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A differential, developmental pattern of spinal interneuron apoptosis during synaptogenesis: Insights from genetic analyses of the protocadherin-γ gene cluster

Tuhina Prasad[1], **Xiaozhong Wang**[2], **Paul A. Gray**[3], and **Joshua A. Weiner**[1,4,*]

[1]Department of Biology, The University of Iowa, Iowa City, IA 52242

[2]Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208

[3]Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110

[4]Neuroscience Graduate Program, The University of Iowa, Iowa City, IA 52242

SUMMARY

While the role of developmental apoptosis in shaping the complement and connectivity of sensory and motor neurons is well documented, the extent to which cell death affects the 13 cardinal classes of spinal interneurons has been unclear. Using a series of genetic manipulations in vivo, we demonstrate for the first time a differential pattern of developmental apoptosis in molecularly-identified spinal interneuron populations, and implicate the adhesion molecule family encoded by the 22-member *Protocadherin-γ* (*Pcdh-γ*) gene cluster in its control. In constitutive *Pcdh-γ* null mutant mouse embryos, many interneuron populations undergo increased apoptosis, but to differing extents: For example, over 80% of En1-positive V1 neurons are lost, while only 30% of Chx10-positive V2a neurons are lost and there is no reduction in the number of V1-derived Renshaw cells. Using two complementary methods, we show that this represents an exacerbation of a normal, underlying developmental pattern: The extent of each population's decrease in *Pcdh-γ* mutants is precisely commensurate both with the extent of its loss during normal embryogenesis and with the extent of its increase in $Bax^{-/-}$ mice, in which apoptosis is genetically blocked. Interneuron apoptosis begins during the first wave of synaptogenesisis in the spinal cord, occuring first among ventral populations (primarily between E14 and E17), and only later among dorsal populations (primarily after P0). Utilizing a new, conditional *Pcdh-γ* mutant allele, we show that the γ-Pcdhs can promote survival non-cell autonomously: mutant neurons can survive if they are surrounded by normal neurons, while normal neurons can undergo apoptosis if they are surrounded by mutant neurons.

Keywords

ventral horn; spinal cord; apoptosis; synapse formation; interneurons; programmed cell death

INTRODUCTION

In the embryonic central nervous system (CNS), there is a vast overproduction of neurons, with 50% or more undergoing apoptosis during the developmental period (Oppenheim, 1991;

*Corresponding author: Joshua A. Weiner, Ph.D., Department of Biology, The University of Iowa, 143 Biology Building, Iowa City, IA 52242, Phone: 319-335-0091, Fax: 319-335-1069, E-mail: joshua-weiner@uiowa.edu.

Nijhawan et al., 2000; Buss et al., 2006). Apoptosis has been shown to occur in proliferative zones, where it may act as a selection mechanism to remove aberrant progenitors (Blaschke et al., 1996; Blaschke et al., 1998; Kuida et al., 1996; Kuida et al., 1998), as well as in cell types that are needed only during a restricted developmental period, such as subplate neurons in the cerebral cortex and cells of the roof and floor plates in the spinal cord (Allendoerfer and Shatz, 1994; Buss and Oppenheim, 2004; Homma et al., 1994; Buss et al., 2006). The best characterized role of apoptosis in the developing nervous system, however, occurs during synaptogenesis, where competition for synaptic activity and/or trophic factors leads to the loss of excess neurons and the size matching of afferent and efferent populations (Buss et al., 2006; Lowrie and Lawson, 2000; Mennerick and Zorumski, 2000; Oppenheim, 1991). The demonstration of developmental apoptosis has been clearest in experimentally accessible, clearly identifiable neuronal populations with defined patterns of connectivity in which the target cells can also be examined.

Less clear has been the extent to which developmental apoptosis occurs in populations that are intermixed and that project widely or diffusely within a given region of the CNS (Lowrie and Lawson, 2000). Spinal interneurons make up > 95% of the spinal cord (Hochman, 2007) and can be grouped into at least 13 cardinal populations (9 dorsal and 4 ventral), which are derived from distinct progenitor domains and which differ in terms of neurotransmitter phenotype, somal location, axonal projection pattern, and expression of identified transcription factor markers (Goulding et al., 2002; Goulding and Pfaff, 2005; Helms and Johnson, 2003; Lewis, 2006) (see Fig. 2A). Dorsal interneurons are involved primarily in the processing and relaying of sensory information from the trunk and limbs, while the primary function of ventral interneurons is to coordinate motor output via modulation of motor neurons. Spinal interneuron populations receive inputs from diverse sources, including dorsal root ganglia (DRG) sensory afferents, descending axons from the brain, interneurons within the same or different spinal cord segments, and, in the case of the ventral Renshaw cells, motor neurons. These diffuse patterns of connectivity make it more difficult to conceptualize the role of developmental cell death, and studies examining whether spinal interneurons, like sensory and motor neurons, undergo a period of naturally occurring apoptosis have produced conflicting results.

Using pyknosis as a hallmark of cell death, McKay and Oppenheim (1991) found no evidence that chick spinal interneurons die during development or following loss of afferent and efferent connections by limb removal or spinal cord transection. Using the TUNEL method, however, it was subsequently shown that a large number of cells, presumed to be interneurons based on their location, undergo apoptosis in the rat spinal cord between embryonic day (E) 20 and postnatal day (P) 4 (Lawson et al., 1997). Spinal interneuron apoptosis, postulated to be due to loss of DRG afferents and/or target motor neurons, was also observed in neonatal rats following sciatic nerve crush (Lawson and Lowrie, 1998) or axotomy (Oliveira et al., 1997, 2002). A study using embryonic rat spinal cord explants in vitro suggested that neurotrophin-3 released by motor neurons promotes the survival of Pax-2-expressing spinal interneurons (Béchade et al., 2002). Mice with massive motor neuron loss due to genetic ablation of muscles did not, however, exhibit obviously increased interneuron apoptosis (Grieshammer et al., 1998; Kablar and Rudnicki, 1999). None of these studies, however, systematically analyzed apoptosis with respect to the many molecularly-identified interneuron populations. Understanding the role of developmental cell death in shaping these populations will be important for determining how early patterns of fate specification mediated by transcription factors relate to the connectivity and mature function of spinal interneurons, a major goal towards which progress has begun to accelerate (Alvarez et al., 2005; Cheng et al., 2005; Gosgnach et al., 2006; McLean et al., 2007; Mizuguchi et al., 2006; Pillai et al., 2007).

Our previous work (Wang et al., 2002b; Weiner et al., 2005) has implicated the γ-protocadherins (γ-Pcdhs), a family of 22 putative adhesion molecules, in the development of

spinal interneurons. The γ-Pcdhs are expressed throughout the embryonic CNS and are found at some, but far from all, developing synapses (Wang et al., 2002b; Frank et al., 2005; Phillips et al., 2003). Mice in which the entire *Pcdh-γ* gene cluster has been deleted (*Pcdh-γ*$^{del/del}$) lack voluntary movements and spinal reflexes, display an alternating tremor of fore- and hindlimbs, and die several hours after birth (Wang et al., 2002b). In the *Pcdh-γ*$^{del/del}$ spinal cord, massive interneuron apoptosis, neurodegeneration, and synapse loss are observed in the late embryonic period (Wang et al., 2002b). When apoptosis is genetically blocked by the additional deletion of the pro-apoptotic protein Bax, the loss of γ-Pcdhs still results in significant reductions of spinal cord synaptic density, and *Pcdh-γ*$^{del/del}$; *Bax*$^{-/-}$ double mutants pups do not survive (Weiner et al., 2005). Spinal interneurons with reduced levels of γ-Pcdhs can survive in vitro, but make fewer synapses, at which both excitatory and inhibitory spontaneous currents are significantly reduced in amplitude (Weiner et al., 2005; Weiner, 2006).

Here, we use both *Pcdh-γ*$^{del/del}$ mice and a new conditional *Pcdh-γ* mutant allele, along with wild-type mice, *Bax* mutants and four Cre transgenic lines, to demonstrate that molecularly-distinct spinal interneuron populations exhibit a normal period of differential, developmental cell death. Interneuron apoptosis proceeds in a ventral-to-dorsal temporal gradient and is associated with the first wave of synaptogenesis in the spinal cord. The phenotype of *Pcdh-γ* null mice represents an exacerbation of this developmental pattern, as the extent of increased apoptosis within each population is commensurate with its levels of apoptosis in wild-type mice. By selectively mutating the *Pcdh-γ* locus in discrete interneuron populations, we further show that the γ-Pcdhs can promote survival non cell-autonomously, consistent with their roles at cell-cell contacts, including developing synapses.

MATERIALS AND METHODS

Mouse Strains

The *Pcdh-γ*^{del} and *Pcdh-γ*^{fus} alleles (Wang et al., 2002b) and *Bax*$^{-/-}$ mutants (Deckwerth et al., 1996; Knudson et al., 1995; White et al., 1998) were described previously. *Actin-Cre* (Lewandoski et al., 1997), *Wnt1-Cre* (Danielian et al., 1998), and *Atoh1*^{tm2Hzo} (referred to here as *Atoh1*$^{-/-}$;Ben-Arie et al., 2000) mouse lines were obtained from The Jackson Laboratories (Bar Harbor, ME). *Pax2-Cre* mice (Ohyama and Groves, 2004) were the kind gift of Dr. Andy Groves (House Ear Institute, Los Angeles, CA), and *Hb9-Cre* mice (Umemori et al., 2004) were the kind gift of Dr. Joshua Sanes (Harvard University, Cambridge, MA). The *Pcdh-γ*^{fcon3} allele was generated in mouse ES cells by homologous recombination. The targeting vector was modified from that used to create the *Pcdh-γ*^{fus} allele (Wang et al., 2002b), in which the EGFP coding sequence was fused in-frame with constant exon 3. A loxP sequence was inserted into an NheI site in the 5'homology arm of the targeting vector by using a pair of oligonucleotides (5779:CTAGATAACTTCGTATAGCATACATTATACGAAGTTAT; 5780:CTAGATAACTTCGTATAATGTATGCTATACGAAGTTAT). The orientation and sequence of the loxP site was confirmed by direct sequencing (using primer 5781:CTGTGCCAAGCCTTGGTTAGGGA). To confirm the presence of the first loxP site in the targeted ES cells, we used primers 5781 and 5782:GCTTCCAAAGTGCCTAGACTAGAG. The resulting *Pcdh-γ*^{fcon3} allele contains the following elements at the 3' end of the *Pcdh-γ* cluster: constant exon2-loxP-constant exon3/EGFP fusion-loxP-PGK/Neo-loxP. *Pcdh-γ*$^{fcon3/fcon3}$ homozygous mice were viable and fertile in the absence of Cre.

