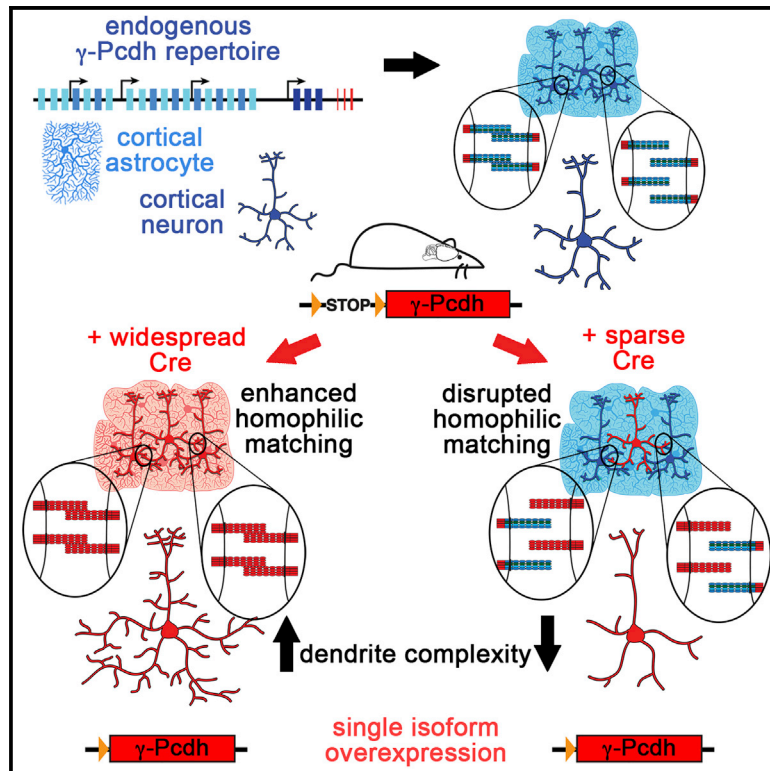


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Homophilic Protocadherin Cell-Cell Interactions Promote Dendrite Complexity

Graphical Abstract



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In Brief

Molumby et al. manipulate the expression of γ -Protocadherin adhesion molecule isoforms in the mouse cerebral cortex to demonstrate that the complexity of a neuron's dendritic arbor is promoted by local homophilic interactions with other neurons and with astrocytes.

Highlights

- Increasing γ -Pcdh homophilic matching promotes dendrite complexity in the cortex
- Inducing γ -Pcdh homophilic mismatching reduces dendrite complexity in the cortex
- Astrocytes regulate dendrite complexity cell non-autonomously via γ -Pcdh interactions
- γ -Pcdhs promote dendrite complexity through local interactions



Homophilic Protocadherin Cell-Cell Interactions Promote Dendrite Complexity

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SUMMARY

Growth of a properly complex dendrite arbor is a key step in neuronal differentiation and a prerequisite for neural circuit formation. Diverse cell surface molecules, such as the clustered protocadherins (Pcdhs), have long been proposed to regulate circuit formation through specific cell-cell interactions. Here, using transgenic and conditional knockout mice to manipulate γ -Pcdh repertoire in the cerebral cortex, we show that the complexity of a neuron's dendritic arbor is determined by homophilic interactions with other cells. Neurons expressing only one of the 22 γ -Pcdhs can exhibit either exuberant or minimal dendrite complexity, depending only on whether surrounding cells express the same isoform. Furthermore, loss of astrocytic γ -Pcdhs, or disruption of astrocyte-neuron homophilic matching, reduces dendrite complexity cell non-autonomously. Our data indicate that γ -Pcdhs act locally to promote dendrite arborization via homophilic matching, and they confirm that connectivity *in vivo* depends on molecular interactions between neurons and between neurons and astrocytes.

INTRODUCTION

The chemoaffinity hypothesis, that establishment of neuronal connectivity is aided by matching of diverse molecular cues between cells, has been very influential (Sperry, 1963; Zipursky and Sanes, 2010). A select group of immunoglobulin (Sidekicks, DSCAMs) and cadherin (Cadherin-8 and -9) cell adhesion molecules, as well as some semaphorins (Sema5A, 5B, and 6A), has been shown to specify dendritic targeting and/or formation of particular circuits (Duan et al., 2014; Krishnaswamy et al., 2015; Matsuoka et al., 2011a, 2011b; Williams et al., 2011; Yamagata and Sanes, 2008; Yamagata et al., 2002). Candidates for generating the extraordinary molecular diversity that would be required for cell-cell interactions throughout the brain include *Drosophila* Dscam, with over 38,000 homophilically interacting

isoforms generated by alternative splicing (mammalian DSCAM does not exhibit such diversity), and mammalian clustered protocadherins (α -, β -, and γ -Pcdhs), which can form thousands of distinct homophilically interacting *cis*-multimers (Garrett et al., 2012b; Hattori et al., 2007; Kise and Schmucker, 2013; Schreiner and Weiner, 2010; Thu et al., 2014; Wojtowicz et al., 2004; Rubinstein et al., 2015; Nicoludis et al., 2015).

In contrast to the chemoaffinity hypothesis as originally conceived (Zipursky and Sanes, 2010), fly Dscam controls the formation of dendritic arbors by mediating self-avoidance: homophilic isoneuronal matching between a neuron's own dendrites results in repulsion, preventing self-crossing and fasciculation and allowing proper dendritic field spread (Garrett et al., 2012b; Kise and Schmucker, 2013; Zipursky and Grueber, 2013). The 22 mammalian γ -Pcdhs, encoded by the *Pcdhg* gene cluster (Wu and Maniatis, 1999; Figure S1A), have been shown to play an analogous, cell-autonomous self-avoidance role in retinal starburst amacrine cells (SACs), preventing isoneuronal dendrite fasciculation while allowing heteroneuronal overlap of their planar dendritic arbors (Kostadinov and Sanes, 2015; Lefebvre et al., 2012). In other CNS populations, including cortical and hippocampal neurons, hypothalamic neurons, retinal ganglion cells, and spinal interneurons, the γ -Pcdhs have been shown to regulate neuronal survival, synapse maturation, and/or axon and dendrite morphology (Garrett et al., 2012a; Garrett and Weiner, 2009; Keeler et al., 2015; Lefebvre et al., 2008; Prasad et al., 2008; Prasad and Weiner, 2011; Su et al., 2010; Suo et al., 2012; Wang et al., 2002; Weiner et al., 2005). Several of these studies indicated that the γ -Pcdhs play these roles, at least in part, cell non-autonomously via heteroneuronal and neuron-astrocyte interactions (Su et al., 2010; Garrett and Weiner, 2009; Prasad and Weiner, 2011). This indicates that cell-autonomous self-avoidance is not the only mechanism through which the γ -Pcdhs regulate neuronal development.

Cell aggregation assays have found that the clustered Pcdhs exhibit strictly homophilic *trans*-interactions between their extracellular domains, with specificity mediated by extracellular cadherin domains 2 and 3 (EC2/3) (Schreiner and Weiner, 2010; Thu et al., 2014; Rubinstein et al., 2015). Based on size-exclusion chromatography and apparent molecular weight of complexes on western blots, Schreiner and Weiner (2010) proposed that the unit of γ -Pcdh *trans*-interaction was a *cis*-tetramer.



