

Published in final edited form as:

*Neuron*. 2012 August 9; 75(3): 402–409. doi:10.1016/j.neuron.2012.06.039.

## FUNCTIONAL SIGNIFICANCE OF ISOFORM DIVERSIFICATION IN THE PROTOCADHERIN GAMMA GENE CLUSTER

Weisheng V. Chen<sup>1</sup>, Francisco J. Alvarez<sup>2,5</sup>, Julie L. Lefebvre<sup>3</sup>, Brad Friedman<sup>1,6</sup>, Chiamaka Nwakeze<sup>1</sup>, Eric Geiman<sup>2</sup>, Courtney Smith<sup>2</sup>, Chan Aye Thu<sup>1</sup>, Juan Carlos Tapia<sup>1</sup>, Bosiljka Tasic<sup>4</sup>, Joshua R. Sanes<sup>3</sup>, and Tom Maniatis<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biophysics, Columbia University Medical Center, 701 W. 168<sup>th</sup> Street, New York, NY 10032

<sup>2</sup>Department of Neuroscience, Physiology and Cell Biology, Wright State University School of Medicine, 3640 Colonel Glenn Highway, Dayton, OH 45435

<sup>3</sup>Center for Brain Science and Department of Molecular and Cellular Biology, Harvard University, 52 Oxford Street, Cambridge, MA 02138

<sup>4</sup>Allen Institute for Brain Science, 551 N. 34<sup>th</sup> Street, Seattle, WA 98103

### SUMMARY

The mammalian Protocadherin (*Pcdh*) alpha, beta, and gamma gene clusters encode a large family of cadherin-like transmembrane proteins that are differentially expressed in individual neurons. The 22 isoforms of the *Pcdhg* gene cluster are diversified into A-, B- and C-types, and the C-type isoforms differ from all other clustered *Pcdhs* in sequence and expression. Here we show that mice lacking the 3 C-type isoforms are phenotypically indistinguishable from the *Pcdhg* null mutants, displaying virtually identical cellular and synaptic alterations resulting from neuronal apoptosis. By contrast, mice lacking 3 A-type isoforms exhibit no detectable phenotypes. Remarkably, however, genetically blocking apoptosis rescues the neonatal lethality of the C-type isoform knockouts, but not that of the *Pcdhg* null mutants. We conclude that the role of the *Pcdhg* gene cluster in neuronal survival is primarily, if not specifically mediated by its C-type isoforms, whereas a separate role essential for postnatal development, likely in neuronal wiring, requires isoform diversity.

### INTRODUCTION

Protocadherins (*Pcdhs*) are the largest subgroup of the cadherin superfamily of cell adhesion proteins. Of the ~70 *Pcdh* genes identified in mammalian genomes, over 50 are located in three tightly linked gene clusters (*Pcdha*, *Pcdhb*, and *Pcdhg*) on a single chromosome (Wu

© 2012 Elsevier Inc. All rights reserved.

\*Correspondence: tm2472@columbia.edu; 212-305-3669.

<sup>5</sup>Present address: Department of Physiology, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322

<sup>6</sup>Present address: Department of Bioinformatics and Computational Biology, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

### SUPPLEMENTAL DATA

The Supplemental Data include the Supplemental Experimental Procedures, 5 Figures, 2 Tables, and 2 Movies and can be found with this article online.

and Maniatis, 1999). These clustered *Pcdh* genes are found exclusively in vertebrates, and are predominantly expressed in the nervous system. Distinct subsets of *Pcdh* genes are differentially expressed in individual neurons, and enormous cell surface diversity may result from combinatorial expression (Esumi et al., 2005; Kaneko et al., 2006; Kohmura et al., 1998; Wang et al., 2002a). A subset of *Pcdhg* isoforms have been shown to engage in intercellular interactions that are strictly homophilic (Schreiner and Weiner, 2010). The molecular diversity as well as the binding specificity of clustered *Pcdhs* has led to the proposal that they provide a synaptic address code for neuronal connectivity, or a single cell barcode for self-recognition and self-avoidance similar to that ascribed to *Dscam1* proteins of invertebrates (Junghans et al., 2005; Serafini, 1999; Shapiro and Colman, 1999; Zipursky and Sanes, 2010).

Genetic manipulations of individual *Pcdh* gene clusters in mice have provided functional evidence that the clustered *Pcdhs* are required for normal development of the nervous system. Mutations in the *Pcdha* gene cluster have been reported to result in defects in olfactory sensory neuron axon coalescence and serotonergic axonal arborization as well as behavioral perturbations (Fukuda et al., 2008; Hasegawa et al., 2008; Katori et al., 2009). By contrast, abolishing *Pcdhg* function leads to neuronal apoptosis and synaptic loss in the spinal cord and retina (Lefebvre et al., 2008; Prasad et al., 2008; Wang et al., 2002b; Weiner et al., 2005). Although these genetic studies have provided interesting insights into the roles of clustered *Pcdhs* in the nervous system, the functional significance of the diverse isoforms encoded by the three gene clusters is not understood. For example, it is unclear whether individual *Pcdh* isoforms within each cluster are functionally equivalent, or whether certain isoforms may play distinct roles. The unique and highly conserved genomic organization of *Pcdh* gene clusters suggests that the isoform diversity and evolutionary diversification of *Pcdh* genes are central to understanding their function.

In mice, the three *Pcdh* gene clusters each contain 14–22 homologous “variable” exons arrayed in tandem. Each variable exon is transcribed from its own promoter, and encodes the entire extracellular domain, a transmembrane domain, and a short intracellular domain of the corresponding *Pcdh* protein. In *Pcdha* and *Pcdhg* clusters (but not *Pcdhb* cluster), these variable exons are followed by a set of three “constant” exons, which are joined to each variable exon via *cis*-splicing to encode a common distal intracellular domain (Tasic et al., 2002; Wang et al., 2002a). An interesting feature of the genomic organization of the *Pcdh* gene clusters is that the last two variable exons in the *Pcdha* cluster, as well as the last three variable exons in the *Pcdhg* cluster, are more similar to each other than to other variable exons within their respective cluster (Wu et al., 2001). These 5 *Pcdh* genes (*Pcdhac1*, *Pcdhac2*, *Pcdhgc3*, *Pcdhgc4*, and *Pcdhgc5*) are designated C-type genes, to be distinguished from A-type and B-type genes of the *Pcdhg* cluster. The C-type isoforms bear several unique features among all *Pcdhs*: 1) While all other *Pcdhs* are more closely related to members within their own cluster, C-type isoforms are evolutionarily divergent, forming a separate branch in the phylogenetic tree (Wu and Maniatis, 1999; Wu et al., 2001); 2) Three out of the five C-type genes (*Pcdhac2*, *Pcdhgc4* and *Pcdhgc5*) lack the conserved sequence element (CSE) found in the promoters of all other *Pcdh* genes (except *Pcdhb1*), suggesting that these genes are regulated differently (Wu et al., 2001). 3) Single cell RT-PCR experiments indicated that, while other *Pcdh* genes are stochastically and monoallelically expressed in Purkinje neurons, every neuron expresses all five C-type genes from both chromosomes (Esumi et al., 2005; Kaneko et al., 2006). Taken together, these observations suggest that the C-type isoforms play unique and essential roles among all clustered *Pcdhs*. To investigate this possibility, we generated mutant mice lacking the 3 C-type genes (*Pcdhgc3*, *Pcdhgc4*, *Pcdhgc5*) in the *Pcdhg* cluster.

