Neuroscience

# Combinatorial expression of gamma-protocadherins regulates synaptic connectivity in the mouse neocortex

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## Abstract

In the process of synaptic formation, neurons must not only adhere to specific principles when selecting synaptic partners but also possess mechanisms to avoid undesirable connections. Yet, the strategies employed to prevent unwarranted associations have remained largely unknown. In our study, we have identified the pivotal role of combinatorial clustered protocadherin gamma (γ-PCDH) expression in orchestrating synaptic connectivity in the mouse neocortex. Through 5-prime end single-cell sequencing, we unveiled the intricate combinatorial expression patterns of γ-PCDH variable isoforms within neocortical neurons. Furthermore, our whole-cell patch-clamp recordings demonstrated that as the similarity in this combinatorial pattern among neurons increased, their synaptic connectivity decreased. Our findings elucidate a sophisticated molecular mechanism governing the construction of neural networks in the mouse neocortex.

**eLife assessment**

The authors used an innovative modified 10X genomic sequencing method to detect cPCDHg is-forms in pyramidal neurons. With **solid** electrophysiological recordings, they showed that neurons expressing the same sets of cPCDHg isoforms are less likely to form synapses with each other. These **valuable** findings confirms previous results and extend our understanding of cPCDHg diversity and neuronal connectivity.

## Introduction

The precision of synaptic connections is vital for the functioning of neural circuits(Yogev and Shen, 2014). Cell adhesion molecules play crucial roles in the specificity of synapse formation(Duan et al., 2014, Serizawa et al., 2006, Tan et al., 2015, Rawson et al., 2017, Sytnyk et al., 2017, Berns et al., 2018, Jang et al., 2017, Courgeon and Desplan, 2019). However, how to achieve such specificity at the microcircuit level remains an open question. The unique expression pattern of clustered protocadherins (cPCDH) leads to millions of possible

combinations of cPCDH isoforms on the neuron surface(Kaneko et al., 2006, Esumi et al., 2005), effectively serving as a distinctive barcode for each neuron(Yagi, 2012). Notably, the absence of γ-PCDH does not induce general abnormalities in the development of the cerebral cortex, including cell differentiation, migration, and survival(Wang et al., 2002, Garrett et al., 2012). However, γ-PCDH presence has been detected at synaptic contacts(Fernandez-Monreal et al., 2009, Phillips et al., 2003, LaMassa et al., 2021), and its absence has substantial effects on neuronal connectivity(Tarusawa et al., 2016, Kostadinov and Sanes, 2015, Lv et al., 2022). While the homophilic properties of γ-PCDH promote dendritic complexity(Molumby et al., 2016), emerging evidence suggests that it might hinder synapse formation. Previous studies indicate that homophilic interactions, facilitated by large overlapping patterns of cPCDH isoforms on opposing cell surfaces, may lead to intercellular repulsion(Rubinstein et al., 2015, Brasch et al., 2019, Honig and Shapiro, 2020, Lefebvre et al., 2012). Consistent with the repulsion concept, the absence of γ-PCDH results in significantly more dendritic spines and inhibitory synapse densities in neocortical neurons(Molumby et al., 2017, Steffen et al., 2021). Paralleling this, neurons overexpressing one of the γ-PCDH isoforms exhibit significantly fewer dendritic spines(Molumby et al., 2017). Furthermore, the absence of the clustered PCDH augments local reciprocal neural connection between lineage-related neurons in the neocortex(Tarusawa et al., 2016, Lv et al., 2022), even when sister cells exhibit more similar expression patterns of γ-PCDH isoforms(Lv et al., 2022).

Intriguingly, while γ-PCDH appears to have “contradictory” effects on dendritic complexity and dendritic spines, it negatively influences synapse formation in the forebrain. It’s important to note that each neuron expresses multiple isoforms of γ-PCDH(Kaneko et al., 2006, Lv et al., 2022). What is the impact of this combinatorial expression on synapse formation? In this study, using 5-prime end (5’-end) single-cell sequencing, we revealed the diversified combinatorial expression of γ-PCDH isoforms in neocortical neurons. Through multiple whole-cell patch-clamp recordings after the sequential *in utero* electroporation, we discovered that the combinatorial expression of γ-PCDHs empowers neurons to decide which partners to refrain from forming synapses with, rather than merely determining which ones to engage in synaptogenesis with.

# Results

## The diversified combinatorial expression pattern of γ-PCDHs in neocortical neurons revealed by 5’-end single-cell sequencing

The gamma isoform of cPCDHs (γ-PCDHs) is critical for synaptic connectivity(Kostadinov and Sanes, 2015, Tarusawa et al., 2016, Lv et al., 2022). To determine the role of γ-PCDH in the neocortex, we examined their expression in the neocortical neurons of postnatal mice. Existing research has suggested that clustered protocadherins are expressed stochastically in Purkinje cells and olfactory sensory neurons(Hirayama et al., 2012, Toyoda et al., 2014, Mountoufaris et al., 2017). In this study, we harnessed the power of 5’-end single-cell RNA sequencing to precisely identify γ-PCDH isoforms by focusing on their variable exon, exon 1, where they differ from each other(Kohmura et al., 1998, Wu and Maniatis, 1999). Given that the second postnatal week is the critical stage for synapse formation in the rodent neocortex(Lendvai et al., 2000, Holtmaat and Svoboda, 2009), we chose postnatal day 11 (P11) as the time point for our examination. Following reverse transcription and cDNA amplification (**Fig. 1**-S1A, B), we divided the cDNA into two segments: one designed for the specific amplification of *Pcdhg* mRNAs and the other for the construction of a 5’ gene expression library (**Fig. 1**-S1C). After the cluster analysis (**Fig. 1**-S2-4), we collected 6505 neurons from an initial pool of 17438 cells (**Fig. 1A** and **Fig. 1**-S1D). For in-depth analysis, we focused on neurons expressing more than 10 unique molecular identifiers (UMI) for all γ-PCDH isoforms (cutoff >1 for each type of individual isoform) (**Fig. 1B, C**). We observed the near-ubiquitous expression of “C-type” isoforms, specifically C3, C4, and C5

(**Fig. 1D**). It's important to note that the fraction of cells expressing "C-type" isoforms was significantly higher when compared to "variable" isoforms (**Fig. 1D** and **Fig. 1**-S1E), which is consistent with findings from a previous study(Toyoda et al., 2014).

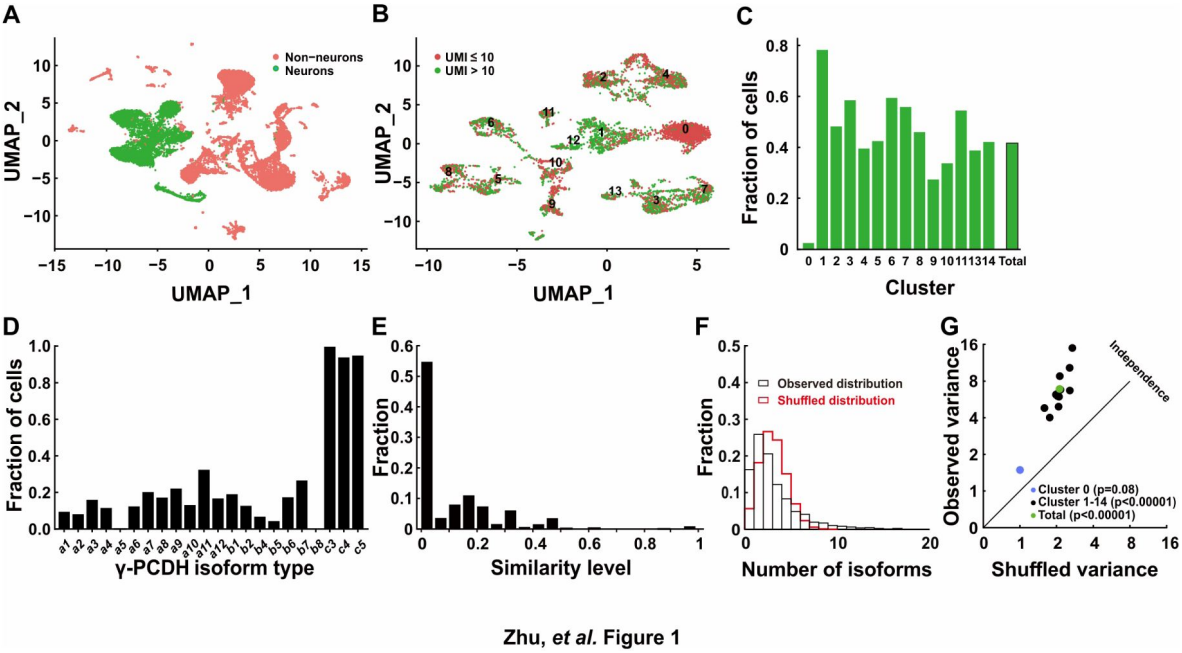
We proceeded to conduct a pairwise analysis to assess the similarity of "variable" isoforms among neurons regarding the single-cell expression pattern of γ-PCDHs. This extensive analysis revealed that the majority of neocortical neurons from all clusters exhibited very low similarity level (**Fig. 1E**, **Fig. 1**-S5 and 1-S6). This finding strongly suggests distinct combinatorial expression patterns among these neurons. Given that all our electrophysiological recordings were carried out on pyramidal neurons in the layer 2/3 of the neocortex, we conducted more detailed analysis, including the examination of detailed expression of γ-PCDHs in individual neurons, specifically in the corresponding cluster 7. All these analyses consistently revealed a diverse range of combinatorial expression patterns among neurons in this cluster, a phenomenon in alignment with the general population of neocortical neurons (**Fig. 1**-S5).

