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Peer Review File

Protocadherin γC4 regulates neuronal survival and dendritic self-avoidance

Corresponding Author: Professor Takeshi Yagi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The clustered protocadherins are a family of 58 genes in mice, all at a single locus but grouped into three subfamilies called alpha, beta and gamma (a,b,g). Yagi and his colleagues were the first to identify the clustered protocadherins (Neuron, 1998) and have studied them intensively over the past 30 years. In this paper, they report generation and analysis of two new mutant alleles of the gamma (g) cluster. In one, only the gC4 gene is disrupted, while in the other only the gC4 gene is intact. The methods used to generate and validate the alleles are excellent. They used these tools to obtain three sets of results.

First, they show that the gC4 isoform is uniquely required for neuronal survival. This confirms earlier findings that the gC3-5 isoforms are required (Chen, Neuron, 2012) and the more recent discovery that gC4 is particularly essential (refs 25 and 26, and McLeod, Genesis, 2025 [not cited but should be]).

Second, they investigated PYK2 and FAK, which have been reported to be regulated by Pcdhs and to be parts of their signal transduction pathway (refs 10 and 11 and Fan, eLife 2018 [not cited but should be]). They show that gC4 is one of the isoforms that regulate this pathway, but probably not the only ones.

Third, they examined the phenomenon of dendritic self-avoidance, in which dendritic branches of a single neuron appear to be repelled by each other. This process has been shown to require Pcdhs in retinal starburst cells and cerebellar Purkinje cells (refs. 14 and 15). Here, they study Purkinje cells and show that self-avoidance is diminished in mice that lack gC4 but not in mice that express only gC4. This established a critical role for gC4. They also show that virus-mediated introduction of gB2 fails to rescue the defect in the absence of other isoforms, and conclude that gC4 is unique in this respect.

Although the role of gC4 in self-avoidance is clear, there are problems with the conclusion that it is unique. First, they only tested one other isoform, gB2 so it could either be that gC4 is unusually potent or that gB2 is unusually impotent. Second, they verify expression of gC4 using an antibody to an epitope tag that had been fused to the cDNA in the vector. However, even though the gB2 was similarly tagged, there is no evidence that it is expressed. Third, the virally-expressed gC4 protein seems to be confined to the cell body, whereas it presumably acts in the dendrites. We have to assume that some of it got to the dendrites – but if less gB2 got there, it might not have been in the proper position to function. The result that virally expressed gC4 only partially rescues (Figure 6n) while endogenous gC4 (“C4 only; Figure 5m) rescues fully supports this possibility. Fourth, these results contrast with those in retina, where either gA1 or gC3 was able to rescue self-avoidance in the absence of other gPcdhs. More recently, Garrett has shown that neither gC4 or gC5 is required for self-avoidance in starbursts (McLeod…Garrett, biorxiv). Of course it could easily be that the two cell types have different requirements; in fact ref. 15 showed that although both alpha and gamma clusters regulate self-avoidance in both cell types, they appear more redundant in one than the other. However, that is a rather minor difference and given the other issues, I do not think the authors have made a compelling case.

In summary, the first two results (survival and PYK2/FAK) are largely confirmatory. For the third, the demonstration that gC4 is involved in cerebellar self-avoidance is convincing, but the argument that it is unique in this regard is not.

Reviewer #2

(Remarks to the Author)
The manuscript investigates the role of Pcdh-γC4 in the development and dendritic self-avoidance of Purkinje cells using diverse CRISPR/Cas9 editing approaches. The study provides compelling evidence for the multifaceted functions of Pcdh-γC4, contributing valuable insights to the field of neurodevelopment.

The results presented in this study are highly interesting. However, several issues require further clarification or investigation to enhance the overall impact of the manuscript.

Major concerns:
(1) The authors suggest that different mutation types may lead to distinct cellular and organ abnormalities. However, it remains unclear whether the specific mutation types described in the manuscript have been identified in patients with developmental disorders?
(2) The manuscript does not clarify whether Pcdh-γC4 exhibits brain region-specific or cell type-specific expression. Additionally, the rationale for focusing on the cerebellum and Purkinje cells requires further justification.
(3) The authors propose that Pcdh-γC4 is critical for Purkinje cell morphology and dendritic self-avoidance in the cerebellum, as well as for motor dysfunctions and seizures. However, it remains unclear whether rescue of these abnormal phenotypes was observed in the PV-Cre; Pcdh-γflox/ΔC4 model.
(4) In Figure 1, the authors demonstrate reduced whole-brain and cerebellar volumes in Pcdh-γΔC4 mice. However, additional data on changes in other key brain regions are needed to clarify whether the cerebellum exhibits the most severe abnormalities.
(5) Whether sex differences were examined or observed in the phenotypes of Pcdh-γΔC4 mice?

Minor concerns
(1) The figure description on page 8, line 128, may be inaccurate. The Western blot data for Pcdh-γB2 and Pcdh-γC3 seem to be presented in Figure 1f, not as currently indicated.
(2) For better clarity and accuracy, it is recommended that the figure description of the superior colliculus on page 13, line 213, be specified as Fig. 2c-(i).

Reviewer #3

(Remarks to the Author)
This manuscript by Higuchi et al. utilizes clever frame-shifting mutations introduced into the Pcdhgc4 gene in mice via CRISPR-Cas9 to create three new lines aiming to add to our understanding of the Pcdhg gene cluster and thus gamma-protocadherin function. In the first, a 2 bp deletion is introduced into the variable cytoplasmic domain (VCD) of the Pcdhgc4 variable exon, such that an early stop codon occurs within the first constant exon (i.e., the 2nd exon of the complete transcript), potentially leading to truncated proteins, albeit at a low level. In the second line, the authors introduced into these same mice a 1 bp deletion leading to a frameshift that puts the complete C4 transcript back in frame, albeit with a point mutation in C4 and with the other 21 Pcdhg isoforms now truncated within the constant domain, complicating interpretation. In the third line, the authors introduce a frame-shifting (apparently, though see below about ambiguous discussion of this line) mutation of 79 basepairs in the 5' end of the Pcdhgc4 gene, creating a complete null of this isoform. They then utilize these lines to examine postnatal survival, neuronal apoptosis, and cerebellar Purkinje cell dendrite self-crossings. While the approach is novel and clever, unfortunately most of the manuscript's claims are overstated and poorly-supported. Several prior results are not taken into account, many experiments are not quantified at all (it is not even clear if the authors examined multiple mice), some results are puzzling based on extensive prior literature and the discrepancies are unexplained, and conclusions are not supported by the data. The manuscript also contains too many grammatical errors and errors in the writing to be certain what the authors mean to say at times, which makes it difficult to interpret what is meant. Much more data would be needed to support the claims made by the authors.

Detailed concerns:

1. The authors refer to the Pcdh-g deltaC4 (DC4 hereafter) line alternately as C4 deficient or a knockout. It is not a knockout. The line likely does express truncated C4 with its extracellular domain, transmembrane domain, and most of its VCD. There is no reason why this protein might not be made in small amounts and be incorporated into protocadherin lattices at the membrane, as dimerization occurs within EC5 and 6, which could still be expressed. The western in figure 1b is not entirely clear—it looks like there may be faint bands present. Indeed, the fact that 10 of 23 pups survived postnatally indicates that some C4 remains. It was conclusively shown by Garret et al 2019 that a clean knockout of the C4 gene results in complete neonatal lethality paralleling the complete gamma cluster KO, as did deletion of C3/4/5 in the Maniatis lab's work (Chen et al 2012). The authors' own (similarly clean) KO, N delta 79 (N79 hereafter), replicates this. The obvious conclusion is that the DC4 line retains some truncated C4. If so, this complicates interpretation of the results using this mouse line. The authors' explanation of NMD differences is not well supported. This line should not be called a null allele unless it can be proven there is no truncated C4 protein. It would be helpful to show multiple individual mouse brain western blots from WT and DC4 mice to show whether there is any variability in possible levels of truncated C4 proteins.

