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

Yi-Jun Zhu[1, 2, 3], Cai-Yun Deng[1], Liu Fan[2], Ya-Qian Wang[2], Hui Zhou[2], Hua-Tai Xu*[2]

1 Institute of Neuroscience and State Key Laboratory of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, 200031, China.

2 Lingang Laboratory, Shanghai Center for Brain Science and Brain-Inspired Intelligence Technology，Shanghai 201210, China.

3 University of Chinese Academy of Sciences, Beijing 100049, China.

* Correspondence should be addressed to H. -T. X. (xuht@lglab.ac.cn)

Abstract

During the process of synaptic formation, neurons require not only certain principles for selecting partners to form synapses but also additional mechanisms to avoid undesired cells. However, the means to get around unwanted partners remains largely unknown. In this study, we have identified that the combinatorial expression of clustered protocadherin gammas (γ-PCDHs) is essential in regulating such specificity in the mouse neocortex. Using single-cell sequencing from the 5-prime end, we revealed the combinatorial expression pattern of γ-PCDH variable isoforms in neocortical neurons. Whole-cell patch-clamp recordings demonstrated that increasing the similarity level of this combinatorial pattern in neurons reduced their synaptic connectivity. Our findings reveal a delicate molecular mechanism for assembling the neural network in the mouse neocortex.

Introduction

The specificity of synaptic connections is essential for the function 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), but how to achieve specificity at the microcircuit level remains to be determined. 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), suggesting cPCDHs function as a unique barcode for each neuron(Yagi, 2012). 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). However, the presence of γ-PCDH has been detected at the 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). The homophilic property of γ-PCDH allows them to promote exuberant dendritic complexity(Molumby et al., 2016). However, more evidence suggests that it

might be detrimental to synapse formation. Previous studies indicated that homophilic interactions mediated by large overlapping patterns of cPCDH isoforms on opposing cell surfaces might lead to intercellular repulsion(Rubinstein et al., 2015, Brasch et al., 2019, Honig and Shapiro, 2020, Lefebvre et al., 2012), in which the interaction with the neuroligin family may be involved(Molumby et al., 2017, Steffen et al., 2021).

Following this repulsion concept, the absence of γ-PCDH caused significantly more dendritic spines and inhibitory synapse densities in neocortical neurons(Molumby et al., 2017, Steffen et al., 2021). Consistently, neurons with overexpressed one of the γ-PCDH isoforms had significantly fewer dendritic spines from the same condition(Molumby et al., 2017). Furthermore, the absence of the clustered PCDH also increased local reciprocal neural connection between lineage-related neurons in the neocortex(Tarusawa et al., 2016, Lv et al., 2022), even though sister cells had more similar expression patterns of γ-PCDH isoforms(Lv et al., 2022).

Although γ-PCDH has these "contradictory" effects on dendrites' complexity and dendritic spines, it is detrimental to synapse formation in the forebrain. However, each neuron expresses multiple isoforms of γ-PCDH(Kaneko et al., 2006, Lv et al., 2022). What could be the impact of this combinatorial expression on synapse formation? Here, using 5-prime end (5'-end) single-cell sequencing, we revealed the diversified combinatorial expression of γ-PCDH isoforms in neocortical neurons. With multiple whole-cell patch-clamp recordings after the sequential *in utero* electroporation, we discovered that the combinatorial expression of γ-PCDHs plays a vital role in synaptic specificity to assemble the neural microcircuit in the mouse neocortex. This is achieved by allowing a neuron to choose which partners not to form a synapse with rather than choosing which ones to make synapses 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

90 determine the role of γ-PCDH in the neocortex, we examined their expression in the
91 neocortical neurons of postnatal mice. Previous studies have suggested that cPCDHs
92 were stochastically expressed in neurons(Hirayama et al., 2012, Toyoda et al., 2014).
93 Here, we applied a 5'-end single-cell RNA sequencing to identify γ-PCDH isoforms by
94 sequencing their variable exon (exon 1), where they differ from each other(Kohmura et
95 al., 1998, Wu and Maniatis, 1999). Since the second postnatal week is the critical stage
96 for synapse formation in the rodent neocortex(Lendvai et al., 2000, Holtmaat and
97 Svoboda, 2009), we chose postnatal day 11 (P11) to examine their expression. After
98 reverse transcription and cDNA amplification (Fig. 1-S1A, B), we split cDNA into two
99 parts: one for specific amplification of *pcdhg* mRNAs and the other for the 5' gene
100 expression library construction (Fig. 1-S1C). After the cluster analysis (Fig. 1-S2-4),
101 we collected 6505 neurons out of 17438 cells (Fig. 1A and Fig. 1-S1D). Neurons with
102 more than 10 UMI (cutoff>1 for each type of individual isoform) for all γ-PCDH (Fig.
103 1B, C) were further analyzed. We found that significantly more cells expressed "C-type"
104 isoforms (C3, C4, and C5) compared to other "variable" isoforms (Fig. 1D and Fig. 1-
105 S1E), which is consistent with a previous study(Toyoda et al., 2014). We performed
106 the pair-wise analysis on similarity between neurons in the single-cell expression
107 pattern of γ-PCDH variable isoforms, which revealed that most neocortical neurons had
108 distinct combinatorial expression patterns (Fig. 1E). Based on the variance
109 analysis(Wada et al., 2018) of fraction distribution of the number of the expressed
110 isoforms per cell for all neurons (Fig. 1F and Fig. 1-S5), we found a weak but significant
111 co-occurrence for the expression of γ-PCDH isoforms in most neurons (Fig. 1G). We
112 did not detect any apparent differences among clusters except for cluster 0, which had
113 much less expression (Fig. 1C) and no co-occurrence of γ-PCDH isoforms (Fig. 1G and
114 Fig. 1-S5). Since all of our electrophysiological recordings were carried out on
115 pyramidal neurons in the layer 2/3 of the neocortex, we analyzed the corresponding
116 cluster 7 in more detail. They also showed distinct expression patterns as a general
117 population of neocortical neurons (Fig. 1-S6). In summary, the data from 5'-end single-
118 cell sequencing reveal that γ-PCDHs are diversely expressed in most neocortical
119 neurons with a combinatorial pattern.

120

121 **The absence of γ-PCDHs led to an increase in local synaptic connectivity among**
122 **pyramidal neurons**