To further characterize this diversity, we employed variance analysis as per Wada et al.(Wada et al., 2018), to examine the distribution of the number of expressed isoforms per cell across all neurons (**Fig. 1F** and **Fig. 1**-S7). As per Wada's definition of 'co-occurrence' (Wada et al., 2018), this primarily indicates potential interactions among different isoform expressions at the population level. This analysis uncovered a subtle but significant co-occurrence of γ-PCDH isoform expression in most neurons (**Fig. 1G**). Notably, we did not detect any discernible differences among clusters, except for cluster 0, which displayed considerably lower expression (**Fig. 1C**) and no co-occurrence of γ-PCDH isoforms (**Fig. 1G** and **Fig. 1**-S7). In summary, the data derived from 5'-end single-cell sequencing underscore the diverse and complex combinatorial expression of γ-PCDHs within the majority of neocortical neurons.

## The absence of γ-PCDHs increases local synaptic connectivity among pyramidal neurons in the neocortex

To delve into the function of γ-PCDH in the synaptic formation among neocortical neurons, we conducted paired recordings on pyramidal neurons in the layer 2/3 of the neocortex from *Pcdhg* conditional knockout (cKO) mice. These genetically engineered mice were created by crossing *Pcdhg* $^{flox/flox}$ mice(Lefebvre et al., 2008, Prasad et al., 2008) with *Nex-cre* mice(Goebbels et al., 2006). This genetic combination specifically removed all variable and C-type γ-PCDH isoforms in pyramidal neurons. Our experimental setup involved multiple whole-cell patch-clamp recordings from cortical slices harvested from P9-32 mice, allowing us to measure the connectivity among nearby pyramidal cells (<200 μm between cell somas) in the layer 2/3 of the neocortex by assessing the presence of evoked monosynaptic responses (**Fig. 2** and **Fig. 2**-S1).

In the sample traces featured in the connectivity matrix obtained from six recorded neurons (**Fig. 2A** and **Fig. 2**-S1), we observed two neuronal pairs (neuronal pairs 4→3 and 5→6) exhibiting unidirectional monosynaptic connections (indicated by orange arrows), and one pair (neuronal pair 1↔3) displaying bidirectional connections (indicated by green arrows) out of 15 possible pairings. Overall, our analysis revealed that the percentage of connected pairs was notably higher in *Pcdhg* cKO mice (20.2%, 103/511) than in wild-type (WT) mice (15.0%, 122/813) (**Fig. 2B**). In light of the distinct roles that vertical vs. horizontal axes might play in synaptic organization within the neocortex(Douglas and Martin, 2004), we conducted an additional set of recordings on P10-20 mice, segregating neuron pairs along these axes. In *Pcdhg* cKO mice, we observed a more significant difference in connectivity for vertically aligned cells (18.3%, 94/515 in *Pcdhg* cKO mice vs. 11.2%, 73/651 in WT mice for vertically aligned neuron pairs; 12.2%, 27/221 in *Pcdhg* cKO mice vs. 9.5%, 28/294 in WT mice for horizontally aligned neuron pairs) (**Fig. 2C**). We also created *Pcdha* cKO mice (**Fig. 2**-S2-4) and carried out similar experiments focused on vertically aligned neurons. In these mice lacking α-PCDH, we didn't observe a significant difference (11.3%, 38/337 in *Pcdha* cKO mice vs. 14.3%, 26/182 in WT mice, **Fig. 2D**).

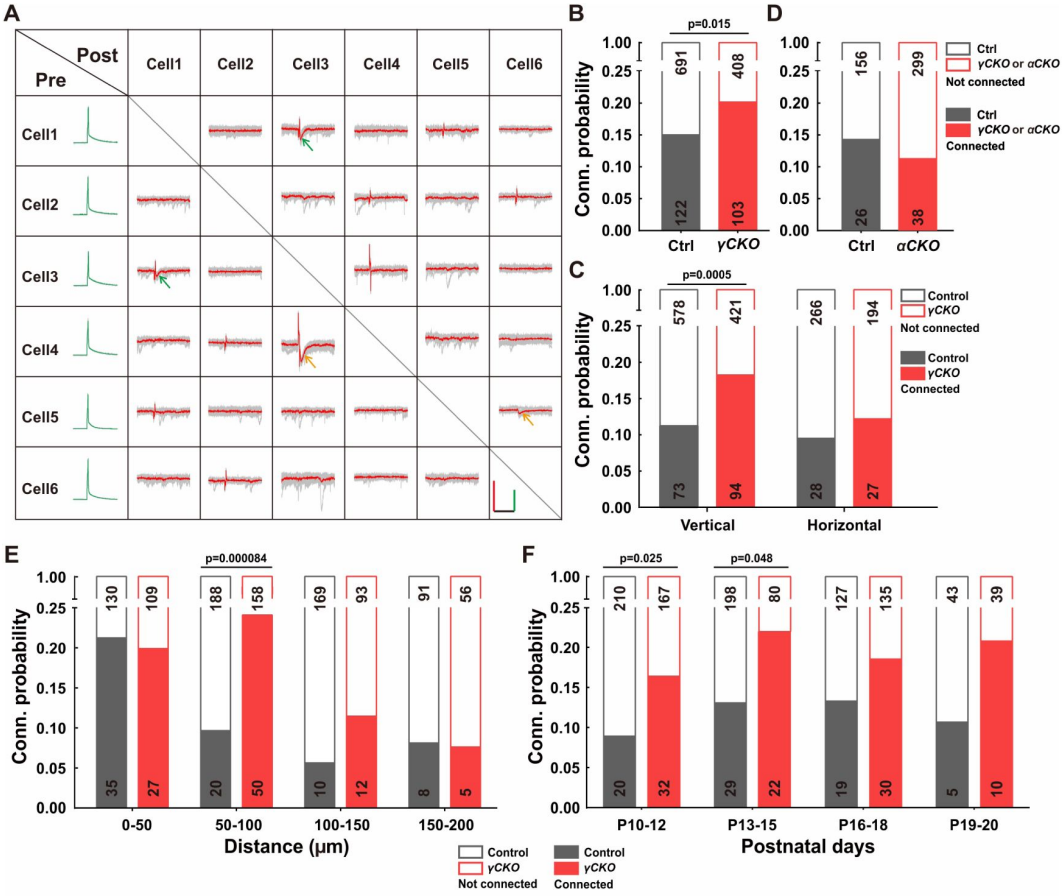



Zhu, *et al.* Figure 1

## Figure 1

### Diversified expression of *Pcdhg* isoforms in neocortical neurons.

(**A**) UMAP analysis displaying 17,438 cells obtained through 5′-end single-cell sequencing after data cleanup and doublet removal. Neurons are depicted as green dots, while non-neuronal cells are marked in red. (**B**) UMAP clusters of all neurons categorized by the UMI cutoff. Red dots denote cells with fewer than 10 total UMIs of *Pcdhg* (n=3,671), and green dots denote cells with more than 10 UMIs (n=2,834). (**C**) Fractions of neurons with more than 10 UMIs in different clusters. (**D**) Distribution of neurons expressing different *Pcdhg* isoforms in the neocortex. (**E**) Fraction distribution illustrating similarity levels in the combinatorial expression of *Pcdhg* variable isoforms among neurons. Similarity levels were calculated as $^{AA\cap BB}$. (**F**) Observed distribution (black) of the fraction of cells expressing varying numbers of isoforms across all neurons. The red curve represents the shuffled distribution generated under the hypothesis of stochastic isoform expression. (**G**) The variance difference between the observed and shuffled fraction distribution of cells from all clusters.




Zhu *et al.*, Figure 2

## Figure 2

### Increased Synaptic Connectivity in the Absence of γ-PCDH

(**A**) Representative traces (red/green) from multiple electrode whole-cell patch-clamp recordings conducted on six neurons in layer 2/3 of the barrel cortex. Average traces are shown in red and green, with ten original traces in gray. Positive evoked postsynaptic responses are indicated by arrows. Orange and green arrows denote unidirectional and bidirectional synaptic connections, respectively. Scale bars: 100 mV (green), 50 pA (red), and 50 ms (black). (**B, D**) Connectivity probability among nearby pyramidal cells in the layer 2/3 of the barrel cortex in *Pcdhg* cKO (**B**), *Pcdha* cKO mice (**D**) and their littermate WT controls. γCKO: *Pcdhg flox/flox*::*nex-cre* mice; αCKO: *Pcdha flox/flox*::*nex-cre* mice; Ctrl: *Pcdhg*+/+::*nex-cre* or *Pcdha*+/+::*nex-cre* mice. (**C**) Connectivity probability among vertically or horizontally aligned neurons in the layer 2/3 of the barrel cortex in *Pcdhg* cKO mice and their littermate WT controls. (**E**) Connectivity probability among vertically aligned pyramidal cells as a function of the distance between recorded pairs in *Pcdhg* cKO mice and WT mice. (**F**) Developmental profiling of connectivity probability among vertically aligned neurons in *Pcdhg* cKO mice and WT mice. Chi-square tests were used in **B**-**F** to calculate statistical differences.

A more detailed analysis of synaptic connections among vertically aligned neurons in *Pcdhg* cKO mice unveiled that the absence of γ-PCDH expression significantly increased synapse formation between cells separated vertically by 50 to 100 μm (24.0%, 50/208 in *Pcdhg* cKO mice *vs.* 9.6%, 20/208 in WT mice) (**Fig. 2E**). Notably, this increased synapse formation was detected starting from P10 (16.1%, 32/199 in *Pcdhg* cKO mice *vs.* 8.7%, 20/230 in WT mice for P10-12; 21.6%, 22/102 in *Pcdhg* cKO mice *vs.* 12.8%, 29/227 in WT mice for P13-15). These time points correspond to the period when chemical synapses between cortical neurons become detectable, and this trend persisted throughout our measurements, spanning up to P20 (**Fig. 2F**). Hence, our findings suggest that γ-PCDHs may play a role in preventing synapse formation from the early stages of neural development.