2. Similarly, though the creation of the C4only line is very clever indeed, this mouse line is not a clean mutant either. In this line, 21 other gamma protocadherin isoforms are expressed, but truncated within the 1st constant exon. This is supported by Figure 1F. These truncated proteins could absolutely have a deleterious effect on gamma cluster protein function by being inserted into lattices (See papers by Brasch et al., Maniatis lab), but not signaling properly due to lack of the constant domain (e.g., through FAK/PYK2). It is also not particularly surprising that these mice survive given that Garett et al 2019 already

demonstrated that discrete loss of C4 leads to lethality and neuronal apoptosis while the 1R1 line, which has only C4 remaining as a functional isoform, albeit at only 10% of normal protein levels, survives and has only mild apoptosis. A paper by Hanes et al. in 2025 in Genesis (not cited by the authors) also reports a cre-activated C4 transgenic mouse line and shows that turning this transgene on in neurons alone rescues both neuronal survival and postnatal survival entirely. The statement in line 142 that C4 "is not strictly required for postnatal survival" is not correct if truncated C4 remains even at a low level in the DC4 line. The authors could say that "full-length C4 is not strictly required for postnatal survival, at least 40% of the time" (10 of 23 mice survived). The statement in line 160 that the C4 only mouse mutation "successfully restricted expression to Pcdhg-C4 alone" is patently false given the western blot in Figure 1F. 21 other gammas are there but truncated (and the western confirms some are).

3. The authors' interpretation (line 129) of the Weiner et al 2005 paper is not correct. In the Pcdhg tr line, all 22 gammas are truncated in the 3rd constant exon. Truncated proteins are still detected, albeit at a lower level than normal. These mice exhibit reduced synaptic density in the absence of any increase in neuronal apoptosis. The authors mis-state this phenotype (line 130). The phenotype of this tr line does not support their conclusions that only C4 is functional in the Pcdhg C4 only mutant. Clearly, in the tr line, C-terminally truncated Pcdhgs can rescue the neuronal apoptosis—though not neonatal lethality. Thus, the 21 truncated gammas in their C4 only line may be contributing to the rescue as well.

4. The way N79 is discussed is very confusing. In line 170 the authors say it is an "in-frame deletion of a portion of EC1". But the Extended Data Figure 4 clearly shows a stop codon is introduced, so it is NOT in frame. Also, the authors interpret extended data figure 4F to say that C4 protein is "hypomorphic", but this makes no sense if the transcript coding frame is disrupted at the very 5' end of the gene. No protein of any significant size could be made. Why do the authors claim that DC4, which is likely NOT a complete KO, is one, while they claim N79, which should be a clean KO, is not but is, rather, hypomorphic? That doesn't fit with the survival or other data. This confusion is very concerning.

5. Figure 2 is unconvincing. One section of one brain is shown, with no quantification. One can conclude nothing from this. The authors would have to quantify many sections from each region, from at least 3-4 embryos per genotype, and use the appropriate statistical test to show any differences in neuronal apoptosis patterns or levels. This figure can't be used as is.

6. Similarly, Figure 3 is not convincing. One western blot of one sample of each genotype is shown. Again, one must analyze several independent samples, often up to 6-8 per genotype given the difficulties of reliably detecting phosphorylated proteins in Western blots. Quantification numbers are shown beneath lanes, but there is no mention of N or statistical tests. Is that quantification of one band from one sample? If so, no conclusions can be drawn. Also, the result is hard to understand. Why would FAK phosphorylation go down but PYK2 go up? In prior work, such as Chen et al 2012 and Suo et al 2012 these proteins follow the same pattern. What is the explanation for why they would be different here? The result also does not make sense in that, in the DC4 line, the other 21 protocadherins are expressed, and thus their constant domains—which is the portion of the gamma proteins that binds to FAK and PYK2—could inhibit FAK and PYK2 phosphorylation. How would the authors explain a mechanism by which C4—which has the same constant domain as the other 21 gamma protocadherins---could "uniquely" interact with and regulate FAK and PYK2? It is not likely to be a unique conformational issue with C4 as prior papers using a variety of gamma and alpha Pcdh constructs (other isoforms) showed inhibition of these proteins.

7. There are N's given for the Purkinje cell analysis, and statistics are done. But the test used is not indicated. This is necessary to determine if the correct tests were used. The differences in arbor size and self-crossings, while significant (albeit without the test being stated), are rather small. From 4 crossings per area in WT to 6 in the DC4 line to 5 in the C4 only line. It would be helpful to compare this to a complete gamma protocadherin null as in the lab's prior papers to determine whether this is a mild phenotype or a severe one. It seems rather mild, which undercuts the claim that "only gC4 is indispensable isoform on dendritic self-avoidance of Purkinje cells". Such a result would also contradict with prior data from Lefebvre et al., with little explanation other than the fact that the mouse lines are not clean mutations but have the complications mentioned above.

8. The experiment in figure 6 is nice, but has a major flaw that prevents the authors' conclusion that C4, but not B2, can rescue the phenotype: nowhere do the authors confirm that the B2 transgene is expressed at the protein level. Without this crucial characterization, no conclusion can be drawn.

9. Characterization of prior literature is not complete. In addition to the missed references and misstatements noted above, some statements in the discussion are unfair. In line 305, 306, the following sentence is not a correct characterization: "While previous studies have suggested that Pcdh-gC4 is essential sole isoform for mouse survival (25—Garrett et al., 2019), our results contradict this notion that Pcdh-gC4 is required for survival". First, that 2019 paper did not "suggest" this—it conclusively showed it by multiple clean mutations in mice in vivo. Second, the authors' results do not contradict it, because as noted the DC4 mutation is not a clean, confirmed null, and because their N79 mutant perfectly matches the similarly clean mutant reported in the Garrett 2019 paper. Again, the 1R1 line reported in that 2019 paper is never mentioned, even though it is relevant to the authors' experiments, nor are the results of Hanes et al 2025 (which is not cited).

10. Though I completely understand that English may not be the authors' first language, the paper is simply too confusingly written and there are too many grammatical mistakes (nearly every line, too many to note here in detail). As noted regarding the explanation of the N79 line, this can result even in the nature of the experiment being confusing or differently described in different parts of the manuscript. A thorough editing for English would be required for the manuscript to be clear.

Minor issue:

• Why is “variable cytoplasmic domain” abbreviated as “VCP”? what is the “P”? Every other paper on these proteins including some of the authors’ own use “VCD”. This should be used throughout in all the figures and text to avoid confusion in the field.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded fully to my criticisms. I have no further comments.

Reviewer #2

(Remarks to the Author)
I have no further comments.

Reviewer #3

(Remarks to the Author)
The authors have addressed the most important reviewer comments and have expanded the data substantially. The English grammar has been improved but the journal should still discuss another round of editing prior to publication as there are many missing articles and other mistakes.