Immunofluorescence

Embryonic and neonatal spinal columns were prepared using one of two methods: 1) Fixation for 2 hours in 4% paraformaldehyde at 4°C, followed by washes with cold PBS, cryoprotection in 30% sucrose at 4°C, and freezing in OCT compound (Sakura); or 2) Snap freezing in OCT

using dry ice/ethanol-cooled isopentane. Transverse cryostat sections were cut at 12 μm. Slides containing fresh-frozen sections were fixed in 100% methanol for 10 minutes at −20°C. Sections were stained as described (Weiner et al., 2005). A list of primary antibodies used is provided in Table S1.

In situ hybridization

In situ hybridization using an antisense riboprobe against the *Pcdh-γ* constant exons was performed as described (Wang et al., 2002b).

TUNEL labeling

The Fluorescein FragEL^{TM} DNA fragmentation Detection Kit (Calbiochem) was used according to manufacturer's instructions.

Image analysis

For interneuron and synaptic puncta counts, each quantification was performed on at least 6 sections from at least three animals (i.e., at least 18 sections for each marker per genotype). Images were taken at equivalent thoracolumbar levels and camera exposures using 10X (cell counts) or 63X (synapses) PlanApo objectives on a Leica DM5000B epifluorescence microscope, or a Leica SP2 AOBS laser scanning confocal microscope. Images were captured in Adobe Photoshop and similarly adjusted for brightness and contrast. Cell counts were performed manually. For synapses, images were thresholded in NIH Image/J and puncta counted by using the Analyze Particles function. Statistical significance was determined by ANOVA followed by Bonferroni post-hoc tests using Prism software.

Western Blotting

Twenty μg of protein from control and mutant brains were resolved on NuPAGE gels (Invitrogen) and blotted using standard methods. Signals were detected using chemiluminescence (SuperSignal West Pico, Pierce).

RT-PCR

Total RNA was extracted from control and mutant tissues using the RNAqueous-4PCR kit (Ambion), and first-strand cDNA synthesized using standard methods. Primer sequences are listed in Table S2. Cycling parameters: 94°C, 1 minute; 55°C, 1 minute; 72°C 3 minutes, for 30 cycles.

RESULTS

Dorsal-ventral disparity of apoptosis in embryonic *Pcdh-γ* null mutant spinal cord

The *Pcdh-γ* gene cluster contains 22 large "variable" exons, each of which encodes a cadherin-like Type I membrane protein consisting of 6 extracellular domains, a transmembrane domain, and a proximal cytoplasmic domain. Each variable exon is expressed from its own promoter (Tasic et al., 2002; Wang et al., 2002a) and spliced to three short "constant" exons, which encode a 125-amino acid shared C-terminal domain (Fig. 1I); all of the 22 possible variable-constant exon spliced transcripts could be detected by RT-PCR analysis of embryonic spinal cord (Fig. 1H). In our initial analysis of *Pcdh-γ*$^{del/del}$ null mutant spinal cord, we noticed that loss of neurons and fiber tracts was most obvious in the ventral horn (Wang et al., 2002b). In sections of E17 or P0 *Pcdh-γ*$^{del/del}$ spinal cords, apoptotic cells detected by using TUNEL (Fig. 1A) or an antibody against cleaved caspase-3 (Fig. 1B) were increased compared to controls primarily in the ventral horn and intermediate grey. Consistent with this, increased numbers of microglia (stained with *Griffonia simplificolia* isolectin B4; Fig. 1C) and reactive astrocytes

(heavily stained by antibodies against GFAP; Fig. 1D) were observed in ventral, but not dorsal, gray matter of mutants.

We asked whether this disparity could be due to restricted expression of *Pcdh-γ* genes in the ventral spinal cord. *In situ* hybridization using an antisense riboprobe corresponding to the *Pcdh-γ* constant exons detected strong expression throughout the developing spinal cord, with no apparent difference in level between dorsal and ventral horns (Fig. 1G). Similarly, immunostaining for γ-Pcdh proteins using antibodies against the constant domain (Phillips et al., 2003; data not shown) or against GFP on tissues from the *Pcdh-γ*^{fus} mouse line, in which the GFP gene is fused to the end of constant exon 3 (Wang et al., 2002b), demonstrated uniform labeling of the neuropil in the spinal cord between E12 (Fig. 1E) and P0 (Fig. 1F). Consistent with a published report on adult spinal cord (Zou et al., 2007), *in situ* hybridization using several riboprobes specific for individual *Pcdh-γ* variable exons did not reveal any obvious dorsal or ventral restriction in neonates (data not shown). Together, these data indicate that the dorsal-ventral disparity in levels of apoptosis seen in mutants is not due to spatially-restricted expression of the γ-Pcdhs during development.

Differential loss of molecularly-defined ventral interneuron populations in *Pcdh-γ* null mutant embryos

Spinal cord neurons are derived embryonically from 11 progenitor domains arrayed along the dorsal-ventral axis, and differentiate to produce 9 cardinal classes of neurons dorsally and 5 ventrally; each neuronal class can be identified by its expression of one or more transcription factors (Lewis, 2006; see Fig. 2A schematic for those used in this study). In the dorsal horn, these classes include the early-born dI1-dI6 interneurons and the later-born dI1B, dIL_A and dIL_B interneurons, while in the ventral horn they include the V0-V3 interneurons as well as motor neurons. We used antibodies against several of the transcription factors that mark distinct spinal interneuron populations to quantify the loss of these populations in *Pcdh-γ*$^{del/del}$ mutant mice (Fig. 2A; Table S1). We focused on markers that are still readily detectable at E17 and P0, relatively late developmental time points in the spinal cord at which neurodegeneration is apparent in *Pcdh-γ*$^{del/del}$ mutants (Wang et al., 2002b). Examples of immunostaining patterns are shown in Fig. 2B–H, and a quantitative summary of our E17 results is presented in Fig. 3. Several important conclusions about the pattern of interneuron loss in the absence of γ-Pcdhs can be drawn from these data.

First, the late embryonic loss of ventral, but not dorsal, interneurons was confirmed. Quantification of molecularly-defined populations derived from the V0 (Evx1-positive; Fig. 2E), V1 (En1-positive; Fig. 2C), V2 (Chx10-positive V2a; Fig. 2D, Gata3-positive V2b; Fig. 3), and V3 (Nkx2.2-positive; Fig. 3) domains all demonstrated a reduction in *Pcdh-γ*$^{del/del}$ mutants, while dorsal horn neurons, including those derived from a single domain (Lmx1b–positive dI5 neurons; Fig. 2G, Lhx2-positive dI1 neurons; data not shown) as well as those more broadly derived (Pax2-positive dI4 and dI6 neurons and Lhx1/5-positive dI2, dI4, and dI6 neurons; Fig. 2B), were normal in number at E17 (Fig. 3). Second, within populations that are broadly distributed across both the dorsal and ventral horns, such as Pax2-positive or Lhx1/5-positive interneurons, only those neurons residing in the ventral horn were lost (Fig. 2B): for example, while those Pax2-positive neurons derived from dI4 and dI6 were present in normal numbers at E17, the V0 and V1-derived Pax2 neurons were decreased by 50% (Fig. 3). Third, the extent of cell loss varied across ventral interneuron populations. Some neuronal types, such as Nkx2.2-positive V3 interneurons and those V1 interneurons that retain En1 expression at E17, were reduced by ~80%, while Chx10-positive V2a interneurons, were reduced by only 30% (Fig. 3). As we previously suggested (Wang et al., 2002b), and confirm here quantitatively, motor neurons (positive for choline acetyltransferase [Chat; Fig. 2H], Isl1 [Fig. 4D] and MNR2 [Fig. 3]) were present in normal numbers in *Pcdh-γ*$^{del/del}$ spinal cords at

all ages examined. Fourth, the extent of cell loss varied even among interneurons derived from the same ventral domain: V1 interneurons that express En1 (Fig. 2C), Pax2 and/or Lhx1/5 (Fig. 2B) were all reduced in *Pcdh-γ*$^{del/del}$ mutants (Fig. 3), but calbindin-positive putative Renshaw cells (identified by their clustered position near the ventral gray-white matter border within laminae VII and IX;Mentis et al., 2006), which have been shown to be V1-derived (Alvarez et al., 2005;Sapir et al., 2004), were normal in number (Fig. 2F, Fig. 3).

Interneurons that are generated in dorsal domains but migrate to the ventral horn are lost in *Pcdh-γ* null mutant embryos

We next asked whether interneurons that are generated in dorsal domains, but which subsequently migrate into the ventral horn, would be affected by loss of the γ-Pcdhs. We analyzed two populations of interneurons: dI3-derived, Isl1-positive interneurons, which settle in the intermediate gray between the dorsal and ventral horns (Gross et al., 2002; Liem et al., 1997), and dI2-derived, FoxP2-positive/Pax2-negative interneurons. Several populations of FoxP2-positive interneurons are produced in the spinal cord and settle in the ventral horn. Those that co-express Pax2 are derived from dI6/V0 and V1 (Geiman, Gray, and Goulding, 2007). A population that is negative for Pax2 is generated dorsally within the Wnt1 expression domain (Fig. S4), appears at E11.5 to be migrating ventrally (Fig. 4A, B), and can be detected at E17 scattered throughout the ventral horn (Fig. 4C). That these FoxP2-positive cells co-express FoxD3 (Fig. 4A) suggests they are derived from dI2 (Gross et al., 2002); confirmation of this was obtained by analyzing this population in *Atoh1*$^{-/-}$ mice (Ben-Arie et al., 2000; Fig. S1).

Having identified these two populations, we quantified them in spinal cords of *Pcdh-γ*$^{del/del}$ mutant and control embryos at E17. Both the dI2-derived FoxP2-positive neurons and the dI3-derived Isl1-positive neurons were reduced by ~50% in the absence of γ-Pcdhs (Fig. 3, Fig. 4C–F). Together with the results presented above, these data suggest that the dorsal-ventral pattern of interneuron loss observed in *Pcdh-γ*$^{del/del}$ mutants is influenced by late embryonic developmental events in the ventral horn, rather than a given interneuron's progenitor domain of origin.