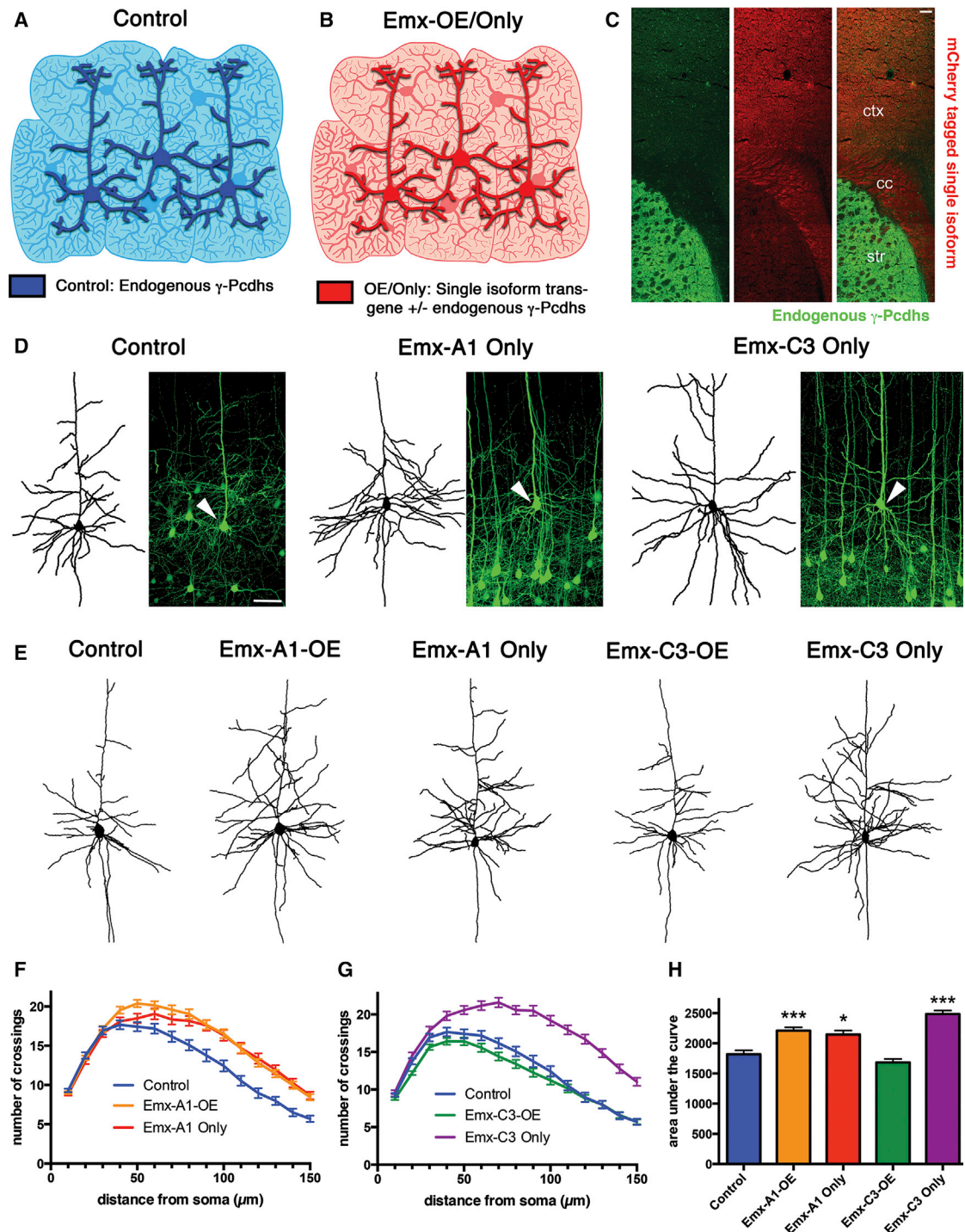


Figure 1. Increased Homophilic γ -Pcdh Matching Promotes Dendrite Arbor Complexity in the Cerebral Cortex

(A and B) Schematics represent the cortex of controls (A) and mice in which the A1-mCherry or C3-mCherry transgene is expressed in all primary neurons (thick-lined cells) and astrocytes (thin-lined cells) using *Emx1-Cre* (B).

(C) A photomontage of microscope fields showing a cryosection from an *Emx1-Cre;Pcdh^{fcen3/ftcn3};C3-mCherry* animal showing GFP-tagged endogenous γ -Pcdhs (green, striatum [str]) and exogenous C3-mCherry (red, cortex [ctx] and corpus callosum [cc]). Cre excision is limited to the cortex, and there is no leaky expression from the non-excised transgene.

(D and E) Representative images (D) and traces (D and E) show *Thy1-YFPH*-labeled layer V pyramidal neurons in the indicated genotypes; arrowheads indicate traced neurons in (D).

(legend continued on next page)

Subsequent crystal structure data indicated, rather, that the clustered Pcdhs form zipper-like assemblies of *cis* homo- or heterodimers interacting in an antiparallel fashion across cell-cell junctions (Rubinstein et al., 2015). All of these studies assaying cell aggregation in non-adhesive K562 cells are in agreement that the introduction of a single mismatching Pcdh isoform into a cell that otherwise matches surrounding cells in Pcdh repertoire is sufficient to disrupt cell aggregation (Schreiner and Weiner, 2010; Thu et al., 2014; Rubinstein et al., 2015). Though cell aggregation in K562 cells is an adhesive, rather than repulsive, phenomenon, neurons may have intracellular signaling pathways that convert an initial γ -Pcdh adhesive event into a repulsive cell response. Such a repulsive response can be inferred from the aberrant dendrite fasciculation and self-crossing in SACs and cerebellar Purkinje cells lacking the γ -Pcdhs (Lefebvre et al., 2012; Kostadinov and Sanes, 2015). However, earlier studies in fibroblast cell lines (Frank et al., 2005) and hippocampal neurons (Fernández-Monreal et al., 2009) also showed that exogenous γ -Pcdhs can aggregate at stable cell-cell junctions and sites of axon and dendrite contact. This suggests that homophilic Pcdh *trans*-interactions between cells also might mediate adhesion or other positive signaling relevant to the growth of some developing neuronal subsets.

We showed previously that loss of the γ -Pcdhs throughout the developing cerebral cortex results in greatly reduced complexity of pyramidal neuron dendrite arbors in the absence of aberrant fasciculation (Garrett et al., 2012a); a similar phenotype also has been observed in hippocampal neurons lacking the α -Pcdhs (Suo et al., 2012). Because the dendrites of pyramidal neurons extend in all directions and thus, during development, will contact many surrounding γ -Pcdh-expressing neurons and astrocytes, a self-avoidance role seems less likely to explain these phenotypes. We tested the hypothesis that homophilic γ -Pcdh *trans*-interactions between neurons, and between neurons and astrocytes, provide a positive signal for dendritic growth and complexity by manipulating γ -Pcdh repertoire in discrete cell types. Across multiple *in vivo* and *in vitro* experiments, we consistently found that increasing homophilic matching between cells leads to greater dendrite complexity, while decreasing homophilic matching between cells leads to reduced dendrite complexity. Our results indicate that, in the cerebral cortex, homophilic γ -Pcdh interactions between cells provide a positive cue that promotes dendrite arborization.

RESULTS

Increasing γ -Pcdh Homophilic Matching Results in Greater Dendrite Complexity in the Cortex *In Vivo*

In the mouse *Pcdhg* cluster, 22 large variable exons encoding six EC domains, a transmembrane domain, and an \sim 90-amino acid cytoplasmic domain are each expressed from their own promoters and spliced to three constant exons that encode a shared 125-amino acid C-terminal domain (Wu and Maniatis, 1999; Fig-

ure S1A). Single-cell RT-PCR (Kaneko et al., 2006; Yagi, 2012), *in situ* hybridization (Wang et al., 2002; Zou et al., 2007), and transcriptome (Zhang et al., 2014) data indicate that each neuron and astrocyte expresses a fraction of the *Pcdhg* isoforms: a few of the 19 stochastically and monoallelically expressed A and B subfamily members plus two or three broadly and biallelically expressed C subfamily members (Figure S1A). Homophilic interaction between γ -Pcdhs on two adjacent cells depends on the degree of isoform repertoire matching (Schreiner and Weiner, 2010; Thu et al., 2014; Rubinstein et al., 2015).

We first sought to increase homophilic γ -Pcdh isoform matching between cells by using *Emx1-Cre*, which is active in all excitatory neurons and astrocytes in the cortex (Garrett et al., 2012a; Gorski et al., 2002), to broadly activate expression of mCherry-tagged γ -Pcdh-A1 or -C3 transgenes (Figures S1B and S1C; previously described and utilized in Lefebvre et al., 2012 and Kostadinov and Sanes, 2015). We generated mice expressing these single-isoform transgenes, either in the presence (A1 or C3 overexpression [OE]) or absence (A1 or C3 only) of endogenous γ -Pcdhs, by additionally employing the *Pcdhg*^{l^{con}3} conditional knockout allele (Figures 1A–1C; Figure S1A; Garrett et al., 2012a; Prasad et al., 2008). The A1 isoform is representative of the sparsely expressed genes situated at the differentially methylated 5' end of the *Pcdhg* cluster, whereas the widely expressed C3 isoform is encoded by a gene situated at the hypomethylated 3' end of the cluster (Kaneko et al., 2006; Zou et al., 2007; Zhang et al., 2014; Toyoda et al., 2014; Lein et al., 2007; Allen Mouse Brain Atlas: <http://mouse.brain-map.org>). Pyramidal neuron dendrite arbors labeled by the addition of the *Thy1-YFPH* allele (Feng et al., 2000) were traced at 5 weeks of age from confocal stacks of somatosensory cortex, and Sholl analysis was performed to measure dendrite complexity. We confirmed that the exogenous A1-mCherry and C3-mCherry proteins can co-immunoprecipitate endogenous γ -Pcdhs, indicating they integrate into multimers as expected (Figure S1D). Additionally, as expected from previous analyses of mice harboring duplications or deletions of the *Pcdha* or *Pcdhg* clusters (Noguchi et al., 2009; Chen et al., 2012), we confirmed that expression of exogenous A1-mCherry did not significantly affect levels of several representative endogenous γ -Pcdh proteins (Figure S1E).

In the *Emx1-Cre* A1-Only and C3-Only single-isoform cortex, all cells should match and thus be able to interact homophilically (Figure 1B). We found that neurons traced from *Emx1-Cre* A1- and C3-Only cortex exhibited greatly enhanced dendrite arbor complexity compared to controls (Figures 1D–1H; Figures S1F–S1H). In the A1-OE cortex, homophilic matching should be significantly increased due to the low, scattered expression of the endogenous A1 isoform (Zhang et al., 2014; Zou et al., 2007). Consistent with this, we observed a similar significant increase in arbor complexity (as measured by Sholl analysis) in A1-OE cortex (Figures 1E, 1F, and 1H). For A1-OE and A1-Only neurons, this reflected a significant increase in both the number

(F–H) Sholl analysis graphs show dendrite crossings of circles drawn at 10- μ m radius intervals from the soma (F and G) and area under the curve graph (H) for indicated genotypes. *n* = 60 (control, A1-Only, and C3-Only) or 120 (A1-OE and C3-OE) neurons per genotype; scale bars, 50 μ m (C) and 60 μ m (D); **p* < 0.05 and ****p* < 0.001.