## RESULTS

### Mice lacking the C-type *Pcdhg* genes are phenotypically indistinguishable from mice lacking the entire *Pcdhg* cluster

The triple C-type isoform knockout (TCKO) allele was generated by deleting the three variable exons (Figures 1A–B and S1A), which specifically removes the C-type genes without affecting the splicing of the remaining 19 A-type and B-type *Pcdhg* variable exons (see below). *Pcdhg<sup>tcko/tcko</sup>* mutants are born alive at the normal Mendelian ratio, but invariably die during the first day after birth. The mutant mice are readily distinguishable from wild type and heterozygous littermates by a characteristic hunched posture and limb tremors, as well as by severely compromised voluntary movements and reflexes (Figure 1C and Movie S1). Remarkably, these phenotypes are identical to those described for the *Pcdhg* full cluster deletion mice (Figure 1D and Movie S1), in which all *Pcdhg* isoforms are abolished (Wang et al., 2002b). In addition to the common phenotypes described above, we found that both lines of mutants also exhibit intense muscle stiffness (Figures 1F–F’’) and umbilical hernia (Figures 1G–G’). Interestingly, these phenotypes closely resemble those of mutant mice deficient in VGAT (Wojcik et al., 2006), GAD67 (Asada et al., 1997), and Gephyrin (Feng et al., 1998), which are essential components for GABA and glycine production and transmission.

While the virtually identical phenotypes of the *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants demonstrate that C-type isoforms are essential, it is also possible that the entire repertoire of *Pcdhg* genes are required; that is, each isoform is indispensable. Indeed, essentially every *Pcdhg* gene in humans has an ortholog in the mouse, in contrast to the *Pcdha* and *Pcdhb* genes (Wu et al., 2001). To address this possibility, we deleted *Pcdhga1*, *Pcdhga2* and *Pcdhga3* genes in the mouse (Figures 1A–B and S1B). The triple A-type isoform knockout (TAKO) mutants are viable and fertile, and survive to adulthood with no discernible abnormalities (Figure 1E and Movie S1).

### *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants display similar levels and patterns of neuronal cell loss in the spinal cord

Previous studies showed that deletion of the *Pcdhg* cluster leads to extensive apoptosis and eventual loss of specific subpopulations of spinal interneurons (Prasad et al., 2008; Wang et al., 2002b; Weiner et al., 2005). To determine whether these changes also occur in TCKO mutants, we labeled cells undergoing apoptosis with anti-cleaved caspase-3 in P0 spinal cords. As expected, the number of apoptotic profiles is markedly increased in the spinal cord of both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants (Figures 2A–A’). Concurrently, the spinal cords of both mutants exhibit similar levels of astrogliosis and microglia activation (Figures S2A), which typically accompany neuronal cell death. To compare the extent of neuronal cell loss in different *Pcdhg* mutant lines, we quantified the surviving NeuN<sup>+</sup> neurons in different spinal regions at P0. The spinal cords of *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants have a similarly reduced cross sectional area compared to those of the wild type littermates, particularly in the ventral horn (LVI–VIII) and in the deep dorsal horn (LIV–V). Superficial dorsal horn (LI–III) and motor pools (LIX), however, appear relatively normal (Figures 2B–B’ and S2B). Consistently, the most severe neuronal loss was detected in the ventral horn and to a lesser extent in the deep dorsal horn (~70% and ~50%, respectively). We also observed ~30% interneuron cell loss in the superficial dorsal horn, which was not reported previously. By contrast, motor neuron (LIX) counts in both mutants are the same as those in wild type controls (Figures 2H and S2B). As expected, *Pcdhg<sup>tako/tako</sup>* spinal cords are indistinguishable from the wild type controls, and neuronal cell counts in each of the 4 specified regions are normal (Figure S2B).

To investigate whether neuronal subpopulations are similarly affected in *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants, we examined several classes of interneurons in the ventral spinal cord at P0. Interestingly, while Pax2+ and Foxp2+ inhibitory interneurons, as well as Chx10+ excitatory interneurons are similarly reduced in number in both mutants, V1-derived Calbindin (CB)+ Renshaw cells and V0-derived cholinergic ChAT+ partition cells are spared (Figures 2C–G, 2I). In conclusion, the *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants display similar levels and patterns of neuronal cell loss in the spinal cord, and interneuron subpopulations are differentially affected in both mutants.

### ***Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants display similar alterations in specific synaptic inputs onto motor neurons**

In addition to neuronal cell loss, a general reduction in the numbers of both excitatory and inhibitory synapses was observed in the neuropil of *Pcdhg<sup>del/del</sup>* spinal cords using generic synaptic markers (Wang et al., 2002b; Weiner et al., 2005). It was unclear, however, whether all synapses are similarly affected, or if certain types are spared or even increased in number. To distinguish between these possibilities, we examined major classes of synaptic inputs onto motor neurons, a cell type that receives defined synaptic inputs and survives in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants. Four type-specific pre-synaptic markers were used, which respectively label synaptic vesicular transporters for the neurotransmitters GABA and glycine (VGAT), glutamate (VGLUT1 and VGLUT2), and acetylcholine (VAcHT). We found that the average linear density of VGAT+ contacts was markedly decreased in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants (Figures 2J–J", 2O), whereas the number of VGLUT1+ proprioceptive primary afferent inputs was surprisingly increased, more than double the number in wild type controls (Figures 2K–K", 2O). By contrast, the densities of VGLUT2+ and VAcHT+ contacts on motor neurons remain constant (Figures 2L–M, 2O). As expected, all four types of synapses are unaltered in *Pcdhg<sup>tako/tako</sup>* mutants (Figures 2O and S2C).

The significant decrease in VGAT+ synapses on motor neurons in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants is consistent with our observation that the two mutants display identical motor defects, which closely resemble those found in the VGAT (Wojcik et al., 2006), GAD67 (Asada et al., 1997), and Gephyrin (Feng et al., 1998) knockouts. Key features of the common phenotypes are muscle stiffness and immobility, which can be explained by tetanic motor neuron activation due to compromised inhibitory neurotransmission. The reduced density of VGAT+ contacts, as well as the normal numbers of VAcHT+ synapses in *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants correlate well with the significant reduction of inhibitory interneurons and unaltered numbers of cholinergic partition cells in both mutants. By contrast, VGLUT2+ synaptic density is normal despite the reduction of certain premotor glutamatergic interneurons (e.g. Chx10+ V2a interneurons), which suggests that alternative neuronal sources or compensatory mechanisms might be involved in the development of these synapses.

The increased densities of VGLUT1+ contacts in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants indicate alterations in the stretch reflex circuit, where proprioceptive sensory afferents (Ia primary afferents, IaPA) establish monosynaptic contacts with spinal motor neurons innervating the same muscle (Chen et al., 2003). Centrally projecting IaPA axons (Parvalbumin+) in wild type spinal cords are distributed in an orderly fashion around motor pools, but in both mutants they appear clumped and more densely surround motor neurons, consistent with the observed increase in the density of VGLUT1+ contacts (Figures 2N–N"). The percentage of Parvalbumin+ neurons in mutant dorsal root ganglia (DRG) is similar to those of wild type animals (L2 DRG, 23.5±1.3% in *Pcdhg<sup>del/del</sup>* and 21.8±1.7% in *Pcdhg<sup>+/+</sup>*, p>0.05), indicating that the increased density of the central projections of IaPAs is not due to increased numbers of proprioceptive neurons, but rather to a defect in their terminal

arborization resulting from the loss of interneurons they interact with (Jankowska, 1992). Taken together, these observations indicate that similar alterations in specific types of synapses on motor neurons occur in both *Pcdhg*<sup>tcko/tcko</sup> and *Pcdhg*<sup>del/del</sup> mutants.