Response Letter to Reviewer's comment

Manuscript Number: COMMSBIO-25-5696-T

Title: Protocadherin γC4 regulates neuronal survival and dendritic self-avoidance

We are deeply grateful to the editor and reviewers for their careful assessment of our manuscript and for their insightful, constructive comments. These suggestions have been invaluable for improving the clarity and overall quality of the study. We have revised the manuscript accordingly and believe that the current version adequately addresses all of the points raised in the reviews. In this response letter, all additions and changes in the revised manuscript are indicated in red text, and the corresponding line numbers are provided for ease of reference.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The clustered protocadherins are a family of 58 genes in mice, all at a single locus but grouped into three subfamilies called alpha, beta and gamma (a,b,g). Yagi and his colleagues were the first to identify the clustered protocadherins (Neuron, 1998) and have studied them intensively over the past 30 years. In this paper, they report generation and analysis of two new mutant alleles of the gamma (g) cluster. In one, only the gC4 gene is disrupted, while in the other only the gC4 gene is intact. The methods used to generate and validate the alleles are excellent. They used these tools to obtain three sets of results.

Response:

We sincerely thank the reviewer for the positive and constructive assessment of our manuscript. We address the specific comments and concerns point-by-point below.

First, they show that the gC4 isoform is uniquely required for neuronal survival. This confirms earlier findings that the gC3-5 isoforms are required (Chen, Neuron, 2012) and the more recent discovery that gC4 is particularly essential (refs 25 and 26, and McLeod, Genesis, 2025 [not cited but should be]).

Response:

We thank the reviewer for this constructive comment. We agree that our findings are consistent with prior work demonstrating the requirement of γC3–γC5 isoforms for neuronal survival (Chen, *Neuron*, 2012) and with more recent studies highlighting the particularly essential role of γC4

(refs. 25 and 26). In addition, as suggested, we have now cited McLeod et al. (*Genesis*, 2025) in the revised manuscript. The following statement was added in the Introduction section:

"And recently, McLeod et al. reported a novel transgenic mouse line that overexpresses Pcdh-γC4 under the CAG promoter, and demonstrated that CAG-driven γC4 overexpression can rescue the spinal cord apoptosis caused by γC4 deficiency." (Line 79-81).

Second, they investigated PYK2 and FAK, which have been reported to be regulated by Pcdhs and to be parts of their signal transduction pathway (refs 10 and 11 and Fan, eLife 2018 [not cited but should be]). They show that gC4 is one of the isoforms that regulate this pathway, but probably not the only ones.

Response:

We thank the reviewer for this comment. As suggested, we have added the citation to Fan et al., eLife (2018) in the revised manuscript. Therefore, we updated the sentence in Introduction section with cited Fan et al., eLife (2018):

"Pcdh-α and Pcdh-γ constant region (CR) negatively regulated FAK or PYK2 for cortical dendrite arborization, neural migration, and cytoskeletal dynamics." (Line 69-71).

In addition, we substantially revised Figure 4 (Figure 3 in the original submission) by increasing biological replication and providing quantitative analysis. Based on the updated quantification, we found that FAK phosphorylation (pY397/total FAK) is elevated in the ΔCR1 and C4only genotype, whereas PYK2 phosphorylation (pY402/total PYK2) does not show a significant change across genotypes. So, we have revised our results and conclusions as follows:

"To examine the specific intracellular signal transduction via γC4 corresponded to neural cell death, we focused on the phosphorylation of FAK and PYK2, which are known to be regulated by Pcdh-γ constant region (γ-CR). We performed western blotting using P0 brain lysates from WT, γΔCR1, γC4nmd, and γC4fl-only mice (Fig. 4a), and quantified phosphorylation as pY397-FAK/total FAK and pY402-PYK2/total PYK2 (n = 6 biological samples per genotype; one-way ANOVA with Tukey's multiple-comparisons test). Total protein levels of FAK and PYK2 were comparable across genotypes (Fig. 4a). In contrast, FAK phosphorylation was significantly increased in γΔCR1 and γC4fl-only compared with WT (Fig. 4b), whereas γC4nmd did not show a significant change relative to WT. This pattern is consistent with prior reports that the γ-CR negatively regulates FAK phosphorylation; notably, in the γC4fl-only line the Pcdh-γ cluster other

than γC4 isoform lack the CR, providing a plausible explanation for elevated pFAK in this genotype. By contrast, we observed no significant differences in PYK2 phosphorylation among genotypes (Fig. 4c), providing no evidence for altered PYK2 phosphorylation under these conditions." (Line 250-264).

Third, they examined the phenomenon of dendritic self-avoidance, in which dendritic branches of a single neuron appear to be repelled by each other. This process has been shown to require Pcdhs in retinal starburst cells and cerebellar Purkinje cells (refs. 14 and 15). Here, they study Purkinje cells and show that self-avoidance is diminished in mice that lack gC4 but not in mice that express only gC4. This established a critical role for gC4. They also show that virus-mediated introduction of gB2 fails to rescue the defect in the absence of other isoforms, and conclude that gC4 is unique in this respect.

Response:

We thank the reviewer for this assessment of our dendritic self-avoidance analyses and for highlighting the significance of γC4 in Purkinje cells. In addition, with regard to the γB2 transgene experiment, we have revised the manuscript in response to the reviewer's subsequent comment (see below) and updated our statement and conclusions accordingly.

Although the role of gC4 in self-avoidance is clear, there are problems with the conclusion that it is unique. First, they only tested one other isoform, gB2 so it could either be that gC4 is unusually potent or that gB2 is unusually impotent. Second, they verify expression of gC4 using an antibody to an epitope tag that had been fused to the cDNA in the vector. However, even though the gB2 was similarly tagged, there is no evidence that it is expressed. Third, the virally-expressed gC4 protein seems to be confined to the cell body, whereas it presumably acts in the dendrites. We have to assume that some of it got to the dendrites – but if less gB2 got there, it might not have been in the proper position to function. The result that virally expressed gC4 only partially rescues (Figure 6n) while endogenous gC4 ("C4 only; Figure 5m) rescues fully supports this possibility. Fourth, these results contrast with those in retina, where either gA1 or gC3 was able to rescue self-avoidance in the absence of other gPcdhs. More recently, Garrett has shown that neither gC4 or gC5 is required for self-avoidance in starbursts (McLeod…Garrett, biorxiv). Of course it could easily be that the two cell types have different requirements; in fact ref. 15 showed that although both alpha and gamma clusters regulate self-avoidance in both cell types, they appear more redundant in one than the other. However, that is a rather minor difference and given the other issues, I do not think the authors have made a compelling case.

Response:

We thank the reviewer for this thorough and insightful critique regarding the interpretation of γC4 “uniqueness” in dendritic self-avoidance. We agree that our original conclusions were overstated and have revised the manuscript accordingly.

First, because we were unable to verify protein-level expression of the gB2 transgene, we agree that no conclusions can be drawn from that experiment. We therefore removed the gB2 transgene data from Figure 7 (Figure 6 in the original submission) and updated the result statement as follows:

"Most notably, dendritic self-crossings per unit area were markedly reduced by γC4 overexpression, consistent with a partial rescue relative to the knockout. Nevertheless, the phenotype was not fully normalized, and self-crossing values remained significantly different from control (Fig. 7l). These results demonstrate that AAV-γC4 expression after birth has the potential to rescue dendritic self-avoidance in Purkinje cells and improves overall arbor organization in the conditional 22-Pcdh-γ knockout background." (Line 330-336).

Second, regarding the subcellular localization of virally expressed γC4, we performed confocal imaging and found that the expressed γC4 signal was detectable not only in the soma but also in dendrites. These data are provided in Supplementary Data Fig. 7 (Supplementary Data Fig. 6 in the original submission). The updated manuscript as follows:

"The high-magnification image showed that PA-tag antibody indicated dot-like Pcdh-γC4 localization (Supplementary Data Fig. 7). The signals were observed not only at Purkinje cell body and dendrite, but also molecular layer interneuron (Supplementary Data Fig. 7b–d)." (Line 320-323).

