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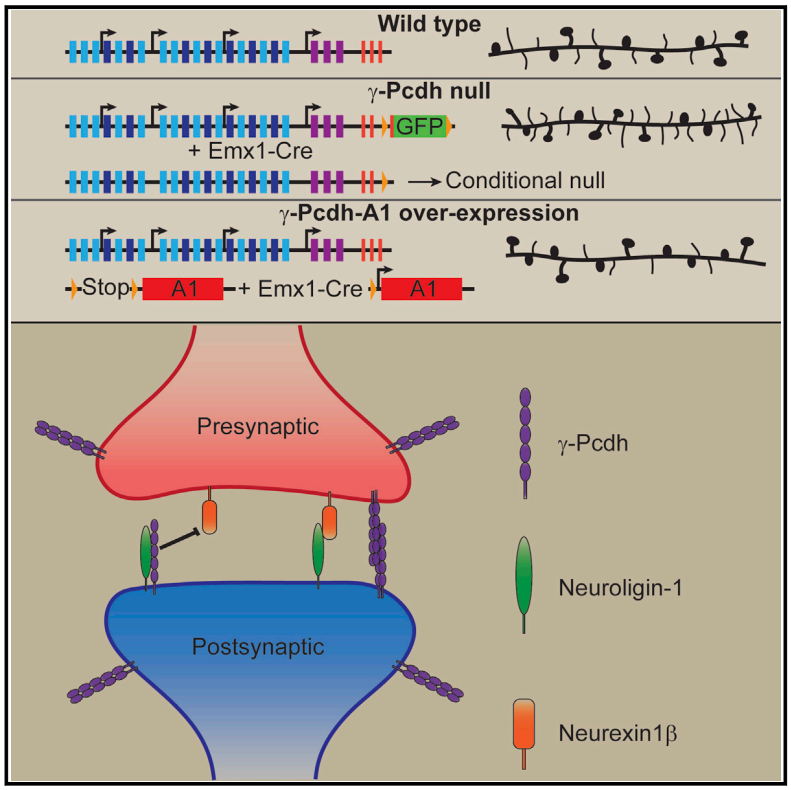
γ-Protocadherins Interact with Neuroligin-1 and Negatively Regulate Dendritic Spine Morphogenesis

Graphical Abstract



Highlights

- γ-Protocadherins (γ-Pcdhs) regulate dendritic spine morphogenesis in the cerebral cortex
- γ-Pcdhs interact with neuroligin-1 (Nlg1) and inhibit its interaction with neurexin1β
- γ-Pcdhs inhibit Nlg1's ability to induce presynaptic differentiation in vitro
- γ-Pcdhs inhibit Nlg1's ability to increase dendritic spine density in vitro

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In Brief

Using conditional mutant and overexpression mouse lines, Molumby et al. demonstrate that γ-Protocadherins negatively regulate cortical dendritic spine morphogenesis in vivo. The γ-Protocadherins interact physically with neuroligin-1 and inhibit its ability to bind neurexin1β, to promote presynaptic differentiation, and to increase dendritic spine density in hippocampal neurons in vitro.

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γ-Protocadherins Interact with Neuroligin-1 and Negatively Regulate Dendritic Spine Morphogenesis

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SUMMARY

The 22 γ-Protocadherin (γ-Pcdh) cell adhesion molecules are critical for the elaboration of complex dendritic arbors in the cerebral cortex. Here, we provide evidence that the γ-Pcdhs negatively regulate synapse development by inhibiting the postsynaptic cell adhesion molecule, neuroligin-1 (Nlg1). Mice lacking all γ-Pcdhs in the forebrain exhibit significantly increased dendritic spine density in vivo, while spine density is significantly decreased in mice overexpressing one of the 22 γ-Pcdh isoforms. Co-expression of γ-Pcdhs inhibits the ability of Nlg1 to increase spine density and to induce presynaptic differentiation in hippocampal neurons in vitro. The γ-Pcdhs physically interact in *cis* with Nlg1 both in vitro and in vivo, and we present evidence that this disrupts Nlg1 binding to its presynaptic partner neurexin1β. Together with prior work, these data identify a mechanism through which γ-Pcdhs could coordinate dendrite arbor growth and complexity with spine maturation in the developing brain.

INTRODUCTION

Proper neural circuit formation requires the arborization of neuronal dendrites and the formation and maturation of excitatory synapses on dendritic spines. These processes are shaped in each neuron by discrete contacts with surrounding neurons and glia during development. At these cell-cell contacts, specific cell adhesion molecules initiate target recognition and promote dendrite growth, pre- and postsynaptic differentiation, and mature synaptic function (de Wit and Ghosh, 2016). The 22 γ-Protocadherins (γ-Pcdhs), cadherin superfamily adhesion molecules encoded by the *Pcdhg* gene cluster, are predominantly expressed in the CNS and play critical roles during neural development (reviewed in Keeler et al., 2015a; Mah and Weiner, 2016). The individual γ-Pcdh isoforms (12 "A" subfamily, seven "B" subfamily, and three "C" subfamily members) engage in promiscuous *cis* multimerization, but interact strictly homophilically in *trans* (Rubinstein et al., 2015; Schreiner and Weiner, 2010; Thu et al., 2014). Due to their adhesive specificity, diversity, and combinatorial expression in neurons (each neuron likely expresses approximately six to seven of the 22 *Pcdhg* genes [Kaneko et al., 2006]), the γ-Pcdhs have been suggested to provide a molecular code that regulates neural circuit formation (Yagi, 2012).

Subcellular fractionation indicates that γ-Pcdh proteins are concentrated in synaptosomes and postsynaptic densities (Wang et al., 2002), and proteomic analysis found them associated with several other synaptic molecules (Han et al., 2010). While immunostaining at both the light and electron microscopy levels detects the γ-Pcdhs at some synaptic contacts (Garrett and Weiner, 2009; Phillips et al., 2003; Wang et al., 2002), much of the protein is localized perisynaptically in both neurons and astrocytes (Garrett and Weiner, 2009) as well as in dendrites and axons (Fernández-Monreal et al., 2009; Phillips et al., 2003), often in intracellular vesicles associated with the secretory pathway (Fernández-Monreal et al., 2010). Mice lacking all γ-Pcdhs die shortly after birth, apparently due to increased neuronal apoptosis of interneurons in the brainstem and spinal cord (Prasad et al., 2008; Wang et al., 2002), accompanied by disrupted progression of embryonic spinal cord synaptogenesis that can be genetically dissociated from apoptosis (Prasad et al., 2008; Wang et al., 2002; Weiner et al., 2005).

Restricted loss or misexpression of the γ-Pcdhs (using conditional knockout or overexpression alleles) in other regions of the CNS, however, has implicated them primarily in the regulation of dendrite arborization. In starburst amacrine cells of the retina, loss of all γ-Pcdhs leads to aberrant dendrite self-crossing and fasciculation that could be rescued cell autonomously by expression of a single γ-Pcdh isoform (Lefebvre et al., 2012). We found that restricted loss of the γ-Pcdhs in the cerebral cortex led to severely reduced dendrite arborization of pyramidal neurons in vivo (Garrett et al., 2012), and a similar simplification of arbors was reported following small hairpin RNA (shRNA)

knockdown of γ-Pcdhs in cultured hippocampal neurons (Suo et al., 2012). Recently, using conditional knockout and single isoform overexpression alleles, we presented evidence that homophilic γ-Pcdh *trans*-interactions between neurons, and between neurons and astrocytes, promote cortical dendrite arbor complexity (Molumby et al., 2016). The extent to which the γ-Pcdhs might also regulate synaptogenesis and dendritic spine formation in the cortex in vivo has not yet been examined.

Here, we show that dendritic spine density is significantly increased in cortically restricted *Pcdhg*-null mice and significantly decreased in cortical neurons overexpressing a γ-Pcdh isoform in vivo. To explain this observation, we asked whether the γ-Pcdhs might inhibit the postsynaptic cell adhesion molecule neuroligin-1 (Nlg1), which binds to presynaptic neurexins and has been shown to promote synaptic differentiation and dendritic spine density in vitro (Boucard et al., 2005; Chih et al., 2005; Dean et al., 2003; Scheiffele et al., 2000), and to be critical for mature synapse function in vivo (Jiang et al., 2016; Varoqueaux et al., 2006). Using a variety of biochemical and neuronal assays, we show that multiple γ-Pcdh isoforms physically interact with Nlg1 via their extracellular domains. In the "artificial synapse" co-culture assay (Biederer and Scheiffele, 2007), Nlg1 expression in non-neuronal COS cells endows them with the ability to induce presynaptic differentiation in contacting hippocampal axons. We find that this ability is almost completely abrogated by co-expression of γ-Pcdhs in COS cells and present evidence that γ-Pcdhs interact in *cis* with Nlg1 to inhibit its interaction with its presynaptic partner, neurexin1β. Finally, we show that co-expression of γ-Pcdhs can also prevent the increase in spine density induced by Nlg1 overexpression in hippocampal neurons. Together with prior work (Garrett et al., 2012; Suo et al., 2012; Molumby et al., 2016), these results suggest that γ-Pcdhs may promote dendrite arborization at the expense of dendritic spine formation and/or stabilization by interacting with, and inhibiting, Nlg1.