Loss of molecularly-defined interneuron populations in *Pcdh-γ*$^{del/del}$ mutants is due to apoptosis

Although our initial analysis of the neonatal *Pcdh-γ*$^{del/del}$ mutant spinal cord suggested that neurogenesis and initial differentiation proceed normally (Wang et al., 2002a), specific interneuron populations were not examined. Thus, it remained possible that some of the results presented above could be due to aberrant interneuron cell fate specification, resulting in loss of molecular markers. We excluded this possibility through three sets of experiments. First, we examined *Pcdh-γ*$^{del/del}$ mutant mice in which apoptosis was blocked by genetic deletion of *Bax* (Weiner et al., 2005). In *Pcdh-γ*$^{del/del}$; *Bax*$^{-/-}$ double mutant mice, the number of interneurons in all ventral populations examined was the same or greater than in control mice (Fig. 5A–C); this is as expected if cell loss in *Pcdh-γ* mutants were due to apoptosis, but not if it were due to cell fate disruptions. Second, through immunostaining we were able directly to identify an increased number of fragmented, apoptotic cells in *Pcdh-γ*$^{del/del}$ spinal cords that were double-positive for cleaved caspase-3 and markers of reduced ventral interneuron populations (Fig. 5D–F). Third, we quantified several ventral interneuron populations at E14, a timepoint after the end of neurogenesis (Nornes and Carry, 1978) but before the onset of neurodegeneration in *Pcdh-γ*$^{del/del}$ mutants, and found that the size of each population did not differ from wild-type (WT) values (Fig. 5G). Together, these data indicate that the loss of molecularly-defined interneuron populations in *Pcdh-γ*$^{del/del}$ mutants is due solely to apoptosis, rather than aberrant cell fate specification.

Apoptosis of spinal interneuron populations in *Pcdh-γ* null mutants reflects an exacerbation of a normal developmental pattern

We next asked whether the differential apoptosis of spinal interneuron populations observed in the absence of γ-Pcdhs might reflect an exacerbation of an existing developmental pattern. We addressed this question by taking complementary two approaches. In the first, we directly quantified the size of eight interneuron populations in WT spinal cords at E14, E17, P0, P2, and P5, taking a decrease in size during development as evidence for apoptosis within that population. We found that each of the populations exhibited developmental reductions of varying extents (Fig. 6A, B). If the increased apoptosis in *Pcdh-γ* null mice represented an exacerbation of an underlying developmental pattern, we would predict that for any given interneuron population, the extent of its increased apoptosis in the mutants (compared to WT, as quantified in Fig. 3) should be proportional to the extent of its normal loss over time in WT mice. We found that, indeed, this was the case: as shown in Fig. 6C, there is a near-perfect correlation ($r=0.93$, $p< 0.005$) between the extent of each population's reduction from E14-E17 in WT mice and the extent of its increased loss in E17 mutants.

While suggestive, these data come with the caveat that the extent of apoptosis within each population might be overestimated due simply to progressive loss of marker expression in older animals, which is known to occur, particularly after P0. Therefore, we took a second approach that obviates this concern. We reasoned that the extent of apoptosis within spinal interneuron populations during normal development could be estimated by determining the extent to which the size of these populations were increased in $Bax^{-/-}$ mice, in which apoptosis is genetically blocked (Fig. 7A–C). As expected, we found that the extent of cell increase in a given population in $Bax^{-/-}$ mice (Fig. 7D) paralleled closely the extent of its decrease in *Pcdh-*$\gamma^{del/del}$ mutants (Fig. 3). For instance, Chx10-positive V2 neurons were reduced by 29.8% in *Pcdh-*$\gamma^{del/del}$ mice and increased by 29.2% in $Bax^{-/-}$ mice, while En1-positive V1 neurons were reduced by 85% in *Pcdh-*$\gamma^{del/del}$ mice and increased by 61.7% in $Bax^{-/-}$ mice; such correlations were statistically significant across all populations examined (Fig. 7F; $r=0.81$, $p < 0.005$).

We also used analysis of both WT mice and *Bax* mutants to probe the significance of the lack of dorsal interneuron apoptosis in *Pcdh-*$\gamma^{del/del}$ neonates. While this could reflect a restricted role for γ-Pcdhs in the ventral spinal cord, two observations suggest otherwise: First, the γ-Pcdh family is expressed uniformly throughout the developing spinal cord (Fig. 1); and second, a ventral-to-dorsal temporal gradient in TUNEL staining was observed in the late embryonic/early postnatal rat (Lawson et al., 1997) suggesting that dorsal interneurons primarily undergo apoptosis after P0, when the death of *Pcdh-*$\gamma^{del/del}$ mice precludes further analysis. Analysis of WT interneuron populations confirms that this is indeed the case: loss of ventral interneuron populations occurred primarily between E14 and E17, and was complete by P0-P2 (Fig. 6B), while dorsal interneurons were lost only after E17, primarily between P0-P5 (Fig. 6A). We also analyzed the size of dorsal interneuron populations in $Bax^{-/-}$ mice, and found that they were increased compared to controls at P5, but not at P0 (Fig. 6D, E) consistent with their apoptosis primarily during the neonatal period, following the late embryonic apoptosis of their ventral counterparts. Intriguingly, calbindin-positive putative Renshaw cells, which are not lost in *Pcdh-*$\gamma^{del/del}$ spinal cords, were correspondingly not increased in $Bax^{-/-}$ mice at P0 or P5 (Fig. 7D, E), suggesting that they may not undergo a period of developmental apoptosis, or at least do so much later than other interneurons.

In previous work, we found that in *Pcdh-*$\gamma^{del/del}$; $Bax^{-/-}$ double mutant neonates, the overall density of synaptic puncta was reduced by 30–50% compared to controls, despite the lack of apoptosis and normal spinal cord size (Weiner et al., 2005). Further, spinal interneurons with hypomorphic levels of γ-Pcdhs can survive in vitro, but make fewer, and physiologically weaker, synapses than do control interneurons (Weiner et al., 2005). These genetic

dissociations suggest that at least one primary function of the γ-Pcdhs is to promote synapse formation or maturation in the spinal cord. It has long been known that neuronal survival depends, in most cases, on synaptic activity (reviewed by Mennerick and Zorumski, 2000). The ubiquity of this mechanism has been confirmed dramatically by genetic deletion of *Munc18-1*, which abolishes vesicular synaptic transmission. In *Munc18-1*$^{-/-}$ mice, neurons differentiate normally, project to their targets, and form synapses, but subsequently undergo massive apoptosis in the late embryonic period throughout the CNS, leading to stillborn pups (Verhage et al., 2000). The temporal and spatial patterns of apoptosis that we have characterized are consistent with the possibility that γ-Pcdh-dependent synaptogenesis helps control interneuron survival. The onset of interneuron apoptosis is coincident with the first wave of synaptogenesis in the rodent spinal cord (May and Biscoe, 1973; May and Biscoe, 1975; Vaughn and Grieshaber, 1973; Vaughn, 1989), which, like the pattern of interneuron apoptosis we describe (Fig. 6), generally proceeds in a ventral-to-dorsal temporal gradient (Vaughn and Grieshaber, 1973; Weber and Stelzner, 1980; Gingras and Cabana, 1999). Because immunostaining for transcription factor markers labels only cell nuclei, it is unfortunately not possible to directly show that synapse loss leads to apoptosis in individual *Pcdh-γ* mutant interneurons belonging to distinct populations. Examination of the spatial pattern of synapse loss in *Pcdh-γ*$^{del/del}$; *Bax*$^{-/-}$ double-mutants, however, did show that significant reductions in both excitatory and inhibitory synaptic puncta density occur only in the ventral horn at P0 (Fig. S2), an age at which interneuron apoptosis is also primarily seen ventrally in both WT (Fig. 6A,B) and *Pcdh-γ*$^{del/del}$ mice (Fig. 3, Fig. 7F).

Pcdh-γ^{fcon3}, a new, conditional mutant allele of *Pcdh-γ*

The few genetic manipulations that have been reported to influence spinal interneuron apoptosis, such as knockout of *Shh* (Borycki et al., 1999) or of *Lbx1* (Gross et al., 2002), do so early in development and appear to result from aberrant cell fate specification. In contrast, the apoptosis we observe in *Pcdh-γ*$^{del/del}$ mutants occurs later, after the normal specification of interneuron populations. Given that γ-Pcdhs are putative adhesion molecules, we reasoned that restricted *Pcdh-γ* mutation in a given spinal interneuron subset might affect not only the mutant neurons, but also their neighbors and synaptic partners. To pursue this question, we created and characterized a new conditional mutant allele, which we term *Pcdh-γ*^{fcon3}.

In this allele, loxP sites flank constant exon 3, which is fused in frame to *GFP* (Fig. S3A). The GFP tag allows γ-Pcdh proteins to be detected using antibodies against GFP, and Cre-mediated deletion of the floxed exon to be confirmed by loss of GFP immunoreactivity. Because transcripts encoding all 22 γ-Pcdh variable exons include the three constant exons, and because deletion of the floxed exon 3-GFP fusion is expected to remove signals for polyadenylation, we asked whether Cre-mediated recombination of *Pcdh-γ*^{fcon3} would result not only in the expected 74 amino acid C-terminal truncation, but in a hypomorphic or null allele. To test this, we crossed *Pcdh-γ*^{fcon3} mice to a line expressing Cre under the ubiquitous β-actin promoter (Lewandoski et al., 1997).