Finally, we appreciate the reviewer’s comments on differences relative to retinal self-avoidance studies and recent work in starburst amacrine cells, and we agree that cell-type-specific requirements and redundancy among isoforms may contribute to the observed differences. In response, we have updated the Discussion to acknowledge these points and to tone down claims of γC4 being uniquely required for Purkinje cell self-avoidance. We added the following statements to the revised Discussion:

"These observations refine prior models by suggesting that, in Purkinje cells, full-length γC4 may provide a key CR-dependent function required for dendritic self-avoidance, whereas the common regions of other Pcdh-γ isoforms are not strictly required for this aspect of dendritic morphogenesis. In retinal starburst amacrine cells, a single Pcdh-γ isoform can be sufficient to support self-avoidance, although isoform diversity is critical for self/non-self discrimination among neighboring cells. Although we did not directly test starburst amacrine cells, our Purkinje cell data are compatible with the concept that a limited set of functional isoforms can sustain self-avoidance in a cell-type–specific manner. Notably, Purkinje cells also show contributions from both Pcdh-α and Pcdh-γ, implying that distinct neuronal populations may integrate protocadherin inputs differently to achieve dendritic patterning." (Lines 424-435).

In summary, the first two results (survival and PYK2/FAK) are largely confirmatory. For the third, the demonstration that gC4 is involved in cerebellar self-avoidance is convincing, but the argument that it is unique in this regard is not.

Response:

We thank the reviewer for this summary assessment. We have revised the manuscript accordingly based on the points raised above.

Reviewer #2 (Remarks to the Author):

The manuscript investigates the role of Pcdh-γC4 in the development and dendritic self-avoidance of Purkinje cells using diverse CRISPR/Cas9 editing approaches. The study provides compelling evidence for the multifaceted functions of Pcdh-γC4, contributing valuable insights to the field of neurodevelopment.

The results presented in this study are highly interesting. However, several issues require further clarification or investigation to enhance the overall impact of the manuscript.

Response:

We appreciate the reviewer's encouraging assessment of our study. We have revised the manuscript to address the issues raised and provide clarifications and additional analyses where appropriate; our detailed responses are provided below.

Major concerns:

(1) The authors suggest that different mutation types may lead to distinct cellular and organ abnormalities. However, it remains unclear whether the specific mutation types described in the manuscript have been identified in patients with developmental disorders?

Response:

We thank the reviewer for this important question. We have revised the Discussion section to clarify the relationship between the mutation types in our mouse models and those reported in patients with neurodevelopmental disorders. Therefore, the following statement was added:

"Recent human genetics studies have identified biallelic PCDHGC4 variants as a cause of a neurodevelopmental syndrome characterized by progressive microcephaly, seizures, and neurodevelopmental impairment, with additional features including joint anomalies. In this context, our γC4nmd mice provide an *in vivo* model that captures key neurological aspects of the human condition. Specifically, γC4nmd mice exhibit reduced brain volume, abnormal gait/locomotor performance, and seizure-like behaviors, consistent with core clinical features reported in individuals with biallelic PCDHGC4 variants (Fig. 1, Supplementary Data Video 3). By contrast, we did not observe overt joint abnormalities in our current analyses; however, musculoskeletal phenotypes were not a primary endpoint and may require dedicated, systematic evaluation in future work.", (Line 364-373).

(2) The manuscript does not clarify whether Pcdh-γC4 exhibits brain region-specific or cell type-specific expression. Additionally, the rationale for focusing on the cerebellum and Purkinje cells requires further justification.

Response:

We thank the reviewer for this important point. We have added text to the manuscript to clarify the expression context and to better justify this focus, revised text provided below:

"Because γC4nmd mice exhibited impaired motor behavior, we reasoned that this newly generated line could serve as a novel model for human neurodevelopmental syndromes (Fig. 1g; Supplementary Video 3). Previous studies indicate that γC4 is broadly expressed across multiple brain regions and cell types. Given that the Pcdh-γ cluster is known to contribute to dendritic self-

avoidance in Purkinje cells, we performed morphological analyses of Purkinje cells in surviving WT, γC4nmd, and γC4fl-only mice.", (Line 267-272).

(3) The authors propose that Pcdh-γC4 is critical for Purkinje cell morphology and dendritic self-avoidance in the cerebellum, as well as for motor dysfunctions and seizures. However, it remains unclear whether rescue of these abnormal phenotypes was observed in the PV-Cre; Pcdh-γflox/ΔC4 model.

Response:

We thank the reviewer for this question. We clarify that PV-Cre; Pcdh-$\gamma^{flox/\Delta C4}$ (PV-Cre; γflox/γC4nmd in revised manuscript) is a conditional knockout model used to induce loss of γC4 in PV-Cre–expressing cells; it is not a rescue paradigm. Accordingly, no rescue (phenotypic recovery) was expected or observed in this model.

(4) In Figure 1, the authors demonstrate reduced whole-brain and cerebellar volumes in Pcdh-γΔC4 mice. However, additional data on changes in other key brain regions are needed to clarify whether the cerebellum exhibits the most severe abnormalities.

Response:

We thank the reviewer for this suggestion. In our current study, MRI volumes were quantified manually (i.e., without automated segmentation/atlas-based tools). We therefore focused on regions whose anatomical boundaries can be consistently and unambiguously delineated across samples—namely the whole brain and the cerebellum—to ensure robustness and reproducibility of the measurements. In addition, the cerebellum was prioritized because we observed cerebellum-linked phenotypes, including behavioral abnormalities and Purkinje cell morphological defects.

For other brain regions, the boundaries were not sufficiently clear in our MRI datasets to support reliable manual region-by-region quantification. We agree that systematic regional segmentation would be valuable, and we consider this an important direction for future work to be addressed in subsequent studies.

(5) Whether sex differences were examined or observed in the phenotypes of Pcdh-γΔC4 mice?

Response:

We thank the reviewer for this question. In this study, we included both male and female mice in our analyses, but we did not perform sex-stratified analyses or formally test for sex-dependent differences in the phenotypes. If needed, we will address potential sex effects in future work using appropriately powered sample sizes. And the following statement was added in the Methods section:

"All mouse experiments were performed using samples from both sexes, and data were collected and analyzed without stratification by sex." (Line 613-615).

Minor concerns

(1) The figure description on page 8, line 128, may be inaccurate. The Western blot data for Pcdh-γB2 and Pcdh-γC3 seem to be presented in Figure 1f, not as currently indicated.

Response:

We thank the reviewer for noting this error. We agree that the description on page 8, line 128 (in the original submission manuscript) was inaccurate, and we have corrected the figure description to indicate that the western blot data for Pcdh-γB2 and Pcdh-γC3 are presented in Figure 1f in revised manuscript.

(2) For better clarity and accuracy, it is recommended that the figure description of the superior colliculus on page 13, line 213, be specified as Fig. 2c-(i).

Response:

We thank the reviewer for this helpful suggestion. However, because Figure 2 has been substantially revised from the original submission (including changes to panel organization and labeling), the specific panel reference "Fig. 2c-(i)" no longer exists in the revised manuscript. We have updated the corresponding figure description accordingly in the revised text.

