SUPPLEMENTAL FIGURES AND LEGENDS

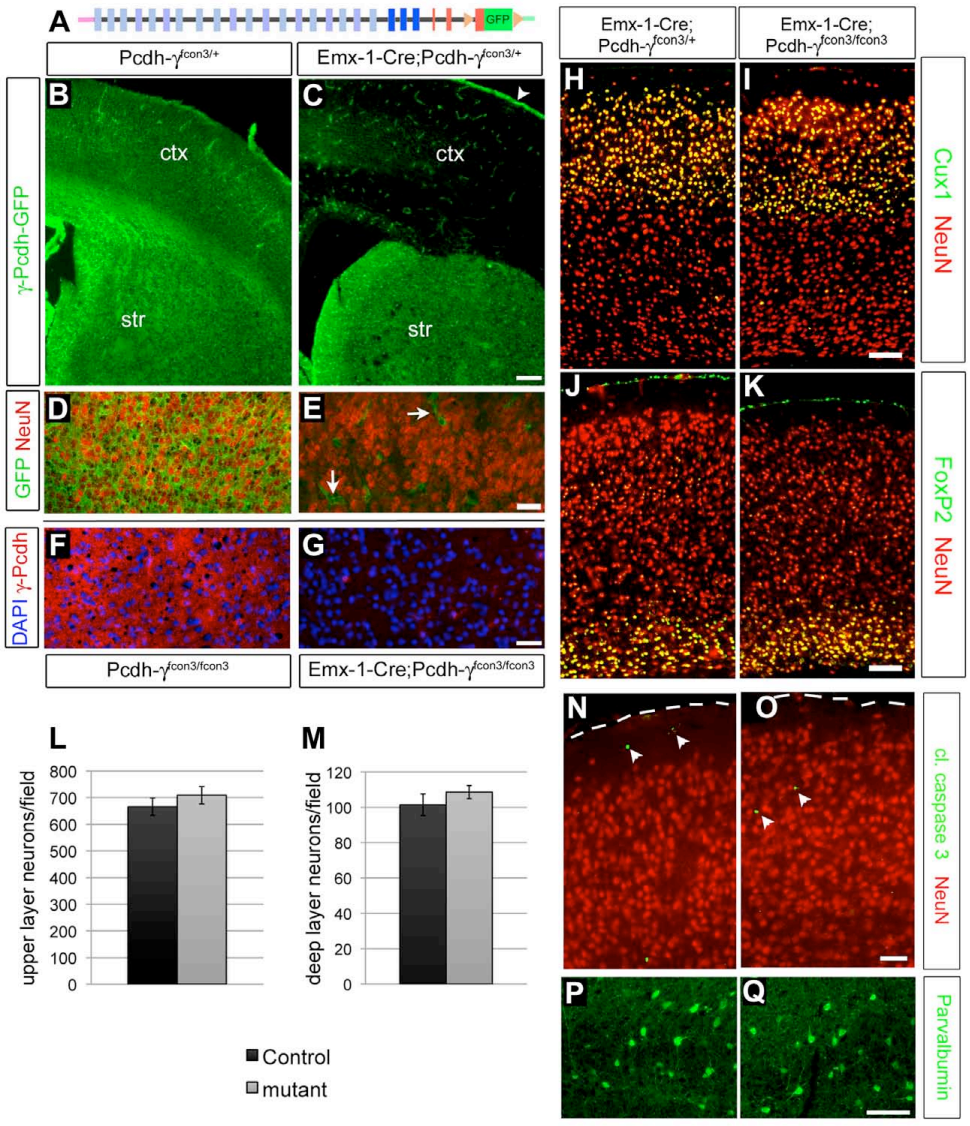

Figure S1 (related to Figure 1): *Emx1-Cre* excision of the *Pcdh-γ^{fcon3}* allele in the cerebral cortex does not increase cell death or disrupt layer formation. A) Diagram of the *Pcdh-γ^{fcon3}* allele illustrating the 22 varible exons (shades of blue correspond to A, B, and C subfamilies) and the constant exons (red), including the GFP-fused third constant exon flanked by loxP sites. B-E) Coronal sections through the cortex and striatum of a neonatal *Emx-1-Cre; Pcdh-γ$^{fcon3/+}$* double-heterozygote (C,E) and a *Pcdh-γ$^{fcon3/+}$* control (no Cre; B,D), stained with anti-GFP antibody. As expected, *Emx1-Cre* results in deletion of the floxed constant exon 3-GFP fusion in the majority of cells in the cortex (ctx), but not in the adjacent striatum (str, C). Residual GFP signal is from blood vessels (arrows, E), which appear to express the γ-Pcdhs, and from scattered ganglionic eminence-derived interneurons. The vast majority of NeuN-positive cells in the cortex of *Emx1-Cre;* Pcdh-γ$^{fcon3/+}$ double-heterozygotes lack GFP staining (E), in contrast