Actin-Cre; Pcdh-γ$^{fcon3/fcon3}$ mutants were recovered at birth, but none survived past P0. Western blotting using an antiserum raised against the entire *Pcdh-γ* constant domain, but affinity purified against constant exons 1 and 2 only (Phillips et al., 2003), did not detect any γ-Pcdh proteins, either full-length or truncated, in *Actin-Cre; Pcdh-γ*$^{fcon3/fcon3}$ brain (Fig. S3B). Using RT-PCR of RNA from mutant brains, we could amplify spliced transcripts containing constant exons 1 and 2, but not exon 3 (Fig. S3C). Transcripts containing variable exons spliced to constant exons 1 and 2 were reduced in Actin-Cre; *Pcdh-γ*$^{fcon3/fcon3}$ brain (Fig. S3C); quantitative real-time PCR indicated that these transcripts were present at about 25% of control levels (data not shown). These data suggest that Cre-mediated excision of the *Pcdh-γ*^{fcon3} allele results in reduced transcript stability and severely hypomorphic levels of γ-Pcdh proteins. In

the absence of antibodies specific for *Pcdh-γ* variable domains, we cannot exclude the possibility that transmembrane proteins lacking the constant domain are produced from the *Pcdh-γ*^{fcon3} allele. Regardless, analysis of P0 spinal cords demonstrated that ubiquitous homozygous deletion of the *Pcdh-γ*^{fcon3} allele precisely phenocopied *Pcdh-γ*$^{del/del}$ mutants (Fig. S3D,E), confirming that excision of the *Pcdh-γ*^{fcon3} allele severely impairs γ-Pcdh expression and function.

Mutation of the *Pcdh-γ* locus in restricted interneuron populations reveals that apoptosis is non-cell autonomous

To recombine the *Pcdh-γ*^{fcon3} allele in restricted interneuron populations, we initially utilized two previously-characterized Cre transgenic lines: *Wnt1-Cre* (Danielian et al., 1998) and *Pax2-Cre* (Ohyama and Groves, 2004). We first confirmed that these lines expressed Cre and deleted the *Pcdh-γ*^{fcon3} floxed exon 3-GFP fusion in the expected interneuron populations. In E12 *Wnt1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ spinal cords, Cre was expressed, and GFP was lost, throughout the superficial dorsal horn, in patches of the deeper dorsal horn, and in a small group of cells at the bottom of the ventral horn (Fig. S4A) that expressed the V3 marker Nkx2.2 (data not shown). Staining for γ-Pcdh-GFP fusion proteins in P0 *Wnt1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ spinal cords confirmed Cre-mediated excision throughout the dorsal horn, with most of the ventral horn being spared (Fig. S4D). Immunostaining of *Pax2-Cre; Pcdh-γ*$^{fcon3/+}$ spinal cords at E11 demonstrated that Cre was faithfully expressed by nearly all Pax2-positive neurons; importantly, no Cre-positive/Pax2-negative cells were observed (Fig. S4E).

We first focused on the dI2-derived interneurons that are derived from within the Wnt1 domain, express FoxP2 but not Pax2, and migrate into the ventral horn (Fig. 4, Fig. S4). Approximately half of these dI2 neurons undergo apoptosis in *Pcdh-γ*$^{del/del}$ (Fig. 3, Fig. 4) and *Actin-Cre; Pcdh-γ*$^{fcon3/fcon3}$ mice (Fig. S3E), in which all spinal cord cells are mutant. In *Wnt1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ neonates, the dI2 FoxP2-positive neurons are mutant (Fig. S4B, C), but the ventral horn into which they migrate is not (Fig. S4D). In this situation, FoxP2-positive/Pax2-negative neurons were present in normal numbers, as were other ventral interneuron populations (Fig. 8A, C). The converse situation obtains in *Pax2-Cre; Pcdh-γ*$^{fcon3/fcon3}$ neonates, in which the dI2 FoxP2-positive neurons are not mutant, as they never express Pax2 (Fig. 4B and data not shown), but many neurons (~20%) surrounding their final position within the ventral horn are. In this case, FoxP2-positive/Pax2-negative neurons were reduced by approximately 30% compared to controls (Fig. 8B, C). A second Pax2-negative interneuron population, those cells that are Pax2-negative/En1-positive, was also significantly reduced in number (Fig. 8C).

Together, these results indicate that disruption of γ-Pcdh function can affect spinal interneuron survival non-cell autonomously: mutant neurons can survive, provided they are surrounded by normal neurons, and conversely, normal neurons can undergo apoptosis if they are surrounded by mutant neurons. To confirm this conclusion, we utilized a third transgenic line, *Hb9-Cre* (Umemori et al., 2004), to restrict *Pcdh-γ*^{fcon3} deletion to motor neurons (Fig. S4F,G). Because motor neurons survive normally in *Pcdh-γ*$^{del/del}$ (Fig. 2, Fig. 3, Fig. 4) and *Actin-Cre; Pcdh-γ*$^{fcon3/fcon3}$ (Fig. S3) mutants, any interneuron apoptosis in *Hb9-Cre; Pcdh-γ*$^{fcon3/fcon3}$ spinal cords would present a clear case in which a mutant neuron can survive and yet surrounding normal neurons can be affected non-cell autonomously. In *Hb9-Cre; Pcdh-γ*$^{fcon3/fcon3}$ neonates, FoxP2-positive/Pax2-negative neurons were indeed significantly reduced in number, as were Pax2-positive/En1-negative neurons (Fig. 8C).

DISCUSSION

Enormous progress has been made in elucidating the genetic program that controls the specification and differentiation of neuronal populations in the developing vertebrate spinal cord (reviewed by Goulding et al., 2002; Goulding and Pfaff, 2005; Helms and Johnson,

2003; Lee and Jessell, 1999; Lewis, 2006; Tanabe and Jessell, 1996). The patterns of transcription factor expression that define spinal interneuron populations are well-described, and roles for many of these transcription factors in the specification of spinal interneurons have been established through the use of gene knockout mice (Gross et al., 2002; Helms et al., 2005; Kriks et al., 2005; Mizuguchi et al., 2006; Moran-Rivard et al., 2001; Pierani et al., 2001; Pillai et al., 2007; Sapir et al., 2004). Because many of the transcription factors that mark spinal interneuron populations are expressed only during a restricted embryonic period, it has been more difficult to determine the patterns of connectivity and physiological function attained by these populations as the spinal cord matures. Recent studies in which discrete spinal interneuron populations are permanently labeled by the use of cell type-specific *Cre* and *LacZ* reporter mouse lines have begun to bridge this conceptual gap (Alvarez et al., 2005; Sapir et al., 2004). Clearly, the identification of molecules that control the connectivity and survival of spinal interneuron populations will be important for a complete understanding of how early patterns of cell type specification relate to the distinct roles these populations play in the mature spinal cord.

Here, we have presented data indicating that spinal interneuron populations undergo a period of differential, developmental apoptosis that proceeds in a ventral-to-dorsal temporal gradient. We have shown that the excessive apoptosis observed in *Pcdh-γ* mutant mice reflects an exacerbation of this underlying wild-type pattern, and have implicated the γ-Pcdhs in non-cell autonomous mechanisms influencing neuronal survival. We further show that ventral interneurons undergo apoptosis in the late embryonic period, followed by dorsal interneurons in the first few postnatal days. This ventral-to-dorsal progression parallels those previously observed for TUNEL staining in rat (Lawson et al., 1997), for spinal cord neurogenesis (Nornes and Carry, 1978), and for synaptogenesis (Vaughn and Grieshaber, 1973; Vaughn, 1989; Gingras and Cabana, 1999). Our extensive quantitative analysis demonstrates, for the first time, that the degree of developmental apoptosis can vary greatly among the many molecularly-identified interneuron populations. Such differential apoptosis presumably provides a mechanism by which neuronal numbers optimal for the establishment of spinal circuitry can be obtained.

What are the mechanisms by which the γ-Pcdhs might control interneuron survival? Our data seem inconsistent with one possibility, which is that γ-Pcdhs, either in addition to or instead of their presumed function as adhesion molecules, act as receptors or co-receptors for a trophic factor; if this were true, we would expect γ-Pcdh disruption to affect apoptosis in a strictly cell-autonomous fashion. As is known to be the case for many neurons, spinal interneuron survival during the perinatal period may be controlled by the formation and maturation of synaptic connections. Under this scenario, if the number of synapses made by an interneuron, or the activity at those synapses, falls below a certain level, that neuron becomes susceptible to apoptosis. In *Pcdh-γ* null spinal cord, the formation and maturation of interneuron synapses is disrupted (Wang et al., 2002b), even when apoptosis is blocked by the additional deletion of the *Bax* gene (Weiner et al., 2005; this study, Fig. S2). One interpretation of our experiments using cell type-restricted *Pcdh-γ* mutants is that the likelihood that any given interneuron will die increases as more and more of its synaptic partners (either interneurons or motor neurons) are mutant, presumably due to reductions in γ-Pcdh-dependent synaptogenesis. The increased apoptosis of each spinal interneuron population in *Pcdh-γ* null mutants is strictly proportional to its normal developmental level (Fig. 6, Fig. 7), and expression of the γ-Pcdh family is ubiquitous in the spinal cord (Fig. 1). Thus, it may be that the γ-Pcdhs function generally to promote survival in all spinal interneurons, but that each interneuron population differs in the threshold of synaptic activity, or other trophic signals, that they require for survival. Interestingly, concurrent studies using the *Pcdh-γ*^{fcon3} line (Lefebvre et al., 2009, this issue), indicate that the γ-Pcdhs are also required for interneuron survival in the postnatal retina. In

this case, however, increased apoptosis of retinal interneurons does not seem to result from synapse loss, suggesting that the γ-Pcdhs can influence survival by multiple mechanisms.

Our analysis of *Pax2-Cre; Pcdh-γ*$^{fcon3/fcon3}$ spinal cords, in which some non-mutant ventral interneurons die while others do not, demonstrates that interneuron survival during embryonic development can be controlled non-cell autonomously by other interneurons, whether via synaptic connections or by other mechanisms. Consistent with this, descending inputs, such as those from the corticospinal tract, do not mature until after birth in rodents (Donatelle, 1977; Gilbert and Stelzner, 1979; Gribnau et al., 1986). While DRG sensory afferent terminals do form during late embryogenesis (Snider et al., 1992; Ozaki and Snider, 1997) and may control spinal interneuron survival to some extent (Oliveira et al., 2002), we found no increase in interneuron apoptosis in *Wnt1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ neonates, in which all DRG neurons are mutant (Fig. 8). Some ventral interneurons, including a subset of Pax2-positive cells, are lost non-cell autonomously in *Hb9-Cre; Pcdh-γ*$^{fcon3/fcon3}$ neonates (Fig. 8), in which many motor neurons are mutant (Fig. S4). This is consistent with the results of Béchade et al. (2002), which suggested that motor neuron-derived neurotrophin-3 is required for the survival of Pax2-expressing interneurons in embryonic spinal cord explants. The signaling pathways in which the γ-Pcdhs participate are, at present, almost entirely unknown; it will be interesting in future studies to examine whether disruption of γ-Pcdh function can affect either the release of trophic factors or the regulation of apoptotic signaling proteins such as the Bcl-2 family.