RESULTS

γ-Pcdhs Regulate Dendritic Spine Morphogenesis In Vivo

In the mammalian forebrain, the γ-Pcdhs are critical for proper dendritic arbor complexity, both in vivo and in vitro (Garrett et al., 2012; Keeler et al., 2015b; Molumby et al., 2016; Suo et al., 2012). We asked whether cortically restricted *Pcdhg* mutants also exhibit impaired synapse and dendritic spine development in vivo. We utilized compound transgenic mice (previously described in Garrett et al., 2012 and Molumby et al., 2016) in which excision of the $Pcdhg^{fcon3}$ conditional null allele (Prasad et al., 2008) was restricted to excitatory neurons and astrocytes in the cortex and hippocampus by inclusion of the *Emx1-Cre* allele (Garrett et al., 2012; Gorski et al., 2002). First, we stained cryosections with antibodies to the presynaptic active zone marker bassoon and the postsynaptic scaffolding protein PSD-95 and estimated the density of excitatory synapses within layer V of 5- to 6-week-old mice by quantifying instances in which these markers overlapped. To our surprise, synapse density measured in this way was not reduced in *Pcdhg* mutants; in fact, cortical *Pcdhg*-null (KO) mice exhibited significantly *increased* synapse density (Figures 1A and 1B), indicating that the γ-Pcdhs may inhibit the formation and/or maturation of excitatory synapses in the cortex. Next, we analyzed dendritic spines on layer V pyramidal neurons labeled by inclusion of the *Thy1-YFPH* allele (Feng et al., 2000). Spines were analyzed for density and morphology from confocal stacks imaged from Vibratome sections through the somatosensory cortex at 5–6 weeks of age using *NeuronStudio* (see Experimental Procedures; Radley et al., 2008; Rodriguez et al., 2008). Consistent with immunostaining results, *Pcdhg* KO neurons exhibited significantly increased total spine density compared to control neurons (Figures 1C and 1D). Augmented spine density in KO neurons was largely accounted for by an increase in thin subtypes, as the density of mushroom and stubby spines was not significantly different (Figure 1E). Whereas immature spines encompass a subset of thin subtypes, their increased numbers in this context could indicate an enhanced spine synapse formation that occurs in the absence of endogenous γ-Pcdhs.

Given that spine density is increased in the absence of any of the 22 γ-Pcdhs, we asked whether overexpression (OE) of an individual γ-Pcdh isoform might lead to decreased spine density. To pursue this, we utilized a mouse line in which an mCherry-tagged γ-Pcdh-A1 isoform is expressed from the ubiquitous Rosa locus only upon Cre excision of a floxed STOP cassette (Lefebvre et al., 2012). As described previously, we activated expression of this transgene using *Emx1-Cre* in the presence (A1-OE) or absence (A1-Only) of endogenous γ-Pcdhs, depending on inclusion of the $Pcdhg^{fcon3}$ conditional knockout allele (Molumby et al., 2016). We hypothesized that γ-Pcdh OE would have the opposite effect as KO and thus decrease dendritic spine density. Indeed, dendritic spine density was significantly decreased on both A1-OE and A1-Only neurons compared to control neurons (Figures 1C and 1D). Interestingly, spine density in A1-OE neurons (which would express their normal complement of endogenous γ-Pcdhs as well as the exogenous A1 isoform) was further decreased from that in A1-Only neurons (which would express only the exogenous A1 isoform), suggesting that the decrease in spine density was proportional to total levels of γ-Pcdhs present (Figures 1C and 1D). Both A1-Only and A1-OE neurons had significantly reduced numbers of mushroom and thin spines, coupled with an increase in stubby spines compared to control neurons (Figure 1E). As stubby spines are highly prevalent in the early postnatal period and steadily decline thereafter to low levels in adulthood (Boyer et al., 1998; Petrak et al., 2005), their increased numbers in the face of overall decrements in other subtypes suggests A1-OE impaired spine maturation.

γ-Pcdhs Inhibit Induction of Presynaptic Differentiation by Neuroligin-1 in the Artificial Synapse Assay

Having found evidence that the γ-Pcdhs negatively regulate spine and synapse density in the cortex in vivo, we next sought to identify the molecular mechanisms involved. One of the most prominent regulators of synapse development and function is the postsynaptic cell adhesion molecule neuroligin-1 (Nlg1), which binds to presynaptic neurexins and has been shown to increase dendritic spine and excitatory synapse density when overexpressed in neurons (Boucard et al., 2005; Chih et al.,

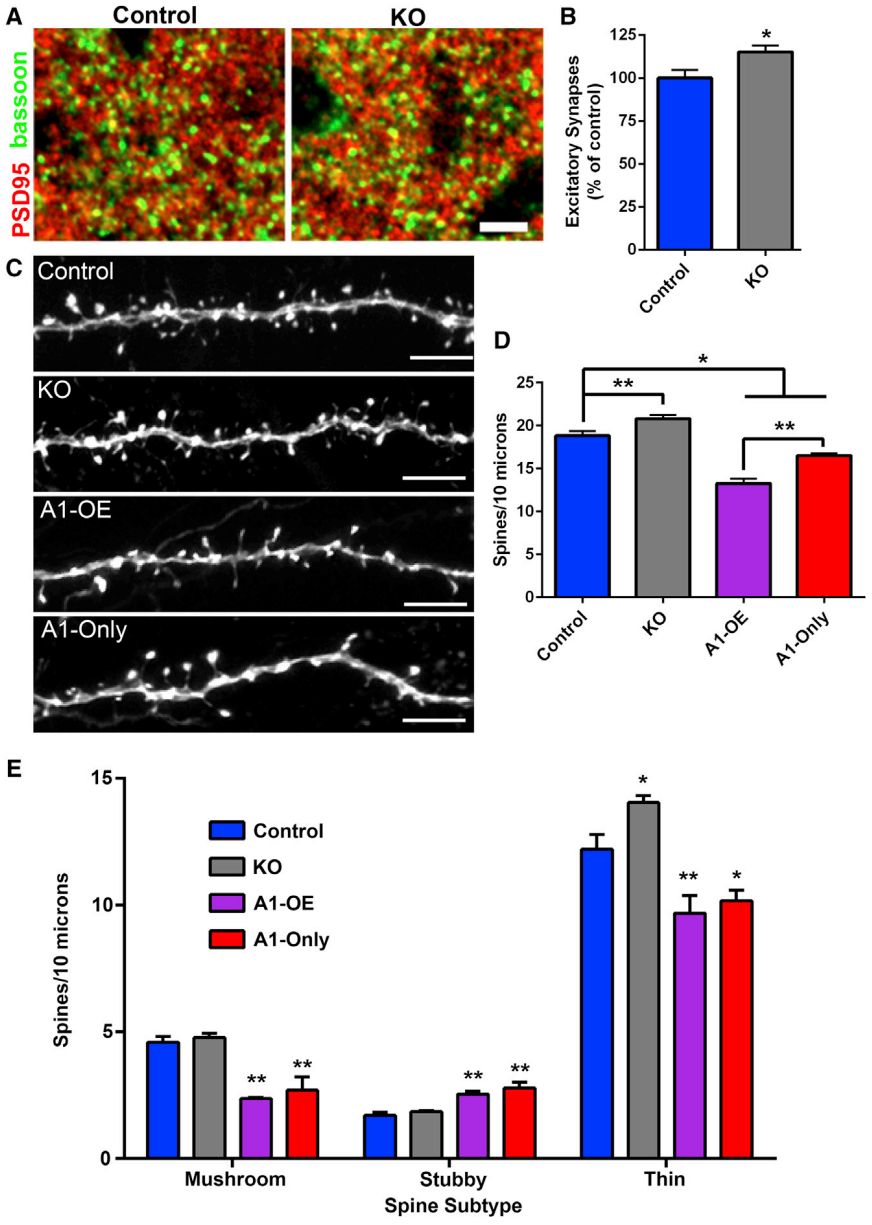


Figure 1. γ-Pcdhs Regulate Spine Morphogenesis In Vivo

(A and B) Cryostat sections, stained with bassoon and PSD95, of *Emx1-Cre;Pcdh-γ*$^{fcon3/fcon3}$ cortex demonstrate increased excitatory synaptic density compared to control *Emx1-Cre;Pcdh-γ*$^{fcon3/+}$ (A, micrographs; B, quantification). Scale bar, 5 μm. n = 18 (control) or 24 (KO) fields of view.

(C) Representative images show Thy1-YFP-labeled layer V pyramidal neuron dendritic spines in the indicated genotypes. Scale bar, 5 μm.

(D) Dendritic spine density quantification graphed as # spines per 10 μm, quantified from 20- to 25-μm dendritic segments per mouse for indicated genotypes. n = 9 control mice (23,537 spines), 8 KO mice (20,800 spines), 9 A1-OE mice (14,917 spines), and 4 A1-Only mice (8,255 spines).

(E) Spine subtype quantification for indicated genotype with significance compared to control for each subtype. Error bars represent the SEM. *p < 0.05, **p < 0.01. Scale bar, 5 μm.