to controls (D). In the *Emx1-Cre; Pcdh-*$\gamma^{fcon3/fcon3}$ adult mutant cortex (G), almost no staining is observed using an antibody recognizing the first two (non-floxed) constant exons (compare to the Cre-negative *Pcdh-*$\gamma^{fcon3/fcon3}$ cortex (F)), confirming that the excised *Pcdh-*γ^{fcon3} allele is a null or near-null. Staining for cell-type specific markers confirms normal lamination of upper layer (Cux1 (Nieto et al., 2004), H, I; quantified in L) and deep layer (FoxP2 staining in J (Ferland et al., 2003), K; quantification of layer V *Thy1-YFPH* neurons in M) neurons. Immunostaining of cortical sections with antibodies against NeuN and cleaved caspase-3 (N, O) demonstrates similar overall neuronal density and lack of neuronal apoptosis in both *Emx1-Cre; Pcdh-*$\gamma^{fcon3/fcon3}$ mutants (O) and Cre-negative controls (N; arrowheads mark the few apoptotic non-neuronal cells observed). There was also no obvious reduction in the number of ganglionic eminence-derived, parvalbumin-positive interneurons (P,Q). Scale bar is 100 μm. Scale bar = 200 μm in (B, C), 50 μm in (D-G), 100 μm in (H-K), and 50 μm in (N-Q).