Although the γ-Pcdhs clearly affect the formation and/or maturation of interneuron synapses (Weiner et al., 2005), it is far from clear that they do so by acting solely, or even primarily, as synaptic adhesion molecules. In immunostaining studies, γ-Pcdh family members are detected at only a fraction (perhaps 25–40%) of CNS synapses (Phillips et al., 2003; Wang et al., 2002b), and only a fraction of γ-Pcdh protein is synaptic. Immunogold-electron microscopy shows that some neuronal γ-Pcdh protein is contained in tubulovesicular structures within axon terminals and dendritic branches (Phillips et al., 2003), which may represent a “reserve pool” that can be inserted at the plasma membrane to stabilize nascent contacts during synapse maturation (Jontes and Phillips, 2006). If this is true, then disruption of γ-Pcdhs in developing interneurons may cause synapses to be unstable or otherwise immature, leading to their subsequent loss. However, even in the adult CNS, many synapses do not appear to accumulate significant γ-Pcdh protein (Wang et al., 2002b; Phillips et al., 2003; data not shown), and localization to non-synaptic regions of dendrites and axons remains extensive. While at least some individual γ-Pcdh family members appear to interact homophilically when expressed in cell lines (Frank et al., 2005; Obata et al., 1995; Sano et al., 1993; but see Morishita and Yagi, 2007); it is still unclear whether γ-Pcdhs primarily mediate cell-cell adhesion in neurons, and heterophilic interactions between γ-Pcdh family members or with other proteins have not been examined.

If all synapses did depend on homophilic adhesion between γ-Pcdhs, then a mutant interneuron would be expected to lose all inputs and undergo apoptosis cell-autonomously, which our analysis suggests is not the case. If, however, only ~25% of interneuron synapses depend on the γ-Pcdhs (as suggested by their localization), then we might expect what we observe in the present study: a mutant neuron can survive if many of its contacting neurons are normal, while a normal neuron can undergo apoptosis if many of its contacting neurons are mutant. The activity levels within each *Pcdh-γ* mutant neuron would be reduced, and thus the greater the number of mutant neurons in a circuit, the lower the overall synaptic activation and the greater the susceptibility to apoptosis. In this way, the γ-Pcdh family could help control the density of synapses in, and thus modulate the function of, developing neuronal circuits. An important outstanding question, which we are now addressing, is whether the diversity of the γ-Pcdh family is required for normal synapse formation and interneuronal survival. If so, then the 22 individual γ-Pcdhs will greatly expand the small coterie of adhesion molecules (Shen and

Bargmann, 2003; Shen et al., 2004; Shen, 2004; Yamagata et al., 2002; Yamagata et al., 2003; Yamagata and Sanes, 2008) that are currently known to mediate the exquisite specificity of synaptic patterning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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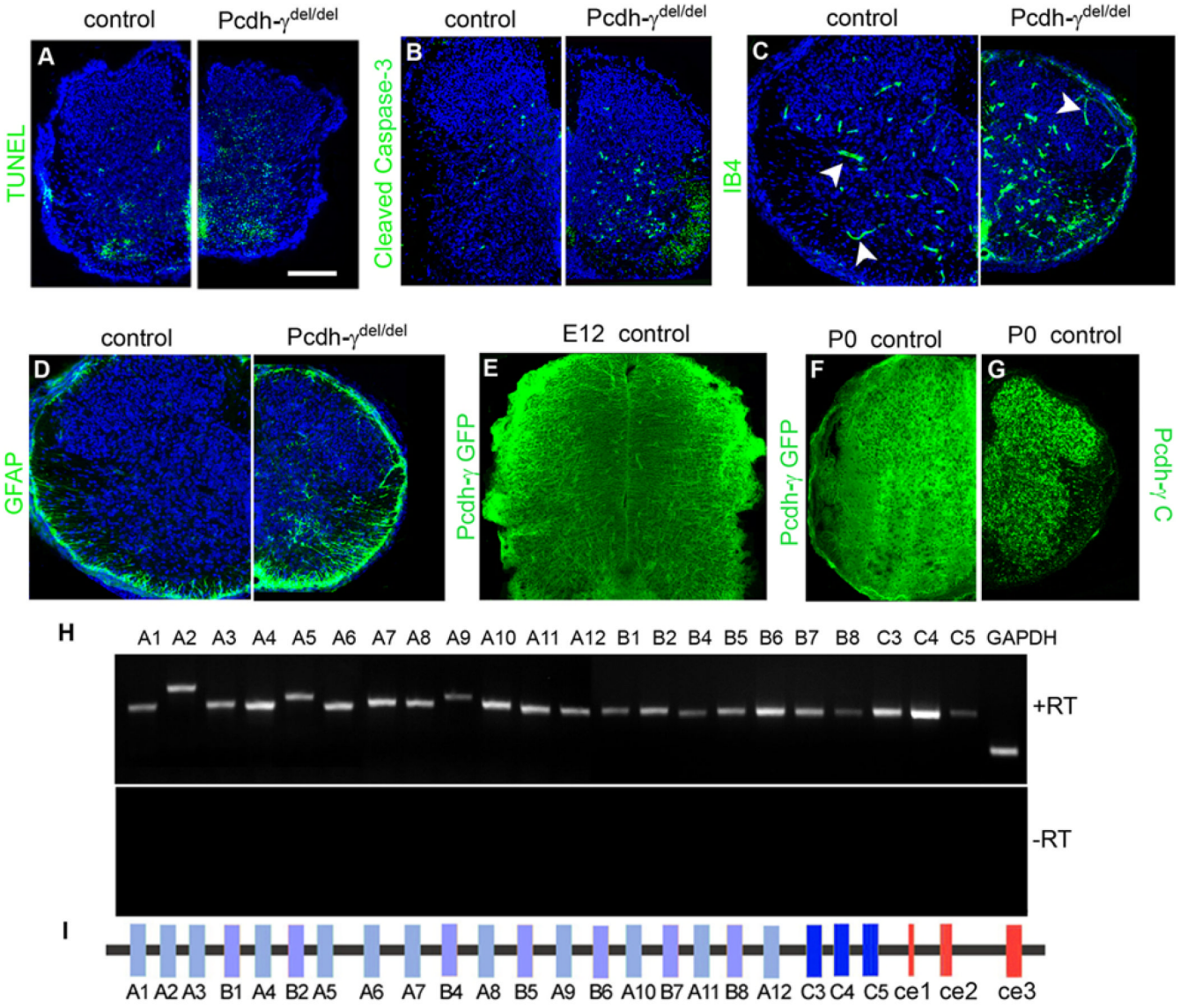

Figure 1. Dorsal-ventral disparity in spinal interneuron apoptosis in *Pcdh-γ* null mutant mice

TUNEL (A), IB4 staining (C), and immunostaining with antibodies against cleaved caspase-3 (B) or GFAP (D) all demonstrate increased signs of apoptosis and neurodegeneration primarily in the ventral horn of *Pcdh-γ*$^{del/del}$ spinal cords, compared to littermate controls, at P0 (arrowheads in C mark blood vessels that, in addition to microglia, are stained by IB4). In E12 (E) and P0 (F) *Pcdh-γ*^{fus} spinal cords, anti-GFP immunostaining demonstrates uniform expression of γ-Pcdh-GFP fusion proteins. In situ hybridization using a riboprobe against the *Pcdh-γ* constant exons also yields uniform labeling of dorsal and ventral horns (G). RT-PCR analysis of E16 spinal cord RNA demonstrates that all 22 variable exon-constant exon spliced transcripts can be detected (H). I) Schematic of the *Pcdh-γ* genomic locus, indicating the 22 variable exons (A, B, and C subfamilies, blue) and 3 constant exons (ce; red). Scale Bar = 100μm.