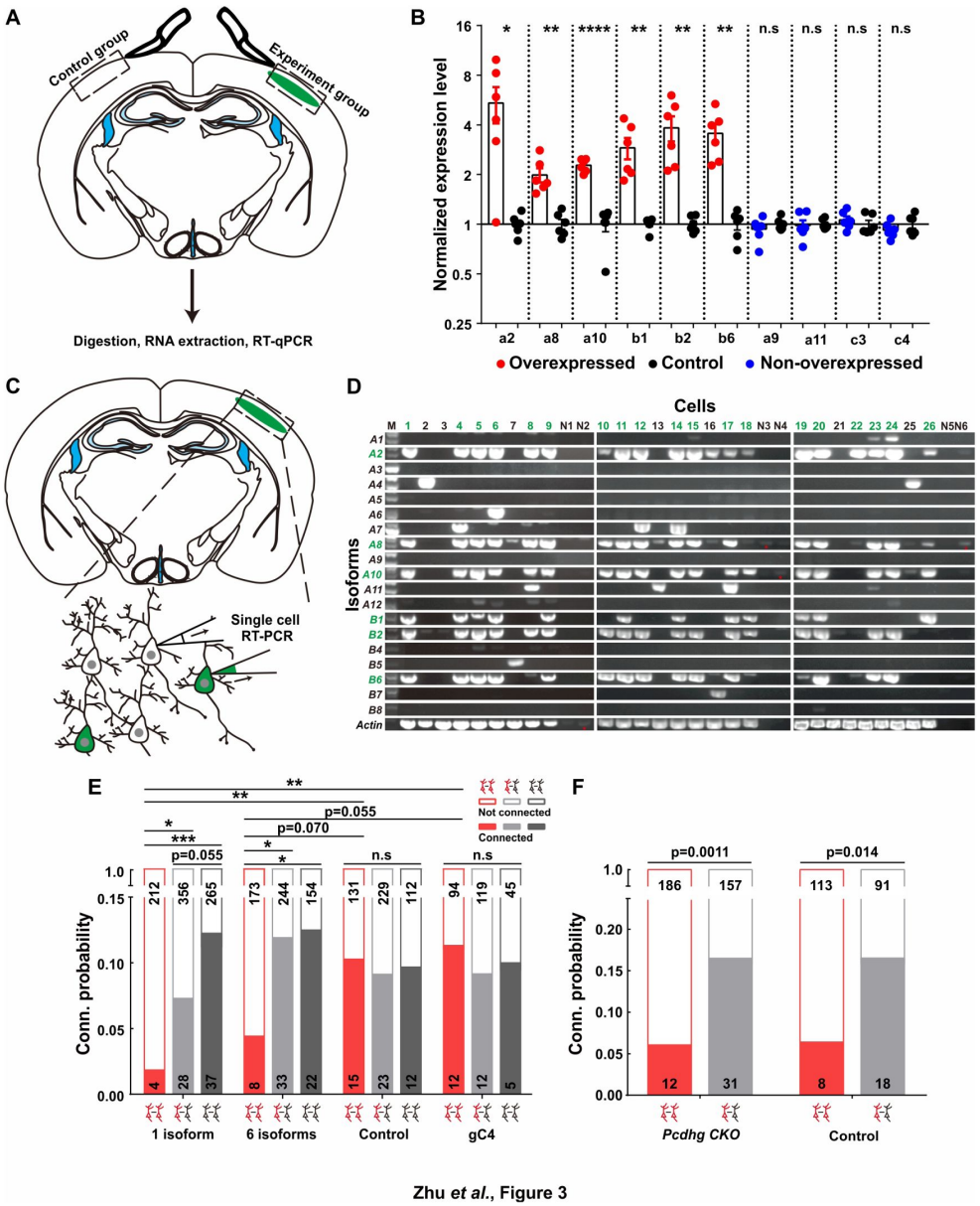
## Overexpression of γ-PCDHs decreases local synaptic connectivity in the mouse neocortex

To further determine the influence of γ-PCDHs on synaptic connectivity, we overexpressed randomly selected single or multiple γ-PCDH isoforms tagged with fluorescent proteins through *in utero* electroporation in mice. Subsequently, we performed a series of multiple whole-cell patch-clamp recordings to unveil how γ-PCDHs affect synaptic connectivity between neurons situated in the layer 2/3 of the neocortex.

Firstly, to validate the overexpression effect, we conducted a battery of assays, including Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) (**Fig. 3A, B**), single-cell RT-PCR (**Fig. 3C, D**), and immunohistochemistry (**Fig. 3**-S1A to C). The qRT-PCR assays unequivocally confirmed that the electroporated isoforms, as opposed to the non-overexpressed ones from the contralateral side, exhibited significantly higher expression levels (**Fig. 3A, B**). To assess how many isoforms were typically expressed in a given neuron when multiple plasmids were electroporated, we strategically tagged the first five isoforms with mNeongreen and the sixth with mCherry (**Fig. 3**-S1A). Employing a probability analysis based on the occurrence of yellow and red-only cells within the total electroporated neuron population (**Fig. 3**-S1B), we ascertained that each positive neuron expressed an average of 5.6 types of isoforms (**Fig. 3**-S1C and the top panel of D). This result harmonized with data obtained from single-cell RT-PCR analysis, wherein an average of 5.3 types out of the six electroporated isoforms was detected from 19 neurons (**Fig. 3C, D** and the bottom panel of **Fig. 3**-S1D). These single-cell RT-PCR findings further substantiate that the electroporation-introduced isoforms predominate within these neurons (**Fig. 3D**).

Since the distribution of neocortical neurons across different layers significantly influences their synaptic connections(He et al., 2015), we meticulously examined the positions of these neurons relative to the pial surface after overexpression. Remarkably, we observed that overexpressing γ-PCDH isoforms did not induce any alterations in cell positioning within the neocortex compared to the control plasmids (**Fig. 3**-S1E, F). Subsequently, through multiple recordings, we embarked on a quest to assess the impact of γ-PCDHs on synapse formation. Intriguingly, overexpressing one or six γ-PCDH variable isoforms in neurons significantly diminished the rate of synaptic connections among them (10.3%, 15/146 in expressing control plasmids; 1.9%, 4/216 in overexpressing one isoform; and 4.4%, 8/181 in overexpressing six isoforms, red bars in **Fig. 3E**). However, when overexpressing γ-PCDH C4, we did not observe any significant effect on the synaptic connection rate (11.3%, 12/106 in neurons overexpressing γ-PCDH C4, **Fig. 3E**).

To further exclude the potential influence of C-type γ-PCDHs in synapse formation, we employed a similar strategy in *Pcdhg* cKO mice, electroporating six variable isoforms. Remarkably, this overexpression also led to a reduction in the connection rate in *Pcdhg* cKO mice (6.1%, 12/198 in overexpressing six isoforms vs. 16.5%, 31/188 in expressing control plasmids), mirroring the outcome observed in WT littermate mice (6.6%, 8/121 in overexpressing six isoforms vs. 16.5%,




## Figure 3

### Overexpressing identical variables, but not C4, γ-PCDHs in neurons decreased their synaptic connectivity.

(**A**) Schematic illustrating the brain regions selected for qRT-PCR in both experimental and control groups. (**B**) qRT-PCR results showing overexpression levels in electroporated regions. Electroporated isoforms are indicated in red, control isoforms in blue, and contralateral sides used as controls are in black. Statistical analysis was conducted using Student's t-test, where * indicates p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001. (**C**) Diagram illustrating the process of cell extraction for single-cell RT-PCR assays. (**D**) Results of single-cell RT-PCR for γ-PCDH isoforms after electroporation. Neurons with fluorescence are highlighted in green, while nearby neurons without fluorescence are in black. Negative controls are labeled as N1-N6. Electroporated isoforms are shown in green, with red stars indicating faint signals in negative controls. (**E**) Impact of overexpressing one or six γ-PCDH isoforms on synaptic connectivity in WT mice. "1 isoform" represents *Pcdhga2*, and "6 isoforms" denote *Pcdhga2*, *Pcdhga8*, *Pcdhga10*, *Pcdhgb1*, *Pcdhgb2*, and *Pcdhgb6*. "gC4" stands for *PcdhgC4*, and "Control" indicates plasmid vector without *Pcdhg* insertion. (**F**) The influence of overexpressing 6 γ-PCDH isoforms on synaptic connectivity in *Pcdhg* cKO mice. The same 6 isoforms used as in (**E**) were employed. *Pcdhg cKO*: *Pcdhg* conditional knockout mice; Control: WT littermates. Statistical differences between groups in (**E**) and (**F**) were determined using the chi-square test and false discovery rate (FDR, Benjamini-Hochberg method) correction.

18/109 in expressing control plasmids) (**Fig. 3F**). These compelling observations collectively underscore that overexpressing the variable isoforms, as opposed to the C-type isoform C4, leads to a decrease in synaptic connectivity within the mouse neocortex.