123 To investigate the function of γ-PCDH in the synaptic formation between neocortical
124 neurons, we conducted paired recordings on pyramidal neurons in the layer 2/3 of the
125 neocortex from γ-PCDH conditional knockout (cKO) mice. These mice were obtained
126 by crossing *Pcdhg*$^{flox/flox}$ mice(Lefebvre et al., 2008) with *Nex-cre* mice(Goebbels et al.,
127 2006), in which all variable and C-type γ-PCDH isoforms were specifically removed in
128 pyramidal neurons. Using multiple whole-cell patch-clamp recordings from cortical
129 slices of P9-32 mice, we measured the connectivity among nearby pyramidal cells (<50
130 μm between cell somas) in the layer 2/3 of the neocortex by the presence of evoked
131 monosynaptic responses (Fig. 2 and Fig. 2-S1). As shown in the sample traces in the
132 connectivity matrix from six recorded neurons (Fig. 2A and Fig. 2-S1), two neuronal
133 pairs (the neuronal pairs 4→3 and 5→6) exhibited unidirectional monosynaptic
134 connections (indicated by orange arrows), and one pair (the neuronal pair 1↔3) with
135 bidirectional connection (indicated by green arrows) out of 15 possibilities. Overall, we
136 found that the percentage of connected pairs was significantly higher in *Pcdhg* cKO
137 mice (20.2%, 103/511) than in wild-type (WT) mice (15.0%, 122/813). Due to their
138 potential different roles in the synaptic organizations in the neocortex(Douglas and
139 Martin, 2004), we performed an extra set of recordings in the layer 2/3 of the neocortex
140 from P10-20 mice to separate neuron pairs along their axes vertical *vs.* horizontal to the
141 pial surface in *Pcdhg* cKO mice. We found a more significant difference in the
142 connectivity for vertically aligned cells (18.3%, 94/515 in *Pcdhg* cKO mice *vs.* 11.2%,
143 73/651 in WT mice for vertically aligned neuron pairs; 12.2%, 27/221 in *Pcdhg* cKO
144 mice *vs.* 9.5%, 28/294 in WT mice for horizontally aligned neuron pairs) (Fig. 2C). We
145 also generated *Pcdha* cKO mice (Fig. 2-S2-4) and performed the same set of
146 experiments for vertically aligned neurons. We did not find a significant difference
147 (11.3%, 38/337 in *Pcdha* cKO mice *vs.* 14.3%, 26/182 in WT mice, Fig. 2D) in the
148 mice without α-PCDH. In a more detailed analysis of synaptic connections among
149 vertically aligned neurons in *Pcdhg* cKO mice, we found that the absence of γ-PCDH

150 expression appeared to significantly increase synapse formation between cells
151 separated vertically by 50 to 100 μm (24.0%, 50/208 in *Pcdhg* cKO mice *vs.* 9.6%,
152 20/208 in WT mice) (Fig. 2E). Furthermore, we found that the absence of γ-PCDH
153 increased synapse formation starting from P10 (16.1%, 32/199 in *Pcdhg* cKO mice *vs.*
154 8.7%, 20/230 in WT mice for P10-12; 21.6%, 22/102 in *Pcdhg* cKO mice *vs.* 12.8%,
155 29/227 in WT mice for P13-15), when chemical synapses between cortical neurons
156 become more detectable, and such tendency maintained for as long as measurements
157 were performed (up to P20) (Fig. 2F). Thus, γ-PCDH may play a role in preventing
158 synapse formation starting from the early stage of neural development.
159

160 **Overexpression of γ-PCDHs decreases local synaptic connectivity in the mouse**
161 **neocortex**

162 To further determine the effect of γ-PCDHs on synapse formation, we overexpressed
163 randomly selected single or multiple γ-PCDH isoforms tagged with fluorescent proteins
164 through *in utero* electroporation in mice. We then performed multiple whole-cell patch-
165 clamp recordings to address the effect of γ-PCDHs on pair-wise synaptic connectivity
166 for neurons in the layer 2/3 of the neocortex.
167 To confirm the overexpression effect, we performed the assays of RT-qPCR (Fig. 3A,
168 B), single-cell RT-PCR (Fig. 3C, D), and immunohistochemistry (Fig. 3-S1A to C).
169 The RT-qPCR assay revealed that the electroporated isoforms, but not the non-
170 overexpressed ones, from the surgical side indeed had significantly higher expression
171 levels than those from the contralateral side (Fig. 3A, B). To estimate how many
172 isoforms were expressed in a given neuron when multiple plasmids were electroporated,
173 we tagged first five isoforms with mNeongreen and the sixth one with mCherry (Fig.
174 3-S1A). Based on the rates of yellow and red-only cells in the total electroporated
175 neuron population (Fig. 3-S1B), the probability analysis revealed that each positive
176 neuron expressed an average of 5.6 types of isoforms (Fig. 3-S1C and the top panel of
177 D). This result was consistent with the data from single-cell RT-PCR analysis in which
178 an average of 5.3 types out of 6 electroporated isoforms was detected from 19 neurons
179 (Fig. 3C, D and the bottom panel of Fig. 3-S1D). The results from single-cell RT-PCR

180 also suggest that electroporation-introduced isoforms are the dominant ones in these
181 neurons (Fig. 3D).
182 Since the layer distribution of neocortical neurons plays a critical role in their synaptic
183 connection(He et al., 2015), we examined their positions relatively to the pial surface
184 after the overexpression. We found that overexpressing γ-PCDH isoforms did not affect
185 cell positions in the neocortex compared to the control plasmids (Fig. 3-S1E, F).
186 We then used multiple recordings to examine the impact of γ-PCDHs on synapse
187 formation. Overexpressing of one or six γ-PCDH variable isoforms in neurons
188 significantly reduced the synaptic connection rate among them (10.3%, 15/146 in
189 expressing control plasmids; 1.9%, 4/216 in overexpressing one isoform; and 4.4%,
190 8/181 in overexpressing six isoforms, red bars in Fig. 3E). However, overexpressing γ-
191 PCDH C4 did not affect the synaptic connection rate (11.3%, 12/106 in overexpressing
192 γ-PCDH C4, Fig. 3E). To exclude further the potential effect of C-types of γ-PCDHs'
193 impacts in synapse formation, we electroporated six variable isoforms in *pcdhg* cKO
194 mice. The overexpression also led to the reduction of the connection rate in *pcdhg* cKO
195 mice (6.1%, 12/198 in overexpressing six isoforms vs. 16.5%, 31/188 in expressing
196 control plasmids), similar to what we observed in WT littermate mice (6.6%, 8/121 in
197 overexpressing six isoforms vs. 16.5%, 18/109 in expressing control plasmids) (Fig.
198 3F). These observations reveal that overexpressing the variables, but not the C-types of
199 γ-PCDHs isoforms, decrease synaptic connectivity in the mouse neocortex.
200

201 **Combinatorial expression of γ-PCDHs regulates synaptic specificity in the mouse**
202 **neocortex**

203 To investigate the impact of the combinatorial expression pattern of γ-PCDH isoforms
204 on synapse formation, we conducted a sequential *in utero* electroporation at E14.5 and
205 E15.5 (Fig. 4A). By electroporating the same or different combinations of γ-PCDH
206 variable isoforms on these two days, we aimed to manipulate the expression similarity
207 level between two groups of neurons. Isoforms were randomly selected based on their
208 comparable expression, except for isoforms A5 and B8, which had low expression
209 levels according to the single cell sequencing data (Fig. 1D and Fig. 1-S1E). We