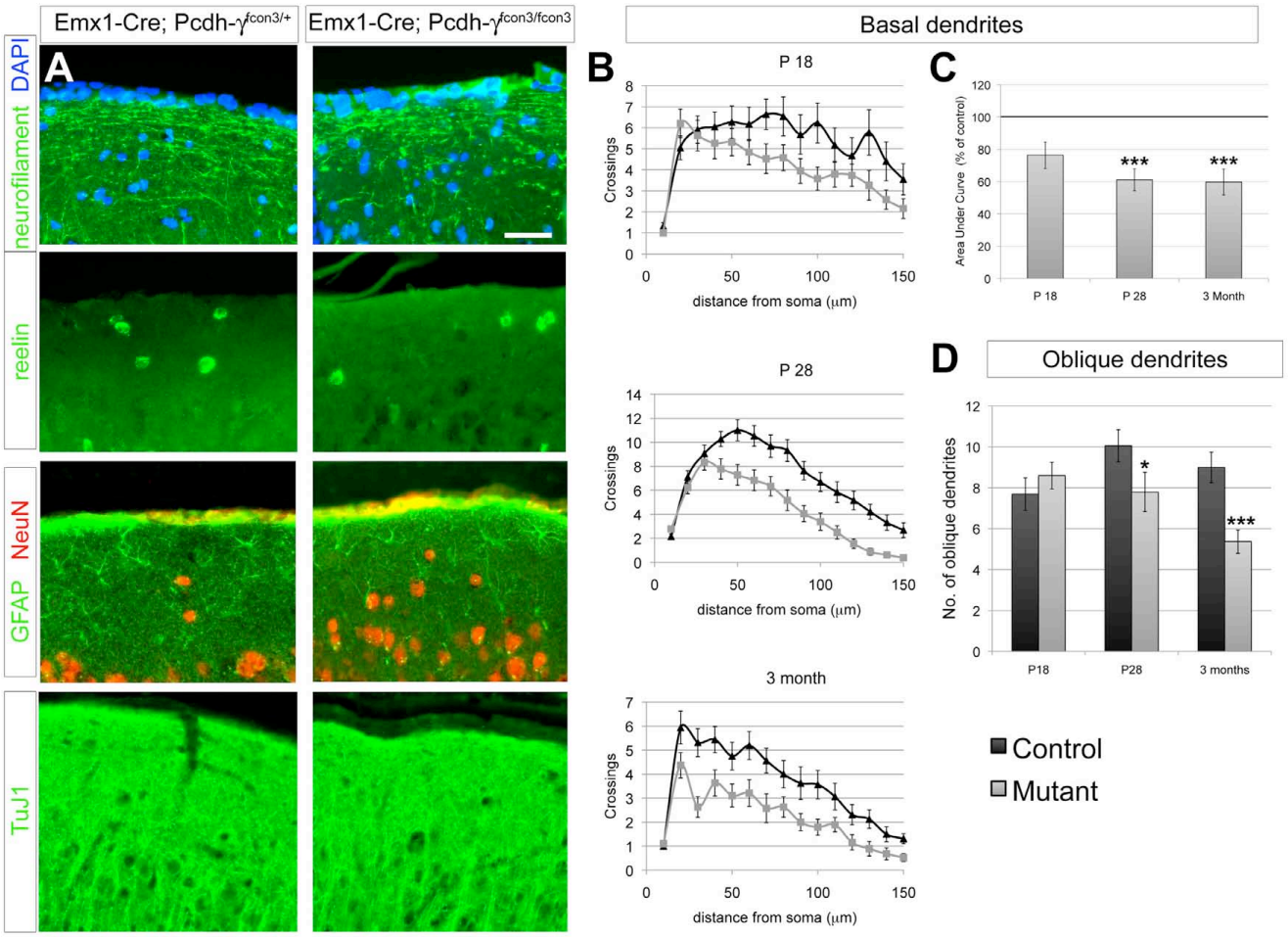

Figure S2 (Related to Figure 2): Disruption of arborization in all dendritic compartments of *Pcdh-γ* mutant cortical neurons. A) Immunostaining analysis shows that cellular components of layer I (axonal neurofilaments, TuJ1-positive processes, reelin-positive Cajal-Retzius cells, and GFAP-positive astrocytes) are unperturbed in *Pcdh-γ* mutants, consistent with the reduction in this layer being due only to loss of apical dendrite branches. Separate analysis of basal (B, C, defined as branches originating from the soma) and oblique (D, defined as branches off of the main apical dendrite below the final tuft) dendrites of YFP-positive layer V neurons shows that all dendrites of mutant neurons are similarly affected. Graphs in B, C and D represent means and SEM. N = 20 neurons from each genotype at each time point. * $p < 0.05$; *** $p < 0.001$.

