production of GST fusion proteins and in vitro verification of interactions

The cDNA sequence corresponding to the variable cytoplasmic domains of Pcdhγ-b1, Pcdhγ-b2, Pcdhγ-b8 and Pcdh γ-c4 was cloned in frame with GST in the pGEX6P1 vector. GST fusion proteins were produced in *E. coli* strain BL21-RIL. In vitro transcription and translation reaction of linearized plasmid was performed using a wheat-germ extract system (Promega) in the presence of T7 RNA polymerase and [^{35}S]methionine. 5 μg of glutathione–agarose beads (Sigma) coupled to the GST fusion proteins was incubated with 7.5 μl of TTIV products in interaction buffer (50 mM Tris, pH 7.2; 10 mM $MgCl_2$; 0.2% Tween, 0.1 mM DTT; 0.1 mM EDTA; and 150 mM NaCl). Three washes were performed with 20 ml of 1 M NaCl and three times with 20 ml PBS. For some clones, we did not obtain correct in vitro transcription and translation and could not test the interaction in vitro.

2.4. Co-immunoprecipitation studies

To drive overexpression of full-length Pcdhγ-b1, cDNA was fused to a FLAG epitope in a pCAGGS vector [12] and SCG10 fused to a myc-tag was overexpressed from a pCDNA3 vector[10]. Three 6-cm dishes containing 2.5×10^5 COS-7 cells were transfected with 1 μg of each plasmid. After 24 h, cells were collected by scraping in 300 μl lysis buffer (50 mM Tris, pH 7, 10 mM $MgCl_2$, and 1% Triton) in the presence of protease inhibitor cocktail (Sigma). Lysates were incubated for 2 h at 4 °C with anti-c-Myc agarose conjugate (Sigma), washed two times in 50 mM Tris, pH 7, 150 mM NaCl, and 1% Triton buffer and two times in 50 mM Tris, pH 7.4, 150 mM NaCl buffer and boiled for 5 min in Laemmli buffer prior to loading on SDS–PAGE gels.

3. Results

3.1. Expression of Pcdhγ-b1 protein

In order to study Pcdhγ-b1 expression, we developed affinity-purified rabbit polyclonal antibodies directed against the variable cytoplasmic region (Fig. 1A). On Western blots, they recognized a 114 kDa band in the extracts of adult cerebral cortex and COS7 cells transfected with Pcdhγ-b1 (Fig. 1B). No staining was observed in mock transfected COS-7 cells. We used these antibodies to study the tissue and developmental expression profiles of Pcdhγ-b1. As expected, Pcdhγ-b1 levels were low compared to that of the entire Pcdhγ family, detected with a pan-Pcdhγ antibody that recognizes all isoforms. Relative levels of Pcdhγ-b1 versus pan-Pcdhγ varied between tissues (Fig. 2A). For instance, in olfactory bulb, relative levels were higher than in cortex. It is not possible to compare the absolute intensities of Pcdhγ-b1 and pan-Pcdhγ bands (Fig. 2A and B) since, to detect Pcdhγ-b1, we needed to use a more sensitive method than for pan-Pcdhγ. To study the developmental regulation of Pcdhγ-b1, we followed its expression levels in embryonic and postnatal spinal cord, which is the tissue affected in *Pcdhγ*$^{-/-}$ mutant mice. Pcdhγ-b1 levels in spinal cord increased from E11 to P5 but decreased substantially thereafter (Fig. 2B).

The weak signal detected in Western blots for Pcdhγ-b1 could be due either to a low level of ubiquitous expression or to selective expression in a subset of cells. We therefore performed immunohistochemistry for Pcdhγ-b1 on sections of spinal cord from newborn mice (Fig. 2C). Whereas Pcdhγ constant region is expressed in the entire spinal cord (Fig. 2D), Pcdhγ-b1 expression was detected in only a limited number of cells. The staining pattern was symmetrical between the right and left halves of the spinal cord, suggesting that the pattern reflects a precise regulation and is not purely stochastic. Positive cells were found not only in the dorsal and medial spinal cord where interneurons are localized (Fig. 2C, arrowheads) but also in the ventral horn. Interestingly, they are interneurons of the medial region that degenerate in Pcdhγ knockout mice [6].