2005; Ichtchenko et al., 1996; Song et al., 1999). Neuroligin-1 also exhibits synaptogenic activity in the "artificial synapse assay": non-neuronal cells expressing Nlg1 can induce presynaptic differentiation, as measured by synaptic vesicle clustering, in contacting axons (Biederer and Scheiffele, 2007; Scheiffele et al., 2000). We utilized this co-culture assay to ask whether γ-Pcdhs might affect synaptogenesis by modulating Nlg1 activity. In this assay, COS7 cells transfected with plasmids encoding hemagglutinin (HA)-tagged Nlg1 (including both the A and B splice site variants; Boucard et al., 2005; Chih et al., 2006; Ichtchenko et al., 1995) plus either a myc-tagged γ-Pcdh isoform (myc-A3 or myc-C3) or RFP-tagged CD4 (an immunoglobulin superfamily molecule negative control) was co-cultured with neonatal hippocampal neurons and incubated for 36 hr to allow for axonal outgrowth. Cultures were fixed and stained with antibodies against the tagged proteins and against synapsin to provide a measure of presynaptic differentiation.

Quantitative analysis revealed significantly reduced synapsin clustering in axons contacting COS cells co-expressing HA-Nlg1 and myc-A3 or myc-C3 compared to those contacting COS cells co-expressing HA-Nlg1 and the CD4-RFP negative control (Figures 2A and 2B). We did not observe any significant synapsin clustering on COS cells transfected with γ-Pcdh constructs alone (compared to the CD4-RFP control), indicating that γ-Pcdhs themselves are not synaptogenic in this assay (see Figure 6B). To control for variation in the levels of overexpressed Nlg1 across different co-transfection conditions, we selected a subset of cells with identical levels of total cellular HA-Nlg1 and separately analyzed these. Those co-expressing myc-A3 or myc-C3 still exhibited significantly reduced synapsin clustering compared to those co-expressing the CD4-RFP control, just as was seen in the entire population of cells (Figure 2C).

As a further control, we performed similar co-culture experiments using antibodies against a different presynaptic marker, synaptotagmin, and controlling for length of tau-positive axon contacts with COS cells. We also used a distinct Nlg1 construct, HA-Nlg1Δcyto-2A-RFP (lacking both the A and B splice variants), that releases RFP into the cytoplasm of the cell after 2A peptide self-cleavage, allowing for accurate quantification of the COS cell area. As before, co-expression of a γ-Pcdh (myc-C3) with Nlg1 significantly reduced presynaptic clustering in contacting axons (Figure 2D), despite the fact that axons grew across COS cells similarly regardless of transfection condition (Figure 2E). Combined, these results provide in vitro evidence

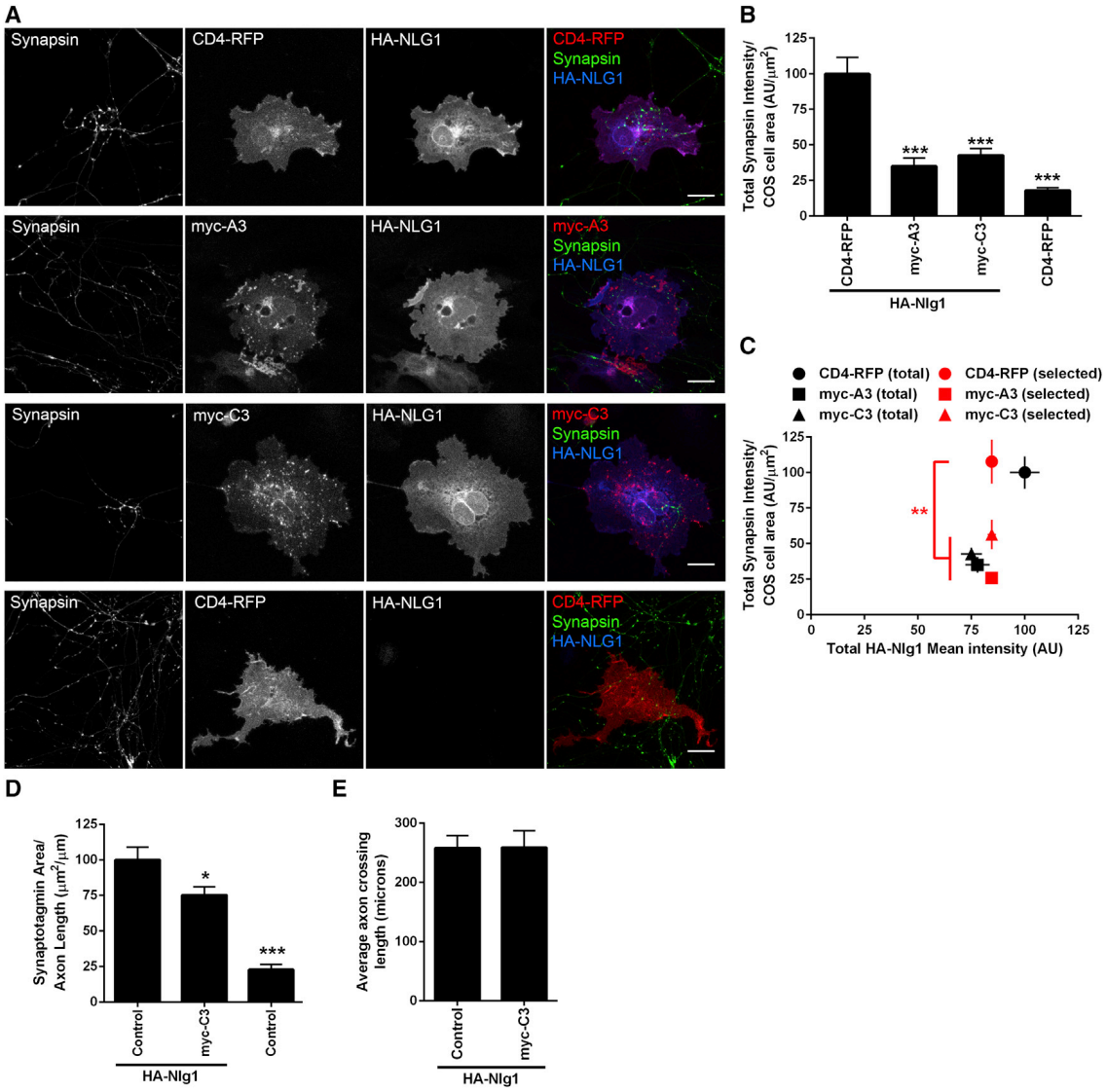


Figure 2. γ-Pcdhs Inhibit Presynaptic Differentiation Induced by Neuroligin-1 In Vitro

(A) COS cells were co-transfected with HA-neuroligin-1 (HA-Nlg1) and either CD4-RFP (control construct) or γ-Pcdh constructs and then co-cultured with wild-type hippocampal neurons. Nlg1 (in the presence of control CD4-RFP) induced robust synapsin clustering on axons contacting the COS cell surface (top). Co-cultured COS cells co-expressing HA-Nlg1 with myc-A3 or myc-C3 induce diminished synapsin clustering compared to HA-Nlg1 and CD4-RFP control (middle). COS cells expressing CD4-RFP alone show no synapsin clustering when cultured with hippocampal neurons (bottom).

(B) Quantification of total integrated intensity of synapsin immunofluorescence associated with COS cells co-expressing the indicated constructs, divided by the COS cell area and normalized to the value of CD4-RFP control.

(C) Comparison of total integrated synapsin intensity of selected (red symbols) COS cells co-expressing HA-Nlg1 and CD4-RFP, myc-A3, or myc-C3 with similar total HA-Nlg1 mean intensity normalized to the total means in (B) and (C). n = 20 per condition. Full datasets are shown for comparison with the selected COS cells (black symbols).

(D) Quantification of synaptotagmin area per tau-positive axon length associated with COS7 cells co-expressing HA-Nlg1Δcyto-RFP2A and myc-C3.

(E) Quantification of tau-positive axon average contact length on COS cells coexpressing HA-Nlg1 or myc-C3. Data are mean with SEM. Ordinary one-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar, 20 μm.

that γ-Pcdhs may inhibit synapse development by reducing the activity of Nlg1.