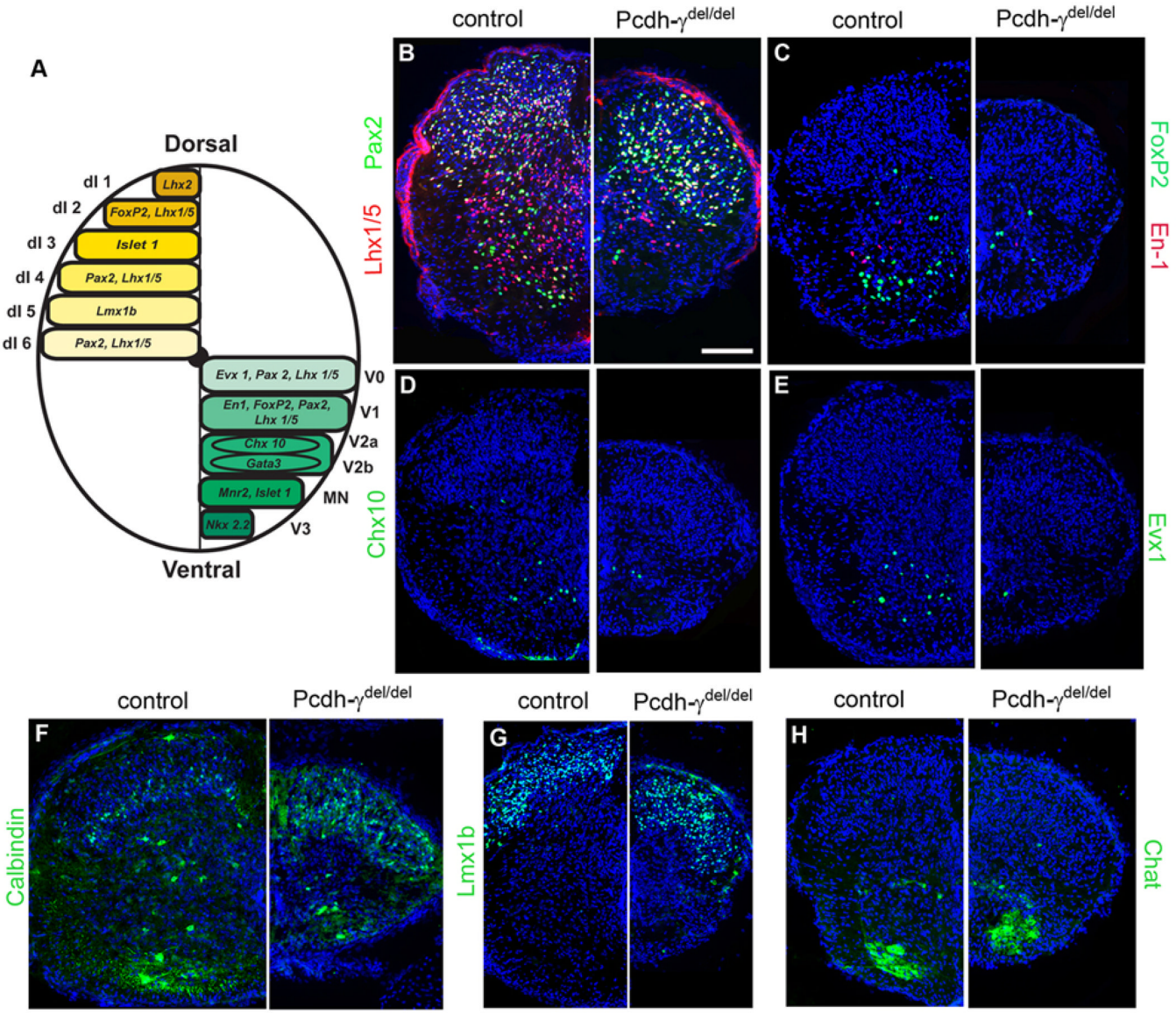

Figure 2. Loss of molecularly-defined spinal interneuron populations in *Pcdh-γ* null mutant mice

A) Schematic of spinal cord domains and the molecular markers used in this study to identify discrete interneuron populations. (B–H) Hemicords from control and *Pcdh-γ*$^{del/del}$ P0 mice immunostained using antibodies against the indicated markers and counterstained with DAPI. At this age, dorsal interneuron populations are not reduced in the *Pcdh-γ*$^{del/del}$ spinal cord (B, G). In contrast, most ventral interneuron populations are reduced, to varying extents (B-E), with the exception of calbindin-positive putative Renshaw cells in the deep ventral horn (F) and Chat-positive cholinergic interneurons at the border of the intermediate gray and the ventral horn (H). Scale Bar = 100 μm.

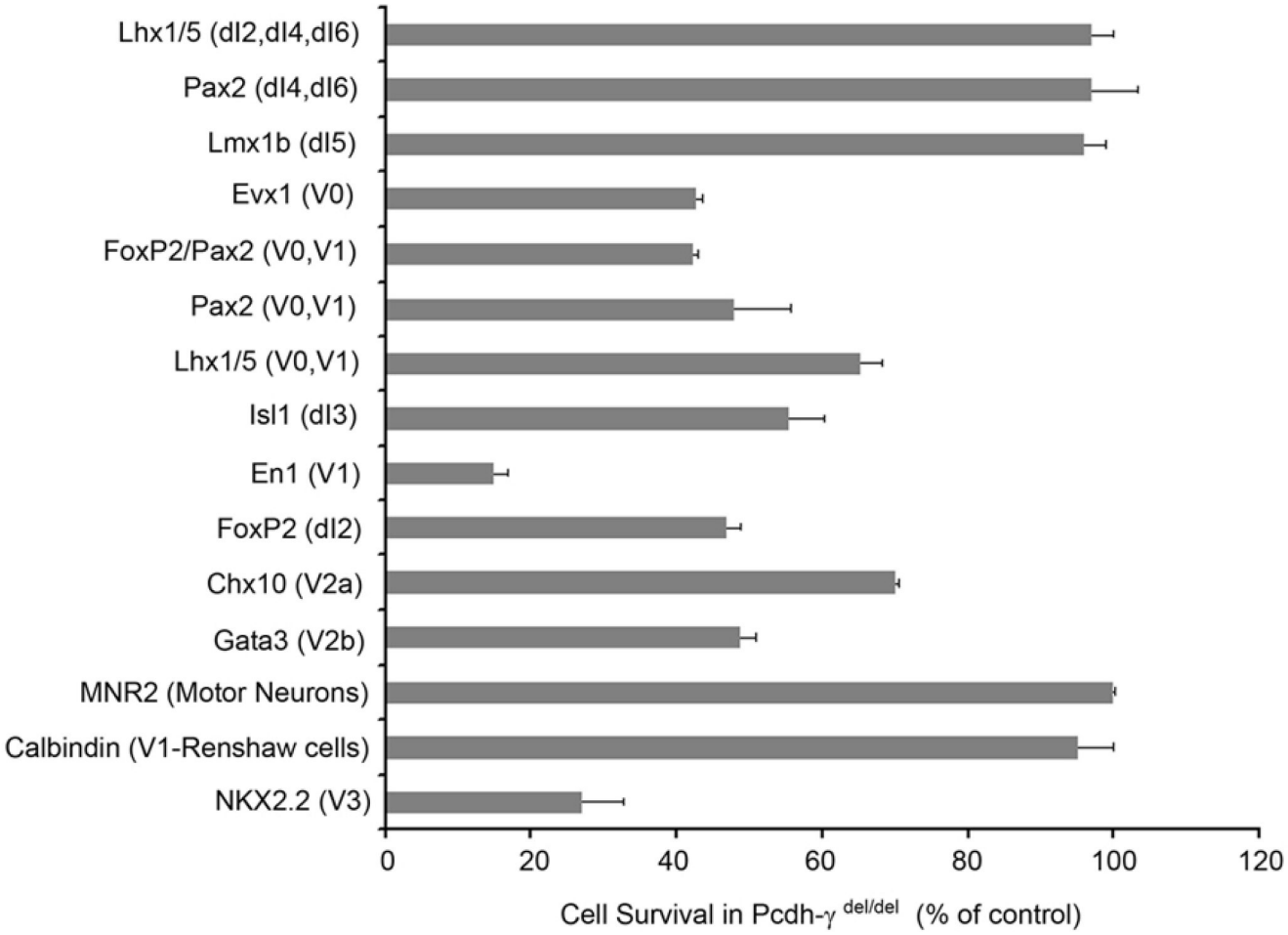

Figure 3. Quantification of differential loss of molecularly-defined spinal interneuron populations in *Pcdh-γ* null mutant mice

Bars (in this and all subsequent graphs) show mean ± SEM of cell counts from 18 sections per molecular marker, taken from 3 animals per genotype. Data are expressed as the percent of control survival of each population in E17 *Pcdh-γ*$^{del/del}$ spinal cords.

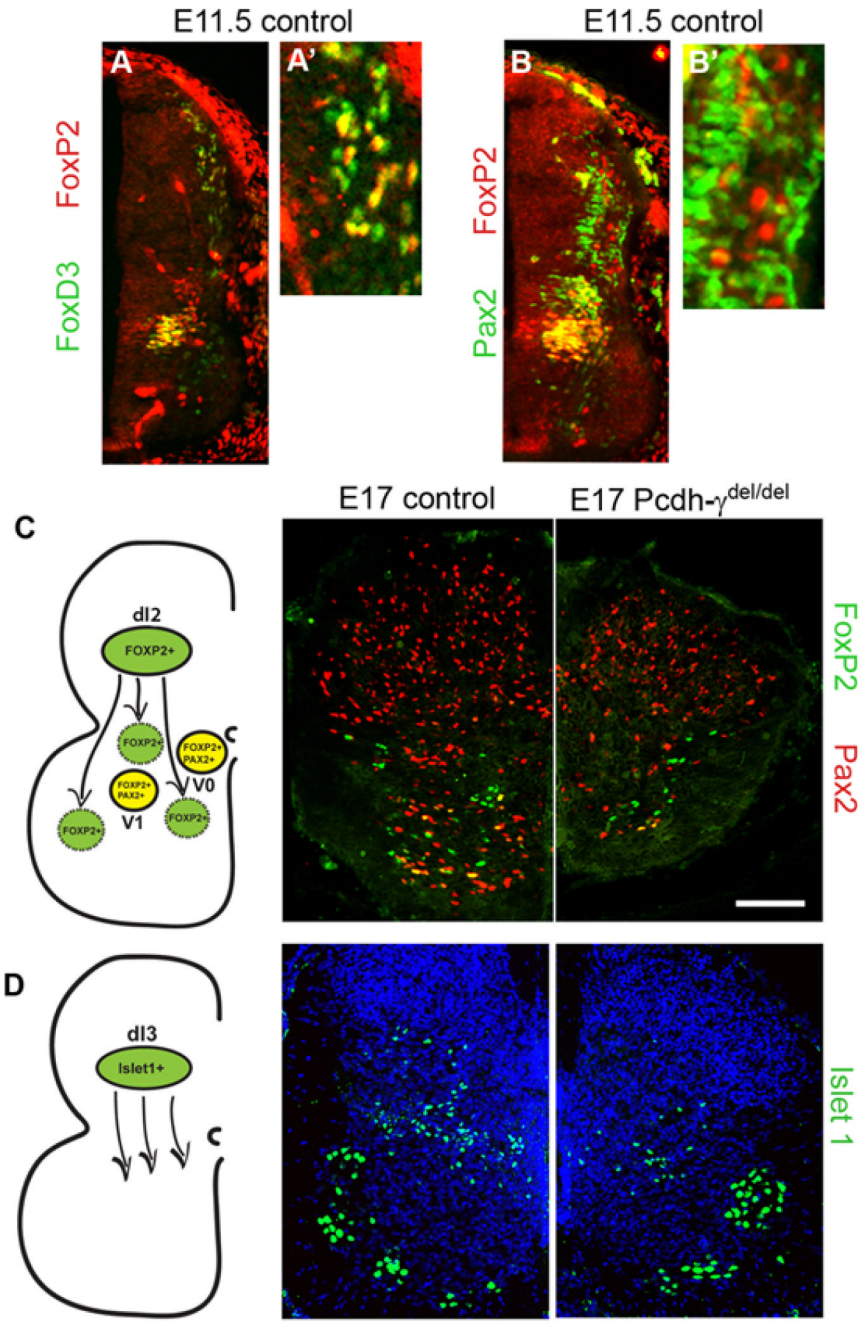

Figure 4. Dorsal interneurons that migrate into the ventral horn are lost in *Pcdh-γ* null mutant embryos

(A, B) E11.5 control hemicords immunostained with antibodies as indicated. Many dI2-derived FoxD3-positive cells that migrate ventrally co-express FoxP2 (A; A' shows a magnified view); double-labeled V1 interneurons are also observed. These dorsally-derived FoxP2-positive interneurons do not co-express Pax2 (B; B'); V0/V1-derived double-positive interneurons are, however observed ventrally. C) Schematic showing the settling patterns of different populations of FoxP2-positive interneurons. Staining of sections from spinal cords of E17 control and *Pcdh-γ*$^{del/del}$ mice shows that ~ 50% of the dI2 population (FoxP2-positive/Pax2-negative) is lost in mutants. D) Isl1-positive dI3 interneurons also migrate ventrally, and ~50%

are lost in *Pcdh-γ*$^{del/del}$ embryos; note there is no reduction in Isl1-positive motor neuron pools in mutants. Scale Bar = 100 μm.