### Combinatorial expression of γ-PCDHs regulates synaptic connectivity in the mouse neocortex

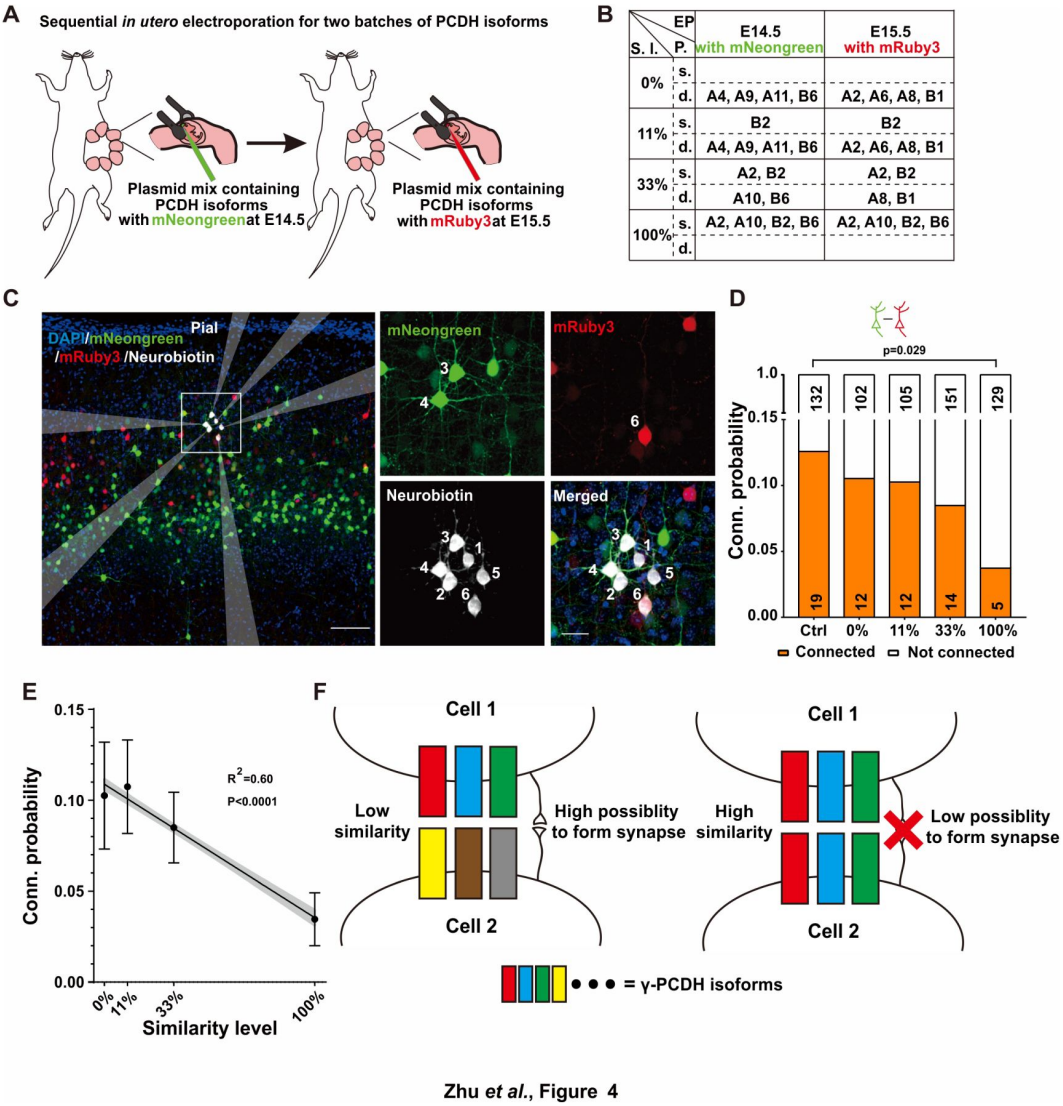
Our quest to understand the influence of combinatorial expression patterns of γ-PCDH isoforms on synapse formation led us to conduct a sequential *in utero* electroporation at E14.5 and E15.5 (**Fig. 4A**). In this intricate procedure, our objective was to deliberately manipulate the degree of similarity in expression between two distinct groups of neurons. To achieve this, we randomly selected isoforms, although with the notable exception of isoforms A5 and B8. These two isoforms were singled out due to their low expression levels as indicated by the single-cell sequencing data (**Fig. 1D** and **Fig. 1**-S1E). The various isoforms were thoughtfully combined to create similarity levels spanning from 0% (indicating no overlap, complete dissimilarity between the two groups) to 100% (representing complete overlap, indicating that the two groups are entirely identical) (**Fig. 4B**).

For the purpose of electroporation at E14.5 and E15.5, we employed fluorescent proteins mNeongreen and mRuby3(Bajar et al., 2016) to tag isoforms, which allowed us to distinguish the cells electroporated on these respective days (**Fig. 4A**). Subsequently, whole-cell patch-clamp recordings were performed on layer 2/3 neurons in the neocortex using acute brain slices containing both green and red cells from P10-14 pups. Each set of recordings encompassed at least one mNeongreen$^{+}$, one mRuby3$^{+}$, and one nearby control neuron without fluorescence (**Fig. 4C**). The results were very clear: neurons sharing the same color displayed significantly lower connectivity (**Fig. 4**-S1A), aligning with our previous findings from single electroporation (as shown in **Fig. 3E**). In the group characterized by a 100% similarity level, the connectivity rate between neurons with different colors that were electroporated on different days was also significantly lower compared to the control (3.7%, 5/134 in the complete-overlap group; 12.5%, 19/151 in control pairs, 100% in **Fig. 4D**). However, as the similarity levels descended from 100% to 0%, the connectivity probabilities progressively reverted to the control level. The likelihood rebounded to 8.4% (14/165) for the pairs with a 33% similarity level and 10.3% (12/117) and 10.5% (12/114) for pairs with 11% or 0% similarity level, respectively (**Fig. 4D**). This compelling observation illuminated a fundamental principle: it is the similarity level of γ-PCDH isoforms shared between neurons, rather than the absolute expression of the protein within individual neurons, that dictates the regulation of synaptic formation.

Remarkably, in line with the single-electroporation experiment (gray bars in **Fig. 3E**), no significant changes were observed in the synaptic connectivity between electroporated neurons and nearby control neurons (**Fig. 4**-S1B). In summation, our findings illuminate a discernible negative correlation between the probability of synaptic connections and the similarity level of γ-PCDH isoforms expressed in neuron pairs (**Fig. 4E**). These discoveries underline the significance of the diversified combinatorial expression of γ-PCDH isoforms in regulating synapse formation between adjacent pyramidal cells. Simply put, the more similar the patterns of γ-PCDH isoforms expressed in neurons, the lower the probability of synapse formation between them (**Fig. 4F**).

## Discussion

Homophilic proteins cPCDHs are strong candidates for promoting synaptic specificity due to their combinatorial and stochastic expression pattern (Yagi, 2012, Kohmura et al., 1998, Toyoda et al., 2014). Our 5'-end single-cell sequencing data provided solid evidence for the combinatorial expression pattern of γ-PCDH isoforms in neocortical neurons. We further demonstrated the critical role of this diversity in synaptic connectivity through three lines of evidence. Firstly, the




Zhu *et al.*, Figure 4

## Figure 4

### Diversified γ-PCDHs are critical for synapse formation in cortical neurons.

(**A**) Diagram illustrating the sequential *in utero* electroporation process at E14.5 and E15.5. (**B**) Overview of the overexpressed γ-PCDH isoforms in different experiments, resulting in varying similarity levels between neurons. S.I.: Similarity level; EP: Electroporation; P.: plasmids mixture; s./d., Same or different isoforms in two electroporations. (**C**) Sample image of recorded neurons after two rounds of electroporation at E14.5 (mNeongreen) and E15.5 (mRuby3). Neurons labeled as positive for mNeongreen (cells 3 and 4), mRuby3 (cell 6), and negative without fluorescence (cells 1, 2, and 5). Neurobiotin was used in the internal solution to label recorded neurons. The translucent arrows show the positions of the electrodes. Scale bar, left: 100 μm, right: 25 μm. (**D**) Connectivity probability for neuron pairs overexpressing different sets of γ-PCDH isoforms (labeled with different fluorescence) following sequential in utero electroporation. Statistical differences were determined using the Chi-square test with FDR (Benjamini-Hochberg method) correction. (**E**) Correlation between the similarity level of overexpressed γ-PCDH combinations and the probability of synaptic connections. Each data point corresponds to the outcome of 100 bootstrapped samples derived from the source data presented in panel **D**. Error bars indicate the standard deviation (S.D) for each data point. The gray shaded area represents the 95% confidence interval of the curve fitting. (**F**) Graph summarizing the effect of γ-PCDHs on synapse formation.

absence of γ-PCDH significantly increased functional connectivity between adjacent neocortical neurons. Secondly, electroporation-induced overexpression of identical γ-PCDH variable isoforms in developing neurons markedly decreased their connectivity. Lastly, using sequential *in utero* electroporation with different batches of isoforms, we found that increasing the similarity level of γ-PCDH variable isoforms expressed in neurons led to a reduction in their synaptic connectivity. These findings suggest that γ-PCDHs regulate the specificity of synapse formation by preventing synapse formation with specific cells, rather than by selectively choosing particular targets. It remains to be studied whether the diversified patterns of γ-PCDH isoforms expressed in different neurons have additional coding functions for neurons beyond their homophilic interaction.

Stochastic and combinatorial expression patterns of cPCDH have been identified in Purkinje cells(Esumi et al., 2005, Toyoda et al., 2014) and olfactory sensory neurons(Mountoufaris et al., 2017). However, it's noteworthy that in serotonergic neurons, only one isoform, Pcdhac2, has been mainly detected (Chen et al., 2017). In our study, utilizing 5'-end single-cell sequencing, we have unveiled the stochastic and combinatorial expression patterns of variable γ-PCDH isoforms in neocortical neurons. These diverse observations across different cell types suggest that cPCDH diversity and the presence of ubiquitous C-type expression are not universal features throughout the brain(Kiefer et al., 2023). These distinct expression patterns of cPCDHs imply that this gene cluster might exert different roles in shaping neural connections in various brain regions. Furthermore, compared to the SMART seq results from the Allen Institute's database and others(Lv et al., 2022) focusing on γ-PCDH, 5'-end single-cell sequencing used in our study not only detected more isoforms in individual cells but also revealed more neurons expressing C-type isoforms. The application of this approach may offer valuable insights for studying the functions of cPCDHs in a broader neurological context.