### Conditionally inactivating the C-type *Pcdhg* genes in the retina recapitulates the *Pcdhg* null phenotype

We next extended the genetic analysis to the retina. We have shown previously that *Pcdhg* genes are required for the survival of many neuronal subpopulations in the developing inner retina, but appear to be dispensable for synapse formation and function (Lefebvre et al., 2008). To generate retina-specific knockouts of TCKO and TAKO mutants, trans-heterozygous animals containing one conditional *Pcdhg*<sup>fcon3</sup> allele (Lefebvre et al., 2008; Prasad et al., 2008) and one *Pcdhg*<sup>tcko</sup> or *Pcdhg*<sup>tako</sup> allele were produced. Upon retina-specific recombination of *Pcdhg*<sup>fcon3</sup> that leads to a functionally null allele, the remaining *Pcdhg* isoforms are only expressed from the *Pcdhg*<sup>tcko</sup> or *Pcdhg*<sup>tako</sup> allele, providing a convenient way to conditionally inactivate the three C-type or A-type isoforms in postnatal animals.

We first examined the retinal architecture in conditional trans-heterozygous and *Pcdhg* null mutant animals. Retina lamination was normal and the ONL and OPL layers were unaffected in all three types of mutants. However, while the INL and IPL layers in *Pcdhg*<sup>fcon3/tako</sup> mutant retinas were indistinguishable from those in control, they were significantly thinner in both *Pcdhg*<sup>fcon3/tcko</sup> and *Pcdhg*<sup>fcon3/fcon3</sup> mutant retinas (Figures 3A-A'', 3D). Quantification of retina interneurons using cell-type specific markers revealed that INL thinning in *Pcdhg*<sup>fcon3/tcko</sup> mutants is due to reduced numbers of bipolar (Chx10+) and amacrine (Pax6+) interneurons (Figures 3B-B'', 3E) as in the null (Lefebvre et al., 2008). Likewise, the loss of RGC projection neurons (Brn3a+) in *Pcdhg*<sup>fcon3/tcko</sup> trans-heterozygous retinas is equal in severity to that of *Pcdhg*<sup>fcon3/fcon3</sup> mutants (Figures 3C-C'', 3F). By contrast, we found no change in retina cell number in conditional *Pcdhg*<sup>fcon3/tako</sup> trans-heterozygous retinas (Figures 3D-F). Like the 3 C-type genes, the 3 A-type genes are also expressed in these cell types in the developing retina (Figures S3A-B), indicating that the full spectrum of *Pcdhg* isoforms are not required for neuronal survival. Increased levels of apoptosis observed in *Pcdhg*<sup>fcon3/tcko</sup> trans-heterozygous retinas (Figures 3G-G') are consistent with the previous finding in *Pcdhg*<sup>fcon3/fcon3</sup> mutants that cell loss and the consequent thinning of IPL is due to elevated programmed cell death (Lefebvre et al., 2008). As with *Pcdhg*<sup>fcon3/fcon3</sup> mutants, targeting of interneurons and RGC dendrites to appropriate IPL laminae was intact in *Pcdhg*<sup>fcon3/tcko</sup> trans-heterozygous mutants (Figures 3H-H'). Therefore, in the retina as in the spinal cord, removal of the 3 C-type isoforms results in elevated neuronal apoptosis and loss of specific neuronal subtypes at similar levels and patterns as deletion of all 22 *Pcdhg* isoforms.

### Expression and function of A-type and B-type *Pcdhg* genes are not appreciably affected in the absence of C-type isoforms

We considered the possibility that the indistinguishable phenotypes of *Pcdhg*<sup>tcko/tcko</sup> and *Pcdhg*<sup>del/del</sup> mutants arise because the deletion of C-type exons, which are located immediately upstream of the constant exons, interferes with transcription and splicing of other *Pcdhg* genes, leading to a severely hypomorphic *Pcdhg* allele. To address this possibility, we first examined the expression of the remaining 19 A- and B-type *Pcdhg* genes in *Pcdhg*<sup>tcko/tcko</sup> brains. RT-PCR using exon-specific primers revealed that all 19 variable exons are expressed and correctly spliced to constant exons (Figures 4A-B). Western blotting indicated that *Pcdhg* total protein levels in *Pcdhg*<sup>tcko/tcko</sup> brains are similar to the wild type and even higher than those in *Pcdhg* full cluster deletion heterozygotes, which are phenotypically normal (Figure 4C). We next asked whether the remaining A- and B-type



proteins in *Pcdhg<sup>tcko/tcko</sup>* mutants are functional. Several studies indicate that Pcdhg and Pcdha proteins interact, and may form multimeric complexes with Pcdhb proteins (Han et al., 2010; Murata et al., 2004; Schalm et al., 2010). Moreover, both Pcdha and Pcdhg proteins are tyrosine phosphorylated in mature neurons, suggesting that they mediate intracellular signaling (Schalm et al., 2010). Co-immunoprecipitation experiments using a pan-Pcdhg antibody in brain lysate indicated that the A- and B-type Pcdhg isoforms in *Pcdhg<sup>tcko/tcko</sup>* mutants still form complexes with Pcdha proteins, they are tyrosine phosphorylated and they interact with Src, suggesting that they are capable of mediating intracellular signaling in the absence of the C-type isoforms (Figures 4D–E). We conclude that *Pcdhg<sup>tcko/tcko</sup>* is not a severe hypomorphic or dominant negative mutant, and that expression and function of the remaining A- and B-type Pcdhg isoforms is not appreciably distinct from those of wild type mice.

To determine whether the expression of a common set of genes is altered in the two phenotypically indistinguishable mutants, we carried out deep sequencing (RNA-Seq) studies using embryonic spinal cords at E13.5, a developmental stage when neurogenesis is near completion (Nornes and Carry, 1978), but elevated apoptosis is not yet detected in the mutants (Prasad et al., 2008). Surprisingly, we observed no striking changes in global gene expression in either of the two mutants other than those in the *Pcdh* gene clusters themselves (Figures S4A–B and Table S1). In the case of *Pcdhg<sup>del/del</sup>* mutants, the majority of *Pcdhb* genes are significantly upregulated, likely the consequence of the closer proximity of a *Pcdhb* cluster enhancer (HS16-20) located downstream of the *Pcdhg* cluster (Yokota et al., 2011), which is now repositioned ~300 kb closer to the *Pcdhb* cluster (Figures 4F–G). The largest increase in expression is observed with an EST gene (*AK149307*), which is located immediately upstream of the *Pcdhg* cluster (Figures 4F–G and Table S1). Sequence analyses revealed that this gene is a relic of the B-type *Pcdhg* isoforms, and its promoter region also contains a conserved sequence element (CSE) found in most *Pcdh* genes (Figures S4C–E). The expression levels of most *Pcdhg* isoforms are not affected by the deletion of C-type genes except for a few neighboring ones which are upregulated, and quantification of constant exon reads indicated that the combinatorial expression levels of the remaining *Pcdhg* genes in *Pcdhg<sup>tcko/tcko</sup>* mice are ~75% of the wild type levels (Figures 4F–G and Table S1). Thus, the loss of function of the C-type isoforms cannot be compensated by other *Pcdhg* isoforms. Many *Pcdhb* genes (as well as *AK149307*) are marginally upregulated in the *Pcdhg<sup>tcko/tcko</sup>* mice, likely also due to the action of the *Pcdhb* cluster enhancer as mentioned above. In addition, no neomorphic *Pcdhg* variants were detected in *Pcdhg<sup>tcko/tcko</sup>* mutants with splice junction analysis of the RNA-Seq data (Table S2). The striking phenotypic similarities in contrast to the vastly distinct Pcdh repertoires in *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants suggest that lack of the C-type *Pcdhg* isoforms themselves, which is common for both mutants, is the primary cause of the common phenotypes.