Reviewer #3 (Remarks to the Author):

This manuscript by Higuchi et al. utilizes clever frame-shifting mutations introduced into the Pcdhgc4 gene in mice via CRISPR-Cas9 to create three new lines aiming to add to our understanding of the Pcdhg gene cluster and thus gamma-protocadherin function. In the first, a 2 bp deletion is introduced into the variable cytoplasmic domain (VCD) of the Pcdhgc4 variable exon, such that an early stop codon occurs within the first constant exon (i.e., the 2nd exon of the

complete transcript), potentially leading to truncated proteins, albeit at a low level. In the second line, the authors introduced into these same mice a 1 bp deletion leading to a frameshift that puts the complete C4 transcript back in frame, albeit with a point mutation in C4 and with the other 21 Pcdhg isoforms now truncated within the constant domain, complicating interpretation. In the third line, the authors introduce a frame-shifting (apparently, though see below about ambiguous discussion of this line) mutation of 79 basepairs in the 5' end of the Pcdhgc4 gene, creating a complete null of this isoform. They then utilize these lines to examine postnatal survival, neuronal apoptosis, and cerebellar Purkinje cell dendrite self-crossings. While the approach is novel and clever, unfortunately most of the manuscript's claims are overstated and poorly-supported. Several prior results are not taken into account, many experiments are not quantified at all (it is not even clear if the authors examined multiple mice), some results are puzzling based on extensive prior literature and the discrepancies are unexplained, and conclusions are not supported by the data. The manuscript also contains too many grammatical errors and errors in the writing to be certain what the authors mean to say at times, which makes it difficult to interpret what is meant. Much more data would be needed to support the claims made by the authors.

Response:

We thank the reviewer for the detailed and constructive critique. We have substantially revised the manuscript, added quantitative analyses and additional data where appropriate, and clarified the text throughout. We address each point in detail below.

Detailed concerns:

1. The authors refer to the Pcdh-g deltaC4 (DC4 hereafter) line alternately as C4 deficient or a knockout. It is not a knockout. The line likely does express truncated C4 with its extracellular domain, transmembrane domain, and most of its VCD. There is no reason why this protein might not be made in small amounts and be incorporated into protocadherin lattices at the membrane, as dimerization occurs within EC5 and 6, which could still be expressed. The western in figure 1b is not entirely clear—it looks like there may be faint bands present. Indeed, the fact that 10 of 23 pups survived postnatally indicates that some C4 remains. It was conclusively shown by Garret et al 2019 that a clean knockout of the C4 gene results in complete neonatal lethality paralleling the complete gamma cluster KO, as did deletion of C3/4/5 in the Maniatis lab's work (Chen et al 2012). The authors' own (similarly clean) KO, N delta 79 (N79 hereafter), replicates this. The obvious conclusion is that the DC4 line retains some truncated C4. If so, this complicates interpretation of the results using this mouse line. The authors' explanation of NMD differences is not well supported. This line should not be called a null allele unless it can be proven there is

no truncated C4 protein. It would be helpful to show multiple individual mouse brain western blots from WT and DC4 mice to show whether there is any variability in possible levels of truncated C4 proteins.

Response:

We thank the reviewer for this important comment and agree that the DC4 (γC4nmd hereafter) line should not be described as a clean knockout/null allele. In response, we have revised the manuscript to clarify the molecular nature of this allele and to provide additional experimental evidence demonstrating the presence and reproducibility of a truncated γC4 product.

First, we updated the western blot analysis and revised the text describing Fig. 1b. We now explicitly state that full-length Pcdh-γC4 is absent in γC4nmd, while a lower-molecular-weight species consistent with a CR-deficient truncated Pcdh-γC4 product is detected using extracellular epitope antibodies. Therefore, we added the following sentences in Results section:

"Next, we examined γC4 protein products in γC4nmd by western blotting using antibodies against the γC4 extracellular domain (Fig. 1b). In contrast to wild-type (WT), the full-length Pcdh-γC4 band was absent, but a lower-molecular-weight band consistent with a CR-deficient truncated Pcdh-γC4 product was detected (Fig. 1b, arrow; Supplementary Data Fig. 3a). ", (Line 110-114).

Second, as suggested, we performed western blotting using multiple independent brains to assess reproducibility and potential variability. So, we added Supplementary Figure S3 and the following sentences in Results section:

"As the truncated γC4 protein was reproducibly detected, we quantified band intensities to compare the expression levels of full-length γC4 in WT with those of truncated γC4 in γC4nmd (Supplementary Data Fig. 3b and 3c, n = 6 animals per genotype). This analysis showed that γC4nmd expresses the truncated γC4 protein at approximately 18% of the WT full-length γC4 level (Supplementary Data Fig. 3c, WT = 100 ± 4.49, γC4nmd = 17.9 ± 2.32, p = 0.0022, Mann-Whitney U-test).", (Line 114-120).

2. Similarly, though the creation of the C4only line is very clever indeed, this mouse line is not a clean mutant either. In this line, 21 other gamma protocadherin isoforms are expressed, but truncated within the 1st constant exon. This is supported by Figure 1F. These truncated proteins could absolutely have a deleterious effect on gamma cluster protein function by being inserted into lattices (See papers by Brasch et al., Maniatis lab), but not signaling properly due to lack of

the constant domain (e.g., through FAK/PYK2). It is also not particularly surprising that these mice survive given that Garett et al 2019 already demonstrated that discrete loss of C4 leads to lethality and neuronal apoptosis while the 1R1 line, which has only C4 remaining as a functional isoform, albeit at only 10% of normal protein levels, survives and has only mild apoptosis. A paper by Hanes et al. in 2025 in Genesis (not cited by the authors) also reports a cre-activated C4 transgenic mouse line and shows that turning this transgene on in neurons alone rescues both neuronal survival and postnatal survival entirely. The statement in line 142 that C4 "is not strictly required for postnatal survival" is not correct if truncated C4 remains even at a low level in the DC4 line. The authors could say that "full-length C4 is not strictly required for postnatal survival, at least 40% of the time" (10 of 23 mice survived). The statement in line 160 that the C4 only mouse mutation "successfully restricted expression to Pcdhg-C4 alone" is patently false given the western blot in Figure 1F. 21 other gammas are there but truncated (and the western confirms some are).

Response:

We thank the reviewer for this detailed and important critique. We agree that neither the γC4nmd allele nor the γC4only allele should be described as "clean" in the sense of a simple null/rescue construct, and we have revised the manuscript to more accurately reflect the molecular nature of these alleles and to avoid overstated conclusions.

Revision of the survival statement (original line 142): We agree that the original wording ("γC4 is not strictly required for postnatal survival") was inappropriate given that the γC4nmd allele retains low-level expression of a CR-deficient truncated γC4 product and shows incomplete penetrance of lethality. Therefor we revised the manuscript as follows:

"These findings indicate that neonatal lethality is not fully penetrant when a γC4 variant lacking γ-CR is expressed." (Line 124-125).

"These findings suggest that the Pcdh-γ constant cytoplasmic region (γ-CR) of γC4 is a key determinant of viability: when γC4 lacks the γ-CR, mice show reduced survival rate and display behavioral abnormalities among survivors. By contrast, whereas loss of the CR across the entire 22-member Pcdh-γ cluster is lethal, retaining the CR only in γC4 is sufficient to support postnatal survival." (Line 162-166).

Revision of the "restricted expression" statement (original line 160): We also agree that the phrase "successfully restricted expression to Pcdhg-C4 alone" was misleading because the C4only allele

retains expression of other γ isoforms as CR-truncated products, as supported by our western blot data. We have revised this sentence to precisely state as follows:

"These results confirm that our genome-editing strategy selectively targeted the intended isoforms and preserved full-length γC4 expression at mRNA levels comparable to WT." (Line 145-147).