γ-Pcdhs Physically Interact with Neuroligin-1

The inhibition of Nlg1's synaptogenic activity by the γ-Pcdhs in vitro indicated a possible physical interaction between these two cell surface molecules. All γ-Pcdhs have an extracellular (EC) domain that contains six cadherin-like repeats (labeled 1–6, with EC1 being most N-terminal and EC6 being membrane-proximal), a transmembrane domain, and a variable cytoplasmic domain (all encoded by a single variable exon unique to each isoform) as well as a C-terminal constant domain shared by

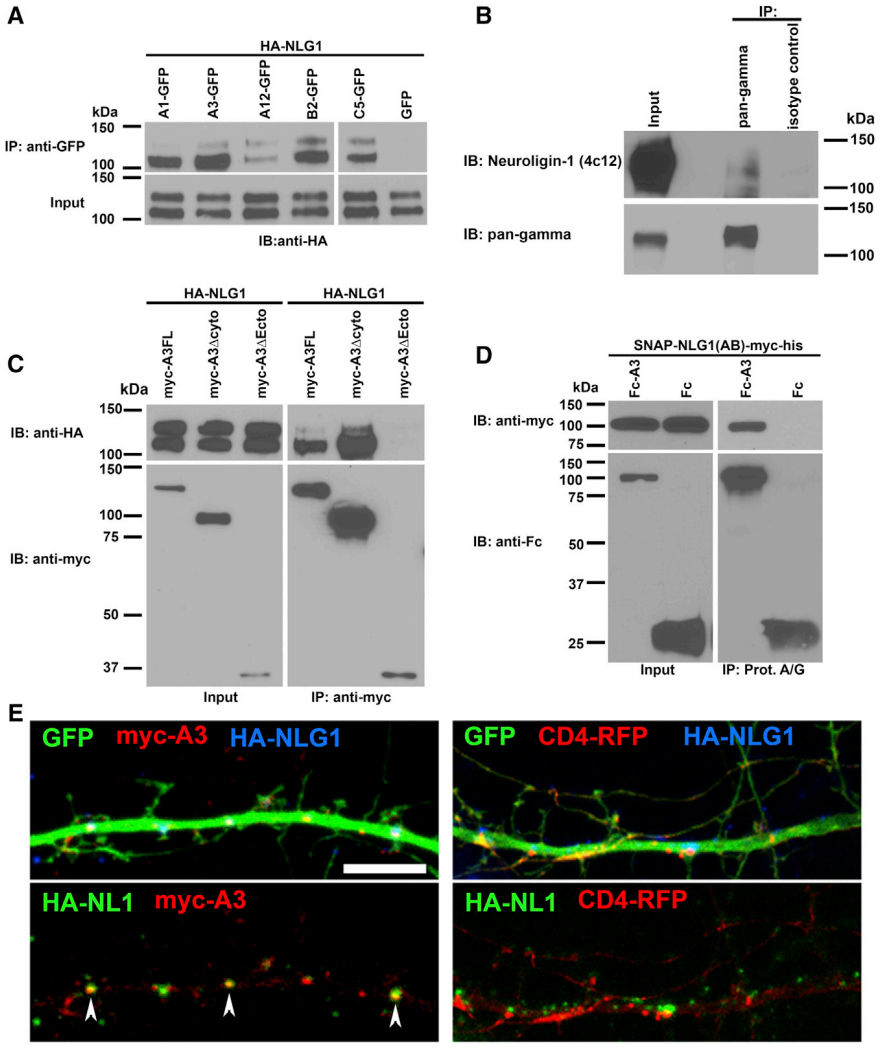


Figure 3. γ-Pcdhs Physically Bind To Neuroligin-1 In *cis*

(A) In co-transfected COS cells, HA-Nlg1 co-immunoprecipitates with multiple full-length γ-Pcdh isoforms C-terminally tagged with GFP, but not with GFP control.

(B) Endogenous Nlg1 co-immunoprecipitates with γ-Pcdhs from P6 cortical lysate in vivo; no Nlg1 was precipitated using a non-specific antibody isotype control.

(C) HA-Nlg1 co-immunoprecipitates with myc-tagged γ-Pcdh-A3 constructs that contain the EC domain (FL, ΔCyto) in COS cells.

(D) Secreted SNAP-myc-his-tagged Nlg1 ectodomain co-immunoprecipitates with secreted Fc-A3 ectodomain (but not the Fc-only negative control) in COS cell conditioned media.

(E) HA-Nlg1 and myc-A3 can interact in *cis* on dendrites (arrowheads). Scale bar, 5 μm.

all 22 γ-Pcdhs (encoded by three small constant exons; Wu and Maniatis, 1999). To test whether individual γ-Pcdh isoforms physically interacted with Nlg1, we co-transfected COS cells with HA-Nlg1 and one of five individual γ-Pcdhs tagged with GFP, and assayed for co-immunoprecipitation using anti-HA antibody. We found that all γ-Pcdh isoforms tested, but not control proteins, were co-immunoprecipitated with HA-Nlg1 (Figure 3A), indicating that the interaction is specific to γ-Pcdhs but not confined to a single isoform. Given this, we also sought to co-immunoprecipitate γ-Pcdhs and Nlg1 from lysates prepared from 1-week-old mouse cortex. Using a monoclonal antibody (Lobas et al., 2012) that recognizes the constant domain shared by all γ-Pcdhs and the 4c12 antibody to detect Nlg1, we were able to co-immunoprecipitate Nlg1 with γ-Pcdhs, indicative of an interaction in the brain in vivo (Figure 3B).

While the fact that multiple γ-Pcdh isoforms could co-immunoprecipitate Nlg1 in vitro might suggest the importance of the constant domain in this interaction, most of the γ-Pcdh constructs that inhibited Nlg1's synaptogenic activity in the artificial synapse assay lacked the cytoplasmic domain (i.e., ΔCyto) for more efficient surface delivery (Schreiner and Weiner, 2010), as did one of the Nlg1 constructs used (i.e., HA-Nlg1Δcyto-2A-RFP; Figures 2D and 2E). Thus, we tested whether the EC or cytoplasmic domain was responsible for γ-Pcdh interaction with HA-Nlg1 by employing γ-Pcdh-A3 deletion constructs lacking the extracellular (ΔEcto) or cytoplasmic (ΔCyto) domains in co-immunoprecipitation experiments. Myc-tagged full length γ-Pcdh-A3 or each deletion construct was transfected into COS cells along with HA-Nlg1 and co-immunoprecipitated using an antibody against the myc-tag. HA-Nlg1 co-immunoprecipitated with full-length and ΔCyto, but not ΔEcto constructs, indicating that the interaction occurs between the ectodomains of the two proteins (Figure 3C).

It is known that EC 2 and 3 are important for the specificity of homophilic *trans*-interactions, while EC6 is required for *cis*-interactions with other clustered protocadherins (Rubinstein et al., 2015; Schreiner and Weiner, 2010). To determine whether the interaction between the ectodomains of Nlg1 and γ-Pcdhs was localized to particular EC repeats, we generated multiple γ-Pcdh-A3 EC deletion constructs and tested them in co-immunoprecipitation assays. Co-immunoprecipitation of Nlg1 was not abolished any of these constructs (ΔEC1-3, ΔEC1-4, ΔEC5-6, and ΔEC6), suggesting that the interaction we observe may be due to multiple sites of contact between the two ectodomains (Figures S1A and S1B).

To further confirm a physical interaction between γ-Pcdhs and Nlg1, we assayed binding using tagged, secreted ectodomain fusion proteins. The ectodomain of γ-Pcdh-A3 was tagged with human Fc (Fc-A3), and the ectodomain of Nlg1 was tagged with SNAP and myc-his (SNAP-NLG1(AB)-myc-his). COS cells were co-transfected with Fc-A3 and SNAP-NLG1(AB)-myc-his,

or Fc-only and SNAP-NLG1(AB)-myc-his, incubated for 36–48 hr, and the conditioned media containing the secreted tagged proteins was collected. To assay a physical interaction between the two secreted ectodomains, we immunoprecipitated using protein A/G beads to pull down the Fc-tag and immunoblotted for the myc tag. Secreted SNAP-NLG1(AB)-myc-his consistently co-immunoprecipitated with Fc-A3, but never with Fc-only, confirming that γ-Pcdh-A3 and Nlg1 interact via their ectodomains, and further suggesting that this interaction is direct (Figure 3D)

Overexpressed HA-Nlg1 appeared in COS cells as two differentially glycosylated forms: a higher apparent molecular weight band at ~130 kDa and a lower band at ~110 kDa (Figures 3A, 3C, S1A, and S1B), consistent with prior results (Comoletti et al., 2003; Ko et al., 2009; Song et al., 1999). We noted that, while both bands co-immunoprecipitated with γ-Pcdhs, the lower band predominated to varying extents (Figures 3A, 3C, S1A, and S1B). The upper band is PNGase F sensitive and considered to be the mature form of Nlg1 most often found at the cell surface, while the lower band is sensitive to Endo H treatment and is thought to be an immature form largely retained within the cell (Figure S1C; Comoletti et al., 2003; Ko et al., 2009). To confirm that the interaction occurs between both differentially glycosylated HA-Nlg1 isoforms, we transfected COS cells with constructs encoding HA-Nlg1 and myc-A3ΔCyto, performed co-immunoprecipitation using antibodies against the myc-tag, and subjected the isolated HA-Nlg1 to Endo H or PNGase F treatment. This experiment confirmed that both forms of HA-Nlg1 interact with the EC domain of myc-A3 (Figure S1D; note that the γ-Pcdh-A3 ectodomain is also differentially glycosylated as demonstrated previously (Schreiner and Weiner, 2010).

We next investigated whether γ-Pcdhs and Nlg1 can interact on the surface of dendrites. Primary hippocampal cultures were transfected at 9 days in vitro (DIV) with constructs encoding HA-Nlg1, GFP, and myc-A3 or CD4-RFP. At 12 DIV, Nlg1 aggregation was induced on the surface of live neurons by the addition to the media of anti-HA antibodies, and recruitment of myc-A3 to the HA-Nlg1 aggregates was assessed compared to CD4-RFP control after 16 hr of incubation. As predicted from the co-immunoprecipitation results, we were able to observe myc-A3, but not CD4-RFP, recruitment to induced HA-Nlg1 aggregates (Figure 3E), indicating that this interaction can occur at the cell surface of developing dendrites.