3.2. Two-hybrid screen for partners of Pcdhγ-b1

In order to gain insight in the cellular functions in which Pcdhγ-b1 is involved, we performed a two-hybrid screen to identify binding partners that might be its effectors. We used as bait the 93-amino acid variable cytoplasmic domain of Pcdhγ-b1. Two million clones of a ventral spinal cord cDNA library were screened for interactions. Plasmid DNAs encoding putative interactors were isolated from the positive clones. We co-transformed yeast again with these plasmids and with the bait vector or several negative controls constructs as described in Section 2. Specific interactions were confirmed for 25 different cDNAs. Eleven of the selected cDNAs encoded unknown proteins; these were not analyzed further as they were not expected to give information about Pcdhγ-b1 function. Six other cDNAs encoded proteins predicted to be

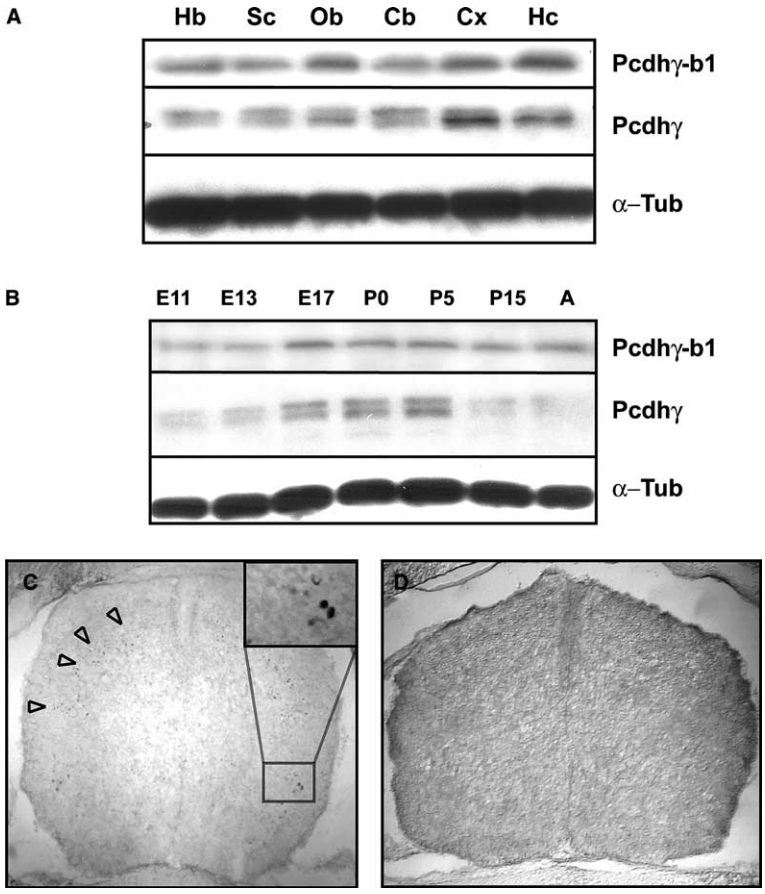

Fig. 2. Pcdhγ-b1 expression in nervous tissue. (A) Pcdhγ-b1 expression in adult neural tissues, the same blot was stripped and probed with pan-Pcdhγ antibodies and finally anti-α-tubulin as a loading control. Pan-Pcdhγ and Pcdhγ-b1 are always co-expressed although the relative levels differ from tissue to tissue (Hb, hindbrain; Sc, spinal cord; Ob, olfactory bulb; Cb, cerebellum; Cx, cortex; Hc, hippocampus). (B) Pcdhγ-b1 expression increases during spinal cord development from embryonic day 11 (E11) to reach a peak around P5 and is only weakly downregulated thereafter. This contrasts with pan-Pcdhγ, which shows sharper variations during development. (C) Immunohistochemical detection of Pcdhγ-b1 on a section of mouse P0 spinal cord. Pcdhγ-b1 is expressed only in a limited number of cells. (D) Pan-Pcdhγ antibodies reveal the ubiquitous expression of Pcdhγ in the P0 spinal cord.