See also Figure S1.

of branchpoints and the total dendrite length (Figures S1F and S1G), without any increase in the average length of each branch (Figure S1H); this latter measure was, however, also significantly increased in C3-Only neurons, suggesting that C3 may play an isoform-specific role in dendritic patterning (Figure S1H). Because most, if not all, cells already highly express the endogenous C3 isoform (Kaneko et al., 2006; Yagi, 2012; Zhang et al., 2014), activation of the C3-mCherry transgene on a wild-type background in the C3-OE cortex would not be expected to shift homophilic matching drastically. Indeed, dendrite arbor complexity in C3-OE cortex was similar to that in controls (Figures 1E, 1G, and 1H; Figures S1F–S1H). Importantly, the normal dendrite arborization observed in C3-OE cortex indicates that a simple increase in total γ -Pcdh levels alone (documented in Figure S1C) cannot account for the enhanced arborization seen in A1-OE, A1-Only, and C3-Only cortex, in which homophilic matching between cells is expected to be increased.

Inducing γ -Pcdh Homophilic Mismatching Results in Reduced Dendrite Complexity in the Cortex In Vivo

Next we asked whether inducing γ -Pcdh homophilic mismatching in the cortex in vivo would reduce dendrite arbor complexity. We utilized the *Sim1-Cre* line (Balthasar et al., 2005), which is active in scattered (<4%) layer V/VI pyramidal neurons in the cortex (Figure 2C; Figure S2E), to knock out endogenous γ -Pcdhs (Figure 2A), or to express the single isoform γ -Pcdh transgenes (Figure 2B), in scattered, isolated neurons (labeled with a tdTomato Cre-reporter allele; Figure 2C). As expected from our prior work (Garrett et al., 2012a), isolated knockout neurons exhibited severely reduced dendrite arborization compared to controls (Figures 2D–2F; Figures S2A–S2C). Strikingly, expressing the A1-mCherry transgene in isolated layer V/VI neurons, either with (Sim-A1-OE) or without (Sim-A1-Only) endogenous γ -Pcdhs, resulted in significant dendrite arborization defects indistinguishable from those of knockout neurons (Figures 2D–2F; Sim-A1-OE and Sim-A1-Only results are combined here, as they were statistically indistinguishable [Figure S2F]). This reduced dendrite complexity was due to severe reductions in the number of branchpoints and total dendrite length, with a mild (but statistically significant) reduction in average branch length as well (Figures S2A–S2C).

As we would predict given the ubiquitous expression of the endogenous C3 isoform and the *Emx1-Cre* results (Figure 1), dendrite arborization of isolated neurons expressing the C3-mCherry transgene was much less affected, though their arbors were still significantly less complex than control (Figures 2D–2F; Figures S2A–S2C). Comparing these *Sim1-Cre* results with the *Emx1-Cre* results (Figure 1) clearly shows that expression of a single γ -Pcdh isoform can be sufficient for a cortical neuron to form a complex dendrite arbor, but only if surrounding cells express the same isoform. In the *Emx1-A1-Only* cortex, neurons exhibited exuberant dendrite arborization above control levels, likely because of perfect homophilic matching with surrounding cells (Figures 1D–1F and 1H). In the *Sim1-A1-Only* cortex, however, neurons activating expression of the exact same transgene failed to arborize normally, because in this case few of the surrounding cells were likely to express endogenous γ -Pcdh-A1 and γ -Pcdh homophilic matching between cells was disrupted

(Figures 2D–2F). It is thus unlikely that our data could reflect isoneuronal dendrite self-avoidance; the dendrites of both *Emx1-A1-Only* and *Sim1-A1-Only* neurons will match themselves perfectly, and yet the results in these two compound transgenics are precisely opposite. To ensure that our results did not reflect changes in isoneuronal self-avoidance within *Sim1-Cre* cortical neurons, we directly quantified the number of dendritic self-crossings from three-dimensional confocal stacks. Sparsely labeled tdTomato+ layer V/VI cortical neurons exhibited few, if any, instances of dendrite self-crossings or fasciculation, and the number of these did not vary between control neurons and any knockout or transgene-expressing neurons (Figure S2D).

Evidence for Local Control of Dendrite Complexity via γ -Pcdh Homophilic Interaction

An intriguing question is whether the effect of γ -Pcdhs on dendrite arborization is local (i.e., specific to the homophilically matching or mismatching dendritic branch) or global (e.g., involving a signaling change throughout the neuron). We took advantage of the varied activity pattern of the *Sim1-Cre* allele to address this question. As discussed above, in layers V and VI of the somatosensory cortex, *Sim1-Cre* is active in only a few, scattered neurons (Figures 2C, 3A, and 3B, purple boxes); these are the neurons that were analyzed in Figure 2 and Figure S2. However, within layer IV, there is an intermittent denser band of *Sim1-Cre*/tdTomato+ cells (Figures 3A and 3B, white boxes), none of which are labeled by the layer V-restricted *Thy1-YFPH* transgene (Figures 3A and 3B). The basal and oblique dendrite arbors of *Sim1-Cre*/tdTomato+ layer V/VI neurons, analyzed in Figure 2, are situated well below this intermittent band in layer IV (Figures 3A and 3B, purple boxes) and thus make no contact with it. However, this is not the case for the more superficially situated *Thy1-YFPH*-labeled neurons: In *Sim1-A1-OE* or *Sim1-A1-Only* cortex, their basal dendrite arbor (Figures 3A and 3B, yellow boxes) will contact neurons that, as they do, express only endogenous repertoires of γ -Pcdh isoforms; however, their oblique dendrites, some of which project off the apical branch as it passes through the *Sim1-Cre*/tdTomato+ layer IV band (Figures 3A and 3B, white boxes), will encounter many cells that express the A1 transgene, and thus mismatch.

If this layer IV-restricted mismatching globally affected dendrite arborization, we would expect YFP+ neurons to exhibit reduced complexity throughout their entire arbor. If, on the other hand, γ -Pcdh matching or mismatching acts locally, we would expect only the oblique dendrites of YFP+ neurons (Figures 3A and 3B, white boxes) to exhibit reduced complexity, with the basal dendritic arbor (Figures 3A and 3B, yellow boxes) remaining unaffected. Sholl analyses clearly support the latter possibility: Oblique dendritic arbors of YFP+ neurons were specifically reduced within the mismatching layer IV band (Figure 3C, red shading), while basal arbors of the same neurons were indistinguishable from controls (Figure 3D), resulting in a significantly decreased oblique/basal complexity ratio in YFP+ neurons within the *Sim1-A1-OE/Only* cortex (Figure 3E shows combined results, which were identical [Figure S3]). We note that a local rather than global effect also is supported by the fact that the scattered layer V/VI *Sim1-Cre*/tdTomato+ neurons (Figures 3A and 3B, purple boxes) exhibit severely reduced arborization