Previous studies by Molumby et al. demonstrated that neurons from the neocortex of *Pcdhg* knockout mice exhibited significantly more dendritic spines, while neurons overexpressing a single γ-PCDH isoforms had fewer dendritic spines in (Molumby et al., 2017). Our recordings are consistent with these previous morphology studies. Tarusawa et al. revealed that the absence of the whole cluster of cPCDH affected synaptic connections among lineage-related cells(Tarusawa et al., 2016). More recently, overexpression of the C-type γ-PCDH isoform C3 also showed a negative effect on synapse formation within a defined clone(Lv et al., 2022). In our study, we further demonstrated an increased synaptic connection rate between adjacent pyramidal neurons in the neocortex of *Pcdhg* knockout mice, while it decreased between neurons overexpressing single or multiple identical γ-PCDH variable isoforms. These effects were not just limited to lineage-dependent cells. Together with previous findings(Molumby et al., 2017, Tarusawa et al., 2016), our observations solidify the negative effect of γ-PCDHs on synapse formation among neocortical neurons. Some subtle differences exist between our findings and previous recordings(Tarusawa et al., 2016, Lv et al., 2022). Tarusawa et al. demonstrated that the connection probability between excitatory neurons lacking the entire cPCDH cluster in the layer 4 was approximately twofold higher at early stage P9-11, significantly lower at P13-16, and similar to control cells at P18-20 compared (Tarusawa et al., 2016). In our study, $Pcdhg^{-/-}$ pyramidal neuronal pairs consistently exhibited a higher connection probability from P10 to P20. Two potential reasons could explain these differences. Firstly, we only removed γ-PCDHs instead of the entire cPCDH cluster, which includes α, β, and γ isoforms. Secondly, γ-PCDH might have different functions in neurons located in the layer 2/3 compared to the layer 4. Lv et al. found that overexpression of C-type γ-PCDH C3 decreased the preferential connection between sister cells(Lv et al., 2022). However, our study demonstrated that only variable, but not C-type isoform C4, had a negative impact on synapse formation. This discrepancy might be attributed to the lineage relationship, which could have an unknown impact on synapse formation. Building upon previous findings, it's becoming increasingly evident that distinct C-type isoforms may play varied roles in shaping neural networks within the brain(Garrett et al., 2019, Steffen et al., 2023, Meltzer et al., 2023, Lv et al., 2022).

Since a single neuron can express multiple isoforms, deleting all γ-PCDH isoforms might mask the role of this combination. In this study, we manipulated the combinatorial expression patterns of γ-PCDH isoforms in nearby neocortical neurons through sequential *in utero* electroporation, expressing different batches of isoforms with adjustable similarities. We observed that when two neurons expressed identical variable isoforms (100% group), the likelihood of synapse formation between them was lowest. As the similarity level between two cells decreased, with fewer shared isoforms, the connectivity probability increased. The connectivity probability between neurons with different variable isoforms (0% group) did not differ from the control pairs (without overexpression). However, the connections between overexpressed and control neurons were not affected under both 100% and 0% similarity conditions. These observations suggest that the similarity level, rather than the absolute expression of the protein, affects synapse formation between neurons. While we observed a negative correlation between expression similarity and the probability of connectivity among neocortical neurons, further investigation is needed to determine the precise cellular mechanisms underpinning this correlation. It's essential to explore whether this correlation arises directly from the formation of synapses or is a secondary effect resulting from cell positioning (Lv et al., 2022), synaptic pruning (Kostadinov and Sanes, 2015) or the influence of γ-PCDHs on the growth of axon/dendrite(Molumby et al., 2017, Molumby et al., 2016). Previous studies have established that the interplay between γ-PCDHs and neuroligin-1 plays a crucial role in the negative regulation of dendritic spine morphogenesis(Molumby et al., 2017). Consequently, exploring whether the similarity in the expression of γ-PCDHs between two neurons influences their interaction with neuroligin-1 could yield valuable insights.

Our findings also demonstrated that the overexpression of multiple γ-PCDH variable isoforms in one neuron only affected its connection if the other neuron overexpressed an identical combination of γ-PCDH isoforms. This highlights the pivotal role of the diversified combinatorial expression of γ-PCDHs of neurons in selecting their synaptic partners in the mouse neocortex. Notably, while the overexpression of the γ-PCDH C4 isoform had no discernible effect on synaptic connectivity, overexpressing six variable isoforms resulted in a reduced connection rate in *Pcdhg* cKO mice. These observations underscore the critical role of variable isoforms, as opposed to the C-type isoform C4, in synapse formation within the mouse neocortex. Although the overexpression of the γ-PCDH C3 isoform has been shown to have a negative effect on synapse formation between sister cells, but no effect on synapses among non-clone cells in the neocortex(Lv et al., 2022), the distinct functions of individual C-type isoforms require further thorough examination. It's worth noting that our observations primarily stem from overexpression assays, providing insights into the effects of γ-PCDHs on synaptic connectivity. Exploring their impact under more physiological conditions using alternative approaches holds significant promise.

Furthermore, while the absence of γ-PCDHs causes significantly more synaptic formation among neocortical pyramidal neurons, evidence supports that their absence also leads to a significant reduction of synapse formation in other brain regions. For example, mice lacking γ-PCDHs exhibit fewer synapses in spinal cord interneurons(Weiner et al., 2005). Knocking down γ-PCDHs causes a decline in dendritic spines in cultured hippocampal neurons(Suo et al., 2012) and diminished astrocyte-neuron contacts in co-cultures from the developing spinal cord(Garrett and Weiner, 2009). The absence of γ-PCDHs leads to reduced dendritic arborization and dendritic spines in olfactory granule cells(Ledderose et al., 2013). Additionally, immuno-positive signals for γ-PCDHs are more frequently detected in mushroom spines than in thin spines(LaMassa et al., 2021). Moreover, our observations revealed that the absence of γ-PCDHs had a more pronounced impact on vertically aligned neurons than on horizontally situated pairs in the neocortex. These findings suggest that different mechanisms may be employed by synapses in different brain regions to achieve their specificity. Notably, different mechanisms have already been proposed for targeting specific inhibitory neural circuits in the neocortex, including "on-target" synapse formation for targeting apical dendrites and "off-target" synapse selective removal for somatic innervations(Gour et al., 2021).

In summary, our data demonstrate that the similarity level of γ-PCDH isoforms between neocortical neurons is critical for their synapse formation. Neurons expressing more similar γ-PCDH isoform patterns exhibit a lower probability of forming synapses with one another. This suggests that the presence of γ-PCDHs enables neocortical neurons to choose which neurons to avoid synapsing with, rather than selecting specific neurons to form synapses with. Whether there are specific attractive forces between cells to promote synaptic specificity remains an open question.

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## Author contributions

H. -T. X. conceived the project. Y. -J. Z. performed most recordings and single-cell analysis. C. -Y. D. and L. F. performed part of recordings. Y. -Q. W. prepared PCDH plasmids. H. Z. prepared mice. Y. -J. Z. and H. -T. X. wrote the manuscript.

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**Reviewer #1 (Public Review):**

The manuscript by Zhu and colleagues aimed to clarify the importance of isoform diversity of PCDHg in establishing cortical synapse specificity. The authors optimized 5' single-cell sequencing to detect cPCDHg isoforms and showed that the pyramidal cells express distinct combinations of PCDHg isoforms. Then, the authors conducted patch-clamp recordings from cortical neurons whose PCDHg diversity was disrupted. In the elegant experiment in Figure 3, the authors demonstrated that the neurons expressing the same sets of cPCDHg isoforms are less likely to form synapses with each other, suggesting that identical cPCDHg isoforms may have a repulsive effect on synapse formation. Importantly, this phenomenon was dependent on the similarity of the isoforms present in neurons but not on the amount of proteins expressed.

The authors have addressed most criticisms raised in the initial review and the manuscript has improved significantly. One of the major concerns in the first review was whether PCDHg isoforms, which are expressed at a much lower level than C-type isoforms, have true physiological significance. The authors conducted additional experiments to address this point by using PCDHg cKO and provided convincing data supporting their conclusion. The results from PCDHg C4 overexpression, showing no impact on synaptic connectivity, further clarified the importance of isoforms. The limitation of the paper is that most experiments relied on overexpression of isoforms. Whether the isoform diversity is necessary for the synapse refinement in a physiological condition remains further clarification.