γ-Pcdh Inhibits Binding of Neurexin1β to Neuroligin-1

Neuroligin-1 binds to neurexin1β on axons to form a *trans*-synaptic complex (Dean et al., 2003; Scheiffele et al., 2000; Südhof, 2008). Given the functional inhibition of Nlg1 by γ-Pcdhs observed in the artificial synapse assay (Figure 2) and the interaction between these two proteins' EC domains (Figure 3), we employed a cell-based protein-binding assay to ask whether γ-Pcdhs can prevent the formation of the Nlg1/neurexin1β complex. COS cells co-expressing HA-Nlg1 and either myc-A3, myc-C3, or CD4-RFP were incubated with a soluble neurexin1β ectodomain-Fc fusion protein (Nrxn1β-Fc), and bound Nrxn was detected by anti-human Fc antibody and quantified by confocal microscopy. While COS cells co-expressing HA-Nlg1 and CD4-RFP exhibited strong Nrxn1β-Fc binding, this was substantially diminished on cells co-expressing either myc-A3 or myc-C3 (Figures 4A and 4B). Reduced Nrxn1β-Fc binding could not be accounted for by any reduction in surface HA-Nlg1 levels; staining of HA-Nlg1 on unpermeabilized COS cells was similar regardless of whether γ-Pcdhs were co-expressed (Figure 4C). Additionally, even when we analyzed a group of cells selected for identical Nlg1 surface levels, Nrxn1β-Fc binding was still significantly reduced when a γ-Pcdh was coexpressed (Figure 4D). These results suggest a mechanism whereby γ-Pcdhs could inhibit Nlg1 activity, by preventing its interaction with presynaptic Nrxns.

γ-Pcdh Does Not Significantly Alter Surface Trafficking of Neuroligin-1

Thus far, our data suggested that the γ-Pcdhs do not significantly disrupt Nlg1 surface trafficking; however, they did preferentially interact with a putative immaturely glycosylated form of Nlg1 that is known to be at least partially intracellular (Comoletti et al., 2003; Ko et al., 2009). Thus, we directly confirmed that γ-Pcdhs do not affect surface trafficking of Nlg1 using a biotinylation approach. Cells were co-transfected with plasmids encoding HA-Nlg1 along with those encoding either CD4-RFP, myc-A3, or myc-C3, and cell-surface proteins were labeled with Sulfo-NHS-SS-Biotin. Biotinylated cell-surface proteins were pulled down using NeutrAvidin beads and analyzed by western blot along with total cell lysate inputs. Compared to the CD4-RFP negative control, cells co-expressing myc-A3 or myc-C3 did not exhibit any significant reduction in biotinylated surface HA-Nlg1 (Figures 5A and 5B). Consistent with earlier results (Ko et al., 2009), we found that the majority of surface Nlg1 comprises the fully glycosylated, mature upper band (short exposure, Figure 5A). However, longer exposures of the same blots did reveal both upper and lower bands in the biotinylated cell-surface fraction (Figure 5C). The presence of this lower band in the cell-surface fraction was not due to cell disruption and aberrant biotinylation of intracellular proteins, as even at maximal exposures no band was observed using anti-β-tubulin (Figure 5C). This suggests the possibility that, at least in COS cells, some of this EndoH-sensitive form of Nlg1 reaches the surface, where it can interact with the EC domains of γ-Pcdhs (see Figure 3). In any case, γ-Pcdh inhibition of Nlg1's ability to bind Nrxn1β and induce presynaptic specializations in vitro does not likely involve altered Nlg1 surface trafficking.

Homophilic Matching In *trans* Does Not Regulate γ-Pcdh Inhibition of Neuroligin-1

A subset of the 22 γ-Pcdhs are stochastically expressed by each neuron and promiscuously form *cis*-multimers that mediate strictly homophilic matching in *trans* between cells (Molumby et al., 2016; Rubinstein et al., 2015; Schreiner and Weiner, 2010; Thu et al., 2014). This suggested to us an elegant potential mechanism by which homophilic γ-Pcdh interactions may allow synapse maturation to proceed by relieving inhibition of Nlg1. Thus, we examined whether increasing homophilic γ-Pcdh matching between axons and COS cells affects γ-Pcdh-mediated inhibition of Nlg1's activity in the artificial synapse assay. To do this, we co-cultured hippocampal neurons harvested from A1-OE transgenic mice (Figure 1) with COS cells expressing

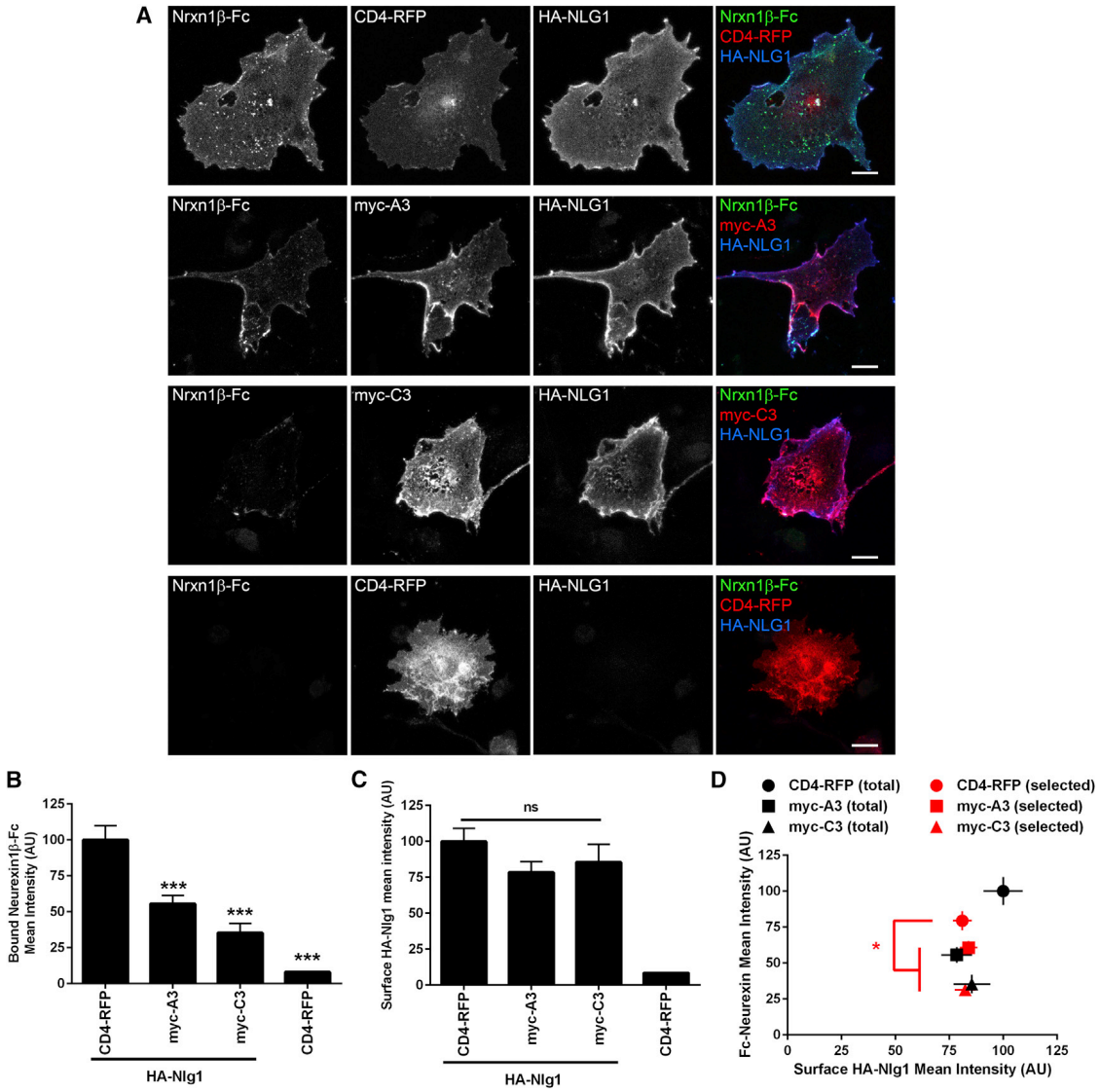


Figure 4. γ-Pcdh Inhibits the Interaction of Neuroligin-1 and Neurexin1β

(A) COS cells were co-transfected with HA-Nlg1 and CD4-RFP (top row), HA-Nlg1 and myc-A3 or myc-C3 (middle rows), or CD4-RFP only (bottom row), incubated with soluble neurexin1β-Fc (Nrxn1β-Fc), fixed, immunostained, and imaged.

(B) Mean intensity quantification of Nrxn1β-Fc bound to COS cells expressing the indicated constructs normalized to HA-Nlg1 and CD4-RFP control show reduced bound Nrxn1β-Fc when myc-A3 or myc-C3 is co-expressed.

(C) Quantification of surface HA-Nlg1 from unpermeablized COS cells co-expressing the indicated constructs normalized to HA-Nlg1 and CD4-RFP control. Expression of γ-Pcdh constructs does not significantly affect surface levels of HA-Nlg1.