### Genetically blocking apoptosis rescues the neonatal lethality of *Pcdhg<sup>tcko/tcko</sup>* mutants but not that of *Pcdhg<sup>del/del</sup>* mutants

Since the primary phenotype observed in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* is neuronal cell death, we crossed both mutant lines to *Bax* knockout mice (Knudson et al., 1995) to compare phenotypes when neuronal apoptosis is genetically blocked. Consistent with previous observations (Weiner et al., 2005), *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* pups show improved neurological function as compared with *Pcdhg<sup>del/del</sup>* mutants, yet they still lack voluntary movements, and despite considerable efforts we were unable to recover any *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* mutants beyond P0 (Table 1 and Movie S2). Surprisingly, however, while some *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* mutants die at P0, many live substantially longer despite being weaker and smaller than wild type and heterozygous pups. By culling littermates we were able to recover a number of *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* mutants at weaning age. Some of

these animals survived up to 6 months, although their persistent ataxia indicates neurological impairment (Table 1 and Movie S2).

As described for the *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* mutants (Prasad et al., 2008; Weiner et al., 2005), the morphology of spinal cord sections of *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* is indistinguishable from that of the *Pcdhg<sup>+/+</sup>;Bax<sup>-/-</sup>* animals, showing no signs of astrogliosis or microglia activation, and the arborization patterns of IaPA terminals appear largely indistinguishable from those of the controls (Figure S5A). Counts of both VGAT+ and VGLUT1+ inputs onto motor neurons were normal in the *Bax<sup>-/-</sup>* genetic background, while VGLUT2+ and VACHT+ synapses remain unchanged (Figure S5B). Therefore, the decreased number of VGAT+ contacts, as well as the increased number of VGLUT1+ contacts and IaPA terminals found in both *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants at P0, are both secondary to the cell death of spinal interneurons. These results are reminiscent of findings in the *Pcdhg* deficient retina in the *Bax<sup>-/-</sup>* genetic background, where retinal architecture, cell numbers, and synaptic densities are restored in the absence of neuronal apoptosis (Lefebvre et al., 2008).

## DISCUSSION

### The role of the *Pcdhg* gene cluster in neuronal survival is mediated by the C-type isoforms

Previous genetic studies using full cluster deletion mutants revealed that *Pcdh*gs are required for neuronal survival, but the underlying mechanism remains elusive. In this study, we generated mice lacking subsets of *Pcdhg* genes and performed quantitative analyses on specific types of neurons and synapses. Mice lacking C-type *Pcdhg* isoforms are phenotypically indistinguishable from *Pcdhg* null mutants, and the cellular and synaptic changes examined in both the spinal cord and retina are essentially identical. By contrast, mice lacking a subset of A-type isoforms are viable and fertile, revealing at least some level of functional redundancy among the alternative *Pcdhg* isoforms. Molecular and biochemical analyses demonstrate that deletion of C-type isoforms does not appreciably alter the expression or function of the A-type and B-type isoforms, indicating that the C-type isoform knockouts are not simply hypomorphic or dominant negative for *Pcdhg* function. Furthermore, transcriptome profiling shows that the *Pcdh* repertoires of the two mutants differ significantly, but no neomorphic *Pcdhg* variants are generated. Therefore, the loss of function of C-type isoforms themselves is most likely responsible for the identical phenotypes observed in both the C-type isoform knockouts and the *Pcdhg* null mutants.

The most remarkable difference between the two types of mutants is that, the neonatal lethality of C-type isoform knockouts can be rescued by genetically blocking apoptosis, while that of the full cluster *Pcdhg* deletion mutants cannot be rescued. The persistence of neonatal lethality in *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* mutants reveals an additional, independent role of *Pcdhg* isoforms that is required for postnatal development. Therefore, the role of *Pcdhg* cluster in neuronal survival is primarily, if not specifically mediated by the C-type isoforms, whereas the requirement of *Pcdhg* cluster for postnatal development appears to be the collective function of all 22 isoforms in neuronal wiring. Indeed, in a parallel study, we have found dendritic arborization defects in *Pcdhg* null mutants that are not observed in the C-type isoform knockouts (Lefebvre et al., 2012). Hence similar phenotypes are observed in the *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants because the C-type genes are deleted in both lines, and the resulting neuronal cell loss dominates the phenotypes. In the absence of apoptosis, however, the neonatal lethality in C-type isoform knockouts is rescued since neural circuitries essential for postnatal survival are largely preserved by the remaining 19 A-type and B-type *Pcdh*gs. By contrast, *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* compound mutants still die at P0 since the entire *Pcdhg* cluster is deleted, which severely compromises critical wiring and synaptic function carried out by all isoforms synergistically. The dual role of *Pcdhg* cluster

in neuronal survival and neuronal wiring is thus elegantly accomplished by functional and regulatory diversification of its isoforms.

### Neuronal apoptosis occurs independently of synaptic defects in *Pcdhg* deficient mice

Because of their differential expression, homophilic affinity and synaptic localization, the clustered *Pcdhs* have been proposed to be the “synaptic adhesive code” that specifies neuronal connectivity (Junghans et al., 2005; Serafini, 1999; Shapiro and Colman, 1999). Therefore, an intuitively attractive hypothesis for the concurrent neuronal apoptosis and synaptic defects in *Pcdhg* deficient mice is that the loss of function of *Pcdhgs* leads to synaptic loss, which in turn compromises neuronal survival (Junghans et al., 2005; Prasad et al., 2008). However, several observations reported here strongly argue against this possibility. 1) Deletion of *Pcdhg* cluster does not lead to a general loss of synapses. Instead, we found that *Pcdhg* deficiency differentially influences cholinergic, glutamatergic, GABAergic and glycinergic synapses on motor neurons. Therefore, the synapse loss observed using generic synaptic markers only reflects the additive effects of alterations in multiple types of synapses, and loss of synaptic contacts, at the very least, cannot explain the loss of neurons in all cases; 2) Consistent with the observations in the retina, the *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* and *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* compound mutants showed preservation of stretch reflex circuits and major synaptic inputs onto motor neurons, indicating that these severe synaptic defects observed are secondary to interneuron loss; 3) Neural circuitries and synaptic functions are restored to a substantial extent in *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* mutants, as shown by the rescue of neonatal lethality. If the synaptic defects are primary, they should remain when neuronal apoptosis is blocked by *Bax* deficiency (Buss et al., 2006). This is most likely the case in *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* mutants, which could not be rescued.

Taken together, these observations strongly suggest that neuronal cell death in *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants does not result from synaptic defects, but occurs independently due to the lack of C-type genes. Although we cannot rule out the possibility that synaptic or wiring defects may have contributed to the apoptosis of certain types of neurons, it cannot account for the massive cell death observed. In fact, loss of synaptic partners does not usually lead to apoptosis, and even grossly aberrant synaptic connections created experimentally are sometimes maintained without affecting the survival of source neurons (Buss et al., 2006; Oppenheim, 1991).