Prior literature and interpretation of γC4fl-only: Finally, we appreciate the reviewer's suggestion to more explicitly integrate prior work. We have updated the Discussion to incorporate the relevance of the Garrett et al., 2019 1R1 allele and the neuronal γC4 transgenic rescue reported by Hanes et al., 2025, which together support the conclusion that γC4 is a key determinant for survival. Our data are consistent with these findings and additionally provide a newly generated model in which γC4 remains the only CR-intact isoform without relying on transgenic overexpression, enabling analysis of postnatal phenotypes in a distinct genetic context. We added the following statements to the Discussion:

"These results indicate that full-length expression of γC4 alone, under the Pcdh-γ cluster constant region truncation, is sufficient to support organismal viability. Recently, large-scale CRISPR/Cas9 screening has generated additional mouse lines, including the 1R1 line in which only the γC4 exon remains intact, as well as a Cre-dependent γC4 overexpression mouse (CAG-C4-GFP)" (Line 382-386).

3. The authors' interpretation (line 129) of the Weiner et al 2005 paper is not correct. In the Pcdhg tr line, all 22 gammas are truncated in the 3rd constant exon. Truncated proteins are still detected, albeit at a lower level than normal. These mice exhibit reduced synaptic density in the absence of any increase in neuronal apoptosis. The authors mis-state this phenotype (line 130). The phenotype of this tr line does not support their conclusions that only C4 is functional in the Pcdhg C4 only mutant. Clearly, in the tr line, C-terminally truncated Pcdhgs can rescue the neuronal apoptosis—though not neonatal lethality. Thus, the 21 truncated gammas in their C4 only line may be contributing to the rescue as well.

Response:

We thank the reviewer for carefully pointing out our incorrect interpretation of Weiner et al., 2005. We apologize for this mistake and have corrected the relevant description of the Pcdh-γ^{tr} phenotype in the revised manuscript. Accordingly, we updated both the Results and Discussion sections, as detailed below.

In result section:

"Since previous studies reported that mice in which all Pcdh-γ proteins are C-terminally truncated exhibit extensive neonatal lethality, we conclude that expression of full-length Pcdh-γC4 alone, among the 22 Pcdh-γ isoforms, is sufficient to support stable postnatal survival in the C4fl-only mutant.", (Lines 149-153).

In discussion section:

"Next, using a series of CRISPR/Cas9-based genetic manipulations, including a newly developed strategy named DOMINO (DOuble Mutation INducing Open reading frame switch), we generated γC4fl-only allele, which only γC4 full-length and 21 other truncated Pcdh-γ (Fig. 1a, Supplementary Data Fig. 4). These mutants were viable and fertile, unlike Pcdh-γ cluster knockout mice and all Pcdh-γ truncated mice. A hypomorphic, C-terminally truncated allele of Pcdh-γ did not show abnormal cell death but died within several hours of birth. Our γC4fl-only allele also reduced embryonic apoptosis to levels comparable to control mice, and the animals survived stably into adulthood (Fig. 2 and Fig. 3). These results indicate that full-length expression of γC4 alone, within the Pcdhγ cluster, is sufficient to support organismal viability.", (Lines 374-382).

4. The way N79 is discussed is very confusing. In line 170 the authors say it is an “in-frame deletion of a portion of EC1”. But the Extended Data Figure 4 clearly shows a stop codon is introduced, so it is NOT in frame. Also, the authors interpret extended data figure 4F to say that C4 protein is “hypomorphic”, but this makes no sense if the transcript coding frame is disrupted at the very 5’ end of the gene. No protein of any significant size could be made. Why do the authors claim that DC4, which is likely NOT a complete KO, is one, while they claim N79, which should be a clean KO, is not but is, rather, hypomorphic? That doesn’t fit with the survival or other data. This confusion is very concerning.

Response:

We apologize for the confusing and inaccurate description of the NΔ79 allele in the original manuscript. We agree with the reviewer that NΔ79 is a clean frameshift knockout allele, and our previous description of the NΔ79 allele as an “in-frame” or “hypomorphic” mutation was incorrect. Consistent with this interpretation, the phenotype of NΔ79 homozygotes matches that reported for the Pcdh-γC4 knockout allele described by Garrett et al., including fully neonatal lethality. Accordingly, we have revised the text throughout to remove the incorrect “in-frame” and “hypomorphic” descriptions and to clearly define NΔ79 as a γC4 knockout allele.

"To examine how the position of coding-sequence disruption influences γC4 transcript fate and organismal outcome, we compared two independently generated alleles: the γC4nmd allele and a signal peptide–targeted frameshift allele (γC4NΔ79) (Supplementary Data Fig. 6). The γC4NΔ79 allele was generated by CRISPR/Cas9-mediated deletion of 79 bp near the 5' end of the coding sequence, within the region encoding the N-terminal signal peptide, resulting in a frameshift and premature termination at EC1 domain (Supplementary Data Fig. 6a and 6b). Consistent with a severe loss-of-function mutation, γC4NΔ79 homozygous pups were not viable and exhibited abnormal gross appearance at birth (Supplementary Data Fig. 6c; Supplementary Data Video 4), in agreement with an independently generated γC4 frameshift knockout allele reported previously (13 bp frame-shifting deletion 3' to the start codon), which also shows fully neonatal lethality.", (Lines 173-183).

5. Figure 2 is unconvincing. One section of one brain is shown, with no quantification. One can conclude nothing from this. The authors would have to quantify many sections from each region, from at least 3-4 embryos per genotype, and use the appropriate statistical test to show any differences in neuronal apoptosis patterns or levels. This figure can't be used as is.

Response:

We thank the reviewer for this important critique and agree that the original version of this figure was insufficient, as it relied on limited representative images without quantitative support. To address this concern, we have substantially expanded the dataset and added rigorous quantification in the revised manuscript. We updated the Figure 2 and the following statements:

"To assess how isoform-specific disruption of γC4 affects neuronal survival in the embryonic brain, we performed cleaved caspase-3 (CC3) immunostaining on E18.5 embryos from WT, γΔCR1 (22 Pcdh-γ knockout), γC4nmd, and γC4fl-only genotypes (Fig. 2 and Fig. 3). In sagittal sections (Fig. 2a–a‴; DAPI/CC3), WT embryos showed sparse CC3 signal, whereas γΔCR1 embryos exhibited prominent apoptosis distributed across broad brain regions. In the γC4nmd embryos, apoptosis was also increased compared to WT but appeared less extensive than in γΔCR1, and the distribution was comparatively more restricted. In contrast, γC4fl-only embryos showed minimal CC3 signal, comparable to WT in sagittal sections. We then analyzed coronal sections matched to the sagittal levels in Fig. 2a and co-stained for GAD67 and CC3 (Fig. 2b–e). In γΔCR1, robust CC3 signal was observed in multiple coronal planes, and apoptotic cells were enriched within GAD67-positive regions, including the reticular formation/midbrain, superior colliculus, periaqueductal gray, and medulla (Fig. 2b′–e′). γC4nmd showed CC3-positive apoptosis in similar anatomical regions, but the overall intensity and extent were clearly milder

than in γΔCR1 (Fig. 2b″–e″). This result indicates that expression of γC4 lacking the γ-CR, accompanied with reduced γC4 expression levels, is sufficient to induce neuronal apoptosis. However, because the extent of cell death is modest, the neuronal apoptosis triggered by Pcdh-γ deficiency may depends on the number of isoforms disrupted. By contrast, γC4fl-only embryos showed minimal CC3 signal across these coronal sections (Fig. 2b‴–e‴), consistent with suppression of embryonic apoptosis when full-length Pcdh-γC4 is the only full-length Pcdh-γ isoform, with the other 21 isoforms truncated." (Line 206-228).