localized in non-cytoplasmic cellular compartments, meaning that their interaction with the cytoplasmic domain of Pcdhγ-b1 is not likely to be biologically relevant; these were not analyzed further. The remaining clones could be classified into different categories according to their properties. Three of the isolated cDNAs encoded proteins associated with axon outgrowth and guidance: SCG10, ULIP and laminin receptor 1. Three others had a described function in RNA metabolism: translation factor eif4g2, poly C binding protein-1 and snRNPU5 40 kDa protein. Of the two remaining, one encoded the intermediate filament α-internexin and the other the Pcdhγ-c4 cytoplasmic variable region.

3.3. In vitro assay shows strong interaction of Pcdhγ-b1 with SCG10

The proteins identified in the two-hybrid screen were further tested for their interaction with Pcdhγ-b1 in an in vitro assay. cDNAs encoding putative interactors were transcribed and translated in vitro in the presence of radiolabeled methionine and used for a GST pull-down assay using either a fusion protein of GST with the variable domain of Pcdhγ-b1 or GST alone. Interaction with Pcdhγ-b1 was confirmed for Pcdhγ-c4 and the RNA binding protein polyC binding protein 1 Pcbp1.

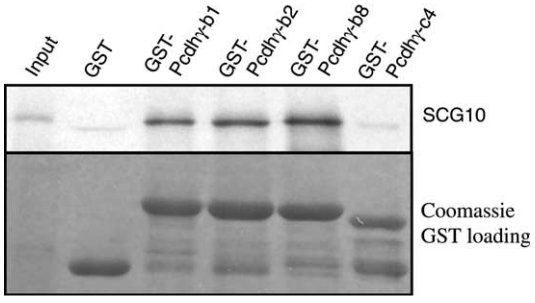

Fig. 3. Pcdhγ-b1 variable cytoplasmic domain interacts with SCG10. Upper panel: autoradiograph of the GST pull-down reactions. In the first lane, 1 μl of radiolabeled SCG10 was deposited. The following lanes show the amounts of radiolabeled SCG10 product pulled down by the GST protein alone or by GST fused to the variable cytoplasmic domain of Pcdhγ-b1, Pcdhγ-b2, Pcdhγ-b8 and Pcdhγ-c4, respectively. Lower panel: Coomassie staining of the SDS–PAGE gel shown in the upper panel, showing the amount of GST product used in each reaction.

However, the strongest interaction was found for SCG10, a microtubule-destabilizing protein found in growth cones of neuronal cells. As shown in Fig. 3, SCG10 was retained at high

levels by a GST::Pcdhγ-b1 fusion protein, while only a small fraction was retained by GST alone. We next asked if the interaction of Pcdhγ-b1 with SCG10 was isoform-specific or if it was a characteristic shared with other Pcdh isoforms. We performed pull-down assays of full-length SG10 with GST fused to either Pcdhγ-b1, Pcdhγ-b2, Pcdhγ-b8 or Pcdhγ-c4. As shown in Fig. 3, a specific interaction was found for the b subclass isoforms tested, which show 34% protein sequence identity in the variable cytoplasmic domain, but not for the c4 isoform whose sequence is very divergent, nor with GST alone.