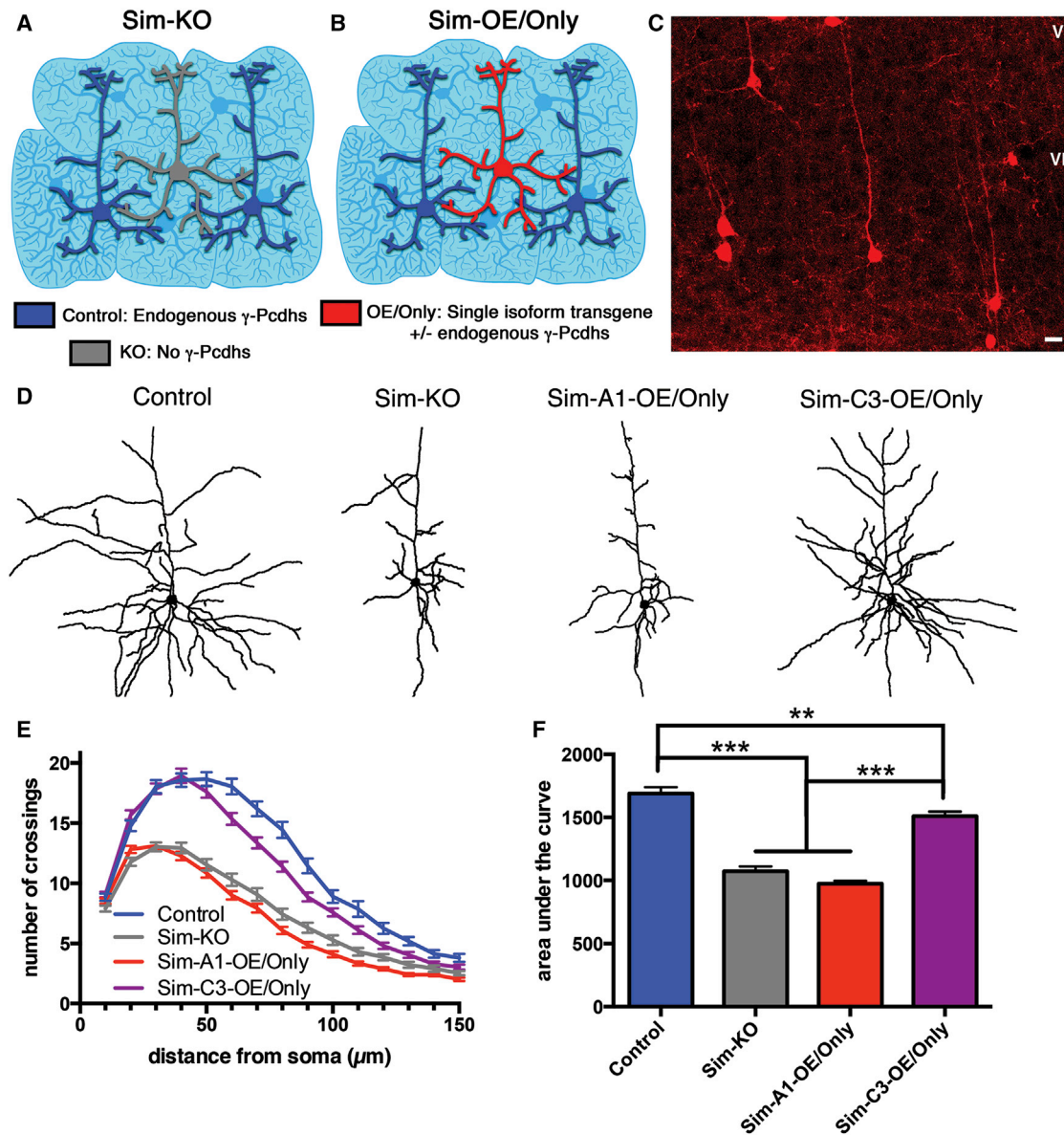


Figure 2. Inducing γ -Pcdh Isoform Mismatching Reduces Dendrite Arbor Complexity in the Cerebral Cortex

(A and B) Schematics represent the cortex of mice in which endogenous γ -Pcdhs are knocked out (gray, Sim-KO; A) and/or the A1-mCherry or C3-mCherry transgene is expressed (red, Sim-OE/Only; B) in scattered deep-layer pyramidal neurons using *Sim1-Cre*.

(C) Confocal image shows isolated tdTomato+ layer V/VI neurons in a *Sim1-Cre;Ai14-tdTomato* cortex.

(D) Representative traces show tdTomato+ layer V/VI pyramidal neurons of the indicated genotypes.

(E and F) Sholl (E) and area under the curve (F) graphs of tdTomato+ layer V/VI pyramidal neurons of the indicated genotypes. Note that results shown here are pooled for OE and Only mice, as the results were identical (Figure S2). $n = 58$ –60 (controls and Sim-KO), 97 (Sim-A1), or 79 (Sim-C3) neurons; scale bar, 20 μm (C); ** $p < 0.01$ and *** $p < 0.001$. See also Figure S2.

throughout their mismatching basal and oblique arbors (Figures 2D–2F), despite the fact that their apical shaft passes through the layer IV band, which contains *Sim1-Cre*/tdTomato+ neurons that match them homophilically. These results strongly imply that γ -Pcdhs regulate dendrite arborization locally via discrete neuron-neuron contacts and/or through spatially contained signaling events.

Astrocytes Regulate Neuronal Dendrite Arborization Cell Non-autonomously via γ -Pcdh Homophilic Interactions

Since our results thus far revealed the importance of homophilic γ -Pcdh interactions between a neuron and its surrounding cells, we next asked whether astrocytic γ -Pcdhs play a role in promoting the proper formation of dendritic arbors. Astrocyte

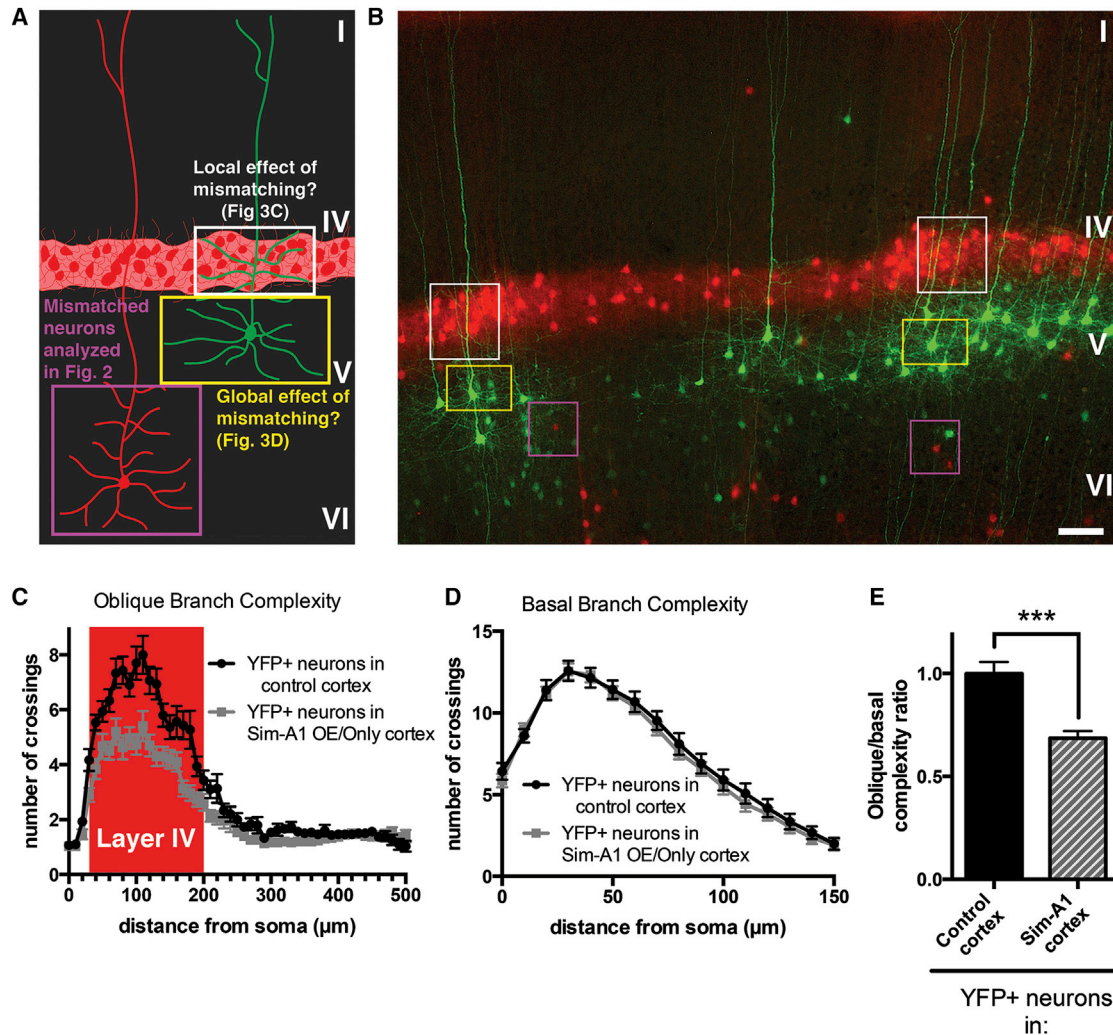


Figure 3. γ -Pcdhs Control Dendrite Complexity through Local Homophilic Interactions

(A and B) Schematic diagram (A) and low-magnification confocal image (B) of a *Sim1-Cre;Thy1-YFPH;Ai14-tdTomato;A1-mCherry* cortex. Regions marked by different color boxes correspond between (A) and (B). Purple boxes mark the basal and oblique arbors of isolated tdTomato⁺ layer V/VI neurons, which were analyzed in Figure 2. Because these neurons overexpress the A1-mCherry transgene while their surrounding cells are wild-type, their dendrites experience mismatches that lead to greatly reduced arborization (Figure 2). Yellow boxes mark the basal arbors of YFP⁺ layer V neurons, which are analyzed in (D). These neurons express endogenous γ -Pcdh repertoires, as do the immediately surrounding cells, so their basal arbors should exhibit normal complexity. White boxes mark the oblique arbors of YFP⁺ layer V neurons, which are analyzed in (C) (red shaded region). These oblique arbors branch off the apical shaft within layer IV, which contains a band of denser *Sim1-Cre* activity and, thus, A1-mCherry expression. These YFP⁺ oblique dendrites express endogenous γ -Pcdh repertoires, but locally encounter layer IV neurons overexpressing the mismatching A1-mCherry isoform.

(C and D) Sholl analyses show the oblique (mismatching, C) and basal (normal matching, D) arbors of YFP⁺ neurons in control (black lines) and Sim-A1-OE or Sim-A1-Only cortex (gray lines; OE and Only results are pooled here, as they were identical; see Figure S3).