Moreover, we analyzed E18.5 embryos from multiple independent litters and quantification of extent of cell death using ImageJ. The quantification protocol was adapted from Kobayashi et al., 2023 iScience, and statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test (also see the revised Methods section). In the revised manuscript, we added a new Figure 3 showing the images used for quantification and the corresponding graphs, and we have included the following text in the Results section:

"We next quantified apoptosis across anatomically defined regions, cortical amygdala (CoA), zona incerta (ZI), reticular formation (RF), superior colliculus (SC), periaqueductal gray (PAG), and gigantocellular nucleus (Gi) (Fig. 3a). We visualized the extent of cell death using two metrics, CC3-positive cell density (cells/mm²) and CC3-positive area (Fig. 3b). Quantification was performed using multiple embryos (WT n = 4, γΔCR1 n = 3, γC4nmd n = 6, and γC4fl-only n = 5), with 2–3 sections per region per embryo and then one-way ANOVA with Tukey's multiple-comparisons test. γΔCR1 embryos showed robust increases in CC3 across all analyzed regions. γC4nmd embryos also exhibited significant increases, but the magnitude and distribution differed from γΔCR1, indicating that loss of full-length Pcdh-γC4 (with residual CR-deficient truncated Pcdh-γC4) does not phenocopy complete loss of all Pcdh-γ isoforms. Importantly, γC4fl-only embryos showed CC3 levels near WT across regions, consistent with Pcdh-γC4 being sufficient to suppress the elevated embryonic apoptosis observed in the pan–Pcdh-γ knockout background." (Line 229-242).

6. Similarly, Figure 3 is not convincing. One western blot of one sample of each genotype is shown. Again, one must analyze several independent samples, often up to 6-8 per genotype given the difficulties of reliably detecting phosphorylated proteins in Western blots. Quantification numbers are shown beneath lanes, but there is no mention of N or statistical tests. Is that quantification of one band from one sample? If so, no conclusions can be drawn. Also, the result is hard to understand. Why would FAK phosphorylation go down but PYK2 go up? In prior work, such as Chen et al 2012 and Suo et al 2012 these proteins follow the same pattern. What is the

explanation for why they would be different here? The result also does not make sense in that, in the DC4 line, the other 21 protocadherins are expressed, and thus their constant domains—which is the portion of the gamma proteins that binds to FAK and PYK2—could inhibit FAK and PYK2 phosphorylation. How would the authors explain a mechanism by which C4—which has the same constant domain as the other 21 gamma protocadherins---could "uniquely" interact with and regulate FAK and PYK2? It is not likely to be a unique conformational issue with C4 as prior papers using a variety of gamma and alpha Pcdh constructs (other isoforms) showed inhibition of these proteins.

Response:

We thank the reviewer for this important critique. We agree that the original version of this figure did not provide sufficient biological replication, did not clearly report the sample size (N), and did not include appropriate statistical analyses. So, we updated the figure (Figure 4 in revised manuscript) and added the following statements:

"We performed western blotting using P0 brain lysates from WT, γΔCR1, γC4nmd, and γC4fl-only mice (Fig. 4a), and quantified phosphorylation as pY397-FAK/total FAK and pY402-PYK2/total PYK2 (n = 6 biological samples per genotype; one-way ANOVA with Tukey's multiple-comparisons test). Total protein levels of FAK and PYK2 were comparable across genotypes (Fig. 4a). In contrast, FAK phosphorylation was significantly increased in γΔCR1 and γC4fl-only compared with WT (Fig. 4b), whereas γC4nmd did not show a significant change relative to WT. This pattern is consistent with prior reports that the γ-protocadherin constant region (CR) negatively regulates FAK phosphorylation; notably, in the γC4fl-only line the Pcdh-γ cluster other than Pcdh-γC4 isoform lack the CR, providing a plausible explanation for elevated pFAK in this genotype. By contrast, we observed no significant differences in PYK2 phosphorylation among genotypes (Fig. 4c), providing no evidence for altered PYK2 phosphorylation under these conditions." (Line 252-264).

As described above, because we observed differences in FAK and PYK2 phosphorylation in the mutant mice, we updated the Discussion section accordingly to reflect these revised findings.

"Furthermore, in relation to the embryonic apoptosis phenotypes, we assessed the phosphorylation levels of FAK and PYK2, kinases implicated in downstream signaling regulated by Pcdh-γ, by western blotting of P2 soluble fractions prepared from neonatal brain lysates. pY397-FAK/FAK was elevated in γΔCR1 and γC4fl-only, whereas pY402-PYK2/PYK2 did not differ across genotypes. In light of models in which Pcdh-γ restrain FAK activation and

downstream PLC/PKC–MARCKS signaling1,2, the elevated pFAK in γC4fl-only may reflect the widespread loss of CR across 21 Pcdh-γ isoforms, thereby weakening cluster-level inhibition of FAK. Recent evidence that PKC phosphorylation of a shared Pcdh-γ C-terminal motif can negatively regulate Pcdh-γ function further raises the possibility of feedback between CR loss–driven changes in FAK/PKC activity and Pcdh-γ regulation. Although protocadherin cytoplasmic domains can inhibit both FAK and PYK2, the absence of PYK2 differences in our bulk P0 dataset may reflect averaging across regions/cell types and developmental context dependence." (Line 405-417).

7. There are N's given for the Purkinje cell analysis, and statistics are done. But the test used is not indicated. This is necessary to determine if the correct tests were used. The differences in arbor size and self-crossings, while significant (albeit without the test being stated), are rather small. From 4 crossings per area in WT to 6 in the DC4 line to 5 in the C4 only line. It would be helpful to compare this to a complete gamma protocadherin null as in the lab's prior papers to determine whether this is a mild phenotype or a severe one. It seems rather mild, which undercuts the claim that "only gC4 is indispensable isoform on dendritic self-avoidance of Purkinje cells". Such a result would also contradict with prior data from Lefebvre et al., with little explanation other than the fact that the mouse lines are not clean mutations but have the complications mentioned above.

Response:

We thank the reviewer for this important comment. We agree that the statistical test used must be clearly stated. We have now explicitly indicated the statistical analyses in Results and Methods section.

In Results section:

"The Purkinje cell morphology and self-crossing quantifications were performed using one-way ANOVA followed by Tukey's multiple-comparisons test." (Line 278-280).

In Methods section:

"Means of multiple samples were compared using one-way ANOVA followed by Tukey's multiple-comparisons test for the Purkinje cell morphology and self-crossing analyses." (Line 772-774)

Regarding the magnitude of the phenotype and comparison to prior work, we note that our Purkinje cell imaging and quantification approach differs substantially from that used by Lefebvre

et al., and thus the values are not directly comparable. In Lefebvre et al., 2012, self-crossings were counted in a 7,225 μm^2 region of interest (ROI) within a single confocal plane, and arbor areas were measured using 2D image-based procedures (convex-hull area measurements in Fiji). In contrast, we cleared cerebellar tissue with SeeDB2 and performed 3D reconstruction and tracing of individual Purkinje cells in Imaris, quantifying morphology and self-crossings across the entire dendritic arbor rather than within a 2D ROI. Because of these methodological differences (2D ROI-based versus 3D whole-cell quantification), we do not consider it appropriate to make a strict quantitative comparison between our values and those reported by Lefebvre et al. We agree that a direct cross-study comparison would require applying the same quantification strategy, which could be addressed in future work if needed.

The goal of our analyses in Figures 5 and 6 (Figure 4 and 5 in original submission) was to determine whether and to what extent γC4 only truncation (γC4nmd) or full-length expression (γC4fl-only) alters Purkinje cell dendritic morphology and self-crossings relative to appropriate controls under our imaging/analysis procedure (described in above). In light of the reviewer's comments and the modest effect sizes, we apologize and agree that our original wording overstated the conclusion. We therefore revised the manuscript in Result and Discussion section for Purkinje cell self-avoidance.