(D) Comparison of mean Nrxn1β-Fc intensity of selected (red symbols) COS cells expressing HA-Nlg1 and CD4-RFP, myc-A3, or myc-C3 with similar surface HA-Nlg1 mean intensity compared to the total means of data shown in (B) and (C) (black symbols). n = 20 per condition. *$p < 0.05$, ***$p < 0.001$, n = 50–60 cells per condition. Scale bar, 20 μm.

either the homophilically matching isoform (V5-tagged A1), or the mis-matching myc-A3, along with HA-Nlg1 (Figure 6). Co-expression of *either* V5-A1 or myc-A3 with HA-Nlg1 in COS cells significantly reduced presynaptic differentiation in contacting axons to the same extent (Figures 6A and 6B), without affecting total expression levels of HA-Nlg1 (Figure 6C).

Interestingly, in some cases when A1-OE axons contacted COS cells expressing the homophilically matching V5-A1, it appeared to cluster at contact sites, along with HA-Nlg1 (Figure 6A). This not only supports the interaction of Nlg1 and γ-Pcdhs at the cell surface (as also shown in Figure 3E), but also makes it all the more remarkable that V5-A1 inhibited Nlg1 activity in this assay regardless of homophilic matching with axons. We thus conclude that homophilic interaction of γ-Pcdh isoforms in *trans* does not affect their ability to bind to and inhibit Nlg1 in *cis*, at least in the artificial synapse assay. To confirm that the presence of Nlg1 does not, itself, disrupt γ-Pcdh homophilic interactions, we performed a K562 cell

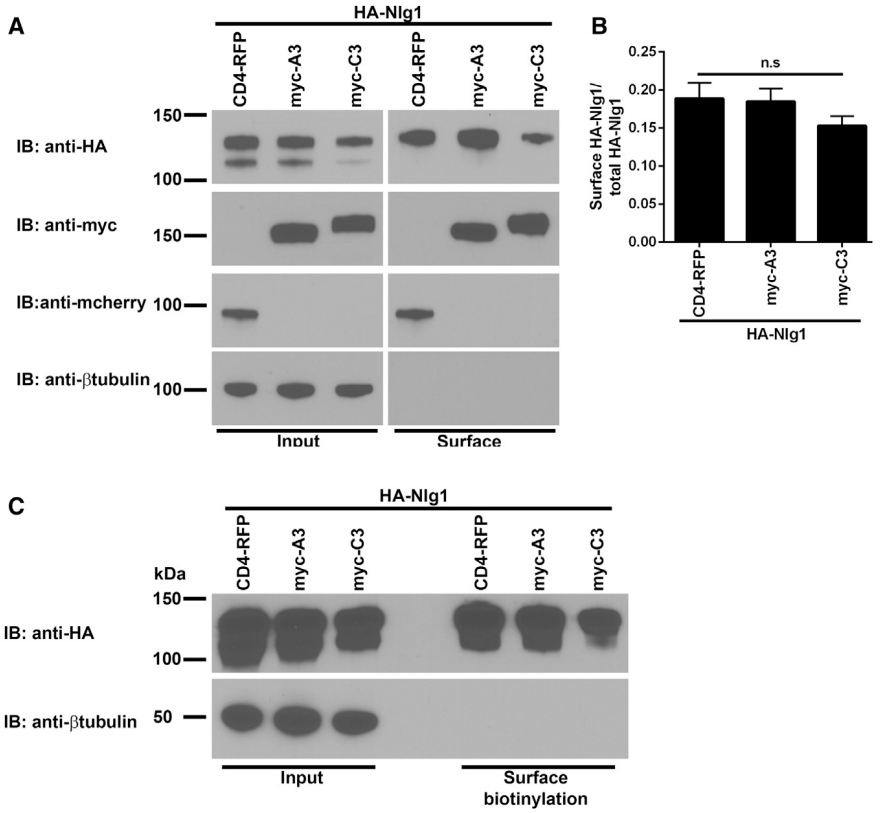


Figure 5. γ-Pcdh Does Not Alter Surface Trafficking of Neuroligin-1

(A) Immunoblotting of surface biotinylated proteins in comparison with input lysates (one of five total HEK293 whole-cell lysate) showed no significant differences in HA-Nlg1 surface expression between indicated co-transfected constructs.

(B) Quantification of the ratio of surface-biotinylated protein to total HA-Nlg1 in cells expressing the indicated constructs. CD4-RFP versus myc-A3 p = 0.9842, CD4-RFP versus myc-C3 p = 0.3386, n = 4. n.s., not significant. Data show mean ± SEM.

(C) Longer exposure of immunoblots show surface biotinylation of the lower molecular weight form of HA-Nlg1, despite a continued lack of signal for β-tubulin (indicating no leakage of biotin reagent into the cell).

aggregation assay as previously described (Schreiner and Weiner, 2010; Thu et al., 2014). For the two γ-Pcdh isoforms tested in this assay, homophilic interaction (i.e., cell aggregation) was observed as expected regardless of whether cells were co-transfected with Nlg1 (Figure S2).

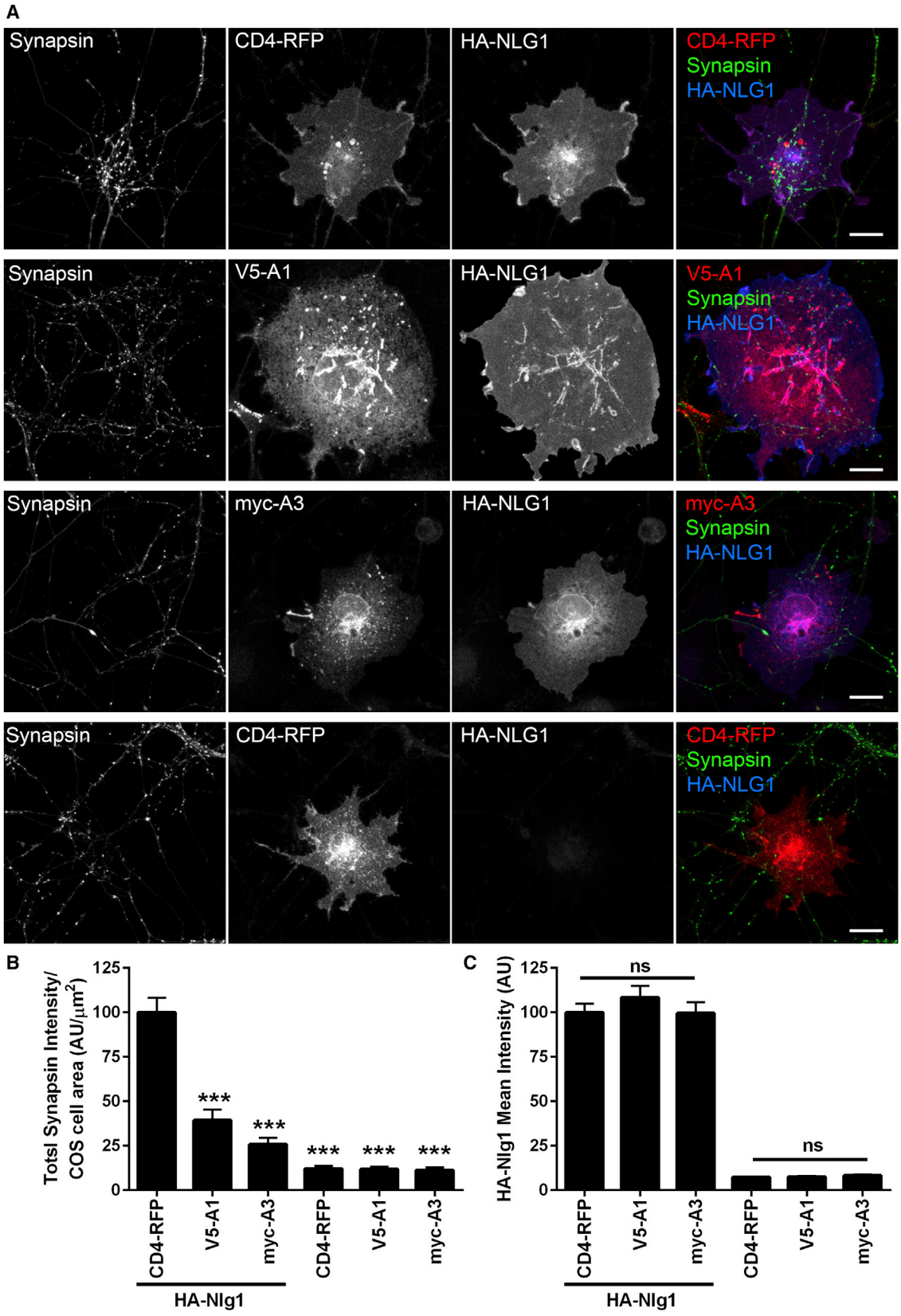
γ-Pcdhs Inhibit the Increase in Spine Density Induced by Neuroligin-1 OE in Cultured Neurons

When overexpressed in hippocampal neurons, Nlg1 can significantly increase the density of dendritic spines (Boucard et al., 2005; Chih et al., 2005; Ko et al., 2009). Based on in vivo phenotypes (Figure 1) and the artificial synapse assay data (Figures 2 and 6), we hypothesized that co-expression of a γ-Pcdh with HA-Nlg1 would inhibit its ability to potentiate spine density. To test this hypothesis, we co-transfected wild-type hippocampal neurons at 9 DIV with constructs encoding HA-Nlg1 and GFP (to fill neurons and reveal their morphology) along with those encoding either CD4-RFP, myc-A3, or myc-C3 and quantified dendritic spine density at 12 DIV. OE of HA-Nlg1 increased spine density in hippocampal neurons compared to controls to an extent similar to that previously described (Figures 7A and 7B; Chih et al., 2005). In contrast, spine density remained at control levels when HA-Nlg1 was cotransfected with either γ-Pcdh isoform (Figures 7A and 7B). These results indicate that γ-Pcdhs can inhibit the activity of Nlg1 in neurons as well as in COS cells and demonstrate the plausibility of an Nlg1-related mechanism underlying the alterations in spine density observed in vivo in *Pcdhg* KO and OE mice (Figure 1).