(E) The calculated ratio of oblique/basal dendrite complexity (oblique area under the curve divided by basal area under the curve; control normalized to 1) confirms a significant localized loss of complexity only within the oblique arbor of YFP⁺ neurons in the Sim-A1 cortex. $n = 28$ YFP⁺ neurons per genotype; scale bar, 100 μm ; *** $p < 0.001$.

processes, which contain γ -Pcdhs and localize them to the sites of neuronal contact (Garrett and Weiner, 2009), are extensive in the cortical neuropil. Restricted mutation of the γ -Pcdhs from astrocytes in the developing spinal cord had no effect on their survival, morphology, or release of thrombospondin, but the loss of astrocyte-neuron γ -Pcdh contacts resulted in delayed synapse development (Garrett and Weiner, 2009). We utilized a *Gfap-Cre* line (77.6; Garcia et al., 2004) that, unlike other lines,

reproducibly restricts excision to astrocytes in the cortex (Figure 4C; Figures S4A and S4B) to generate mice in which wild-type neurons were surrounded by γ -Pcdh-knockout astrocytes (Figure 4A). Compared to controls, neurons surrounded by knockout astrocytes exhibited significantly reduced dendrite complexity (Figures 4D–4F; reductions in branchpoints and total dendrite length, but not average branch length [Figures S4G–S4I]). This occurred in the absence of any changes in the number

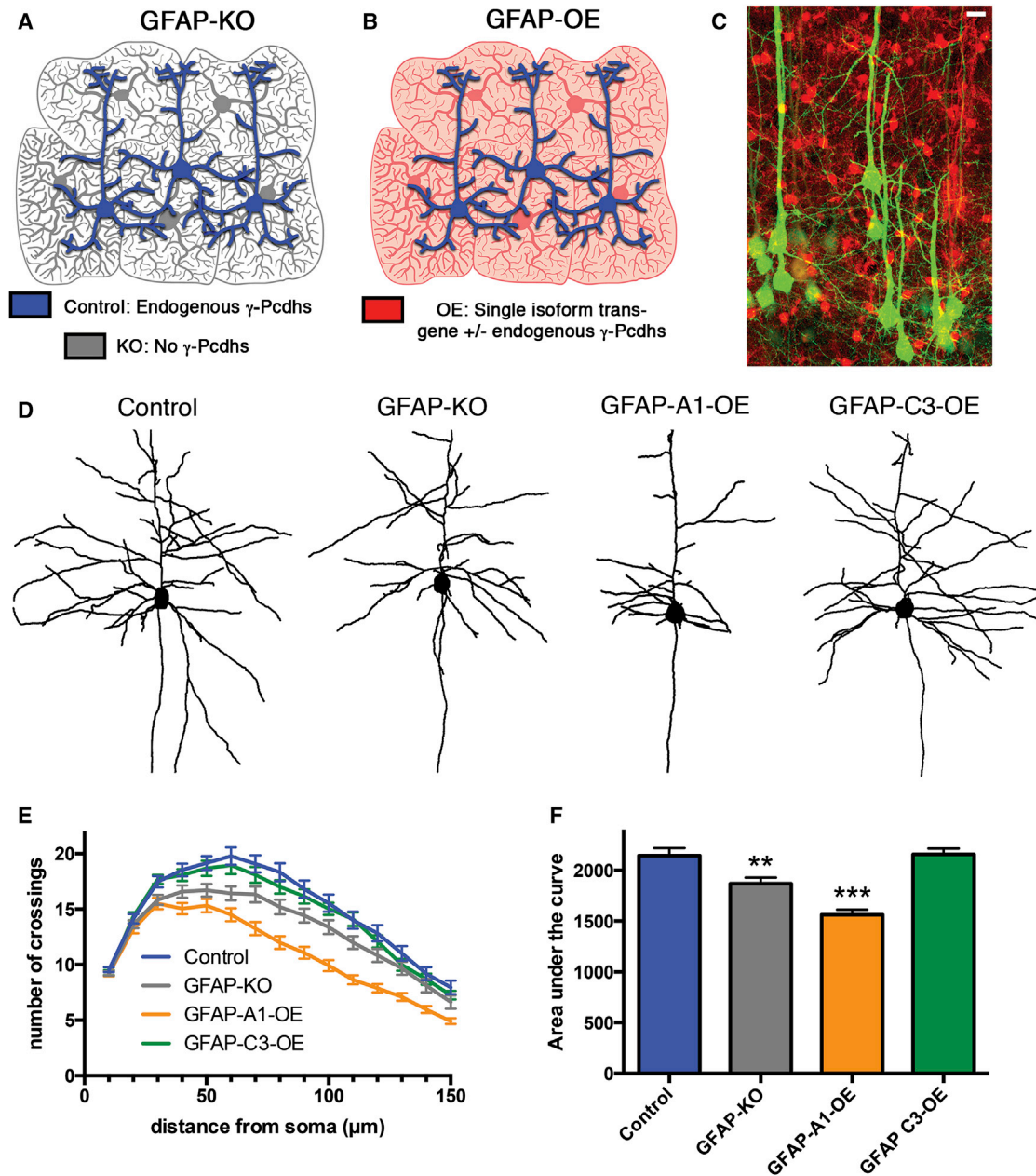


Figure 4. Astrocytic γ -Pcdhs Regulate Dendrite Arborization Cell Non-autonomously in the Cerebral Cortex

(A and B) Schematics represent neurons and astrocytes in the indicated genotypes, as in previous figures.

(C) Maximum projection of a confocal stack through the cortex of a *Gfap-Cre;Thy1-YFPH;Ai-14-tdTomato* mouse, demonstrating astrocyte-restricted excision mediated by the 77.6 Cre line, is shown.

(D–F) Representative traces (D), Sholl analysis (E), and area under the curve graph (F) of *Thy1-YFPH*⁺ layer V pyramidal neurons of the indicated genotypes are shown. Scale bar, 20 μ m (C); n = 60 neurons per genotype; **p < 0.01.

See also Figure S4.

of astrocytes, the size of their somata, the number of their primary branches, and the total area covered by their processes (Figures S4C–S4F). These data indicate that astrocytic γ -Pcdhs regulate neuronal development in a cell-non-autonomous manner; the fact that the traced neurons expressed endogenous γ -Pcdhs demonstrates that isoneuronal and/or

neuron-neuron interactions alone are insufficient for normal arbor complexity in cortical neurons.

We then addressed whether induced mismatching between astrocytic and neuronal γ -Pcdhs negatively affects dendrite complexity. Using *Gfap-Cre*, we generated mice in which astrocytes, but not neurons, expressed the A1-mCherry transgene

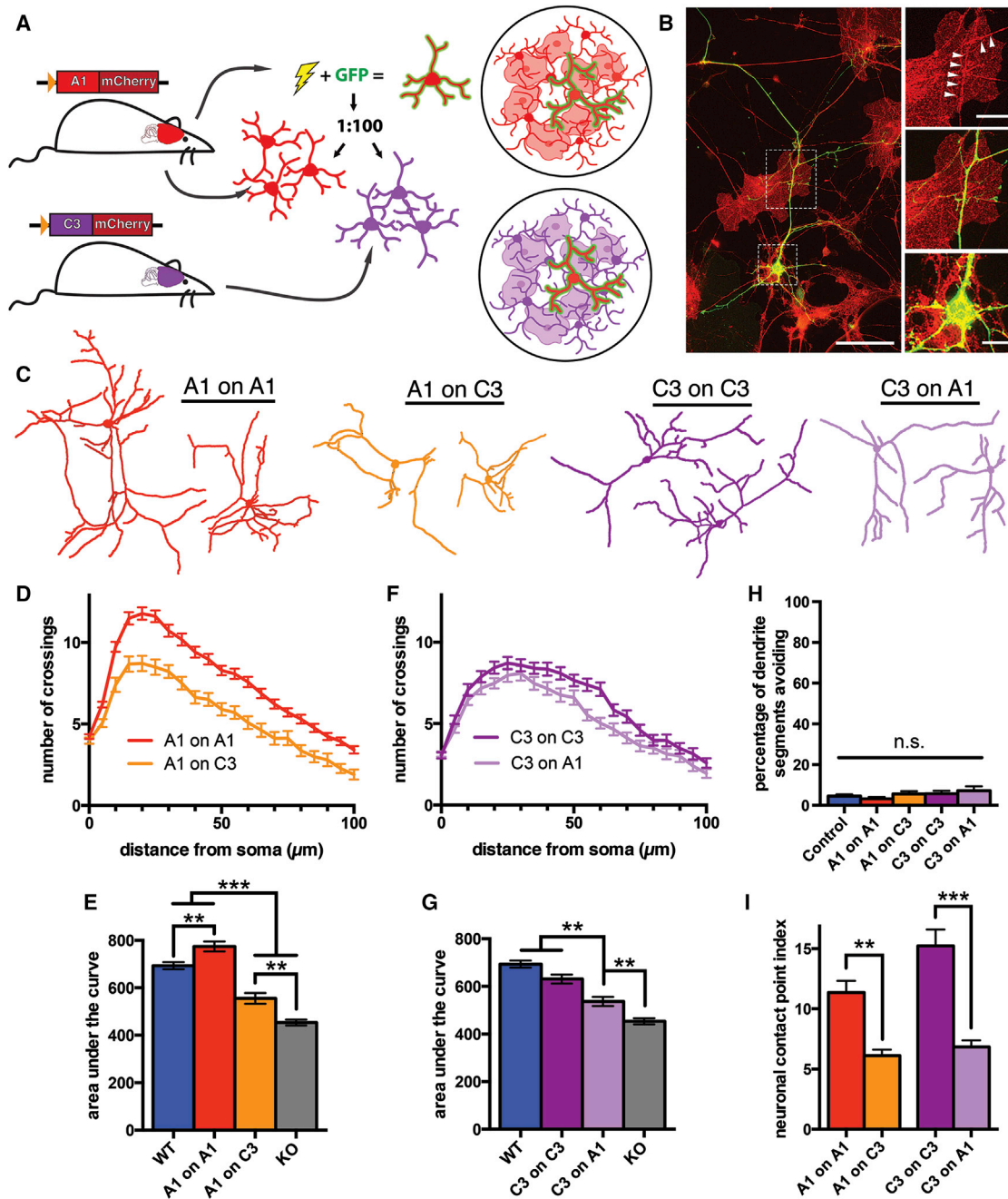


Figure 5. Dendrite Arborization Can Be Increased or Decreased by Manipulating γ -Pcdh Homophilic Matching between a Neuron and Its Environment In Vitro

(A) Schematic shows the neuronal co-culture assay: neonatal cortical cells from one transgenic mouse (red) are nucleofected with a plasmid encoding GFP, diluted at a 1:100 ratio with cells from another cortex expressing the same (red, top dish) or a different (purple) single-isoform transgene.