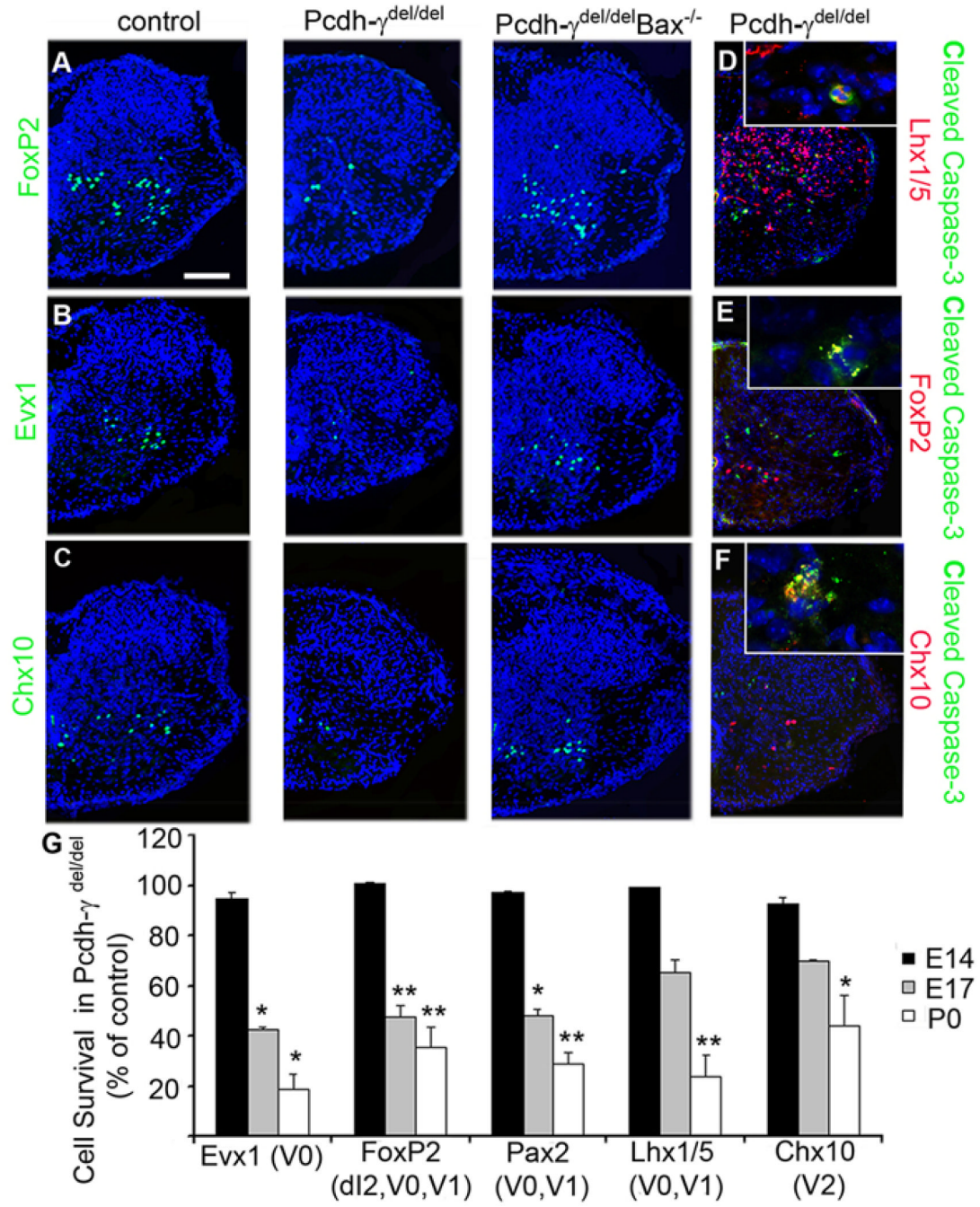

Figure 5. Loss of spinal interneurons in *Pcdh-γ* null mutant mice is due to apoptosis, not aberrant specification

A–C) P0 hemicords from control, *Pcdh-γ*$^{del/del}$ and *Pcdh-γ*$^{del/del}$; *Bax*$^{-/-}$ mice immunostained as indicated. The reductions in ventral populations observed in *Pcdh-γ*$^{del/del}$ mutants are rescued in the *Pcdh-γ*$^{del/del}$; *Bax*$^{-/-}$ double mutants, confirming that they are due to increased apoptosis. D-F) Fragmented ventral interneurons in P0 *Pcdh-γ*$^{del/del}$ spinal cord can be double-labeled (insets) with antibodies against cleaved caspase-3, a marker of apoptotic cells. G) Quantitative analysis of ventral interneuron populations at E14, E17 and P0 demonstrates that a normal number of postmitotic interneurons are produced in *Pcdh-γ*$^{del/del}$ embryos, but that

many are lost between E14 and E17, coincident with the initial period of synaptogenesis; further loss is apparent by P0. Scale Bar = 100 μm.

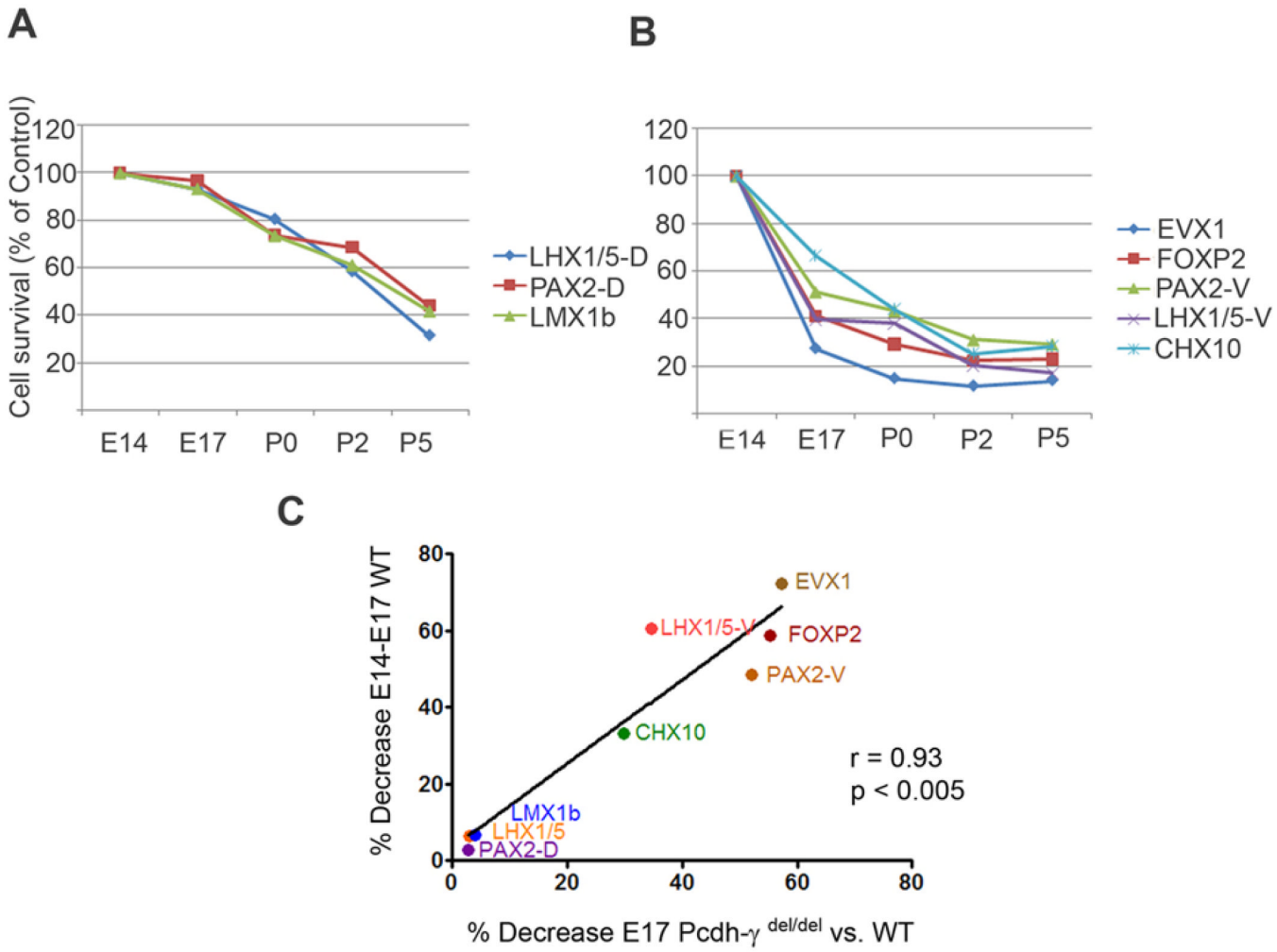

Figure 6. Spinal interneurons undergo a normal, developmental pattern of apoptosis that proceeds in a ventral-to-dorsal temporal gradient

Multiple molecularly-defined interneuron populations were quantified in wild-type (WT) spinal cords at E14, E17, P0, P2, and P5 (at least 6 sections per marker per animal, 3 sets of animals). Means are graphed in (A) and (B) as percent of population size at E14. Ventral interneuron populations are lost, to differing extents, primarily between E14 and P0 (B), while dorsal interneuron populations are lost primarily after P0 (A). The increased apoptosis observed in *Pcdh-γ* null mutant mice likely reflects an exacerbation of this normal pattern, because there is a near-perfect correlation between the extent of cell loss in each population during WT development (y-axis in C) and the extent to which apoptosis is increased in that population in mutants compared to controls (x-axis in C).