3.4. Co-immunoprecipitation of Pcdhγ-b1 and SCG10 in cell extracts

Experiments in yeast and in vitro used the variable cytoplasmic domain of Pcdhγ-b1 fused either to GAL4 or to GST, and thus did not reproduce the normal cellular environment of the protein. We tested the interaction of SCG10 with the entire Pcdhγ-b1 protein after overexpression in COS7 cells. Pcdhγ-b1 was expressed as a fusion with a Flag tag and SCG10 was tagged with myc epitope. Immunoprecipitation with an antibody to myc led to clear co-precipitation of Pcdhγ-b1 (Fig. 4). We also performed the reverse experiment: immunoprecipitation with an anti-FLAG antibody also co-precipitated SCG10 (Fig. 4). Thus, the full-length Pcdhγ-b1 isoform can interact with SCG10 when expressed in mammalian cells.

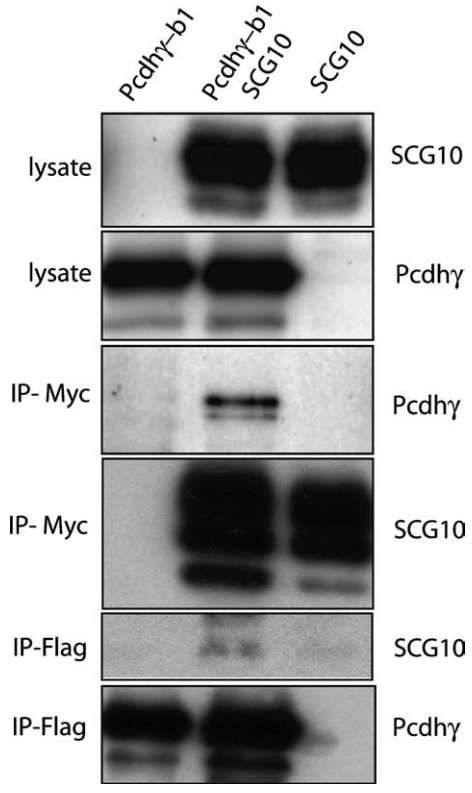

Fig. 4. Co-immunoprecipitation of PCDHγ-b1 and SCG10 in COS cells. Cos-7 cells were transfected with either Pcdhγ-b1-FLAG, Pcdhγ-b1-FLAG and SCG10-myc or SCG10-myc. The upper two panels show Western blots of the lysates. The lower panels show the immunoprecipitation reaction performed with an anti-myc antibody for SCG10-myc or with an anti-Flag for Pcdhγ-b1-Flag. SCG10 was detected with antibody 9E10 against myc-epitope and Pcdhγ with a pan-Pcdhγ antibody.