(B) Image of a nucleofected GFP+/mCherry+ neuron growing on GFP-/mCherry+ astrocytes in an A1-Only (perfectly matching) culture. A1-mCherry protein is concentrated at sites of contact (arrowheads), suggesting the formation of a homophilic *trans*-interaction complex at the cell surface.

(C) Representative traces show GFP+-nucleofected neurons in matching A1 on A1 or C3 on C3 cultures and mismatching A1 on C3 or C3 on A1 cultures.

(D–G) Sholl (D and F) and area under the curve (E and G) graphs showing that dendrite arbor complexity is significantly higher in neurons growing in homophilically matching co-cultures (A1 on A1 or C3 on C3) than in those growing in mismatching co-cultures (A1 on C3 or C3 on A1; all results shown here are OE; OE versus Only results shown in Figure S5). Additionally, comparison to similarly generated wild-type (WT) cultures corroborates the *in vivo* results shown in Figures 1, 2, and 4: A1 on A1 OE (E) neurons exhibit significantly greater arborization than control neurons, while C3 on C3 OE neurons do not (G). C3-Only on C3-Only neurons, again as *in vivo*, exhibit significantly greater arborization than do C3-OE neurons (Figure S5).

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(Figure 4B). Wild-type neurons, few of which express endogenous γ -Pcdh-A1 and none of which overexpress A1-mCherry, indeed exhibited severely reduced dendrite arborization when surrounded by mismatching A1-OE astrocytes (Figures 4D–4F). As we found for mismatched neurons in the *Sim1-Cre* analyses (Figures S2A–S2C), wild-type neurons encountering mismatching astrocytes exhibited reductions in average branch length as well as in total dendrite length and number of branchpoints (Figures S4G–S4I). As a control, we also analyzed mice in which the C3-mCherry transgene was activated in astrocytes using *Gfap-Cre*. Wild-type neurons, most or all of which highly express endogenous γ -Pcdh-C3, exhibited normal dendrite complexity when surrounded by C3-OE astrocytes (Figures 4D–4F; Figures S4G–S4I), a result entirely consistent with the *Emx*- and *Sim*-C3 results (Figures 1 and 2).

An In Vitro System to Manipulate Dendrite Complexity via γ -Pcdh Repertoire Matching or Mismatching

To further explore the role that homophilic γ -Pcdh matching between cells plays in dendrite complexity, we devised an in vitro assay (Figure 5A). Cells from a neonatal *Emx*-A1-OE or *Emx*-C3-OE cortex were labeled by nucleofection with a GFP-encoding plasmid, diluted at a 1:100 ratio with other cortical cells expressing either the matching or mismatching mCherry-tagged isoform, and plated. Isolated GFP+/mCherry+ neurons, surrounded by GFP-/mCherry+ neurons and astrocytes (Figure 5B), were then imaged, traced, and subjected to Sholl analysis after 8 days in vitro (DIV). In all cases, dendrite arborization was significantly higher when the traced neurons were surrounded by cells overexpressing the matching γ -Pcdh (A1 on A1 and C3 on C3) compared to those surrounded by cells overexpressing the mismatching γ -Pcdh (A1 on C3 and C3 on A1; OE results shown in Figures 5C–5G; OE versus Only results shown in Figure S5). As positive and negative controls for γ -Pcdh function, we also generated similar co-cultures using wild-type or knockout neurons (Figures 5E and 5G). Together, our in vitro results remarkably corroborate the in vivo data gathered using *Emx1-Cre* and *Sim1-Cre* as follows: (1) A1-OE on A1-OE neurons exhibited greater dendrite complexity than did wild-type neurons, while C3-OE on C3-OE neurons were similar to wild-type (Figures 5E and 5G; cf. Figure 1H); (2) mismatching neurons (A1 on C3 and C3 on A1) exhibited reduced dendrite complexity compared to wild-type (Figures 5E and 5G; cf. Figure 2F); (3) both A1-Only on A1-Only and A1-OE on A1-OE neurons exhibited similarly heightened dendrite complexity (Figure S5A; cf. Figure 1H); and (4) C3-Only on C3-Only neurons exhibited increased complexity compared to wild-type, while C3-OE on C3-OE neurons did not (Figure S5B; cf. Figure 1H).

We could not find evidence of repulsive avoidance in cortical co-cultures. Because dendrites often encounter astrocytes in vitro (e.g., Figure 5B), we asked whether those contacting a homophilically matching astrocyte ever stop, grow along the astrocyte's edge rather than onto it, or turn and grow away from the contact site. We found very few instances of dendrites that appeared to avoid growing onto astrocytes, regardless of whether they homophilically matched or mismatched (Figure 5H). Next we calculated a neuronal contact point index by counting the number of intersections or fasciculations between nucleofected GFP+ dendrites and other surrounding GFP-/MAP2+ dendrites, normalizing to total dendrite area in the field and multiplying by a constant. We found that the extent of contact between homophilically matching neurons (A1 on A1 and C3 on C3) was actually significantly higher than it was between mismatching neurons (A1 on C3 and C3 on A1) (Figure 5I), again failing to provide evidence for homophilic repulsion and indicating that neuron-neuron contacts contribute to the formation of complex dendritic arbors. Finally, we noted that when a neuron grew onto a homophilically matching astrocyte, mCherry-tagged γ -Pcdhs often concentrated along the length of contacts (Figure 5B, arrowheads), suggestive of homophilic *trans*-interactions at the cell membrane. Together, our data indicate a positive, apparently adhesive, response to γ -Pcdh homophilic matching in cortical neurons that leads to enhanced dendrite arborization.

Although cell aggregation assays have shown that all 22 γ -Pcdhs similarly exhibit strictly homophilic interactions (Schreiner and Weiner, 2010; Thu et al., 2014), it is important to confirm that the disruptive effect of isoform mismatching on dendrite arborization is not confined to the A1 or C3 isoforms for which transgenic lines are available. In vitro assays have demonstrated that introduction of a single mismatching isoform to K562 cells that otherwise match in γ -Pcdh repertoire significantly disrupts homophilic cell aggregation (Schreiner and Weiner, 2010; Thu et al., 2014; Rubinstein et al., 2015). We thus used low-efficiency lipofection of matching A1 on A1 cultures to ask whether introduction of a single mismatching γ -Pcdh isoform to a neuron would affect the formation of its arbor. Neurons transfected with control plasmid (GFP only) or a GFP-tagged γ -Pcdh-A1 (Figures 6A–6C) exhibited similarly high dendrite arbor complexity, confirming the finding that increasing total levels of γ -Pcdhs without modulating matching has little effect (cf. C3-OE results; Figures 1, 2, and 4). Dendrite arborization was, in contrast, drastically and identically reduced to knockout levels in neurons expressing any one of the following four GFP-tagged mismatching γ -Pcdhs: A3, A12, B2, and B6 (Figures 6A–6C; note that N-terminally HA-tagged B2 had the same effect as

(H) Relationship of dendrites to astrocytes was quantified in fixed cultures. Each instance where a dendrite appeared to contact only the edge of an astrocyte or to have turned to grow away from an astrocyte was counted, and this number was divided by the total number of dendrite/astrocyte contacts to obtain the percentage of dendrite segments avoiding astrocytes (H).

(I) An index measuring neuron-neuron contact points was calculated by counting the number of intersections between GFP+-nucleofected dendrites and other surrounding MAP2+ dendrites, normalizing to total dendrite area and multiplying by a constant. $n = 57$ – 60 (A1/C3 on A1/C3 and WT), 180 (KO), or 30 – 36 (avoidance and contact point index) neurons per condition; scale bars, $50 \mu\text{m}$ (B, left), $15 \mu\text{m}$ (B, upper and middle right), and $10 \mu\text{m}$ (B, lower right); ** $p < 0.01$ and *** $p < 0.001$.

See also Figure S5.