We updated the statement in Results section as follows:

"Compared with WT (3.9 ± 0.37), dendritic self-crossings per unit area increased to 6.2 ± 0.62 in γC4nmd ($p = 0.0091$). In contrast, γC4fl-only showed 4.9 ± 0.74 self-crossings per unit area, which was not significantly different from WT ($p = 0.4200$), consistent with preserved Purkinje cell morphology. Together, these results indicate that full-length expression of full-length γC4, including the Pcdh-γ constant region, is required within the 22-member Pcdh-γ cluster to regulate Purkinje cell dendritic architecture, whereas the constant regions of the other Pcdh-γ isoforms are not strictly necessary for this aspect of dendritic morphogenesis." (Line 284-291).

To reflect the results described above, we revised the Discussion section accordingly, as shown below:

"Both global and Purkinje cell–specific truncation of γC4 led to excessive dendritic self-crossing. On the other hand, Purkinje cells which only full-length γC4 expression under the other 21 Pcdh-γ disrupt γ-CR maintained a typical dendritic architecture." (Line 420-422).

"These observations refine prior models by suggesting that, in Purkinje cells, full-length γC4 may provide a key CR-dependent function required for dendritic self-avoidance, whereas the common regions of other Pcdh-γ isoforms are not strictly required for this aspect of dendritic morphogenesis." (Line 424-428).

8. The experiment in figure 6 is nice, but has a major flaw that prevents the authors' conclusion that C4, but not B2, can rescue the phenotype: nowhere do the authors confirm that the B2 transgene is expressed at the protein level. Without this crucial characterization, no conclusion can be drawn.

Response:

We thank the reviewer for highlighting this critical issue. We agree that our original conclusion—that γC4, but not γB2, can rescue the phenotype—requires confirmation that the γB2 transgene is expressed at the protein level. In our current experiments, we were unable to detect γB2 protein expression, despite PA-tag fusion at C-terminal of AAV-γB2 construction, and therefore we cannot support this comparative claim. Accordingly, we have removed the γB2 rescue data from the revised manuscript.

9. Characterization of prior literature is not complete. In addition to the missed references and misstatements noted above, some statements in the discussion are unfair. In line 305, 306, the following sentence is not a correct characterization: "While previous studies have suggested that Pcdh-gC4 is essential sole isoform for mouse survival (25—Garrett et al., 2019), our results contradict this notion that Pcdh-gC4 is required for survival". First, that 2019 paper did not "suggest" this—it conclusively showed it by multiple clean mutations in mice *in vivo*. Second, the authors' results do not contradict it, because as noted the DC4 mutation is not a clean, confirmed null, and because their N79 mutant perfectly matches the similarly clean mutant reported in the Garrett 2019 paper. Again, the 1R1 line reported in that 2019 paper is never mentioned, even though it is relevant to the authors' experiments, nor are the results of Hanes et al 2025 (which is not cited).

Response:

We thank the reviewer for this important comment and agree that our characterization of the prior literature in the original manuscript was incomplete and, in parts, inappropriate. We have therefore revised the relevant Discussion text to more accurately reflect the conclusions of Garrett et al., 2019, and we removed the incorrect implication that our results "contradict" that work. As detailed in our responses above, we now explicitly state that DC4 (γC4nmd in revised manuscript)

is not a clean null allele, and that our NΔ79 allele behaves as a clean γC4 knockout whose phenotype is consistent with the γC4-null alleles reported by Garrett et al., 2019. Therefore, we revised and added the following sentences in Discussion section:

"In our new finding, γC4nmd does not represent a clean γC4 null allele. Rather, full-length γC4 is lost, while a CR-deficient truncated γC4 protein is reproducibly detected at approximately 18% of the WT full-length γC4 level (Fig. 1b; Supplementary Data Fig. 3). We therefore interpret the postnatal neurological phenotypes in γC4nmd mice as consequences of reduced and altered γC4 function in the setting of residual truncated γC4 expression, which may more closely approximate partial loss-of-function (hypomorphic) conditions than complete gene ablation. Consistent with this interpretation, clean γC4 protein-null alleles result in fully neonatal lethality, including the previously reported γC4 knockout allele and our independent γC4NΔ79 allele (Supplementary Data Fig. 6). By contrast, γC4nmd mutants show incomplete penetrance of lethality, with ~40% surviving postnatally. γC4NΔ79 retains ~50% of γC4 mRNA relative to WT, whereas γC4nmd mutants express only ~10% (Supplementary Fig. 6e). Yet, immunoblotting of brain lysates revealed that the ~120 kDa band detected in WT was absent in γC4NΔ79, and the corresponding band was also lost in γC4nmd (Fig. 1b; Supplementary Data Fig. 6f). Notably, γC4nmd consistently showed an additional weak band at ~100 kDa. Based on its reproducibility, the allele structure, and the apparent molecular weight, this band likely represents a truncated γC4 product lacking the Pcdh-γ constant region (Supplementary Data Fig. 1b and 1c; Supplementary Data Fig. 3). We speculate that the presence of this residual truncated γC4 protein in γC4nmd, but not in the protein-null γC4NΔ79 line, may underlie the difference between incomplete versus complete neonatal lethality.", (Line 340-360).

In addition, we have strengthened our discussion of the C4only interpretation by incorporating the relevant prior work, including the 1R1 allele described in Garrett et al., 2019 and the new γC4 transgenic mice reported by Hanes et al., 2025. Accordingly, we updated and added the following statements to the Discussion section:

"Recently, large-scale CRISPR/Cas9 screening has generated additional mouse lines, including the 1R1 line in which only the γC4 exon remains intact, as well as a Cre-dependent γC4 overexpression mouse (CAG-C4-GFP). These models likewise rescue the apoptosis caused by loss of γC4. However, because both 1R1 and CAG-C4-GFP alter γC4 expression levels, they may not reflect the native physiological state. In contrast, γC4fl-only shows no significant differences in γC4 mRNA or protein levels compared with WT, minimizing confounds due to expression-

level changes and providing a complementary platform to interrogate γC4 function from a distinct perspective (Fig. 1f, Supplementary Fig. 5)." (Line 384-392).

10. Though I completely understand that English may not be the authors' first language, the paper is simply too confusingly written and there are too many grammatical mistakes (nearly every line, too many to note here in detail). As noted regarding the explanation of the N79 line, this can result even in the nature of the experiment being confusing or differently described in different parts of the manuscript. A thorough editing for English would be required for the manuscript to be clear.

Response:

We sincerely apologize to the reviewer for the lack of clarity and the numerous grammatical errors in the original submission. We fully agree that the writing issues could obscure the intended meaning and even lead to inconsistencies in how experiments are described (including the N79 line). To address this concern, we have thoroughly revised the manuscript for English language and overall clarity, carefully harmonizing terminology and experimental descriptions across all sections. In addition, the revised text has been edited by a professional English editing service. We believe these changes have substantially improved the readability and precision of the manuscript.

Minor issue:

• Why is "variable cytoplasmic domain" abbreviated as "VCP"? what is the "P"? Every other paper on these proteins including some of the authors' own use "VCD". This should be used throughout in all the figures and text to avoid confusion in the field.

Response:

We thank the reviewer for pointing out the potential confusion regarding our abbreviation. In our original nomenclature, we referred to the common cytoplasmic region as "CP" and therefore denoted the variable portion as "VCP." However, we agree that the term "VCD" (variable cytoplasmic domain) is more widely used in the clustered protocadherin literature and should be adopted to maintain consistency and avoid confusion in the field. Accordingly, we have replaced "VCP" with "VCD" throughout the revised manuscript and all figures.