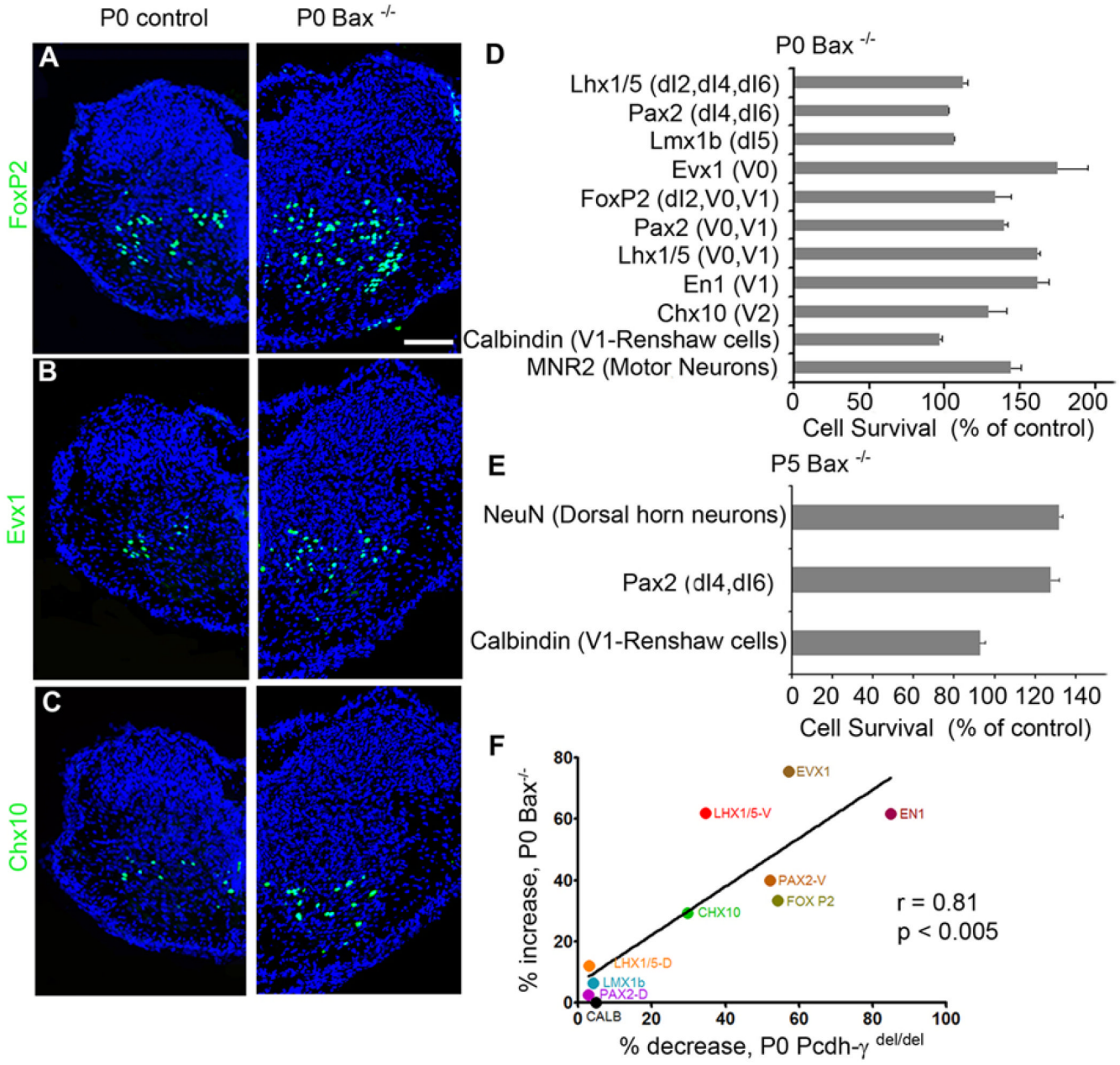

Figure 7. Analysis of *Bax*$^{-/-}$ mice confirms that spinal interneuron apoptosis in *Pcdh-γ* mutant mice represents an exacerbation of an underlying normal developmental pattern

A–C) P0 hemicords from control and *Bax*$^{-/-}$ mice immunostained as indicated. Quantification demonstrates that the sizes of these and other ventral interneuron populations (but not dorsal populations) are increased in the *Bax*$^{-/-}$ spinal cord at P0 (D), indicating that they undergo a normal period of developmental apoptosis. Quantification of dorsal horn interneurons in P5 *Bax*$^{-/-}$ spinal cords confirms that they do undergo a normal period of developmental apoptosis, but only after birth. The increased apoptosis observed in *Pcdh-γ* null mutants represents an exacerbation of an underlying developmental pattern, as the percent decrease in each interneuron population correlates strongly with its percent increase in *Bax*$^{-/-}$ neonates (F). Scale Bar = 100 μm.

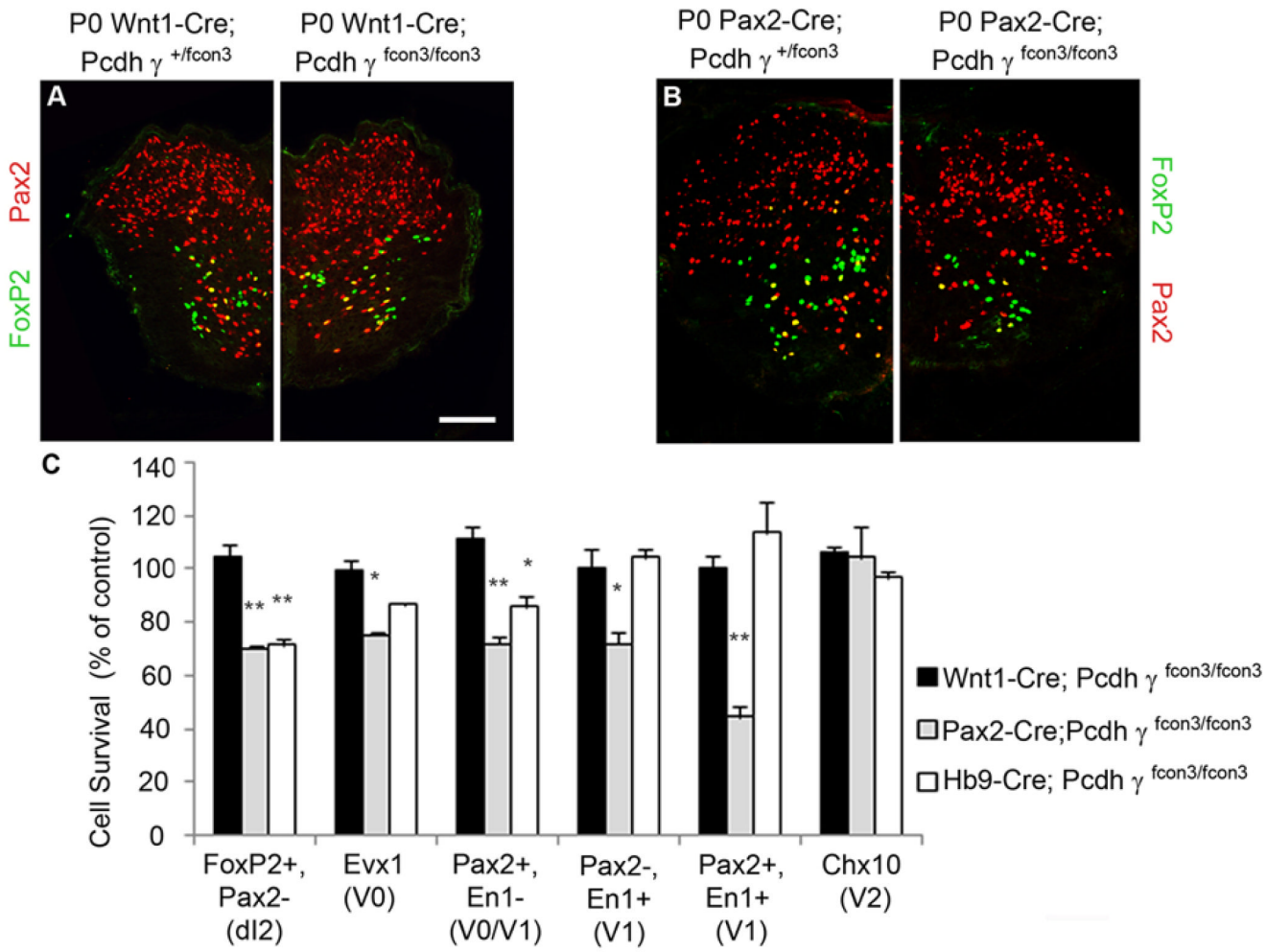

Figure 8. Cell-type restricted *Pcdh-γ* mutation reveals a non-cell autonomous requirement of γ-Pcdh function for neuronal survival

FoxP2-positive, Pax2-negative dI2 interneurons were quantified both in *Wnt1-Cre; Pcdh-γ$^{fcon3/fcon3}$* mice, in which they are mutant, and in *Pax2-Cre; Pcdh-γ$^{fcon3/fcon3}$* mice, in which they are not, at P0 (A, B). There is no reduction in this population in *Wnt1-Cre; Pcdh-γ$^{fcon3/fcon3}$* mice (A, C), but a loss of ~30% is observed in *Pax2-Cre; Pcdh-γ$^{fcon3/fcon3}$* mice compared to controls (B, C). C) Quantitative analysis of ventral interneuron populations in *Wnt1-Cre; Pcdh-γ$^{fcon3/fcon3}$*, *Pax2-Cre; Pcdh-γ$^{fcon3/fcon3}$*, and *Hb9-Cre; Pcdh-γ$^{fcon3/fcon3}$* spinal cords at P0. Data are expressed for mutants as a percent of control values. *$p < 0.05$; **$p < 0.01$. Scale Bar = 100 μm.