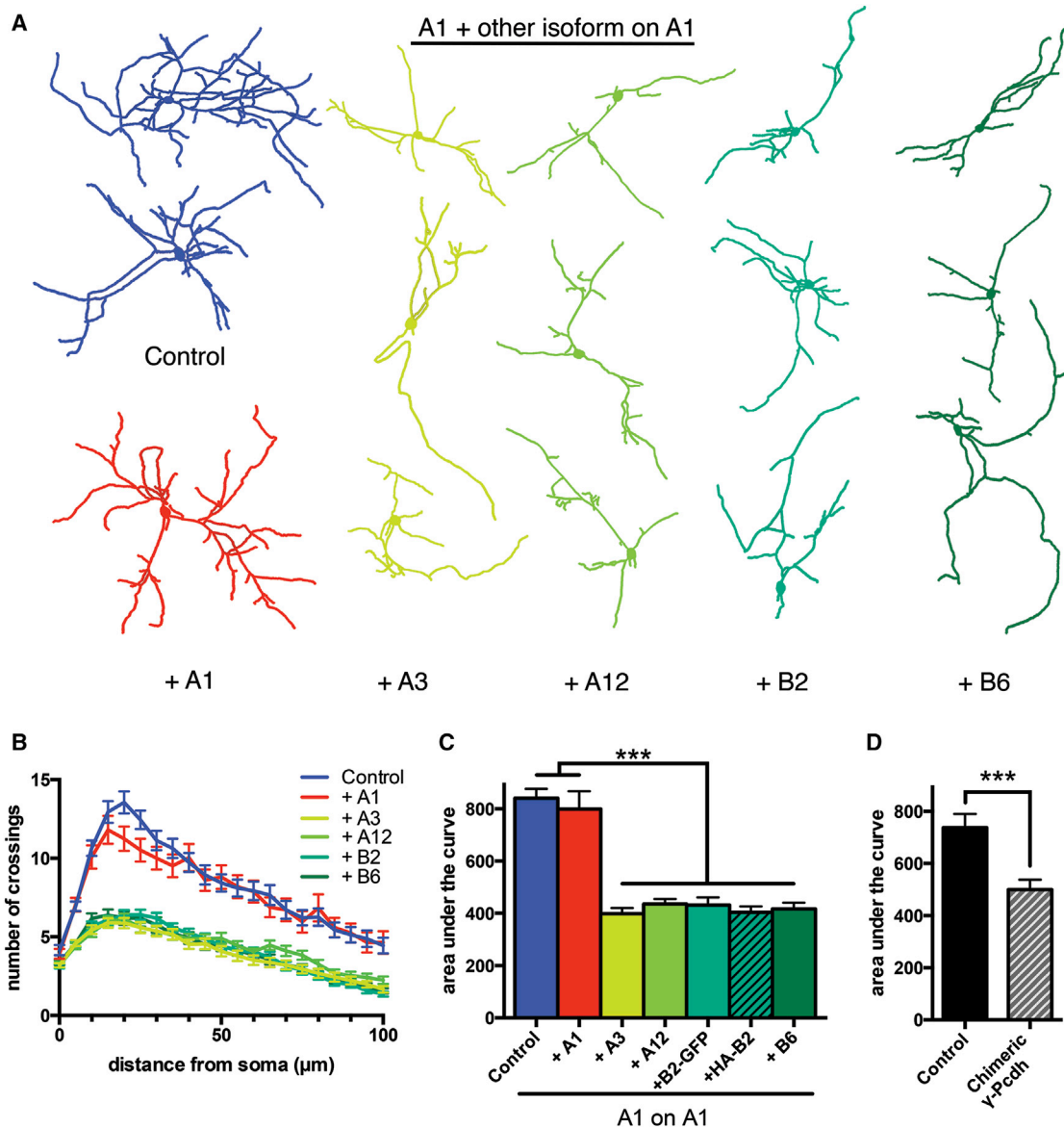


Figure 6. Disrupting γ -Pcdh Homophilic Interactions by Overexpressing a Single Mismatching Isoform Reduces Dendrite Arborization

Neurons in A1 on A1 homophilically matching co-cultures were lipofected at low efficiency (1%–5%) with a plasmid expressing GFP alone (control) or in addition to a plasmid encoding one of several γ -Pcdh isoforms with a C-terminal GFP tag: γ -Pcdh-A1, -A3, -A12, -B2, or -B6 (an N-terminally HA-tagged γ -Pcdh-B2 plasmid was also used to control for effects of the GFP tag).

(A) Representative neuronal traces in each transfection condition are shown.

(B and C) Sholl analysis (B) and area under the curve graphs (C). Addition of a single non-matching γ -Pcdh isoform to a neuron growing in an otherwise homophilically matching co-culture (A1 on A1) is sufficient to reduce arborization to KO levels (cf. Figures 5E and 5G).

(D) Introducing a chimeric γ -Pcdh isoform that can interact with itself, but not with any endogenous γ -Pcdh isoform, into wild-type neurons also significantly reduced dendrite arborization (measured by Sholl analysis at 8 DIV; area under the curve shown) compared to control neurons. $n = 80$ –105 (+additional isoform) or 30 (+chimera) neurons; *** $p < 0.001$.

C-terminally GFP-tagged B2 in this assay). For a final test of the effect of γ -Pcdh mismatching on dendrite arborization, we utilized a chimeric molecule (EC1 and EC3 from γ -Pcdh-A3; EC2 and EC4–6 from γ -Pcdh-C3; Schreiner and Weiner, 2010) that can interact homophilically with itself but not with any endogenous γ -Pcdhs. We lipofected wild-type cortical neuron cultures with a plasmid encoding GFP, either alone

or in addition to a plasmid encoding this chimeric γ -Pcdh, and we assessed dendrite arborization after 8 DIV. As predicted from the prior literature (Schreiner and Weiner, 2010; Thu et al., 2014; Rubinstein et al., 2015) and our other experiments here, disruption of endogenous γ -Pcdh interaction by introduction of this chimeric poison partner significantly reduced dendrite arbor complexity (Figure 6D).

DISCUSSION

Here we have differentially manipulated γ -Pcdh isoform repertoire in cells of the cerebral cortex *in vivo* and shown that increasing the likelihood of homophilic matching between a given neuron and its surrounding neurons and astrocytes promotes dendrite arborization. Conversely, induced mismatching of γ -Pcdh *trans*-interactions results, both *in vivo* and *in vitro*, in simplified arbors that resemble those of *Pcdhg* knockout neurons. Our data indicate that astrocytes regulate dendrite arborization cell non-autonomously *in vivo* via homophilic γ -Pcdh interactions, adding to the growing evidence that these cells play critical roles in neural circuit formation (Clarke and Barres, 2013). Further, we present evidence suggesting that homophilic γ -Pcdh interactions act locally, rather than through global cell signaling, to promote the growth and complexity of dendritic arbors.

Though homophilic γ -Pcdh interactions result in repulsion between adjacent SACs in the retina (Lefebvre et al., 2012; Kostadinov and Sanes, 2015), in the cortex these interactions promote dendrite growth and branching in a way that seems more consistent with an adhesive or otherwise positive signaling effect. In our earlier analysis of cortical *Pcdhg* knockouts, we found no evidence of increased dendrite self-crossing or fasciculation (Garrett et al., 2012a), in contrast to the clear self-avoidance defects shown in SACs and Purkinje cells (Lefebvre et al., 2012). In the present study, we found that neurons sharing expression of a single γ -Pcdh isoform exhibited an *increased* number of contacts compared to neurons that overexpressed mismatching isoforms. Additionally, dendrites did not avoid perfectly matching astrocytes, but rather grew along them and clustered γ -Pcdh proteins along contact sites. These data suggest that homophilic γ -Pcdh *trans*-interactions can result in adhesion, repulsion, or other signaling events relevant to dendrite arborization depending on cell-type-specific attributes. These might include the type and shape of the dendritic arbor (e.g., planar SACs and Purkinje cells versus bushy cortical neurons), expression of distinct *cis*-interactors (including the many α - and β -Pcdhs), or variations in downstream intracellular signaling components. Such context-dependent signaling would not be surprising in a family of molecules as complex as the clustered Pcdhs. In chick retina, Dscams are adhesive rather than repulsive (Yamagata and Sanes, 2008) and guidance molecules, such as netrins, ephrins, and semaphorins (Dickson, 2002), can act as both attractants and repellents depending on the presence of distinct co-receptors or signaling pathway activity states.

Although the most parsimonious interpretation of our data is that homophilic engagement of γ -Pcdhs across cell membranes directly promotes dendrite arborization, we cannot discount the possibility that γ -Pcdh over- or mis-expression affects other proteins involved in regulating arborization. Proteomic analysis of γ -Pcdh-containing protein complexes identified, in addition to other clustered Pcdhs, over 120 putative interacting proteins (Han et al., 2010). These include other adhesion-associated proteins with dendritic roles (e.g., classical cadherins and catenins) as well as proteins regulating trafficking, cytoskeleton, and intracellular signaling. As a portion of γ -Pcdh proteins are found in intracellular vesicular compartments (Fernández-Monreal et al.,

2010), it may well be that γ -Pcdhs regulate the trafficking of other cell surface proteins that are critical for dendrite arborization. Similarly, it is not yet clear whether homophilic *trans*-interaction of γ -Pcdhs in neurons directly induces a dendritic branchpoint or instructs dendrite growth along a contact site. We and others previously showed that the γ -Pcdhs can promote dendrite arborization by regulating signaling pathways involving the adhesion kinases FAK and Pyk2, PKC, MARCKS, and Rho GTPases (Garrett et al., 2012a; Suo et al., 2012; Keeler et al., 2015). These studies did not address whether γ -Pcdh regulation of such signaling required homophilic *trans*-interactions or could take place constitutively. If the latter, then an increase in total γ -Pcdh protein levels, as seen in our studies using the single-isoform transgenics, could conceivably be important. For several reasons noted earlier, we believe that this is unlikely to be a major driver of our results; nevertheless, in the absence of tools to accurately quantify γ -Pcdh surface levels on a cell-by-cell basis, we can't exclude the possibility that subtle variations in γ -Pcdh expression modulate their function. Finally, we note that *in vitro* studies indicate that the γ -Pcdhs can undergo two cleavage events: matrix metalloproteinases such as ADAM10 mediate ectodomain shedding, while γ -secretase cuts near the membrane to release an intracellular fragment that can translocate to the nucleus (Haas et al., 2005; Reiss et al., 2006). It is not yet clear how such cleavage is regulated *in vivo*, whether it differs for different isoforms, or whether *cis*- or *trans*-interactions modulate it. The regulation of γ -Pcdh levels at the dendritic cell surface by this or other mechanisms will be important to address in future studies.