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**Reviewer #2 (Public Review):**

This short manuscript by Zhu et al. describes an investigation into the role of gamma protocadherins in synaptic connectivity in the mouse cerebral cortex. First, the authors conduct a single-cell RNA-seq survey of postnatal day 11 mouse cortical neurons, using an adapted 10X Genomics method to capture the 5' sequences that are necessary to identify individual gamma protocadherin isoforms (all 22 transcripts share the same three 3' "constant" exons, so standard 3'-biased methods can't distinguish them). This method adaptation is an advance for examining individual clustered protocadherin transcripts, and it is helpful to publish the method in this manuscript. The results largely confirm what was known from other approaches, which is that a few of the 19 A and B subtype gamma protocadherins are expressed in an apparently stochastic and combinatorial fashion in each cortical neuron, while the 3 C subtype genes are expressed by most neurons. Second, using elegant paired electrophysiological recordings, the authors show that in gamma protocadherin knockout cortical slices, the likelihood of two neurons on layers 2/3 being synaptically connected is increased. That suggests that gamma protocadherins generally inhibit synaptic connectivity in the cortex; again, this has been reported previously using morphological assays, but it is helpful to see it confirmed here with physiology. Finally, the authors use an impressive sequential in utero electroporation method to provide evidence that the degree of isoform matching between two neurons negatively regulates their reciprocal synaptic connectivity. These are difficult experiments to do, and while some caveats remain (e.g., lack of demonstration of protein levels in electroporated neurons, lack of resolution of the differences between the present results and those of other papers, a focus on C4 rather than C3 or C5 when considering the highly expressed C-type isoforms), the main result is consistent. Strengths of this manuscript include the impressive methodology and improved demonstration of the previously-reported finding that gamma protocadherins work via homophilic matching to put a brake on synapse formation in the cortex. Because of the unique organization and expression pattern of the gamma protocadherins, it is not likely that these results will be broadly applicable to the general understanding of the role of cell

adhesion molecules in synapse development. However, the methodology, which is now better described, should be applicable more broadly and the improved demonstration of the role of gamma protocadherin's negative role in cortical synaptogenesis is helpful in confirming earlier studies. There are several differences between the results here and those of other papers on the cortex, as well as those examining other neuronal populations such as spinal cord. The present findings do not resolve them, but adopting genetic approaches rather than overexpression in the future should help.

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**Author Response**

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *The manuscript by Zhu and colleagues aimed to clarify the importance of isoform diversity of PCDHg in establishing cortical synapse specificity. The authors optimized 5' single-cell sequencing to detect cPCDHg isoforms and showed that the pyramidal cells express distinct combinations of PCDHg isoforms. Then, the authors conducted patch-clamp recordings from cortical neurons whose PCDHg diversity was disrupted. In the elegant experiment in Figure 3, the authors demonstrated that the neurons expressing the same sets of cPCDHg isoforms are less likely to form synapses with each other, suggesting that identical cPCDHg isoforms may have a repulsive effect on synapse formation. Importantly, this phenomenon was dependent on the similarity of the isoforms present in neurons but not on the amount of proteins expressed.*
>
> *One of the major concerns in an earlier version was whether PCDHg isoforms, which are expressed at a much lower level than C-type isoforms, have true physiological significance. The authors conducted additional experiments to address this point by using PCDHg cKO and provided convincing data supporting their conclusion. The results from PCDHg C4 overexpression, showing no impact on synaptic connectivity, further clarified the importance of isoforms. I have no further concerns, however, I would like to point out that the evidence for the necessity of the PCDHg isoform is still lacking because most experiments were done by overexpression. It would be helpful for the readers if the authors could add this point to the discussion.*

Thank you for the positive feedback on our work. We have now incorporated a discussion of the limitations associated with overexpression.

> ***Reviewer #2 (Public Review):***
>
> *This short manuscript by Zhu et al. describes an investigation into the role of gamma protocadherins in synaptic connectivity in the mouse cerebral cortex. First, the authors conduct a single-cell RNA-seq survey of postnatal day 11 mouse cortical neurons, using an adapted 10X Genomics method to capture the 5' sequences that are necessary to identify individual gamma protocadherin isoforms (all 22 transcripts share the same three 3' "constant" exons, so standard 3'biased methods can't distinguish them). This method adaptation is an advance for examining individual gamma transcripts, and it is helpful to publish the method, the characterization of which is improved in this revised manuscript. The results largely confirm what was known from other approaches, which is that a few of the 19 A and B subtype gamma protocadherins are expressed in an apparently stochastic and combinatorial fashion in each cortical neuron, while the 3 C*

> *subtype genes are expressed ubiquitously. Second, using elegant paired electrophysiological recordings, the authors show that in gamma protocadherin cortical slices, the likelihood of two neurons on layers 2/3 being synaptically connected is increased. That suggests that gamma protocadherins generally inhibit synaptic connectivity in the cortex; again, this has been reported previously using morphological assays, but it is important to see it confirmed here with physiology. Finally, the authors use an impressive sequential in utero electroporation method to provide evidence that the degree of isoform matching between two neurons negatively regulates their reciprocal synaptic connectivity. These are difficult experiments to do, and while some caveats remain, the main result is consistent. Strengths include the impressive methodology and improved demonstration of the previously-reported finding that gamma protocadherins work via homophilic matching to put a brake on synapse formation in the cortex. Weaknesses include the writing, which even in the revision fails to completely put the new results in context with prior work, which together has largely shown similar results; a still-incomplete characterization of a new alpha protocadherin KO mouse (a minor point but it should still be addressed); and a lack of demonstration of protein levels in electroporated brains. Because of the unique organization and expression pattern of the gamma protocadherins, it is unlikely that these results will be directly applicable to the broader understanding of the role of cell adhesion molecules in synapse development. However, the methodology, which is now better described, should be applicable more broadly and the improved demonstration of the role of gamma protocadherin's negative role in cortical synaptogenesis is helpful.*

Thank you for the positive comments on our work. We have taken your suggestion into account and expanded our discussion to contextualize our research within the broader field of PCDH. Additionally, we have included more data to further illustrate the decrease in αPCDH expression in Pcdha conditional knockout mice. Your feedback has been invaluable in enhancing our manuscript.

> ***Reviewer #3 (Public Review):***
>
> *In this study, Zhu and authors investigate the expression and function of the clustered Protocadherins (cPcdhs) in synaptic connectivity in the mouse cortex. The cPcdhs encode a large family of cadherin-related transmembrane molecules hypothesized to regulate synaptic specificity through combinatorial expression and homophilic binding between neurons expressing matching cPcdh isoforms. But the evidence for combinatorial expression has been limited to a few cell types, and causal functions between cPcdh diversity and wiring specificity have been difficult to test experimentally. This study addresses two important but technically challenging questions in the mouse cortex: 1) Do single neurons in the cortex express different cPcdh isoform combinations? and 2) Does Pcdh isoform diversity or particular combinations among pyramidal neurons influence their connectivity patterns? Focusing on the Pcdh-gamma subcluster of 22 isoforms, the group performed 5'end-directed single-cell RNA sequencing from dissociated postnatal (P11) cortex. To address the functional role of Pcdhg diversity in cortical connectivity, they asked whether the Pcdhgs and isoform matching influence the likelihood of synaptic pairing between 2 nearby pyramidal neurons. They performed simultaneous whole-cell recordings of 6 pyramidal neurons in cortical slices, and measured paired connections by evoked monosynaptic responses. In these experiments, they measured synaptic connectivity between pyramidal neurons lacking the Pcdhgs, or overexpressing dissimilar or matching sets of Pcdhg isoforms introduced by electroporation of plasmids encoding Pcdhg cDNAs.*

Overall, the study applies elegant methods that demonstrate that single cortical neurons express different combinations of Pcdh-gamma isoforms, including the upper layer

Pyramidal cells that are assayed in paired recordings. The electrophysiology data demonstrate that nearby Pyramidal neurons lacking the entire Pcdhg cluster are more likely to be synaptically connected compared to the control neurons, and that overexpression of matching isoforms between pairs decreases the likelihood to be synaptically connected. These are important and compelling findings that advance the idea that the Pcdhgs are important for cortical synaptic connectivity, and that the repertoire of isoforms expressed by neurons influence their connectivity patterns potentially through a self/nonself discrimination mechanism. However, the findings are limited to probability in connectivity and do they do not support the authors' conclusions that Pcdhg isoforms regulate synaptic specificity, 'by preventing synapse formation with specific cells' or to 'unwanted partners'. Characterizations of the cellular basis of these defects are needed to determine whether they are secondary to other roles in cell positioning, axon/dendrite branching and synaptic pruning, and overall synaptic formation. Claims that Pcdh-alpha and Pcdhg C-type isoforms are not functionally required are premature, due to limitations of the experiments. Moreover, claims that 'similarity level of γPCDH isoforms between neurons regulate the synaptic formation' are not supported due to weak statistical analyses presented in Fig4. The overstatements should be corrected. There was also missed opportunity to clearly discuss these results in the context of other published work, including recent publications focused on the cortex.

Thank you for your feedback on the strengths and weaknesses of our work. In terms of the cellular basis of affected synaptic connectivity caused by γ-PCDH isoforms, we have compared the probability of connectivity for neuronal pairs with similar range of distance. Our findings indicate that the manipulation primarily affects pairs within the 50-150 micrometer range, suggesting that cell positioning might be a critical factor for the impact of γ-PCDH on synapse formation. However, we acknowledge that we couldn't definitively determine whether the negative effect on synaptic connectivity stems directly from impaired synapse formation or indirectly from synaptic pruning or the influence of PCDHγ on axon/dendritic branching. We've added these limitations to our discussion to provide a more comprehensive view of our research. Furthermore, we've adjusted our statements to better reflect the significance of our findings. Your feedback has been instrumental in improving the clarity and depth of our manuscript.

> *Strengths:*
>
> - *The 5' end sequencing with a Pcdhg-amplified library is a technical feat and addresses the pitfall of conventional scRNA-Seq methods due to the identical 3'sequences shared by all Pcdhg isoform and the low abundance of the variable exons. New figures with annotated cell types confirm that several pyramidal and inhibitory cortical subpopulations were captured.*

Statistical assessment of co-occurrence of isoform expression within clusters is also a strength.