DISCUSSION

The interplay between the complexity of a neuron's dendritic arbor and the development of its synaptic connectivity is regulated by cues in its molecular environment, including cell-cell interactions mediated by adhesion molecules (de Wit and Ghosh, 2016). In neurons of the cerebral cortex and hippocampus, the γ-Pcdhs were previously shown to be critical for the development of a properly complex dendritic arbor (Garrett et al., 2012; Molumby et al., 2016; Suo et al., 2012). Here, we present evidence that they negatively regulate dendritic spine density in the cerebral cortex in vivo. Multiple γ-Pcdhs can bind to Nlg1 and inhibit its ability to bind neurexin-1β, induce presynaptic differentiation, and promote dendritic spine density in in vitro assays. Together, these results suggest that the γ-Pcdhs, through both *cis*- and *trans*-interactions, may regulate a balance between dendrite arborization and spine development in the developing forebrain.

The negative regulation of spine density observed in this study contrasts with our prior analysis of *Pcdhg* mutant embryonic spinal cords, which indicated that the γ-Pcdhs provide positive regulation of early synaptogenesis there (Garrett and Weiner, 2009; Weiner et al., 2005). This may reflect different roles for the γ-Pcdhs in interneurons (the predominant neurons of the spinal cord) versus projection neurons (as in those analyzed in the forebrain), or in embryonic synaptogenesis versus postnatal synapse maturation. Our observation of increased spine density in *Pcdhg* KO cortical neurons also contrasts with reports of reduced spine density following *Pcdhg* KO in olfactory granule cells (Ledderose et al., 2013), or following shRNA-mediated knockdown of *Pcdhg* constant exons in cultured hippocampal neurons (Suo et al., 2012). Given the known off-target effects of many shRNA vectors on dendritic spine density (Alvarez et al., 2006), and the impossibility of rescuing the phenotype by re-expressing each neuron's original *Pcdhg* repertoire, it is difficult to interpret the knockdown phenotype in light of our



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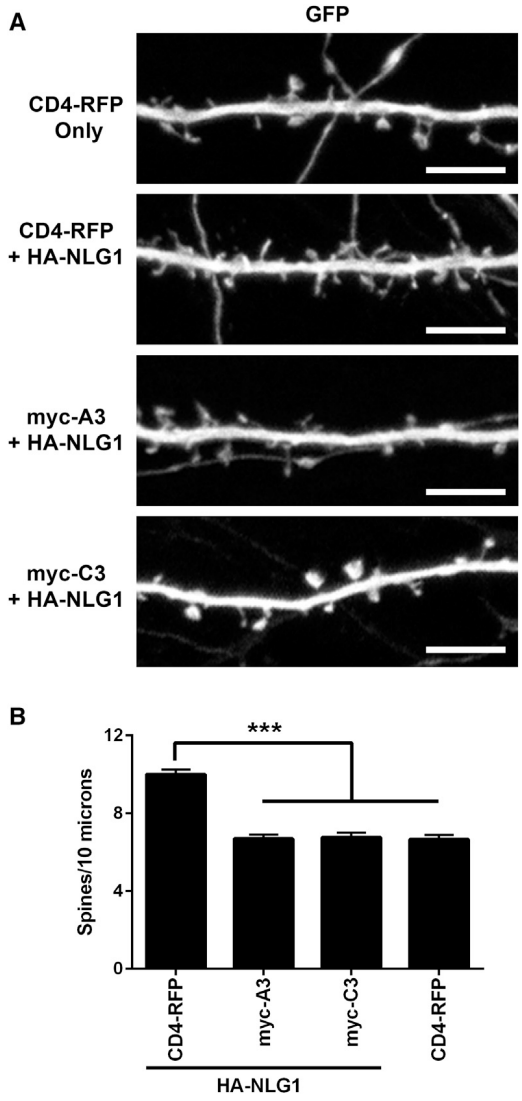

Figure 7. γ-Pcdh OE Inhibits the Increase in Spine Density Induced by Neuroligin-1 OE

(A) Cultured hippocampal neurons were co-transfected at 9 DIV with GFP and the indicated constructs and then fixed for spine analysis at DIV 12.

(B) Quantification of the number of spines per 10-μm dendritic length for each condition show that HA-Nlg1 increases spine density when co-transfected with CD4-RFP control as expected, but this increase is reduced back to CD4-RFP-only control levels when myc-A3 or myc-C3 is co-expressed. ***$p < 0.001$, n = 30 per condition. Scale bar, 5 μm.

genetic data. In any case, our in vivo KO results are strongly supported by the demonstration that that OE of γ-Pcdhs in vivo has the opposite effect on cortical spine density as does KO, and that γ-Pcdhs can suppress the potentiation of spine density by Nlg1 OE in hippocampal neurons. We suggest that γ-Pcdh function may be differentially regulated by distinct *cis*-interaction partners, either through their EC domains as observed here for Nlg1 or through intracellular signaling partners of their cytoplasmic domains, and that these may vary across neuronal subsets or developmental stages (Keeler et al., 2015a; Mah and Weiner, 2016; Mah et al., 2016).

The landscape of spine morphologic profiles in *Pcdhg* KO and OE mice may provide clues into the roles of γ-Pcdhs in the development of spine synapses. Thin and stubby spines are generally representative of immature spine morphologies, as these are highly abundant in the developing brain (Fiala et al., 1998; Petrak et al., 2005). Filopodial subtypes are abundant postnatally; their numbers decline thereafter, and subsequent spinogenesis that has been observed is generally of the thin subtype (Boyer et al., 1998; Dailey and Smith, 1996; Holtmaat et al., 2005; Knott et al., 2006). Post mortem anatomical and time-lapse sampling of cortical dendritic spines also supports the idea that spine synapse maturation is typified by expansion of the spine head, with mushroom subtypes lying at the farthest end of this maturational continuum (Bloss et al., 2011; Holtmaat et al., 2005; Matsuzaki et al., 2004). In this regard, the fact that the increase in spine density in adult *Pcdhg* KO mice is in thin subtypes supports the interpretation that γ-Pcdhs can negatively regulate new spine formation while generally leaving other subtypes unaffected. By contrast, γ-Pcdh-A1 OE not only induced profound decrements in overall spine density, but also completely altered the landscape of spine subtypes. A1-OE mice show decreases in thin and mushroom spines, whereas stubby spine densities are elevated. Although the functional significance of stubby spines in the adult brain has remained elusive, their numbers are estimated to be at least 2-fold higher in the early postnatal period as compared with the adult, and this same period is marked by substantially fewer thin and mushroom spines as compared with the adult cortex (Petrak et al., 2005). Although more work is needed to definitively address this issue, the similarities between A1-OE adult and wild-type postnatal spine profiles suggest that γ-Pcdh OE may not simply prevent spine formation or increase elimination but could impede the maturation of spine synapses in the cortex, consistent with inhibition of Nlg1.

Our demonstration that γ-Pcdhs can inhibit the activity of Nlg1 in the artificial synapse co-culture assay places them in the company of other membrane associated molecules shown to interact with and regulate neuroligins in this assay (Pettem et al., 2013; Woo et al., 2013). Such molecular interactions provide an additional neuroligin regulatory mechanism, along with phosphorylation affecting surface expression (Bemben et al., 2014) and splice site insertions affecting binding to neurexin-1α and -1β (Boucard et al., 2005; Chih et al., 2006). We present evidence

Figure 6. Homophilic Interaction Does Not Alter γ-Pcdh Inhibition of Nlg1 Synaptogenic Activity

(A) Co-cultured COS cells co-expressing HA-Nlg1 and a V5-tagged γ-Pcdh-A1 that homophilically matched A1-OE crossing axons induced similarly reduced synapsin clustering as did cells co-expressing myc-A3, which did not homophilically match A1-OE axons. COS cells expressing CD4-RFP alone show no synapsin clustering when cultured with hippocampal neurons (bottom).

(B) Quantification of total integrated intensity of synapsin immunofluorescence associated with COS cells co-expressing the indicated constructs, divided by the COS7 cell area and normalized to the value of CD4-RFP control.