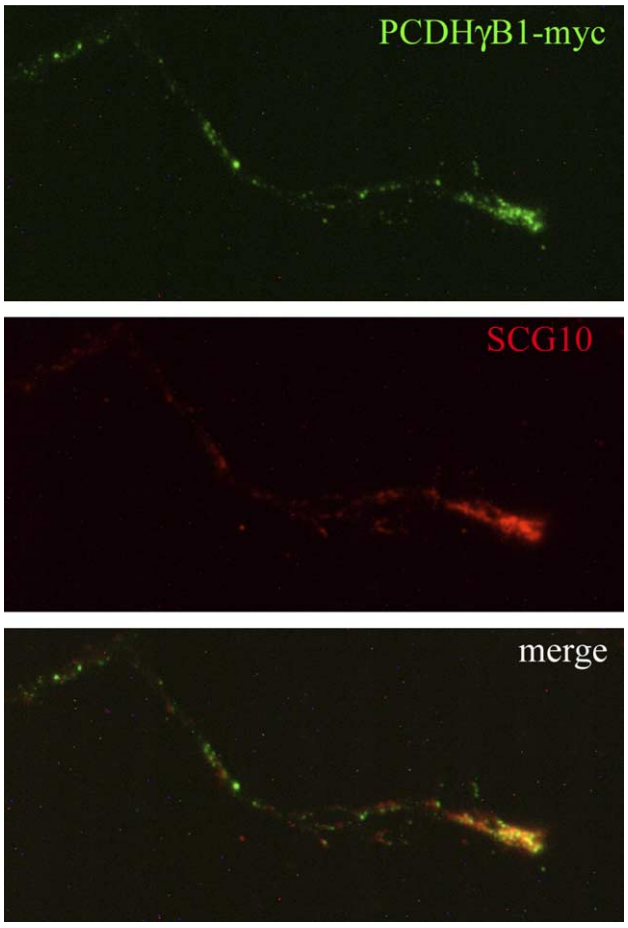

Fig. 5. Pcdhγ-b1 is found together with SCG10 in a subset of growth cones of spinal interneurons. A growth cone of a spinal interneuron transfected with Pcdhγ-b1-myc. Pcdhγ-b1 was visualized with antibodies against the myc-tag (green) and endogenous SCG10 (red) using specific antibodies.

3.5. SCG10 and Pcdhγ-b1 are both localized in growth cones

We previously reported the localization of Pcdhγ in growth cones of developing neurons [4]. However, the use of pan-Pcdhγ antibodies does not allow us to extrapolate this result to the Pcdhγ-b1 isoform which might have a different localization. As the antibodies specific for the Pcdhγ-b1 isoform do not detect endogenous protein in cultured cells, we transfected myc-tagged Pcdhγ-b1 into primary mouse spinal interneurons and compared its localization with that of endogenous SCG10. As shown in Fig. 5, Pcdhγ-b1 and SCG10 did not colocalize in the axon shaft but, in a subset of neurons, their fluorescence patterns in the growth cone could be superimposed, suggesting that at some stages of the growth cone progression Pcdhγ-b1 and SCG10 are appropriately placed to interact.

4. Discussion

Our study is the first to focus on the b1 isoform of Pcdhγ. We show that it is expressed in a discrete population of neurons during spinal cord development. Moreover, in order to identify downstream effectors of Pcdhγ-b1 protein we performed a two-hybrid screen using its variable cytoplasmic domain as bait and identified SCG10 as a potentially relevant

intracellular effector. We confirmed the interaction using two further independent assays, GST pull down and co-immunoprecipitation. We also showed that other Pcdhγ-b subtype proteins, but not a c-subtype control, can interact with SCG10.

Our observations of the highly localized pattern of Pcdhγ-b1 in spinal cord are in line with the work of Wang et al. [8] who found that two individual isoforms, A11 and C4, were always co-expressed with the constant region and, depending on the neural tissues examined, were expressed in more or less overlapping sets of cells, thus supporting the idea of combinatorial expression patterns to distinguish neuronal subtypes. Distinct but related promoters upstream of each variable region exon would allow for such individual transcriptional regulation [8,9].

SCG10 is a microtubule-destabilizing protein of the stathmin family whose expression is restricted to the nervous system (for review, see [13]). It is associated with membranes and is regulated through phosphorylation [14]. SCG10 and Pcdhγ-b1 show similar developmental expression profiles and both localize to growth cones [15,16]. However, although Pcdhγ-b1 and SCG10 are both found in all growth cones examined, they show close superposition only in a subset; this might reflect a modulation of the interaction depending on the phase of progression of the growth cone. Both Pcdhγ and SCG10 are expressed at high levels in the dorsal root entry zone of the spinal cord at developmental stages when sensory axons tips reach this region and stop to make a long pause before entering the spinal cord [4,15].

The interaction of Pcdhγ-b1 and other members of the Pcdhγ-b subfamily with SCG10 could lead to modifications of the microtubule cytoskeleton in response to extracellular cues. These cues remain to be identified but could be homophilic interactions, as described for Pcdhγ-c3 [17,18]. The loss of this regulatory signaling would be expected to perturb neurite guidance and lead to establishment of erroneous connections, as indeed observed in mice lacking the entire Pcdhγ gene cluster [6].

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