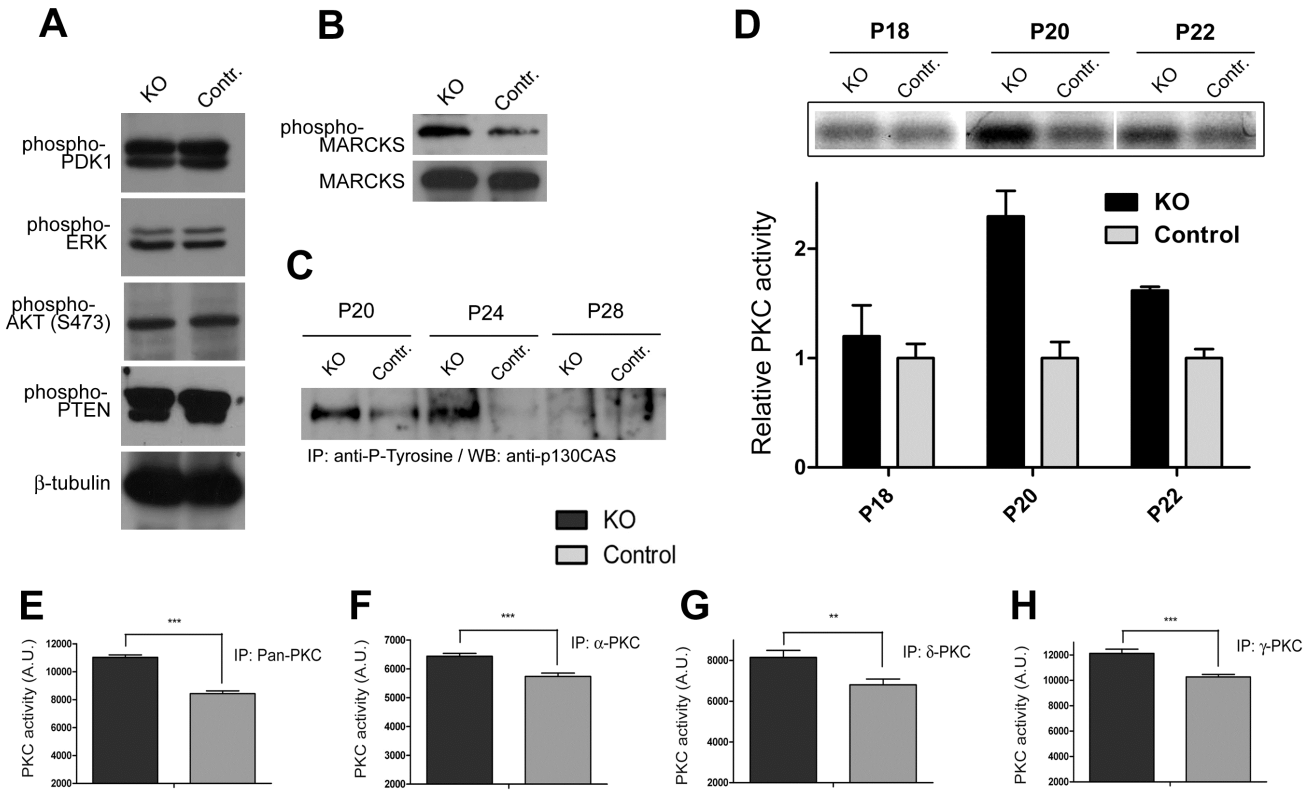

Figure S3 (Related to Figure 3): Analysis of signaling molecules in cortex-restricted *Pcdh-γ* mutants. (A) Western blots of *Emx1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ mutant or control littermate cortical lysates using the indicated phospho-specific antibodies. No differences in the levels of phosphorylated PDK1, ERK, AKT, or PTEN were observed. β-tubulin was used as a loading control. (B) Levels of phospho-MARCKS are elevated in mutant cortical neurons *in vitro*, just as they are *in vivo.* (C) Tyrosine-phosphorylated proteins were immunoprecipitated from *Emx1-Cre; Pcdh-γ*$^{fcon3/fcon3}$ mutant (KO) or control littermate cortical lysates at P20, P24, and P28 and analyzed by western blot using an antibody to p130CAS, an adaptor protein regulated by FAK (Cary et al., 1998). Mutant cortex exhibited higher levels of phosphorylated p130 CAS at P20 and P24, providing further confirmation that FAK activity is aberrantly high. (D) Levels of PKC enzymatic activity were measured from membrane preparations of mutant and control cortices at P18, P20, and P22 to confirm that PKC activity becomes aberrantly

high in mutants only at P20. Individual PKC isoforms (F-H), or all PKC isoforms (E), were isolated by immunoprecipitation (IP) with the appropriate antibodies and activity measured. In all cases, PKC activities were significantly higher in the mutant cortical samples (All graphs except for (D) show means and SEM of 3 experiments; **$p<0.05$; ***$p<0.01$).