We previously identified a role for γ -Pcdh-mediated astrocyte-neuron contacts in the regulation of synaptogenesis in the embryonic spinal cord (Garrett and Weiner, 2009). Here we extend the *in vivo* functions of astrocytic γ -Pcdhs, showing that either loss of endogenous γ -Pcdhs or overexpression of a sparsely expressed γ -Pcdh isoform (A1) in astrocytes cell non-autonomously disrupted dendrite arborization in cortical neurons. Several lines of evidence indicate that these results are due to loss of γ -Pcdh homophilic interactions between astrocytes and neurons, rather than undetected cellular or molecular defects in GFAP-KO or GFAP-A1 astrocytes: (1) complete loss of γ -Pcdhs in both astrocytes and neurons using *Emx1-Cre* did not result in any increase in apoptosis of either cell type (Garrett et al., 2012a); (2) overexpression of the A1 transgene in both astrocytes and neurons in EMX-A1-OE cortex (Figure 1) led to increased dendrite arborization, while restricted expression of the exact same transgene in astrocytes only (GFAP-A1-OE cortex; Figure 4) resulted in reduced dendrite arborization; and (3) astrocyte number, soma size, number of primary branches, and area covered by astrocyte branches were all identical between controls and GFAP-KO, GFAP-A1-OE, and GFAP-C3-OE cortex (Figure S4). It is important to note that the reduction in dendrite complexity observed in the *Gfap-Cre* knockout cortex is not as severe as that previously reported in the *Emx1-Cre* knockout cortex (Garrett et al., 2012a). This indicates that, while astrocyte-neuron γ -Pcdh interactions are necessary for normal dendritic arbor complexity, neuron-neuron interactions must contribute as well. This is further supported by other *in vivo* (Figure 3C) and *in vitro* (Figure 5I) data in the present study.

It is interesting that dendrite complexity in neurons encountering astrocytes overexpressing a mismatching γ -Pcdh isoform was lower than in those encountering knockout astrocytes (Figure 4F). Though the reason for this is not clear, it might imply the collaboration of α - and/or β -Pcdhs, which have been found to interact physically and functionally in *cis* with γ -Pcdhs (Murata et al., 2004; Han et al., 2010; Thu et al., 2014), in homophilic adhesion between neurons and astrocytes. If adhesive interfaces in vivo indeed incorporate α -, β -, and γ -Pcdhs, introduction of a mismatching γ -Pcdh in astrocytes could act as a poison partner (analogous to the experiments shown in Figure 6) and affect neuronal dendrite arborization more than simple loss of astrocytic γ -Pcdhs would. Hippocampal neurons lacking α -Pcdhs exhibit reduced dendrite complexity in vivo (Suo et al., 2012), though as yet there are no reports of phenotypes in mice lacking the β -Pcdhs. Clearly, it will be important in the future to examine neurons lacking all clustered Pcdhs to determine whether α -, β -, and γ -Pcdhs act through the same pathways to regulate neuronal morphology.

A recent model based on structural and biochemical data (Rubinstein et al., 2015) suggests that the unit of clustered Pcdh interaction in *trans* is a dimer of dimers and that many of these units together could form a zipper-like structure. Cells with identical isoform repertoires would be able to form larger zipper-like Pcdh assemblies, while even a small number of mismatches would be enough to severely abrogate such *trans*-interactions (Rubinstein et al., 2015). Though this model was developed to explain Pcdh-mediated self-avoidance, our results are not inconsistent with such a mismatch mechanism. Overexpression in a given neuron of a single isoform unlikely to be found in surrounding cells (Sim-A1 mice, Figure 2; transfection of A1 on A1 neurons with other isoforms, Figure 6) led to a severe reduction in dendrite arborization, while overexpression of a single isoform likely to be shared by all cells (Sim-C3 mice, Figure 2; transfection of A1 on A1 neurons with A1-GFP, Figure 6) affected arborization mildly or not at all. One caveat of our study is that we manipulated only two γ -Pcdh isoforms as proof-of-principle exemplars in vivo. While it is possible that manipulation of other isoforms could give different results than we see for A1 and C3, this is unlikely given the following: (1) all γ -Pcdhs can interact in *cis*- and their *trans*-interactions are all strictly homophilic in K562 assays (Schreiner and Weiner, 2010; Thu et al., 2014); and (2) we showed directly that four other γ -Pcdh isoforms (A3, A12, B2, and B6) can, when introduced into otherwise-matching neurons, significantly disrupt dendrite arborization (Figure 6).

Given the apparently stochastic expression of the 19 endogenous γ -Pcdh A and B subfamily isoforms, on a background of near-ubiquitous expression of the three C subfamily isoforms (Yagi, 2012), there should be a particular statistical chance, albeit a small one, of cells with similar repertoires being in the vicinity of a neuron's growing dendrites. To determine precisely how our proof-of-principle results shown here, utilizing single overexpressed isoforms, relate to the normal developmental interactions between endogenous repertoires of γ -Pcdhs, we need a better understanding of many variables. These include but are not limited to the following: (1) the extent to which isoform protein levels vary cell by cell and, if so, whether this can significantly modulate interactions; (2) whether any isoform-specific rules

govern the formation of *cis*-complexes in vivo (e.g., Must every dimer of dimers include a C subfamily isoform? Are homodimers favored over heterodimers?); (3) whether all possible combinations of isoforms, given a cell's transcript repertoire, are translated, delivered to the plasma membrane, and localized equivalently; (4) whether individual isoforms, through their variable cytoplasmic domains, activate unique intracellular signaling partners; and (5) whether partial homophilic matches within a dimer or other multimer (i.e., only one of two isoforms in a dimer of dimers being shared), while not allowing stable interaction, might nevertheless transiently activate a signaling event with a distinct outcome.

In any case, it seems unlikely that homophilic *trans*-interactions between endogenous γ -Pcdhs would provide directional or instructive signals to particular individual cells; rather, the statistical likelihood of enough homophilic interactions occurring (however many enough is) might place a permissive bound on the extent of a neuron's dendrite arbor that would normalize arbor complexity across a large population. Our results allow us to predict that reduction in functional *Pcdhg* gene repertoire in the cortex, either through mutation or hypermethylation, should result in an increased chance of homophilic matching between cells and an increase in the complexity of dendritic arbors. A key future goal will be to demonstrate this experimentally using new animal models, as well as to consider epigenetic changes in the *Pcdhg* cluster that could contribute to developmental disorders in which altered dendrite morphology is observed.

EXPERIMENTAL PROCEDURES

Mouse Strains

All animal procedures were approved by the University of Iowa's institutional animal care and use committee and performed in compliance with university and NIH guidelines for the use of animals. The A1-mCherry and C3-mCherry transgenic lines (Lefebvre et al., 2012) were the kind gift of Julie Lefebvre and Joshua Sanes (Harvard University). The *Pcdhg*^{con3} conditional mutant allele has been described (Garrett et al., 2012a; Prasad et al., 2008). The *Gfap-Cre*, *Emx1-Cre*, *Sim1-Cre*, *Thy1-YFPH*, and *Ai14-TdTomato* lines were obtained from The Jackson Laboratory.

Cortical Neuron Cultures

Cultures were prepared as described (Garrett et al., 2012a). For co-cultures, cells harvested from individual animals were plated at a density of 200,000 cells per coverslip. Remaining cells were nucleofected (Amaxa) with N1-EGFP plasmid and plated at 2,000 cells per coverslip onto the previously plated high-density cells from the same, or another, individual animal. In other experiments, low efficiency (~1%–5%) transfection using Lipofectamine 2000 (Invitrogen) was performed at 2 DIV.

Analysis of Dendrite Arborization

Transgenic mice were perfused with 4% paraformaldehyde (PFA) at 5 weeks of age. Confocal images of Vibratome sections were taken using a 20 \times objective on Leica SP2 AOBs or Leica TCS SPE microscopes. All collected images were blinded to condition. The 50- to 60- μ m z stacks were collected with a 0.5- μ m step size, imported into FIJI (NIH ImageJ), and dendrites were traced in 3D using the Simple Neurite Tracer plugin. Epifluorescence images cultured neurons (8 DIV) were taken using a 20 \times objective on a Leica TCS SPE microscope and traced in 2D. The Sholl analysis function of Simple Neurite Tracer was used. Comparisons were performed in Prism by one-way ANOVA and corrected for multiple comparisons with Tukey's test, and we reported multiplicity-adjusted p values for each comparison (or by unpaired, two-tailed

t test when comparing only two samples). Asterisks denote the following significance levels: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.03.093>.

AUTHOR CONTRIBUTIONS

M.J.M, A.B.K, and J.A.W. conceived the experiments, collected and analyzed data, and wrote the manuscript.

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