210 assigned their combinations to achieve similarity levels ranging from 0% (no overlap,
211 completely different) to 100% (complete overlap, all identical) (Fig. 4B). We used
212 fluorescent proteins mNeongreen and mRuby3(Bajar et al., 2016) to tag isoforms for
213 electroporation at E14.5 and E15.5 respectively (Fig. 4A). Subsequently, whole-cell
214 patch-clamp recordings were conducted on layer 2/3 neurons in the neocortex from
215 acute brain slices containing both green and red cells from P10-14 pups. Each set of
216 recordings included at least one mNeongreen^{+}, one mRuby3^{+}, and one nearby control
217 neuron without fluorescence (Fig. 4C). Our results showed that neurons with the same
218 color exhibited significantly lower connectivity (Fig. 4-S1A), which aligns with the
219 findings from single electroporation (as shown in Fig. 3E). In the complete overlap
220 (100%) group, the connectivity rate between neurons electroporated at different days
221 was also significantly lower compared to the control (3.7%, 5/134 in the complete-
222 overlap group; 12.5%, 19/151 in control pairs, 100% in Fig. 4D). However, as the
223 similarity levels decreased from 100% to 0%, the connectivity probabilities
224 progressively returned to the control level. The likelihood recovered to 8.4% (14/165)
225 for the pairs with a 33% similarity level and 10.3% (12/117) and 10.5% (12/114) for
226 pairs with 11% or 0% similarity level, respectively (Fig. 4D). These observations
227 suggested that it is the similarity level of γ-PCDH isoforms between neurons, but not
228 the absolute expression of the protein in individual neurons, that regulate the synaptic
229 formation. Similar to the single electroporation experiment (gray bars in Fig. 3E), there
230 were no significant changes in the synaptic connectivity between electroporated and
231 nearby control neurons (Fig. 4-S1B). Overall, our findings demonstrate a negative
232 correlation between the probability of forming synaptic connections and the similarity
233 level of γ-PCDH isoforms expressed in neuron pairs (Fig. 4E). These findings strongly
234 suggest that the diversified combinatorial expression of γ-PCDH isoforms is crucial for
235 synapse formation between nearby pyramidal cells. The more similar the γ-PCDH
236 isoforms patterns expressed in neurons, the lower the probability of forming synapses
237 between them (Fig. 4F).
238
239 **Discussion**

240 Homophilic proteins cPCDHs are strong candidates for promoting synaptic specificity
241 due to their combinatorial and stochastic expression pattern(Yagi, 2012, Kohmura et
242 al., 1998, Toyoda et al., 2014). Our 5'-end single-cell sequencing data provided insights
243 into the combinatorial expression pattern of γ-PCDH isoforms in neocortical neurons.
244 We further demonstrated the critical role of this diversity in synaptic specificity through
245 three lines of evidence. Firstly, the absence of γ-PCDH significantly increased
246 functional connectivity between adjacent neocortical neurons. Secondly,
247 electroporation-induced overexpression of identical γ-PCDH variable isoforms in
248 developing neurons markedly decreased their connectivity. Lastly, using sequential *in*
249 *utero* electroporation with different batchs of isoforms, we found that increasing the
250 similarity level of γ-PCDH variable isoforms expressed in neurons led to a reduction in
251 their synaptic connectivity. These findings suggest that γ-PCDHs regulate the
252 specificity of synapse formation by preventing synapse formation with specific cells,
253 rather than by selectively choosing particular targets. It remains to be studied whether
254 the diversified patterns of γ-PCDH isoforms expressed in different neurons have
255 additional coding functions for neurons beyond their homophilic interaction.
256 Previous studies by Molumby et al. demonstrated that neurons from the neocortex of
257 *pcdhg* knockout mice exhibited significantly more dendritic spines, while neurons
258 overexpressing a single γ-PCDH isoforms had fewer dendritic spines in (Molumby et
259 al., 2017). Our recordings are consistent with these previous morphology studies.
260 Tarusawa et al. revealed that the absence of the whole cluster of cPCDH affected
261 synaptic connections among lineage-related cells(Tarusawa et al., 2016). More recently,
262 overexpression of the C-type γ-PCDH isoform C3 also showed a negative effect on
263 synapse formation within a defined clone(Lv et al., 2022). In our study, we further
264 demonstrated an increased synaptic connection rate between adjacent pyramidal
265 neurons in the neocortex of *pcdhg* knockout mice, while it decreased between neurons
266 overexpressing single or multiple identical γ-PCDH variable isoforms. These effects
267 were not just limited to lineage-dependent cells. Together with previous
268 findings(Molumby et al., 2017, Tarusawa et al., 2016), our observations solidify the
269 repulsion effect of γ-PCDH on synapse formation among neocortical neurons. Some

270 subtle differences exist between our findings and previous recordings(Tarusawa et al.,
271 2016, Lv et al., 2022). Tarusawa et al. demonstrated that the connection probability
272 between excitatory neurons lacking the entire cPCDH cluster in the layer 4 was
273 approximately twofold higher at early stage P9-11, significantly lower at P13-16, and
274 similar to control cells at P18-20 compared (Tarusawa et al., 2016). In our study, *pcdhg*$^{-}$
275 $^{/-}$ pyramidal neuronal pairs consistently exhibited a higher connection probability from
276 P10 to P20. Two potential reasons could explain these differences. Firstly, we only
277 removed γ-PCDH instead of the entire cPCDH cluster, which includes α, β, and γ
278 isoforms. Secondly, γ-PCDH might have different functions in neurons located in the
279 layer 2/3 compared to the layer 4. Lv et al. found that overexpression of C-type γ-PCDH
280 C3 decreased the preferential connection between sister cells(Lv et al., 2022). However,
281 our study demonstrated that only variable, but not C-type isoforms had a negative
282 impact on synapse formation. This discrepancy might be attributed to the lineage
283 relationship, which could have an unknown impact on synapse formation. Since a single
284 neuron can express multiple isoforms, deleting all γ-PCDH isoforms might mask the
285 role of this combination. In this study, we manipulated the combinatorial expression
286 patterns of γ-PCDH isoforms in nearby neocortical neurons through sequential *in utero*
287 electroporation, expressing different batches of isoforms with adjustable similarities.
288 We observed that when two neurons expressed identical variable isoforms (100%
289 group), the likelihood of synapse formation between them was lowest. As the similarity
290 level between two cells decreased, with fewer shared isoforms, the connectivity
291 probability increased. The connectivity probability between neurons with different
292 variable isoforms (0% group) did not differ from the control pairs (without
293 overexpression). However, the connections between overexpressed and control neurons
294 were not affected under both 100% and 0% similarity conditions. These observations
295 suggest that the similarity level, rather than the absolute expression of the protein,
296 affects synapse formation between neurons.
297 Our findings also demonstrated that the overexpression of multiple γ-PCDH variable
298 isoforms in one neuron only affected its connection if the other neuron overexpressed
299 an identical combination of PCDHg isoforms. This indicates that the expression of

300 multiple PCDHg variable isoforms in a neuron assists in selecting its synapse partner,
301 emphasizing the essential role of diversified combinatorial expression of γ-PCDHs in
302 synaptic specificity in the mouse neocortex. Moreover, the overexpression of the γ-
303 PCDH C4 isoform did not affect synaptic connections, while overexpression of six
304 variable isoforms led to a reduction in the connection rate in *pcdhg* cKO mice. These
305 findings suggest that the variables, but not the C-type isoforms of γ-PCDHs, play
306 crucial roles in the synapse formation in the mouse neocortex.
307 While the absence of γ-PCDHs causes significantly more synaptic formation among
308 neocortical pyramidal neurons, evidence supports that their absence also leads to a
309 significant reduction of synapse formation in other brain regions. For example, mice
310 lacking γ-PCDHs exhibit fewer synapses in spinal cord interneurons(Weiner et al.,
311 2005). Knocking down γ-PCDHs causes a decline in dendritic spines in cultured
312 hippocampal neurons(Suo et al., 2012) and decrease the astrocyte-neuron contacts in
313 the coculture from the developing spinal cord(Garrett and Weiner, 2009). The absence
314 of γ-PCDHs leads to the reduction of dendritic arborization and dendritic spines in
315 olfactory granule cells(Ledderose et al., 2013). Additionally, immuno-positive signals
316 for γ-PCDHs are more frequently detected in mushroom spines than in thin
317 spines(LaMassa et al., 2021). Moreover, we observed that the absence of γ-PCDHs
318 impacted vertical-aligned neurons more than horizontal-situated pairs in the neocortex.
319 These findings suggest that different mechanisms may be employed by synapses in
320 different brain regions to achieve their specificity. Notably, different mechanisms have
321 already been proposed for targeting specific inhibitory neural circuits in the neocortex,
322 including "on-target" synapse formation for targeting apical dendrites and "off-target"
323 synapse selective removal for somatic innervations(Gour et al., 2021).
324 In summary, our data demonstrate that the similarity level of γ-PCDH isoforms between
325 neocortical neurons is critical for their synapse formation, with neurons expressing
326 more similar γ-PCDH isoforms patterns exhibiting a lower probability of forming
327 synapses between them. This suggests that the presence of γ-PCDHs enables
328 neocortical neurons to choose which neurons to avoid synapsing with, rather than
329 selecting specific neurons to form synapses with. Whether there are specific attractive