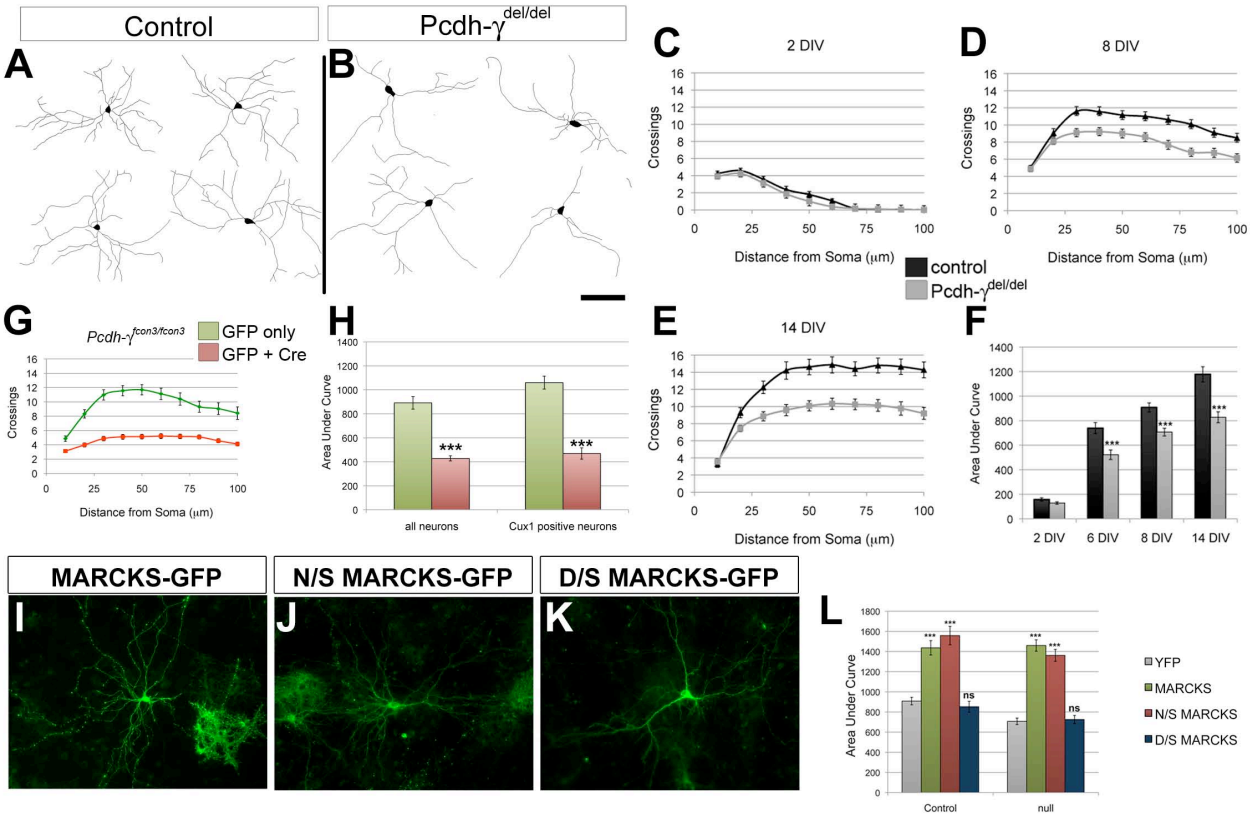

Figure S4 (Related to Figure 4): Cell-autonomous dendrite arborization defects in *Pcdh-γ* mutant cortical neurons *in vitro.* Cortical neurons were cultured from neonatal control and *Pcdh-γ*$^{del/del}$ mice and transfected with a plasmid expressing YFP at 1 DIV. Cultures were fixed at 2, 8, and 14 DIV and dendritic arborization was measured by Sholl analysis. While initial neurite outgrowth appeared nearly normal (C, F) mutant neurons displayed significantly reduced dendritic arbor complexity at all later time points (D-F). Several typical arbor reconstructions are shown in (A, B). Neurons cultured from *Pcdh-γ*$^{fcon3/fcon3}$ conditional mutant mice were co-transfected at 1 DIV with plasmids encoding Cre and GFP (or GFP only as a control), and measured by Sholl analysis at 8 DIV. Individual mutant neurons respond to γ-Pcdh loss with cell-autonomous dendrite arborization defects (G) that were similar between Cux1-positive and -negative neurons (H). Fluorescence micrographs show that GFP-tagged wild type (I), unphosphorylatable (J, N/S), and pseudophosphorylated (K, D/S) MARCKS were each

expressed at similar levels when transfected into neurons. Wild type and N/S MARCKS greatly increased dendrite arborization in control and *Pcdh-γ* null neurons, while D/S MARCKS had no effect (L). Scale bar is 100 μm in (A,B). Graphs represents means and SEM. *** $p<0.001$.

Supplemental Experimental Procedures

Mouse strains: *Pcdh-γ^{del}* (Wang et al., 2002b), and *Pcdh-γ^{fcon3}* alleles (Prasad et al., 2008) have been described elsewhere. The *Cre-ER* line used is described in (Guo et al., 2002) and was obtained from Dr. Joshua Sanes, Harvard University. The *Emx1-Cre* line (Gorski et al., 2002) and the *Thy1-YFPH* line (Feng et al., 2000) were obtained from the Jackson Laboratory (Bar Harbor, ME). For all experiments, at least three animals per genotype, per time point were analyzed for each measure.