(C) Quantification of total Nlg1 mean fluorescence intensity in COS cells analyzed in (B). n = 40 per condition. ***$p < 0.001$. Scale bar, 20 μm.

that the mechanism of inhibition involves a physical *cis*-interaction between γ-Pcdh and Nlg1 EC domains that, in turn, disrupts the formation of the Nlg1/neurexin1β *trans*-complex. Cell imaging and biochemical studies have indicated that γ-Pcdhs are present in PSD fractions but are also prominently featured perisynaptically and in the dendrite shaft (Fernández-Monreal et al., 2009; Garrett and Weiner, 2009; Wang et al., 2002). The predominantly perisynaptic localization of γ-Pcdhs suggests that they could sequester Nlg-1 away from postsynaptic sites. Although mutations in Nlg1 associated with autism (Chubykin et al., 2005) and phosphorylation by CaMKII (Bemben et al., 2014) have been shown to significantly alter surface trafficking and inhibit synaptogenesis, we found no evidence that γ-Pcdhs alter trafficking of Nlg1. Nevertheless, the interaction of γ-Pcdhs with immaturely glycosylated forms of Nlg1, and the demonstration that a significant fraction of γ-Pcdh proteins is found in intracellular vesicles (Fernández-Monreal et al., 2009, 2010) means we cannot exclude a possible effect on Nlg1 trafficking in neurons and dendrites.

Despite Nlg1's synaptogenic activity in the artificial synapse assay, and spine-promoting activity in neurons in vitro, Nlg1, 2, and 3 triple-knockout mice exhibited normal synapse density in the brainstem in vivo and in cultured hippocampal neurons (Varoqueaux et al., 2006). Cortical spines and synapses could not be examined in vivo, however, because of the perinatal lethal phenotype due to disrupted synapse maturation and transmission (Varoqueaux et al., 2006). Subsequent conditional ablation of Nlg1, 2, and 3 in postnatal CA1 pyramidal hippocampal neurons disrupted long-term potentiation without having any effect on spine density, indicating that Nlg1 is indeed not required for synapse and spine formation (Jiang et al., 2016). However, it has also been shown that intercellular variation of Nlg1 protein levels across cortical neurons altered synaptogenesis and spine density (Kwon et al., 2012). By using sparse Nlg1 knockdown in vivo and mixed co-cultures of control and Nlg1 knockout neurons, Kwon et al. (2012) showed that neurons with reduced Nlg1 compared to surrounding neurons exhibited reduced spine density. This may be most relevant to our data, because the degree to which γ-Pcdhs modulate Nlg1 function could vary in a given neuron depending on γ-Pcdh surface expression, or subcellular localization; the regulation of these parameters in neurons is still unclear.

Nlg1/Nrxn1β interactions in the embryonic *Xenopus* brain have been implicated in "synaptotropic" regulation of dendrite growth (Chen et al., 2010). The "synaptotropic hypothesis" posits that nascent *trans*-synaptic contacts between growing dendrites and axons prevent retraction of dendritic filopodia and ultimately stabilize dendritic regions of proper innervation while destabilizing others (Cline and Haas, 2008; Vaughn, 1989). The results of Chen et al. (2010), in which Nlg1 function was disrupted by in vivo administration of Nrxn-Fc, expression of mutant Nlg1 constructs, or knockdown, led to a modified model in which immature synapses stabilize filopodia enough to prevent collapse but allow for continued dendrite arbor growth; further synapse maturation stabilizes the dendrite arbor and ends the period of growth and plasticity. The γ-Pcdhs may thus prevent synapse maturation by inhibiting Nlg1, while at the same time promoting elaboration of dendritic complexity, a possibility in line with the in vivo phenotypes shown here and in Molumby et al. (2016).

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 NIH guidelines for the use of animals. The *Pcdh-γ*^{fcon3} conditional deletion allele (Garrett et al., 2012; Garrett and Weiner, 2009; Prasad et al., 2008) and the OE *A1-mCherry* transgenic mouse line (Kostadinov and Sanes, 2015; Lefebvre et al., 2012; Molumby et al., 2016) have been previously described. The *Emx1-Cre* line (stock #005628) and *Thy1-YFPH* line (stock #003782) were obtained from The Jackson Laboratory. All in vivo spine analyses were performed on both male and female littermates at 5–6 weeks of age. All hippocampal cultures were generated from entire litters of either C57BL/6 wild-type mice or *A1-mCherry* OE mice at P0.

Cell and Hippocampal Neuron Cultures

Wild-type neuronal cultures were performed using pooled hippocampal tissue from P0 C57BL/6 pups as described previously (Garrett et al., 2012; Keeler et al., 2015b; Molumby et al., 2016). Transgenic hippocampal cultures were prepared from individual A1-mCherry OE pups as previously described (Molumby et al., 2016). Neurons, plated onto 12-mm German glass coverslips (coated with Matrigel [Corning] diluted 1:50 in Neurobasal media [Invitrogen]) at a density of 30,000 cells per coverslip, were maintained in Neurobasal supplemented with GlutaMAX, B27 supplements (Invitrogen), and penicillin/streptomycin. In some studies, neurons were transfected with indicated constructs using Lipofectamine 2000 at 9 DIV, and cultures were fixed and analyzed at 12 DIV.

For neuron-fibroblast co-culture assays (performed essentially as described in Biederer and Scheiffele, 2007), separately transfected COS7 cells were seeded onto wild-type hippocampal cultures supplemented with fresh Neurobasal and Ara-C (2 μM) at 8 DIV and fixed with 4% paraformaldehyde 36 hr later. For transgenic cultures, genotypes from tail preps were determined by PCR, and desired cultures were kept for neuron-fibroblast co-culture assays as described above.

Production of Neurexin1β-Fc and Binding Assay

HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) with the neurexin1β-Fc plasmid. The medium on transfected cells was replaced with a minimal volume of DMEM with 10 mM HEPES, 1% FBS, and penicillin/streptomycin for collection of the secreted neurexin1β-Fc fusion protein. After 96 hr of incubation, media containing the fusion protein were collected and centrifuged at 3,200 rpm for 10 min to remove debris. Tris-HCl (pH 8.0) was added to the collected media to a final concentration of 25 mM. Collected media was added to a Protein A Hitrap (GE Healthcare) column at a flow rate <1 mL/min. The column was washed subsequently with 100 mL of 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mL of 5 mM Tris-HCl (pH 8.0). The protein was eluted in 5 mL of 100 mM glycine-HCl (pH 3.0), 100 mM NaCl, followed by 5 mL of 100 mM glycine-HCl (pH 2.5), 100 mM NaCl, and collected in 500 μl fractions containing 75 μl of Tris-HCl (pH 8.0) to neutralize the protein solution. Samples were checked by SDS/PAGE and peak fractions of eluted protein were pooled and concentrated in a 30K cutoff Amicon ultrafiltration unit (EMD-Millipore).

COS7 cells were plated onto Matrigel-coated 12-mm coverslips and transfected using Lipofectamine 2000; 36 hr later, cells were washed with DMEM supplemented with 20 mM HEPES (pH 7.4) and 0.1% BSA followed by incubation with purified neurexin1β-Fc (0.15 μM) for 1 hr at 20°C. Cells were washed three times with PBS, fixed with 4% paraformaldehyde, and blocked using DMEM supplemented with 20 mM HEPES (pH 7.4) and 4% BSA. Primary antibodies against EC tags were incubated overnight at 4°C, washed three times with PBS, and detected with fluorescently tagged secondary antibodies as previously described (Molumby et al., 2016).

Co-aggregation Assay

Hippocampal neurons were transfected as above. At 12 DIV, neurons were incubated with rat anti-HA (Roche 3F10) for 30 min, followed by a 30 min

incubation with 647 Donkey anti-Rat (Jackson Labs). Previously collected neuronal-conditioned media supplemented with fresh Neurobasal was returned to the wells for 16 hr. Neurons were fixed, washed, and prepared as previously described (Molumby et al., 2016).

Surface Biotinylation

Total lysate and biotinylated surface proteins (labeled with Sulfo-NHS-SS-Biotin according to manufacturer's procedures [Pierce]) were examined by SDS-PAGE and western blot essentially as previously described (Schreiner and Weiner, 2010). See Supplemental Experimental Procedures for details.

In Vivo Synapse and Dendritic Spine Image Collection and Analysis

Vibratome sectioning, cryostat sectioning, immunostaining, and confocal imaging were performed as described (Garrett and Weiner, 2009; Garrett et al., 2012; Molumby et al., 2016). Assessment of dendritic spine density and morphology were performed as described (Anderson et al., 2014; Radley et al., 2013). See Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.060.

AUTHOR CONTRIBUTIONS

Conceptualization, M.J.M., D.S., A.M.G., and J.A.W.; Methodology, M.J.M., R.M.A., D.S., A.M.G., J.J.R., and J.A.W.; Investigation, M.J.M., R.M.A., D.J.N., N.K.K., A.M.G., and D.S.; Writing – Original Draft, M.J.M., R.M.A., D.S., J.J.R., and J.A.W.; Writing – Review & Editing, M.J.M., D.S., J.J.R., and J.A.W.; Funding Acquisition, J.A.W.

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