330 forces between cells to promote synaptic specificity remains an open question.

331

332 **Acknowledgements**

333 We thank Dr. Yifeng Zhang for providing the *Pcdhg flox/flox* mouse line and Dr. Zilong
334 Qiu for *Nex-cre* mouse line, Dr. Jun Chu for sharing plasmids with mNeongreen and
335 mRuby3, and other members in Xu lab for their discussions and technique supports. We
336 are grateful to Prof. Mu-ming Poo and Song-hai Shi for critical reading of the
337 manuscript. This work was supported by grants from the Training Program of the Major
338 Research Plan of the National Natural Science Foundation of China, grant No.
339 91632101; Strategic Priority Research Program of the Chinese Academy of Sciences,
340 grant No. XDB32010100; National Natural Science Foundation of China project
341 31671113; Shanghai Municipal Science and Technology Major Project，grant No.
342 2018SHZDZX05，the State Key Laboratory of Neuroscience and the Lingang
343 Laboratory, grant No. LG-GG-202201-01.

344

345 **Author contributions**

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

349

350 **References**

351 BAJAR, B. T., WANG, E. S., LAM, A. J., KIM, B. B., JACOBS, C. L., HOWE, E. S., DAVIDSON, M. W., LIN, M.
352 Z. & CHU, J. 2016. Improving brightness and photostability of green and red fluorescent
353 proteins for live cell imaging and FRET reporting. *Sci Rep,* 6**,** 20889. doi: 10.1038/srep20889.
354 BERNS, D. S., DENARDO, L. A., PEDERICK, D. T. & LUO, L. 2018. Teneurin-3 controls topographic circuit
355 assembly in the hippocampus. *Nature,* 554**,** 328-333. doi: 10.1038/nature25463.
356 BRASCH, J., GOODMAN, K. M., NOBLE, A. J., RAPP, M., MANNEPALLI, S., BAHNA, F., DANDEY, V. P.,
357 BEPLER, T., BERGER, B., MANIATIS, T., POTTER, C. S., CARRAGHER, B., HONIG, B. & SHAPIRO, L.
358 2019. Visualization of clustered protocadherin neuronal self-recognition complexes. *Nature,*
359 569**,** 280-283. doi: 10.1038/s41586-019-1089-3.
360 COURGEON, M. & DESPLAN, C. 2019. Coordination between stochastic and deterministic specification
361 in the Drosophila visual system. *Science,* 366doi: 10.1126/science.aay6727.
362 DOUGLAS, R. J. & MARTIN, K. A. 2004. Neuronal circuits of the neocortex. *Annu Rev Neurosci,* 27**,** 419-

363 51. doi: 10.1146/annurev.neuro.27.070203.144152.
364 DUAN, X., KRISHNASWAMY, A., DE LA HUERTA, I. & SANES, J. R. 2014. Type II cadherins guide assembly
365 of a direction-selective retinal circuit. *Cell,* 158**,** 793-807. doi: 10.1016/j.cell.2014.06.047.
366 ESUMI, S., KAKAZU, N., TAGUCHI, Y., HIRAYAMA, T., SASAKI, A., HIRABAYASHI, T., KOIDE, T., KITSUKAWA,
367 T., HAMADA, S. & YAGI, T. 2005. Monoallelic yet combinatorial expression of variable exons of
368 the protocadherin-alpha gene cluster in single neurons. *Nat Genet,* 37**,** 171-6. doi:
369 10.1038/ng1500.
370 FERNANDEZ-MONREAL, M., KANG, S. & PHILLIPS, G. R. 2009. Gamma-protocadherin homophilic
371 interaction and intracellular trafficking is controlled by the cytoplasmic domain in neurons.
372 *Mol Cell Neurosci,* 40**,** 344-53. doi: 10.1016/j.mcn.2008.12.002.
373 GARRETT, A. M. & WEINER, J. A. 2009. Control of CNS synapse development by {gamma}-
374 protocadherin-mediated astrocyte-neuron contact. *J Neurosci,* 29**,** 11723-31. doi:
375 10.1523/JNEUROSCI.2818-09.2009.
376 GOEBBELS, S., BORMUTH, I., BODE, U., HERMANSON, O., SCHWAB, M. H. & NAVE, K. A. 2006. Genetic
377 targeting of principal neurons in neocortex and hippocampus of NEX-Cre mice. *Genesis,* 44**,**
378 611-21. doi: 10.1002/dvg.20256.
379 GOUR, A., BOERGENS, K. M., HEIKE, N., HUA, Y., LASERSTEIN, P., SONG, K. & HELMSTAEDTER, M. 2021.
380 Postnatal connectomic development of inhibition in mouse barrel cortex. *Science,* 371doi:
381 10.1126/science.abb4534.
382 HE, S., LI, Z., GE, S., YU, Y. C. & SHI, S. H. 2015. Inside-Out Radial Migration Facilitates Lineage-
383 Dependent Neocortical Microcircuit Assembly. *Neuron,* 86**,** 1159-66. doi:
384 10.1016/j.neuron.2015.05.002.
385 HIRAYAMA, T., TARUSAWA, E., YOSHIMURA, Y., GALJART, N. & YAGI, T. 2012. CTCF is required for neural
386 development and stochastic expression of clustered Pcdh genes in neurons. *Cell Rep,* 2**,** 345-
387 57. doi: 10.1016/j.celrep.2012.06.014.
388 HOLTMAAT, A. & SVOBODA, K. 2009. Experience-dependent structural synaptic plasticity in the
389 mammalian brain. *Nat Rev Neurosci,* 10**,** 647-58. doi: 10.1038/nrn2699.
390 HONIG, B. & SHAPIRO, L. 2020. Adhesion Protein Structure, Molecular Affinities, and Principles of Cell-
391 Cell Recognition. *Cell,* 181**,** 520-535. doi: 10.1016/j.cell.2020.04.010.
392 JANG, S., LEE, H. & KIM, E. 2017. Synaptic adhesion molecules and excitatory synaptic transmission.
393 *Curr Opin Neurobiol,* 45**,** 45-50. doi: 10.1016/j.conb.2017.03.005.
394 KANEKO, R., KATO, H., KAWAMURA, Y., ESUMI, S., HIRAYAMA, T., HIRABAYASHI, T. & YAGI, T. 2006.
395 Allelic gene regulation of Pcdh-alpha and Pcdh-gamma clusters involving both monoallelic
396 and biallelic expression in single Purkinje cells. *J Biol Chem,* 281**,** 30551-60. doi:
397 10.1074/jbc.M605677200.
398 KOHMURA, N., SENZAKI, K., HAMADA, S., KAI, N., YASUDA, R., WATANABE, M., ISHII, H., YASUDA, M.,
399 MISHINA, M. & YAGI, T. 1998. Diversity revealed by a novel family of cadherins expressed in
400 neurons at a synaptic complex. *Neuron,* 20**,** 1137-51. doi: 10.1016/s0896-6273(00)80495-x.
401 KOSTADINOV, D. & SANES, J. R. 2015. Protocadherin-dependent dendritic self-avoidance regulates
402 neural connectivity and circuit function. *Elife,* 4doi: 10.7554/eLife.08964.
403 LAMASSA, N., SVERDLOV, H., MAMBETALIEVA, A., SHAPIRO, S., BUCARO, M., FERNANDEZ-MONREAL,
404 M. & PHILLIPS, G. R. 2021. Gamma-protocadherin localization at the synapse is associated
405 with parameters of synaptic maturation. *J Comp Neurol,* 529**,** 2407-2417. doi:
406 10.1002/cne.25102.