### Functional similarities and differences between vertebrate *Pcdhs* and invertebrate *Dscams*

The vertebrate-specific *Pcdh* gene cluster shares remarkable resemblance with *Drosophila Dscam1* gene in that they both have a complex genomic structure, which encodes a large number of distinct isoforms of cell adhesion molecules with homophilic binding affinity. These parallels have led to the hypothesis that *Pcdhs*, like *Dscams*, may provide a source of cell surface diversity for neurite self-recognition and self-avoidance (Zipursky and Sanes, 2010). This possibility is supported by our recent finding of dendritic self-avoidance defects in *Pcdhg* deficient mice (Lefebvre et al., 2012). A fundamental difference, however, resides in the fact that each *Dscam1* isoform appears to be functionally equivalent, whereas certain *Pcdhs*, such as the C-type isoforms, have unique roles as we show here. Unlike *Dscam1* where isoform diversity is generated by alternative splicing, differential expression of *Pcdh* isoforms is regulated by alternative promoter choice (Tasic et al., 2002; Wang et al., 2002a), which provides precise spatial and temporal controls over gene expression. As noted earlier, the C-type isoforms are phylogenetically unique among *Pcdhs* and exhibit distinct expression patterns. It remains to be seen whether just one of the three C-type genes is solely responsible for this function, or whether they work synergistically. We note that the two other C-type isoforms in the *Pcdha* cluster (*Pcdhac1* and *Pcdhac2*) are dispensable for neuronal survival (Hasegawa et al., 2008; Katori et al., 2009), but they may play other



specific roles yet to be identified. Further functional studies specifically targeting each of these C-type isoforms would be required to address these possibilities.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

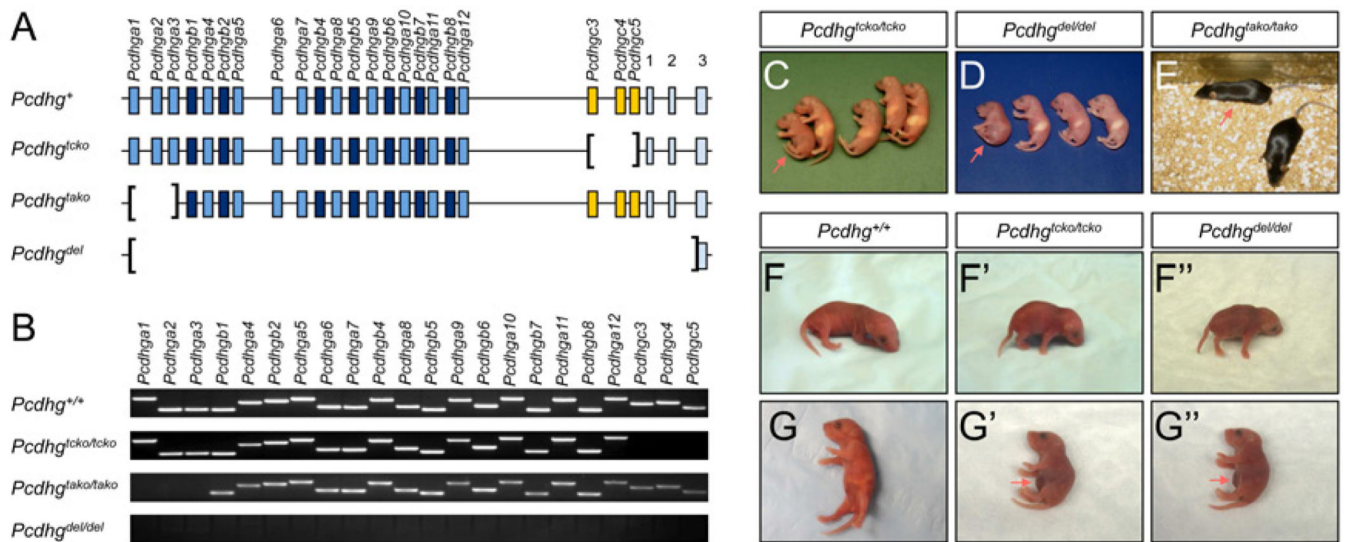
## Acknowledgments

We thank Rick Myers, Rolf Kemler, Greg Philips, Andreas Kolb and Philippe Soriano for providing essential reagents, Monica Carrasco, Flo Pauli, Jiangwen Zhang, Hilary Bowden, and Amy Kirner for technical assistance, and Tom Jessell, George Mentis, Angel de Blas, Larry Shapiro, and members of the Maniatis laboratory for advice and discussion. This work is funded by NIH grants NS047357 to F. J. A., R01NS029169 to J. R. S., and R01NS043915 to T. M.

## REFERENCES

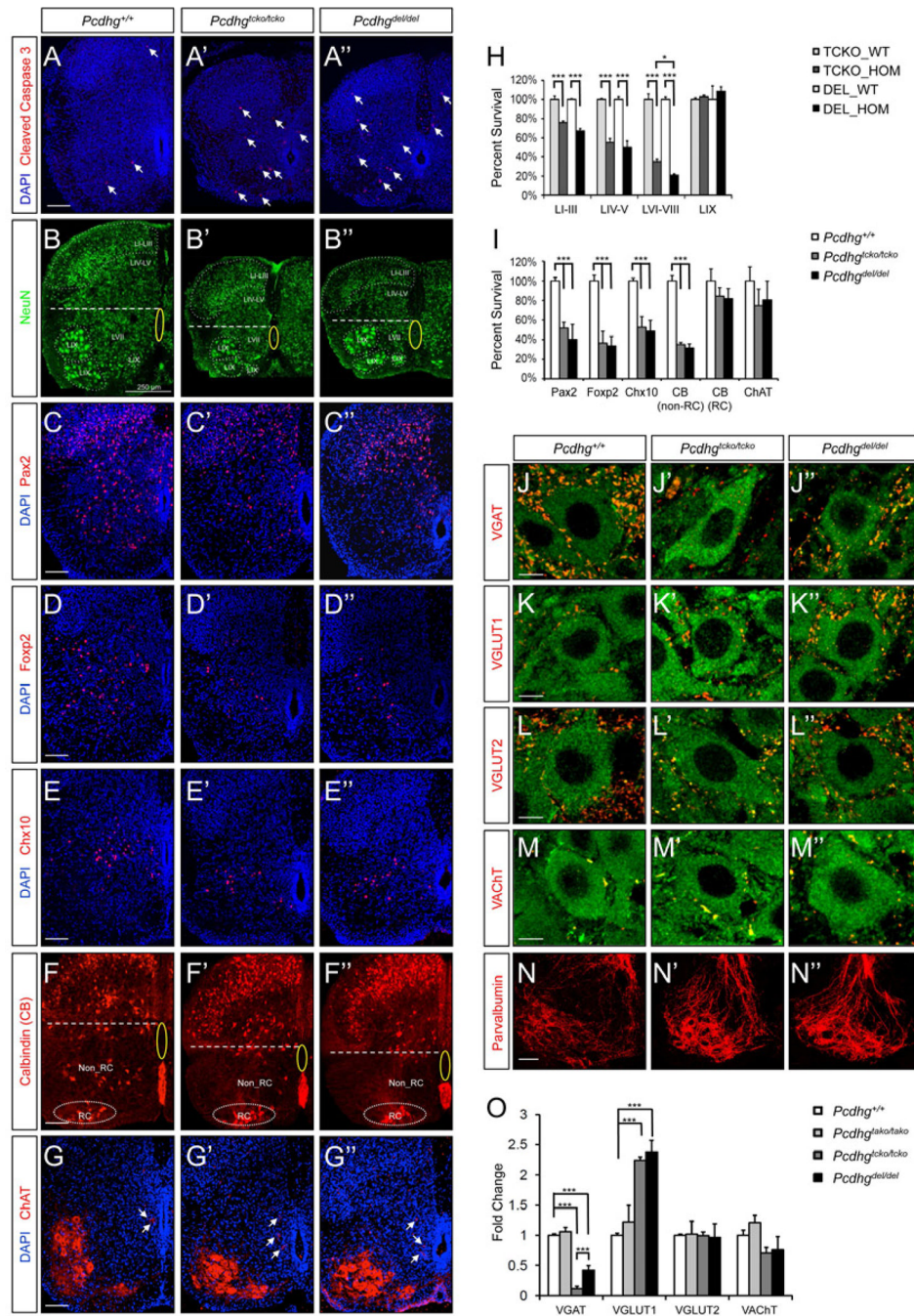
- Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci U S A*. 1997; 94:6496–6499. [PubMed: 9177246]
- Buss RR, Sun W, Oppenheim RW. Adaptive roles of programmed cell death during nervous system development. *Annu Rev Neurosci*. 2006; 29:1–35. [PubMed: 16776578]
- Chen HH, Hippenmeyer S, Arber S, Frank E. Development of the monosynaptic stretch reflex circuit. *Curr Opin Neurobiol*. 2003; 13:96–102. [PubMed: 12593987]
- Esumi S, Kakazu N, Taguchi Y, Hirayama T, Sasaki A, Hirabayashi T, Koide T, Kitsukawa T, Hamada S, Yagi T. Monoallelic yet combinatorial expression of variable exons of the protocadherin-alpha gene cluster in single neurons. *Nat Genet*. 2005; 37:171–176. [PubMed: 15640798]
- Feng G, Tintrop H, Kirsch J, Nichol MC, Kuhse J, Betz H, Sanes JR. Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science*. 1998; 282:1321–1324. [PubMed: 9812897]
- Fukuda E, Hamada S, Hasegawa S, Katori S, Sanbo M, Miyakawa T, Yamamoto T, Yamamoto H, Hirabayashi T, Yagi T. Down-regulation of protocadherin-alpha A isoforms in mice changes contextual fear conditioning and spatial working memory. *Eur J Neurosci*. 2008; 28:1362–1376. [PubMed: 18973563]
- Han MH, Lin C, Meng S, Wang X. Proteomics analysis reveals overlapping functions of clustered protocadherins. *Mol Cell Proteomics*. 2010; 9:71–83. [PubMed: 19843561]
- Hasegawa S, Hamada S, Kumode Y, Esumi S, Katori S, Fukuda E, Uchiyama Y, Hirabayashi T, Mombaerts P, Yagi T. The protocadherin-alpha family is involved in axonal coalescence of olfactory sensory neurons into glomeruli of the olfactory bulb in mouse. *Mol Cell Neurosci*. 2008; 38:66–79. [PubMed: 18353676]
- Jankowska E. Interneuronal relay in spinal pathways from proprioceptors. *Prog Neurobiol*. 1992; 38:335–378. [PubMed: 1315446]
- Junghans D, Haas IG, Kemler R. Mammalian cadherins and protocadherins: about cell death, synapses and processing. *Curr Opin Cell Biol*. 2005; 17:446–452. [PubMed: 16099637]
- Kaneko R, Kato H, Kawamura Y, Esumi S, Hirayama T, Hirabayashi T, Yagi T. Allelic gene regulation of Pcdh-alpha and Pcdh-gamma clusters involving both monoallelic and biallelic expression in single Purkinje cells. *J Biol Chem*. 2006; 281:30551–30560. [PubMed: 16893882]
- Katori S, Hamada S, Noguchi Y, Fukuda E, Yamamoto T, Yamamoto H, Hasegawa S, Yagi T. Protocadherin-alpha family is required for serotonergic projections to appropriately innervate target brain areas. *J Neurosci*. 2009; 29:9137–9147. [PubMed: 19625505]
- Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. 1995; 270:96–99. [PubMed: 7569956]