> - *By establishing the combinatorial expression of Pcdhgs by maturing pyramidal cells, the study further substantiates the 'single neuron combinatorial code for cPcdhs' model. Although combinatorial expression is not universal (ie. serotonergic neurons), there was limited evidence. The findings that individual pyramidal neurons express ~1-3 variable Pcdhg transcripts plus the Ctype transcripts aligns with single RT-PCR studies of single Purkinje cells (Esumi et al 2005; Toyoda et al 2014). They differ from the findings by Lv et al 2022, where C-type expression was lower among pyramidal neurons. OSNs also do not substantially express C-type isoforms (Mountoufaris et al 2017; Kiefer et al 2023).*

*Differences, and the advantages of the 5'end -directed sequencing (vs. SmartSeq) could be raised in the discussion.*

- *Simultaneous whole-cell recordings and pairwise comparisons of pyramidal neurons is a technically outstanding approach. They assess the effects of Pcdhg OE isoform on the probability of paired connections.*

- *The connectivity assay between nearby pairs proved to be sensitive to quantify differences in probability in Pcdhg-cKO and overexpression mutants. The comparisons of connectivity across vertical vs lateral arrangement are also strengths. Overexpressing identical Pcdhg isoform (whether 1 or 6) reduces the probability of connectivity, but there are caveats to the interpretations (see below).*

*Weaknesses:*

*n earby pairs but are not sufficient evidence for synapse specificity. The cPcdhs play multiple roles in neurite arborization, synaptic density, and cell positioning. Kostadinov 2015 also showed that starburst cells lacking the Pcdhgs maintained increased % connectivity at maturity, suggesting a lack of refinement in the absence of Pcdhgs. The known roles raise questions on how these manipulations might have primary effects in these processes and then subsequently impact the probability of connectivity. Investigations of morphological aspects of pyramidal development would strengthen the study and potentially refine the findings. The authors should more clearly relate their findings to the body of cPcdh studies in the discussion.*

Previous studies revealed the adverse effects of γ-PCDHs on dendritic spines, demonstrating that their absence results in increased dendritic spines density, while overexpression leads to a reduction. In our study, we consistently observed that γ-PCDHs exert a negative influence on synaptic connectivity. This consistency strengthens the overall body of evidence in support of the role of γ-PCDHs in synaptic connectivity and dendritic spine regulation. While we have previously mentioned this point in our discussion to highlight the concordance between our findings and prior research, your input is greatly appreciated in reinforcing the scientific context of our work.

- *Pcdhg cKO-dependent effects on connectivity occur between closely spaced soma (50-100um - Figure 2E), highlighting the importance of spatial arrangement to connectivity (also noted by Tarusawa 2016). Was distance considered for the overexpression (OE) assays, and did the authors note changes in cell distribution which might diminish the connectivity? Recent work by Lv et al 2022 reported that manipulating Pcdhgs influences the dispersion of clonally-related pyramidal neurons, which also impacts the likelihood of connections. Overexpression of Pcdhgc3 increased cell dispersion and decreased the rate of connectivity between pairs. Though these papers are mentioned, they should be discussed in more detail and related to this work.*

Our data indicated that variable γ-PCDH isoforms primarily influence synaptic connectivity in neuronal pairs within the 50-150 micrometer range. Notably, as the distance between neurons increases, we observed a corresponding reduction in synaptic connectivity, as illustrated in Figure 2E. We have also included additional discussion regarding potential variances among different C-type isoforms.

- *Though the authors added suggested citations and improved the contextualization of the study, several statements do not accurately represent the cited literature. It is at the expense of crystalizing the novelty and importance of this present work. For instance, Garrett et al 2012 PMID: 22542181 was the first to describe roles for Pcdhgs in cortical pyramidal cells and dendrite arborization, and that pyramidal cell migration and survival are intact. Line 52 cited Wang et al 2002, but this was limited to gross inspection. Garrett et al is the correct citation for: 'The absence of γ-PCDH does not cause general abnormality in the development of the cerebral cortex, such as cell differentiation, migration, and survival (Wang et al., 2002).' Second, single cell cPcdh diversity is introduced very generally, as though all neuron types are expected to show combinatorial variable expression with ubiquitous C-Type expression. But those initial studies were limited to Purkinje cells (Esumi 2005 and Toyoda 2014). Profiling of serotonergic neurons and OSN reveals different patterns (citations needed for Chen 2017 PMID: 28450636; Mountofaris et al PMID: 2845063; Canzio 2023 PMID: 37347873), raising the idea that cPcdh diversity and ubiquitous Ctype expression is not universal. Thus, the authors missed the opportunity to emphasize the gap regarding cPcdh diversity in the cortex.*

We would like to extend our gratitude to the reviewer for pointing out the citation related to the roles of γ-PCDHs in the neocortex. After a thorough review of both papers, Wang et al., 2002 and Garrett et al., 2012, we concur that it would be more appropriate to cite both of these papers here. Your suggestion to underscore the diverse expression patterns of γPCDHs in neocortical neurons is well-received, and we have integrated this aspect of our findings with previous observations into a new paragraph within the discussion section. Your insights have greatly enriched the depth of our paper, and we genuinely appreciate your contribution.

- *They have not shown rigorously and statistically that the rate of connectivity changes with% isoform matching. In Figure 4D, comparisons of % isoform matching in OE assays show a single statistical comparison between the control and 100% groups, but not between the 0%, 11% and 33% groups. Is there a significant difference between the other groups? Significant differences are claimed in the results section, but statistical tests are not provided. The regression analysis in 4E suggests a correlation between % isoform similarity and connectivity probability, but this is not sound as it is based on a mere 4 data points from 4D. The authors previously explained that they cannot evaluate the variance in these recordings as they must pool data together. However, there should be some treatment of variability, especially given the low baseline rate of connectivity. Or at the very least, they should acknowledge the limitations that prevent them from assessing this relationship. Claims in lines 230+ are not supported: ' Overall, our findings demonstrate a negative correlation between the probability of forming synaptic connections and the similarity level of γPCDH isoforms expressed in neuron pairs (Fig. 4E)".*

We employed a bootstrap method to estimate the potential variance in the analysis presented in Fig. 4E. It's important to note that due to methodological limitations, a comprehensive assessment of variance based solely on recordings from a single animal is challenging. As such, we have adjusted our claims to be more aligned with our observations.

- *Figure 4 provides connectivity probability, but this result might be affected by overall synapse density. Did connection probability change with directionality (e.g*

> *between red to green cells, or green to red cells).*

As suggested by the reviewer, we have conducted an analysis to assess the directionality of connections under different conditions. This analysis involved comparing the directionalities of connections following the overexpression of six variable isoforms, as depicted in Fig. 3E. Upon examining 33 connected OE-Ctrl pairs following the electroporation of these 6 isoforms, we observed 3 pairs with bidirectional connections, 19 pairs with connections from OE to Ctrl, and 11 with connections from Ctrl to OE. To assess the statistical significance of these observations, we applied a Chi-square test. The results from this analysis indicated that there was no significant difference in the directionality of connections. These findings offer further support for the idea that overexpressing multiple γ-PCDH isoforms within a single neuron might not be sufficient to alter its connections with other neurons.

> - *Generally, the statistical approaches were not sufficiently described in the methods nor in the figure legends, making it difficult to assess the findings. They do not report on how they calculated FDR for connectivity data, when this is typically used for larger multivariate datasets.*

We employed the False Discovery Rate (FDR) correction, specifically the BenjaminiHochberg method, to determine which values remained statistically significant. This method is widely accepted and involves inputting all the p-values and the total number, 'n.' Additional details about this correction are now provided in the Method section for clarity.

> - *The possibility that the OE effects are driven by total Pcdhg levels, rather isoform matching, should be examined. As shown by qRT-PCR in Fig. 3, expression of individual isoforms can vary. It is reasonable that protein levels cannot be measured by IHC, although epitope tags could be considered as C-terminal tagging of cPcdhs preserves the function in mice (see Lefebvre 2008). Quantification of constant Pcdhg RNA levels by qRT-PCR or sc-RT-PCR would directly address the potential caveat that OE levels vary with isoform combinations.*

Through a series of multiple whole-cell recordings, we examined neuronal pairs within the 0% group, where both neurons exhibited overexpression of different combinations of γPCDH isoforms. What we discovered is that the connectivity level within pairs of neurons where both neurons overexpressed γ-PCDH isoforms, pairs with only one neuron overexpressing these isoforms, and pairs with two control neurons (lacking overexpression) was remarkably similar. However, as we incrementally raised the similarity level between the recorded neurons by increasing the overlap in the combinatorial expression of γ-PCDH isoforms, we observed a gradual decrease in the connectivity probability between these neurons. Notably, the connectivity probability reached its minimum when the recorded cells had the exact same combinatorial expression of γ-PCDH isoforms at the 100% similarity level. These findings suggest that the similarity level between neurons, rather than the absolute expression level of γ-PCDH isoforms, plays a critical role in affecting synapse formation.

> *-A caveat for the relative plasmid expression quantifications in Figure 3-S1 is that IHC was used to amplify the RFP-tagged isoform, and thus does not likely preserve the relationship between quantities and detection.*

We attempted to enhance the mNeongreen signal, known for its exceptional signal-tonoise ratio, by utilizing the 32f6-100 antibody from Chromotek. However, our observations did not reveal any additional cells through immunostaining compared to the images obtained solely based on the mNeongreen signal. This indicates that the application of the available antibody

did not yield a significant improvement in cell detection.
It's important to emphasize that if the RFP signal is overvalued, it would result in an increase in both the "red only" and "red in total" categories. However, it's worth noting that the "red only" category is more sensitive to the outcome than the "red in total" category. Therefore, an overvaluation of the RFP signal would lead to an underestimation of the total estimated plasmid content in electroporated neurons. Consequently, this would result in a lower estimate for the proportion of co-expression cells rather than a higher estimate. We have updated the calculation method in the "Estimating the numbers of overexpressed γPCDH isoform" section to reflect these considerations.