407 LEDDEROSE, J., DIETER, S. & SCHWARZ, M. K. 2013. Maturation of postnatally generated olfactory bulb
408 granule cells depends on functional gamma-protocadherin expression. *Sci Rep,* 3**,** 1514. doi:
409 10.1038/srep01514.
410 LEFEBVRE, J. L., KOSTADINOV, D., CHEN, W. V., MANIATIS, T. & SANES, J. R. 2012. Protocadherins
411 mediate dendritic self-avoidance in the mammalian nervous system. *Nature,* 488**,** 517-21.
412 doi: 10.1038/nature11305.
413 LEFEBVRE, J. L., ZHANG, Y., MEISTER, M., WANG, X. & SANES, J. R. 2008. gamma-Protocadherins
414 regulate neuronal survival but are dispensable for circuit formation in retina. *Development,*
415 135**,** 4141-51. doi: 10.1242/dev.027912.
416 LENDVAI, B., STERN, E. A., CHEN, B. & SVOBODA, K. 2000. Experience-dependent plasticity of dendritic
417 spines in the developing rat barrel cortex in vivo. *Nature,* 404**,** 876-81. doi:
418 10.1038/35009107.
419 LV, X., LI, S., LI, J., YU, X. Y., GE, X., LI, B., HU, S., LIN, Y., ZHANG, S., YANG, J., ZHANG, X., YAN, J., JOYNER,
420 A. L., SHI, H., WU, Q. & SHI, S. H. 2022. Patterned cPCDH expression regulates the fine
421 organization of the neocortex. *Nature,* 612**,** 503-511. doi: 10.1038/s41586-022-05495-2.
422 MOLUMBY, M. J., ANDERSON, R. M., NEWBOLD, D. J., KOBLESKY, N. K., GARRETT, A. M., SCHREINER, D.,
423 RADLEY, J. J. & WEINER, J. A. 2017. gamma-Protocadherins Interact with Neuroligin-1 and
424 Negatively Regulate Dendritic Spine Morphogenesis. *Cell Rep,* 18**,** 2702-2714. doi:
425 10.1016/j.celrep.2017.02.060.
426 MOLUMBY, M. J., KEELER, A. B. & WEINER, J. A. 2016. Homophilic Protocadherin Cell-Cell Interactions
427 Promote Dendrite Complexity. *Cell Rep,* 15**,** 1037-1050. doi: 10.1016/j.celrep.2016.03.093.
428 PHILLIPS, G. R., TANAKA, H., FRANK, M., ELSTE, A., FIDLER, L., BENSON, D. L. & COLMAN, D. R. 2003.
429 Gamma-protocadherins are targeted to subsets of synapses and intracellular organelles in
430 neurons. *J Neurosci,* 23**,** 5096-104. doi: 10.1523/JNEUROSCI.23-12-05096.2003.
431 RAWSON, R. L., MARTIN, E. A. & WILLIAMS, M. E. 2017. Mechanisms of input and output synaptic
432 specificity: finding partners, building synapses, and fine-tuning communication. *Curr Opin*
433 *Neurobiol,* 45**,** 39-44. doi: 10.1016/j.conb.2017.03.006.
434 RUBINSTEIN, R., THU, C. A., GOODMAN, K. M., WOLCOTT, H. N., BAHNA, F., MANNEPALLI, S., AHLSEN,
435 G., CHEVEE, M., HALIM, A., CLAUSEN, H., MANIATIS, T., SHAPIRO, L. & HONIG, B. 2015.
436 Molecular logic of neuronal self-recognition through protocadherin domain interactions. *Cell,*
437 163**,** 629-42. doi: 10.1016/j.cell.2015.09.026.
438 SERIZAWA, S., MIYAMICHI, K., TAKEUCHI, H., YAMAGISHI, Y., SUZUKI, M. & SAKANO, H. 2006. A
439 neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting.
440 *Cell,* 127**,** 1057-69. doi: 10.1016/j.cell.2006.10.031.
441 STEFFEN, D. M., FERRI, S. L., MARCUCCI, C. G., BLOCKLINGER, K. L., MOLUMBY, M. J., ABEL, T. &
442 WEINER, J. A. 2021. The gamma-Protocadherins Interact Physically and Functionally with
443 Neuroligin-2 to Negatively Regulate Inhibitory Synapse Density and Are Required for Normal
444 Social Interaction. *Mol Neurobiol,* 58**,** 2574-2589. doi: 10.1007/s12035-020-02263-z.
445 SUO, L., LU, H., YING, G., CAPECCHI, M. R. & WU, Q. 2012. Protocadherin clusters and cell adhesion
446 kinase regulate dendrite complexity through Rho GTPase. *J Mol Cell Biol,* 4**,** 362-76. doi:
447 10.1093/jmcb/mjs034.
448 SYTNYK, V., LESHCHYNS'KA, I. & SCHACHNER, M. 2017. Neural Cell Adhesion Molecules of the
449 Immunoglobulin Superfamily Regulate Synapse Formation, Maintenance, and Function.
450 *Trends Neurosci,* 40**,** 295-308. doi: 10.1016/j.tins.2017.03.003.