- Kohmura N, Senzaki K, Hamada S, Kai N, Yasuda R, Watanabe M, Ishii H, Yasuda M, Mishina M, Yagi T. Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. *Neuron*. 1998; 20:1137–1151. [PubMed: 9655502]
- Lefebvre JL, Kostadinov D, Chen WV, Maniatis T, Sanes JR. Protocadherins mediate dendritic self-avoidance in the mammalian nervous system. *Nature*. 2012 in press.
- Lefebvre JL, Zhang Y, Meister M, Wang X, Sanes JR. gamma-Protocadherins regulate neuronal survival but are dispensable for circuit formation in retina. *Development*. 2008; 135:4141–4151. [PubMed: 19029044]
- Murata Y, Hamada S, Morishita H, Mutoh T, Yagi T. Interaction with protocadherin-gamma regulates the cell surface expression of protocadherin-alpha. *J Biol Chem*. 2004; 279:49508–49516. [PubMed: 15347688]
- Nornes HO, Carry M. Neurogenesis in spinal cord of mouse: an autoradiographic analysis. *Brain Res*. 1978; 159:1–6. [PubMed: 728790]
- Oppenheim RW. Cell death during development of the nervous system. *Annu Rev Neurosci*. 1991; 14:453–501. [PubMed: 2031577]
- Prasad T, Wang X, Gray PA, Weiner JA. A differential developmental pattern of spinal interneuron apoptosis during synaptogenesis: insights from genetic analyses of the protocadherin-gamma gene cluster. *Development*. 2008; 135:4153–4164. [PubMed: 19029045]
- Schalm SS, Ballif BA, Buchanan SM, Phillips GR, Maniatis T. Phosphorylation of protocadherin proteins by the receptor tyrosine kinase Ret. *Proc Natl Acad Sci U S A*. 2010; 107:13894–13899. [PubMed: 20616001]
- Schreiner D, Weiner JA. Combinatorial homophilic interaction between gamma-protocadherin multimers greatly expands the molecular diversity of cell adhesion. *Proc Natl Acad Sci U S A*. 2010; 107:14893–14898. [PubMed: 20679223]
- Serafini T. Finding a partner in a crowd: neuronal diversity and synaptogenesis. *Cell*. 1999; 98:133–136. [PubMed: 10428025]
- Shapiro L, Colman DR. The diversity of cadherins and implications for a synaptic adhesive code in the CNS. *Neuron*. 1999; 23:427–430. [PubMed: 10433255]
- Tasic B, Nabholz CE, Baldwin KK, Kim Y, Rueckert EH, Ribich SA, Cramer P, Wu Q, Axel R, Maniatis T. Promoter choice determines splice site selection in protocadherin alpha and gamma pre-mRNA splicing. *Mol Cell*. 2002; 10:21–33. [PubMed: 12150904]
- Wang X, Su H, Bradley A. Molecular mechanisms governing Pcdh-gamma gene expression: evidence for a multiple promoter and cis-alternative splicing model. *Genes Dev*. 2002a; 16:1890–1905. [PubMed: 12154121]
- Wang X, Weiner JA, Levi S, Craig AM, Bradley A, Sanes JR. Gamma protocadherins are required for survival of spinal interneurons. *Neuron*. 2002b; 36:843–854. [PubMed: 12467588]
- Weiner JA, Wang X, Tapia JC, Sanes JR. Gamma protocadherins are required for synaptic development in the spinal cord. *Proc Natl Acad Sci U S A*. 2005; 102:8–14. [PubMed: 15574493]
- Wojcik SM, Katsurabayashi S, Guillemin I, Friauf E, Rosenmund C, Brose N, Rhee JS. A shared vesicular carrier allows synaptic corelease of GABA and glycine. *Neuron*. 2006; 50:575–587. [PubMed: 16701208]
- Wu Q, Maniatis T. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell*. 1999; 97:779–790. [PubMed: 10380929]
- Wu Q, Zhang T, Cheng JF, Kim Y, Grimwood J, Schmutz J, Dickson M, Noonan JP, Zhang MQ, Myers RM, et al. Comparative DNA sequence analysis of mouse and human protocadherin gene clusters. *Genome Res*. 2001; 11:389–404. [PubMed: 11230163]
- Yokota S, Hirayama T, Hirano K, Kaneko R, Toyoda S, Kawamura Y, Hirabayashi M, Hirabayashi T, Yagi T. Identification of the cluster control region for the protocadherin-beta genes located beyond the protocadherin-gamma cluster. *J Biol Chem*. 2011; 286:31885–31895. [PubMed: 21771796]
- Zipursky SL, Sanes JR. Chemoaffinity revisited: dscams, protocadherins, and neural circuit assembly. *Cell*. 2010; 143:343–353. [PubMed: 21029858]



**Figure 1. *Pcdhg* mutant alleles and phenotypes**

(A) Schematic representations of *Pcdhg* wild type and mutant alleles. (B) Validation of deletions in homozygous animals by genomic PCR using exon-specific primers. (C–E) Gross phenotypes of *Pcdhg*<sup>tcko/tcko</sup> (P0), *Pcdhg*<sup>del/del</sup> (P0) and *Pcdhg*<sup>tako/tako</sup> (P60) mutants as compared to littermate controls. Homozygous mutants are indicated with a red arrow. (F–G) Striking phenotype similarities between *Pcdhg*<sup>tcko/tcko</sup> and *Pcdhg*<sup>del/del</sup> mutants at P0. Both have hunched posture and lack of voluntary movements and reflexes, exhibiting limb tremor, intense muscle stiffness and umbilical hernia (arrow), and die within hours after birth.