> - *Figure 1 didn't change in response to reviews to improve clarity. New panels relating to the scRNASeq analyses were added to supplementary data but many are central and should be included in Figure 1 (ie. S1-Fig6D). In the Results, the authors state that neuronal subpopulations generally show a combinatorial expression of some variable RNA isoforms and near ubiquitous C-type expression. But they only show data for the Layer 2/3 neuron-specific cluster in S1-Fig-6D, and so it is not clear if this pattern applies to other clusters. Fig. S1-5 show a low number of expressed isoforms per cell, but specific descriptions on whether these include C-type isoforms would be helpful. Figure 1F showing isoform profile in all neurons is not particularly meaningful. There is a lot of interest in neuron-type specific differences in cPcdh diversity, and the authors could highlight their data from S1-5 accordingly.*

In addition to the layer 2/3 cluster, we observed a diverse combinatorial expression of various variable γ-PCDH isoforms alongside nearly ubiquitous C-type expression in all other clusters of cells. We have now explicitly mentioned this observation in the main text. To underscore this point further, we have included a new figure, Fig. 1-S6, which provides information on the similarity analysis for all other clusters. It's important to note that the data in previous Fig. S1-5 (now renumbered as S1-7) were solely related to "variable" isoforms. We apologize for any confusion and have made this clarification by including it in the title of the figure.

> - *The concept of co-occurrence and results should be explained within the results section, to more clearly relate this concept to data and interpretations. Explanations are now found in the methods, but this did not improve the clarity of this otherwise very interesting aspect of the study.*

Thanks for your suggestion. We have incorporated some of the explanations from the methods section into the main text t, mainly for the concept of “co-occurence”.

> - *The claim that C-type Pcdhgs do not functionally influence connectivity is premature. Tests were limited to PcdhgC4, which has unique properties compared to the other 2 C-type isoforms (Garrett et al 2019 PMID: 31877124; Mancia et al PMID: 36778455). The text should be corrected to limit the conclusion to PcdhgC4, and not generally to C-type. The authors should test PcdhgC3 and PcdhgC5 isoforms.*

We have changed the claim for PcdhgC4, but not generally for C-type to better reflect our observation.

> - *The group generated a novel conditional Pcdh-alpha mouse allele using CRISPR methods, and state that there were no changes in synaptic connectivity in these Pcdh-alpha mutants. But this claim is premature. The Southern blots validate the*

> *targeting of the allele. But further validations are required to establish that this floxed allele can be efficiently recombined, disrupting Pcdha protein levels and function. Pcdha alleles have been validated by western blots and by demonstration of the prominent serotonergic axonal phenotype of Pcdha-KO (ie. Chen 2017 PMID: 28450636; IngEsteves 2018 PMID: 29439167).*

We have obtained a new set of qRT-PCR data that confirms the decreased expression of α-PCDH in Pcdha CKO mice. These data have been integrated into Figure 2-S2D.

> - *The Discussion would be strengthened by a deeper discussion of the findings to other cPcdh roles and studies, and of the limitations of the study. The idea that the Pcdhgs are influencing the rate of connectivity through a repulsion mechanism or synaptic formation (ie through negative interactions with synaptic organizers such as Nlgn - Molumby 2018, Steffen 2022) could be presented in a model, and supported by other literature.*

I would like to express my sincere appreciation to the reviewer for their invaluable comments and suggestions, which have led to extended discussions within our work. We have incorporated these suggestions into our paper to establish stronger connections between our observations and prior research findings.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> 1. *In Figure S6, the authors measured Euclidean distance from the single cell data to take account of the isoform expression levels in explaining diversity. However, it is hard to interpret the data without any control. The authors could measure the same value from a shuffled /randomized dataset for comparison (similarly to Fig 1F).*

We understand the reviewer's concern about the significance of the Euclidean distance analysis without an appropriate control. The inclusion of the Euclidean distance metric was initially a response to suggestions from other reviewers who recommended incorporating diverse methods for analyzing expression patterns among neurons.

In response to your valuable feedback, we have taken measures to address these concerns. We have introduced shuffled data for comparison, thus enhancing the meaningful context for interpreting the results derived from the Euclidean distance analysis.

> 1. *The authors need to clarify which cortical regions were used for electrophysiological experiments.*

Apologies for any confusion. To clarify, all recordings were conducted on neurons located in layer 2/3 of the neocortex without further discrimination. We have reinstated this information in both the main text and the methods section to ensure its clarity.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *There are still some issues that must be addressed.*

> 1. *The references to gamma protocadherin repulsion are not correct in context. A repulsive role of homophilic interaction has been inferred from certain knockout phenotypes in a subset of neurons (not in cortical neurons). However, repulsion has never been shown to follow gamma protocadherin engagement. The authors present no new evidence that their results are attributable to cellular repulsion at nascent synaptic contacts. The mechanism is unknown. The references to repulsion to explain their results should make it clear that this is one possible explanation, but it is not shown. Also some references in the text are not correct. For example, line 63/64: the results of Molumby and Steffen are not involving homophilic adhesion or repulsion, but rather a cis interaction with neuroligins. Those papers should not be discussed as involving repulsion as in the reference to Lefebvre 2012. Also line 268/269 "Together with previous findings (Molumby,,,Tarusawa), our observations solidify repulsion effect of g-PCDH on synapse formation. . .". This is not the case. Neither Molumby nor Tarusawa demonstrated any such repulsion.*

Thank you to the reviewer for pointing out the errors in our citations. We have made the necessary corrections to the citations and have also refined the descriptions of our observations to improve clarity and accuracy.

> 1. *The discussion of the results when C4 is overexpressed must also be greatly toned down. C4 is a strange C-type protein--it cannot get to the cell surface alone but relies on other cPCDHs for this, and its primary role is in preventing cell death. It is odd that the authors used this isoform to represent C-types. They should have used C3, which two recent papers showed have specific roles at some synapses (Meltzer et al 2023, Ginty lab) and in dendrite branching (Steffen et al 2023, Weiner lab) , or C5. It is entirely possible that just C4 has no role in synaptic matching--but C3 and C5 might. They should not conclude that the C-types have no such role and only A and B types do. That must be toned down (e.g., line 198/199, line 281).*

We acknowledge that using C4 to represent all three C-types (C3, C4, and C5) is not accurate. We have now modified the statement in the main text to rectify this.

> 1. *For the citation of Pcdhg flox/flox mice (line 126), Prasad et al., Development, 2008, Weiner lab, should also be cited as it fully characterized that line that was also used in Lefebvre et al 2008. They were co-published.*

Thank you for highlighting the missing citation, and we have now included it in the relevant section.

> 1. *the Pcdh alpha KO Mouse characterization is still insufficient. The authors must show that alpha expression is gone following introduction of Cre, either by RT-PCR using alpha constant domain primers, or an alpha antibody on Western. blot. The southern and off-target sequencing do not confirm that all alpha gene expression is gone.*

Thank you for your feedback. We have conducted the qRT-PCR analysis as per your suggestion. The results clearly indicate a substantial reduction in α-PCDH expression within the neocortex of Pcdha cKO mice. We have thoughtfully incorporated this data into the manuscript, and it is visually represented in the new panel of Figure 2-S2D. Your valuable input has contributed to enhancing the quality of our work, and we sincerely appreciate the opportunity to address this important aspect.

> 1. *I do not understand something in Figure 4-S1A. Why with 0% matching is synaptic connectivity so low? This is not the same as in Figure 3E. This has to be explained because it does suggest that overexpression of ANY isoforms can inhibit synapse formation, which is consistent with Molumby 2017, even though this paper says it is not just the levels but the isoform specificity.*

The panel of Fig.4-S1A illustrates the connection rate between neurons with the same color (icons in upper left), representing cells that express the same combination of γ-PCDHs (100% of similarity). The X-axis (0%, 11%, 33%, and 100%) reflects the similarity level between the 2 populations of cells (GFP and RFP).

> 1. *There are still issues with the English grammar in the paper. It is not too bad in the main text but someone should re-edit it. However, the figure legends are indeed much worse and truly must be edited professionally before they are acceptable.*

We apologize for our English writings in the paper. We have now polished most part of the manuscript, especially the parts for figure legends.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> - *This study has many strengths and innovative findings. Most comments above included suggestions to strengthen the paper. The overall message that Pcdhgs influence the rate of synaptic connectivity between nearby cells is compelling. How this Pcdhg-isoform-dependent process could influence synaptic specificity can be explored in a model in the discussion. But this study did not test a role in 'synaptic specificity'; this term should be removed from the title and line 81 in the intro.*

Thank you for your invaluable comments aimed at improving our paper. Regarding the title, we believe that "synaptic connectivity" might be a more suitable choice than "synaptic specificity." However, we're open to considering other alternatives as well.

> - *The manuscript and overall quality of the science will be improved by removing those sections that are not adequately investigated (ie.Pcdh-a cKO; PcdhgC4 is assessed but findings can't be extended to other C-type isoforms) and by outlining limitations of the study.*

We have modified the related claim mentioned in the main text.

> - *The studies negatively correlating between isoform matching and connectivity are not robust. Additional approaches are needed if the authors want to make this claim.*

In Figure 4E, we have implemented a bootstrapping method. Bootstrapping is a statistical technique falling under the broader category of resampling methods. It involves random sampling from the observed data with replacement, enabling the calculation of standard errors, confidence intervals, and supporting hypothesis testing.

> - *Statistical approaches should be described in methods, figure legends.*

More information about statistical approaches has been added in the figure legends.

- *The discussion should elaborate on the limitations of the study, and relate to other studies, including Lv et al 2022.*

We have added more discussion to relate our observations to previous findings.