451 TAN, L., ZHANG, K. X., PECOT, M. Y., NAGARKAR-JAISWAL, S., LEE, P. T., TAKEMURA, S. Y., MCEWEN, J.
452 M., NERN, A., XU, S., TADROS, W., CHEN, Z., ZINN, K., BELLEN, H. J., MOREY, M. & ZIPURSKY, S.
453 L. 2015. Ig Superfamily Ligand and Receptor Pairs Expressed in Synaptic Partners in
454 Drosophila. *Cell,* 163**,** 1756-69. doi: 10.1016/j.cell.2015.11.021.
455 TARUSAWA, E., SANBO, M., OKAYAMA, A., MIYASHITA, T., KITSUKAWA, T., HIRAYAMA, T., HIRABAYASHI,
456 T., HASEGAWA, S., KANEKO, R., TOYODA, S., KOBAYASHI, T., KATO-ITOH, M., NAKAUCHI, H.,
457 HIRABAYASHI, M., YAGI, T. & YOSHIMURA, Y. 2016. Establishment of high reciprocal
458 connectivity between clonal cortical neurons is regulated by the Dnmt3b DNA
459 methyltransferase and clustered protocadherins. *BMC Biol,* 14**,** 103. doi: 10.1186/s12915-
460 016-0326-6.
461 TOYODA, S., KAWAGUCHI, M., KOBAYASHI, T., TARUSAWA, E., TOYAMA, T., OKANO, M., ODA, M.,
462 NAKAUCHI, H., YOSHIMURA, Y., SANBO, M., HIRABAYASHI, M., HIRAYAMA, T., HIRABAYASHI, T.
463 & YAGI, T. 2014. Developmental epigenetic modification regulates stochastic expression of
464 clustered protocadherin genes, generating single neuron diversity. *Neuron,* 82**,** 94-108. doi:
465 10.1016/j.neuron.2014.02.005.
466 WADA, T., WALLERICH, S. & BECSKEI, A. 2018. Stochastic Gene Choice during Cellular Differentiation.
467 *Cell Rep,* 24**,** 3503-3512. doi: 10.1016/j.celrep.2018.08.074.
468 WANG, X., WEINER, J. A., LEVI, S., CRAIG, A. M., BRADLEY, A. & SANES, J. R. 2002. Gamma
469 protocadherins are required for survival of spinal interneurons. *Neuron,* 36**,** 843-54. doi:
470 10.1016/s0896-6273(02)01090-5.
471 WEINER, J. A., WANG, X., TAPIA, J. C. & SANES, J. R. 2005. Gamma protocadherins are required for
472 synaptic development in the spinal cord. *Proc Natl Acad Sci U S A,* 102**,** 8-14. doi:
473 10.1073/pnas.0407931101.
474 WU, Q. & MANIATIS, T. 1999. A striking organization of a large family of human neural cadherin-like
475 cell adhesion genes. *Cell,* 97**,** 779-90. doi: 10.1016/s0092-8674(00)80789-8.
476 YAGI, T. 2012. Molecular codes for neuronal individuality and cell assembly in the brain. *Front Mol*
477 *Neurosci,* 5**,** 45. doi: 10.3389/fnmol.2012.00045.
478 YOGEV, S. & SHEN, K. 2014. Cellular and molecular mechanisms of synaptic specificity. *Annu Rev Cell*
479 *Dev Biol,* 30**,** 417-37. doi: 10.1146/annurev-cellbio-100913-012953.