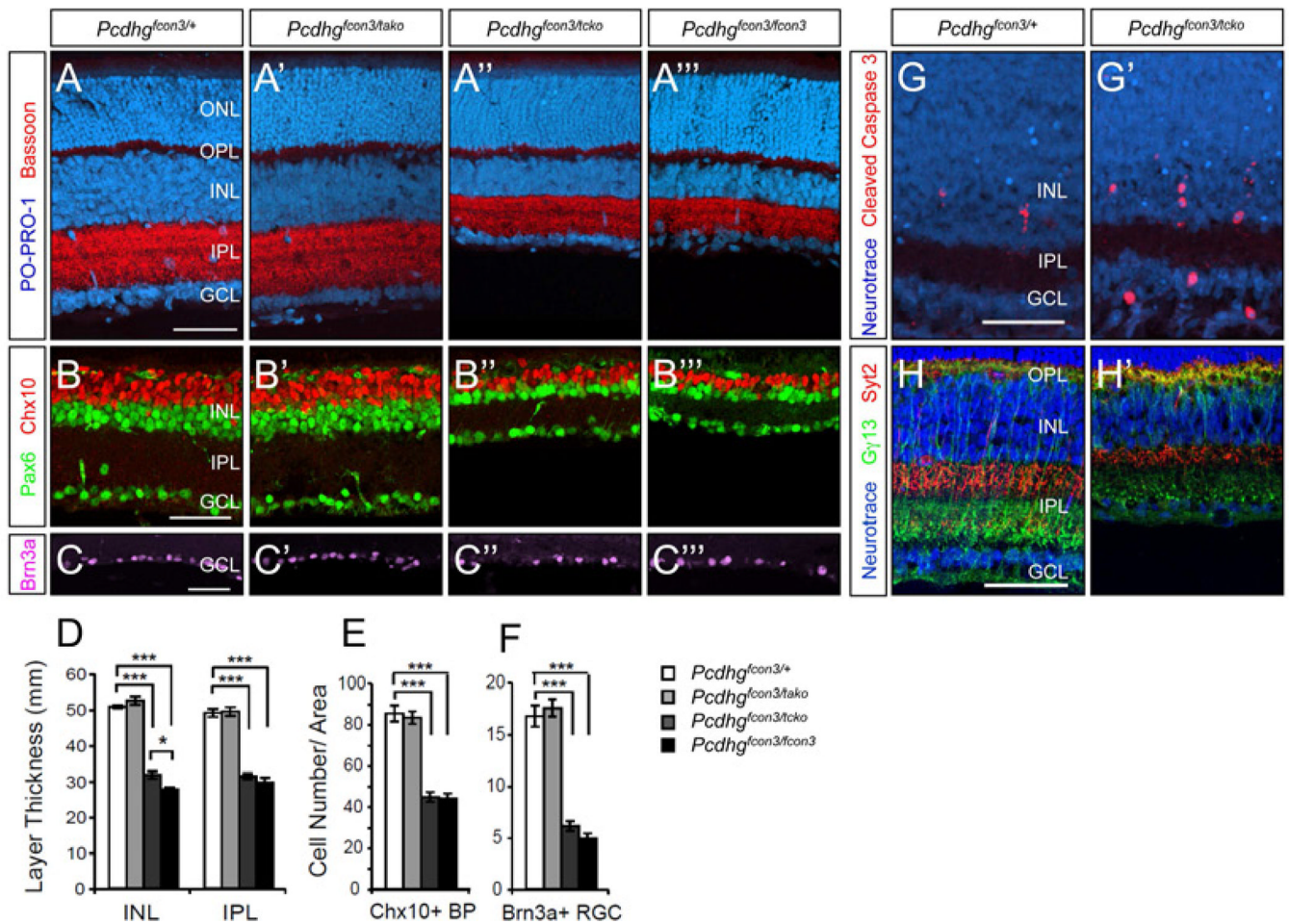
**Figure 2. Similar levels and patterns of neuronal loss and synaptic changes in the spinal cord of *Pcdhg<sup>tcko/tcko</sup>* and *Pcdhg<sup>del/del</sup>* mutants**

(A–A'') Both mutants exhibit increased levels of apoptosis as indicated by the increased numbers of cleaved caspase 3+ profiles (arrows). Scale bar: 100  $\mu$ m. (B–B'') Quantitative analyses of surviving neuronal populations in different lamina. NeuN+ neurons of distinct laminar regions are indicated. The central canal area is highlighted with a yellow oval, and the spinal cord is horizontally bisected with a dashed line to indicate the dorsal horn and the ventral horn areas. Scale bar: 250  $\mu$ m. (C–G) Interneuron subpopulations are differentially affected to similar extents in both mutants. Shown are representative images of Pax2 (C–C''), Foxp2 (D–D''), Chx10 (E–E''), CB (F–F'') and ChAT (G–G'') immunolabeled P0 spinal cords