480

481

482

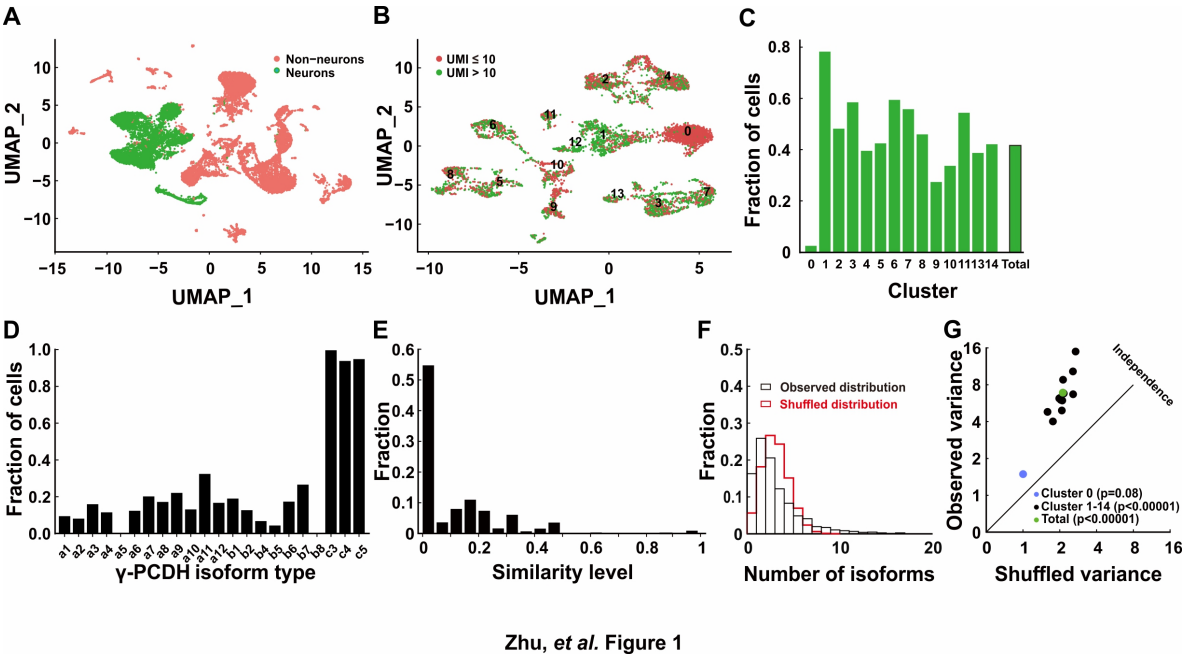
483

484

485

486

487 **Figures:**

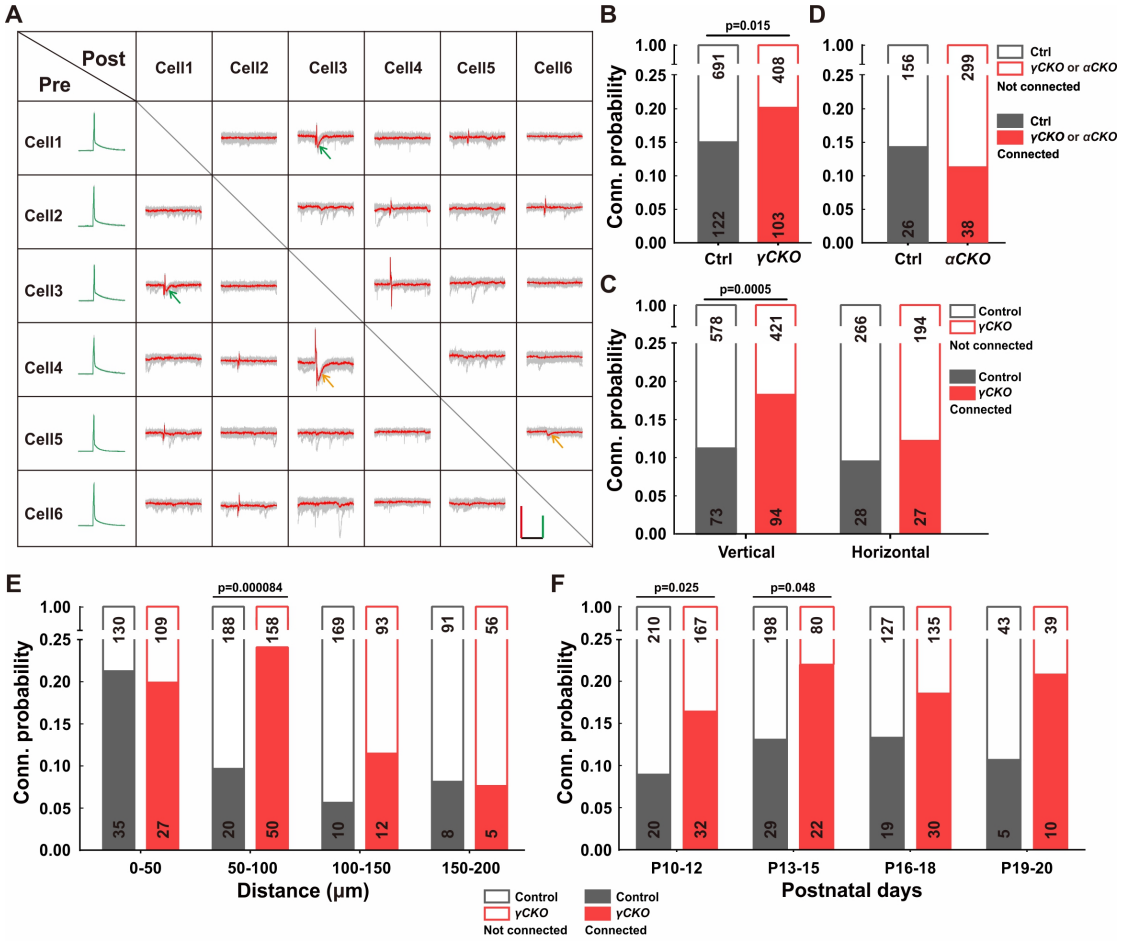


Zhu, *et al.* Figure 1

488 **Figure 1: Diversified expression of *pcdhg* isoforms in neocortical neurons.**

489 (**A**) UMAP analysis of all cells (n=17438) collected from 5'-end single-cell sequencing after the
490 cleanup and doublets removing. Neurons were labeled as green dots and non-neurons as red. (**B**)
491 UMAP clusters of all neurons further labeled by the UMI cutoff. Red dots represented cells with no
492 more than 10 total UMIs of *pcdhg* (n=3671), and green dots represented cells with more than 10
493 UMIs (n=2834). (**C**) Fractions of neurons with more than 10 UMIs in different clusters. (**D**)
494 Fractions of neurons expressing different *pcdhg* isoforms in the neocortex. (**E**) Fraction distribution
495 for different similarity levels in the combinatorial expression of *pcdhg* variable isoforms among
496 neurons. The similarity level was calculated as $\frac{A \cap B}{A \cup B}$. (**F**) The observed distribution (black) for the
497 fraction of cells with a various number of isoforms for all neurons. The shuffled distribution (red)
498 was generated by the hypothesis that all the isoform expressed stochastically. (**G**) The difference
499 between observed and shuffled variance of the fraction distribution of cells from all clusters.

500



Zhu *et al.*, Figure 2

Figure 2: The absence of γ-PCDH increased the synaptic connectivity.

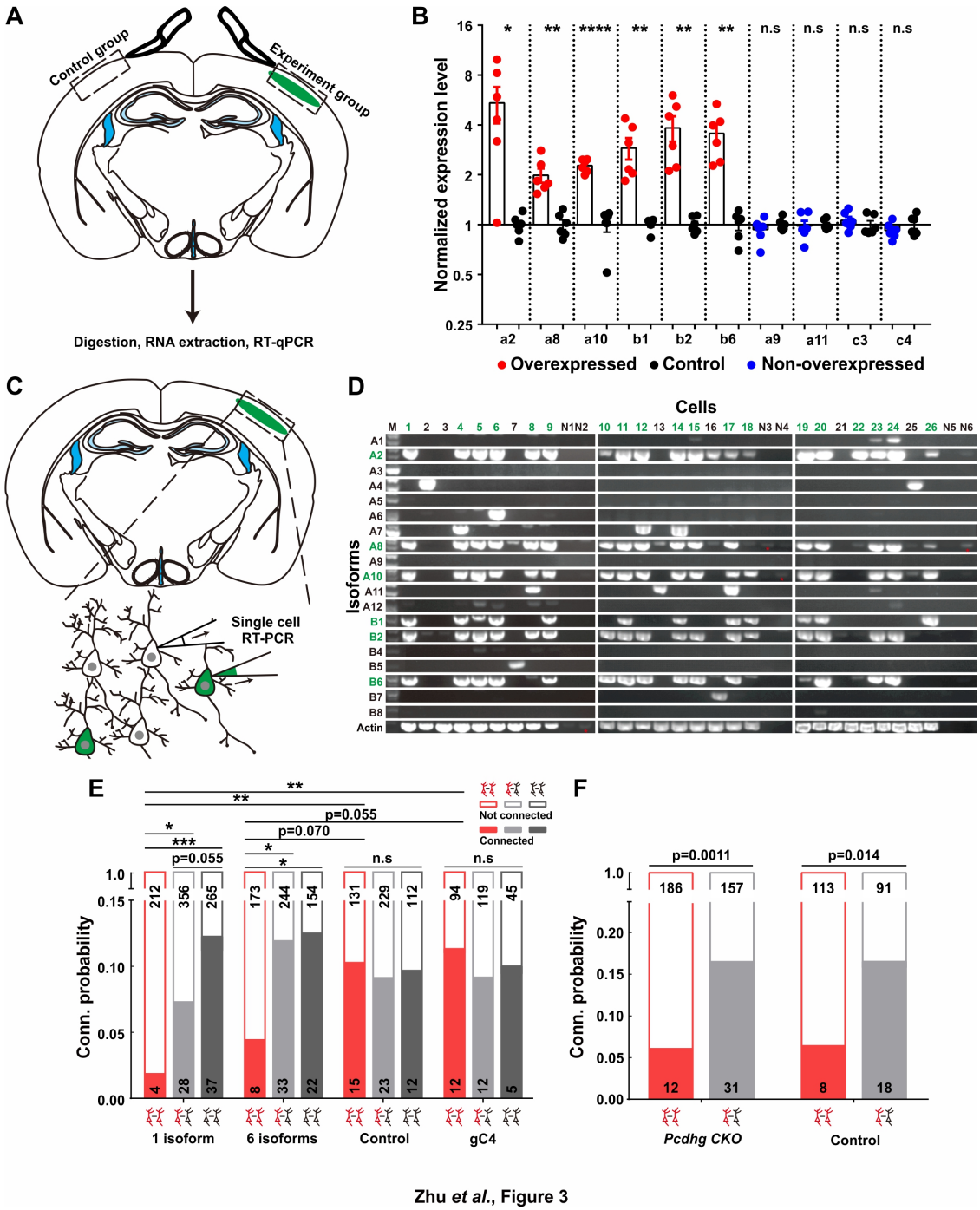
(**A**) Sample traces (red/green, average traces; gray, 10 original traces) of a multiple electrode whole-cell patch-clamp recording on six neurons in the layer 2/3 of the barrel cortex. Positive evoked postsynaptic responses were indicated by arrows. Orange/green arrows: unidirectional/ bidirectional synaptic connections. Scale bars, 100 mV (green), 50 pA (red), and 50 ms (black). (**B**) Connectivity probability among nearby pyramidal cells in the layer 2/3 of the barrel cortex in *pcdhg* cKO mice and their littermate WT controls. γKO: *pcdhg flox/flox*::*nex-cre* mice; Ctrl: *pcdhg*+/+::*nex-cre*. (**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. (**D**) Connectivity probability among vertically aligned pyramidal cells in the layer 2/3 of the barrel cortex in *pcdha* cKO mice and their littermate WT controls. αKO: *pcdha flox/flox*::*nex-cre* mice; Ctrl: *pcdha*+/+::*nex-cre* mice. (**E**) Connectivity probability among vertically aligned pyramidal cells along with the distance

513 between recorded pairs in *pcdhg* cKO mice or WT mice. (**F**) Developmental profiling of the

514 connectivity probability among vertically aligned neurons in *pcdhg* cKO mice or WT mice. Chi-

515 square tests were used in **B-F** to calculate the statistical difference.

516

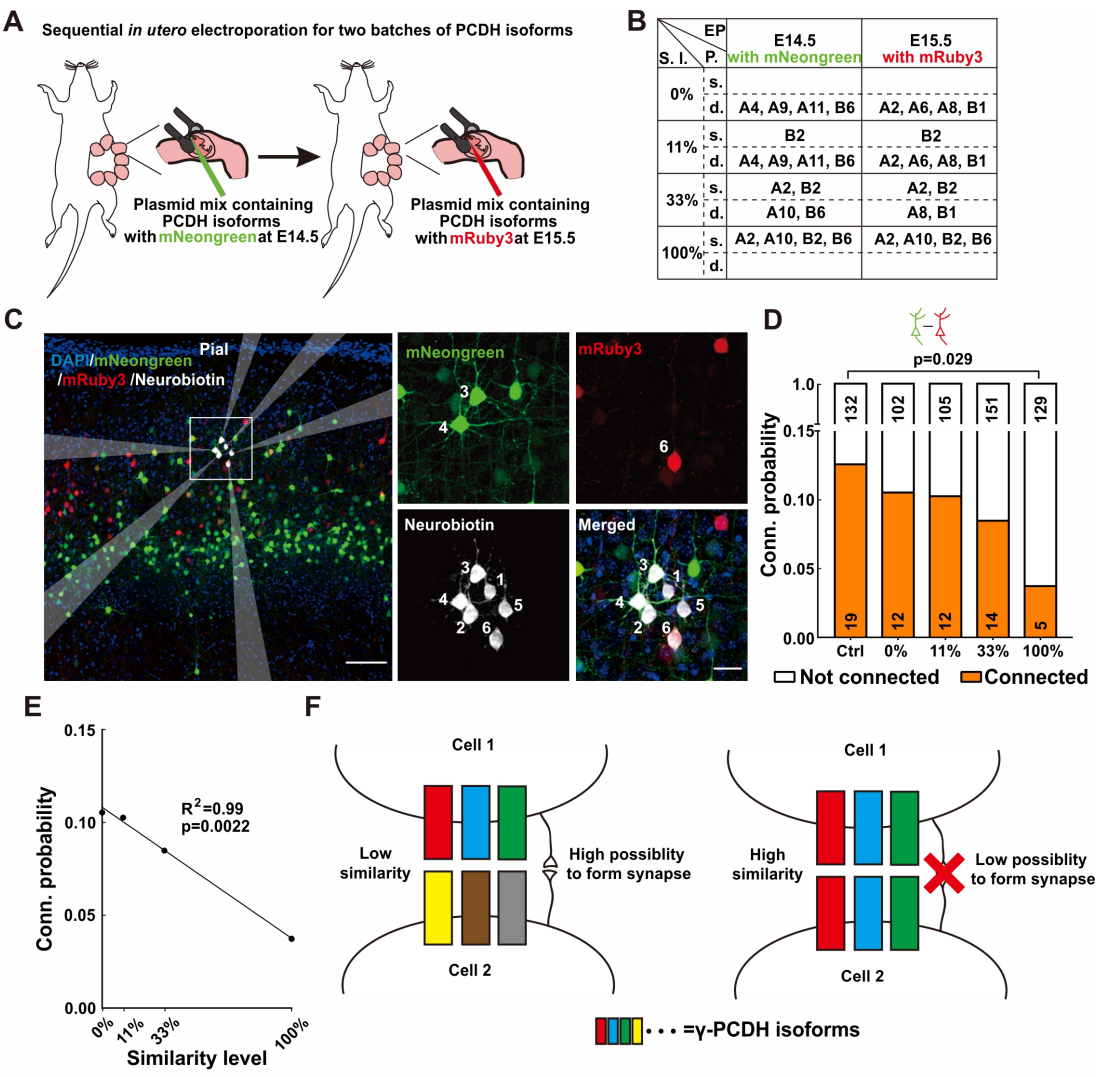


Zhu *et al.*, Figure 3

517 **Figure 3: Overexpressing identical variables, but not C-types γ-PCDHs in neurons decreased**

518 **their synaptic connectivity.**

519 (**A**) The sketch to show the brain regions used for RT-qPCR for both experimental and control
520 groups. (**B**) The overexpression levels were tested by RT-qPCR of electroporated regions.
521 Electroporated isoforms: red; control isoforms: blue. The contralateral sides were used as controls
522 shown in black. Student's *t test* was used, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. (**C**)
523 The diagram of extracting cells for the single-cell RT-PCR assay. (**D**) Single-cell RT-PCR for γ-
524 PCDH isoforms after the electroporation. Green numbers indicated neurons with fluorescence, and
525 black ones represented nearby neurons without fluorescence. N1-N6 were negative controls for the
526 PCR. Isoforms used in the electroporation were shown in green. Red stars indicate faint signals in
527 negative controls. (**E**) The effect of overexpressing one or six γ-PCDH isoforms on the synaptic
528 connectivity in WT mice. 1 isoform: *pcdhga2*; 6 isoforms: *pcdhga2*, *pcdhga8*, *pcdhga10*, *pcdhgb1*,
529 *pcdhgb2*, and *pcdhgb6*; gC4: *pcdhgC4*; Control: the plasmid vector without *pcdhg* insertion. (**F**)
530 The effect of overexpressing 6 γ-PCDH isoforms on the synaptic connectivity in *pcdhg* cKO mice.
531 6 used isoforms were same with the ones in (**E**). *pcdhg cKO*: *pcdhg* conditional knockout mice;
532 Control: WT littermates. Chi-square test and false discovery rate (FDR) correction were used to
533 determine the statistical differences between groups in (**E**) and (**F**).



Zhu *et al.*, Figure 4

Figure 4: Diversified γ-PCDHs were critical for synapse formation in cortical neurons. (**A**) The diagram of a sequential *in utero* electroporation at E14.5 and E15.5. (**B**) The overexpressed γ-PCDH isoforms in different sets of experiments to achieve different similarity levels between neurons. S.l., Similarity level; EP, Electroporation; P., plasmids mixture; s./d., same/different isoforms in two electroporations. (**C**) A sample image of recorded neurons after two times of electroporation at E14.5 with plasmids carrying mNeongreen and E15.5 with plasmids carrying mRuby3. In this sample, cells 3 and 4 are mNeongreen positive, cell 6 is mRuby3 positive, and cells 1, 2, and 5 are negative ones without fluorescence. Neurobiotin was added into the internal solution to indicate recorded neurons. The translucent arrows showed the positions of electrodes. Scale bar, left, 100μm, right, 25μm. (**D**) The connectivity probability for neuron pairs overexpressed different batch of γ-PCDHs isoforms (labeled with different fluorescence) after a sequential *in utero*

545 electroporation. Chi-square test and false discovery rate (FDR) correction were used to determine
546 the statistical difference. (**E**) The correlation between the similarity level of overexpressed γ-PCDHs
547 combination and the probability of synaptic connection rate (The source data were the same as d).
548 (**F**) The graph summary of γ-PCDHs' effect on synapse formation.