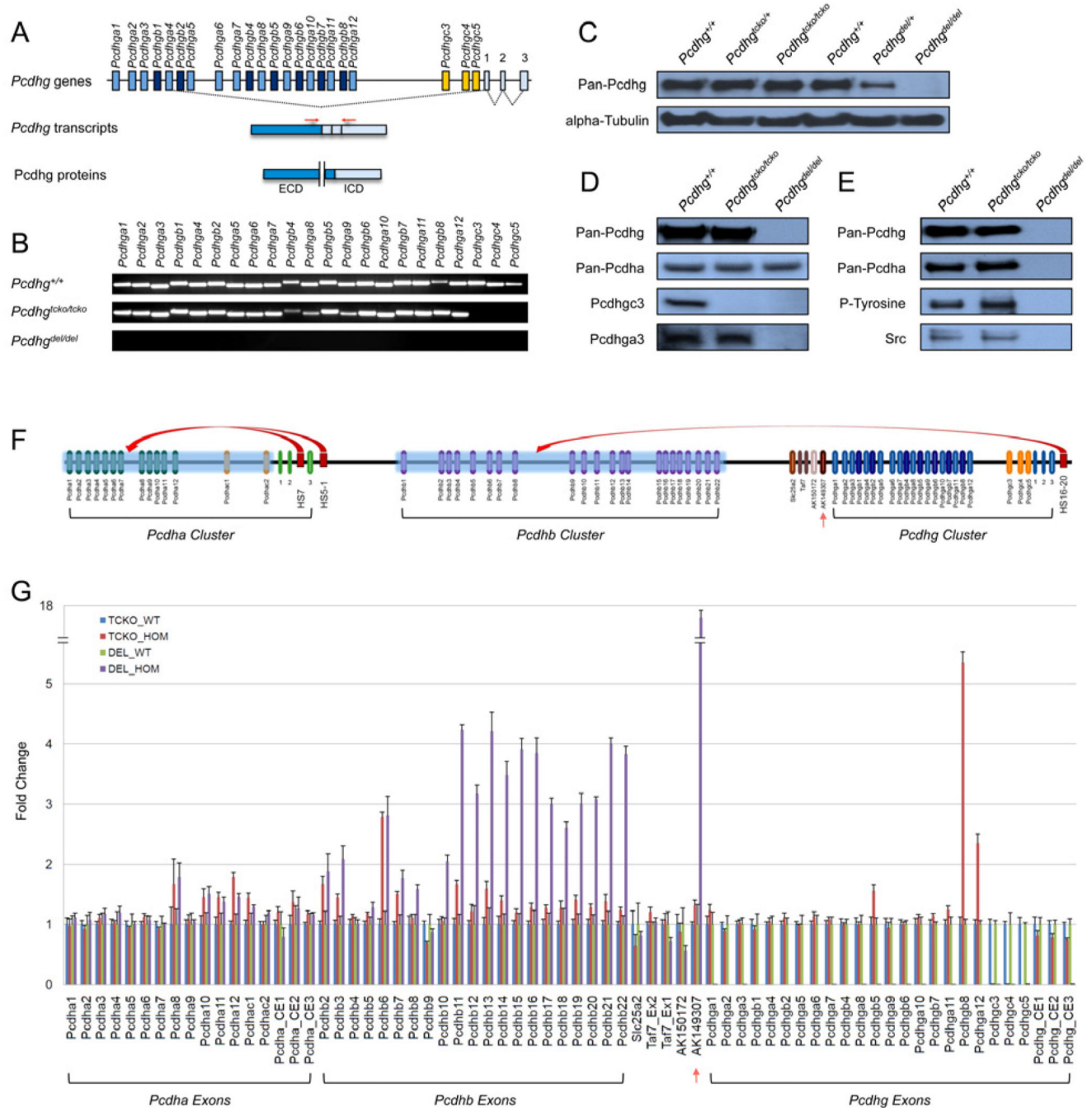
of *Pcdhg*<sup>+/+</sup>, *Pcdhg*<sup>tcko/tcko</sup>, *Pcdhg*<sup>del/del</sup> mice. Clusters of Renshaw cells (RC, F–F'') are encircled, and partition cells (G–G'') are indicated with arrows. Note the motor neurons are prominently labeled with anti-ChAT (G–G''). Scale bars: 100  $\mu$ m. (H) Percent survival of NeuN<sup>+</sup> neuronal populations according to their laminar locations (LI–LIX) in homozygous mutants (TCKO\_HOM or DEL\_HOM), calculated by normalizing neuronal counts to wild type littermate controls (TCKO\_WT and DEL\_WT). Details of the quantitative analyses can be found in Figure S2B. \* $p < 0.05$ , \*\*\* $p < 0.001$ . (I) Percent survival of interneuron subpopulations in the ventral horn. \*\*\* $p < 0.001$ . (J–M) Specific types of synaptic inputs onto motor neurons of wild type and mutant spinal cords at P0. Motor neurons are labeled with anti-ChAT in green, and synaptic inputs with antibodies against different vesicular transporters in red. Scale bars: 5  $\mu$ m. (N–N'') Terminal arborization of Parvalbumin+ Ia primary afferents surrounding motor pools is altered in both lines of mutants. Scale bar: 50  $\mu$ m. (O) Fold changes in synaptic density for each type of synapse in the mutants as calculated by normalizing to wild type controls. Details of the quantitative analyses can be found in Figure S2C. \*\*\* $p < 0.001$ .





**Figure 3. Similar levels and patterns of neuronal loss in the retina of *Pcdhg*<sup>tcko/tcko</sup> and *Pcdhg*<sup>del/del</sup> mutants**

(A-A''') Nuclear and synaptic layers of retina sections from P18 control and mutants are labeled with Po-Pro1 or anti-Bassoon, respectively. Retinal lamination is normal, and the ONL and OPL thickness are unaffected. INL and IPL thickness are similarly reduced in *Pcdhg*<sup>fcon3/tcko</sup> and *Pcdhg*<sup>fcon3/fcon3</sup> mutants but are unaffected in *Pcdhg*<sup>fcon3/tako</sup> mutants. (B–C) Numbers of Chx10+ bipolar (red), Pax6+ GABAergic amacrine interneurons (green), as well as Brn3a+ projection retinal ganglion cells (magenta) are similarly reduced in *Pcdhg*<sup>fcon3/tcko</sup> and *Pcdhg*<sup>fcon3/fcon3</sup> mutants. (D) Quantification of INL and IPL thickness in control and mutant retinas. (E, F) Quantification of bipolar interneurons (Chx10+ BP) and projection retinal ganglion cells (Brn3a+ RGC). (F–F'') Increased number of apoptotic cells (cleaved caspase 3+) is detected in the GCL and INL of the mutant retina (P4), as seen in *Pcdhg* null retinas. (G–G'') Syt2+ OFF bipolar processes (red) and Gy13+ ON bipolar processes (green) ramify in the upper and lower regions of the IPL, respectively (P18). Laminar specificity is retained in mutants deficient in C-type *Pcdhg* isoforms, but marker-laminae are reduced and disrupted, identical as shown for *Pcdhg* null mutants. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. \*p < 0.05, \*\*\* p < 0.001. Scale bars: 50  $\mu$ m.



**Figure 4. Expression and function of A- and B-type Pcdhg isoforms are not appreciably affected in the absence of C-type genes**

(A) Schematic representation of *Pcdhg* genes, transcripts and proteins. Positions of specific primers (red arrows) are indicated. ECD, extracellular domain; ICD, intracellular domain. (B) RT-PCR of individual *Pcdhg* transcripts in wild type and both lines of mutants. (C) Western blot indicates that the expression level of Pcdhg proteins in the *Pcdhg<sup>tcko/tcko</sup>* mutant brain is similar to the wild type level, but higher than that of *Pcdhg<sup>+/del</sup>*. Alpha tubulin is used as loading control. (D–E) A-type and B-type Pcdhg isoforms in *Pcdhg<sup>tcko/tcko</sup>* mutant brains still form complexes with Pcdha proteins, remain tyrosine phosphorylated, and mediate signaling. Western blots of P0 brain lysate from wild type and both mutants are

shown in D, and blots of pan-Pcdhg immunoprecipitates in E. (F) Schematic representation of *Pcdh* clusters. Three identified cluster-wide enhancers (HS5-1, HS7 and HS16-20) and their respectively regulated regions are indicated. (G) Relative expression levels of individual exons in the *Pcdh* cluster region in E13.5 wild type and mutant spinal cords based on RNA-Seq analysis. Several genes with extremely low expression levels are excluded but can be found in Table S1. Significance levels are not indicated but can be found in Table S1. The red arrow in F and G indicates the EST gene *AK149307*.

**Table 1**

Genetically blocking apoptosis rescues the neonatal lethality of *Pcdhg<sup>tcko/tcko</sup>* mutants but not that of *Pcdhg<sup>del/del</sup>* mutants

Parent genotypes	Age	# Animals Genotyped	# Single Homozygotes	# Double Homozygotes
<i>Pcdhg<sup>tcko/+</sup>;Bax<sup>+/-</sup></i>	P0	127	29	16
	P21	191	0	9
<i>Pcdhg<sup>del/+</sup>;Bax<sup>+/-</sup></i>	P0	89	11	9
	P21	193	0	0

Some normal looking littermates (wild type or heterozygous) were not genotyped and therefore correct genotype ratios cannot be calculated based on these numbers. Single homozygotes: *Pcdhg<sup>tcko/tcko</sup>* or *Pcdhg<sup>del/del</sup>* mutants that are either *Bax<sup>+/+</sup>* or *Bax<sup>+/-</sup>*; double homozygotes: *Pcdhg<sup>tcko/tcko</sup>;Bax<sup>-/-</sup>* or *Pcdhg<sup>del/del</sup>;Bax<sup>-/-</sup>* mutants.