Antibodies: The following primary antibodies were used: rabbit anti-FoxP2 (Abcam); goat anti-Cux1 (Santa Cruz); rabbit anti-GFP (Molecular Probes); mouse anti-γ-Pcdh constant domain (NeuroMab; produced with and tested by our lab; Lobas et al., in preparation); mouse anti-NeuN (Chemicon); rabbit anti-cleaved caspase-3 (Cell Signaling Technology); mouse anti-reelin (Abcam); rabbit anti-GFAP (Promega); mouse TuJ1 (Covance); mouse anti-parvalbumin (Sigma); mouse anti-neurofilaments (Sternberger Monoclonals); mouse anti-bassoon (Stressgen); mouse anti-PSD-95 (Affinity BioReagents), anti-α-PKC, anti-δ-PKC, anti-γ-PKC (Santa Cruz). All of the following phospho-specific antibodies (and any corresponding non-phospho-specific control antibodies) were from Cell Signaling Technology: anti-phosphoPDK1, anti-phosphoERK, anti-phosphoAKT, anti-phosphoPTEN, anti-phosphoMARCKS, anti-phosphoPLCγ1, anti-phosphoFAK.

Cortical cultures: Cortical cultures were prepared as described (Ghosh et al., 1995). Briefly, cortices were dissected from E18/P0 animals and the meninges removed. Cortices were cut into small chunks and digested in an enzyme solution (papain, 10 U/ml) 2 X 20 minutes after which tissue was rinsed with increasing concentrations of trypsin inhibitor followed by plating media (Basal Medium Eagle, 5% FBS, Glutamax (Invitrogen), N2 supplements (Invitrogen) and penicillin/streptomycin). Cells were plated onto German coverglass coated sequentially with poly-L-lysine and EHS laminin at a density of 250,000 cells per coverslip. After 2 hours, and every 2 days subsequent, media was changed to Neurobasal media with Glutamax, B27 supplements (Invitrogen), and pen/strep. Transfections with

Lipofectamine 2000 (Invitrogen) were performed after 1 day *in vitro* (DIV). Each coverslip was incubated with 0.5 μg total DNA and 0.5 μl Lipofectamine 2000, mixed in Neurobasal media according to the manufacturer's instructions, for 2 hours before being changed back to regular complete Neurobasal media. The MARCKS constructs were described previously (Swierczynski et al., 1995), and were the kind gift of Dr. Perry Blackshear. In some experiments, pharmacological inhibitors were added with media change at 6 DIV: Gö6983 (Tocris Bioscience) at a concentration of 1 μM, U73122 (Tocris Bioscience) at 100 nM, and PF-228 (Sigma-Aldrich) at 1 μM. These concentrations are in the range most commonly found effective in published assays; higher concentrations proved toxic to neurons in pilot studies (data not shown)

Tissue preparation and immunofluorescence: Tissue was prepared using two different methods: 1) Tissue was dissected, immersed in OCT compound (Sakura; Torrance, CA) and snap frozen in isopentane cooled in a dry ice/ethanol bath; or 2) tissue was fixed in 4% paraformaldehyde (PFA) either by transcardial perfusion followed by immersion overnight at 4°C (adult animals) or direct immersion for 2 hours at 4°C (neonatal animals). Cryostat sections were cut at 12-20 μm and fresh-frozen sections were fixed in 100% methanol for 10 minutes at -20°C. For studies of dendrite branching in mice with the *Thy1-YFPH* transgene, PFA-fixed brains were embedded in 2% agarose and cut into 100 μm sections with a Vibratome. Dissociated cultures were fixed in 4% PFA for 10 minutes at room temperature. Immunostaining was performed as described previously (Prasad et al., 2008; Weiner et al., 2005).

Western blotting and immunoprecipitation: Western blots and immunoprecipitations were performed according to standard procedures as described (Schreiner and Weiner, 2010). Band intensity was quantified using Image/J using the "Analyze Gels" function from scanned images of autoradiographic films. Raw Image/J values are not comparable between gels (See http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/). Therefore, in

all cases the values in each lane for a given phospho-specific antibody were normalized to the values in the same lane of the same blot, subsquently probed using the pan-protein (that is, not phospho-specific) antibody. All mutant data are graphed relative to control levels within each blot, setting the control P20 value at "1". All experiments were performed 3-5 times using 3 sets of independent samples.

PKC activity measurements: Total PKC activity was measured in crude membrane preparations according to the instructions of the PepTag non-radioactive protein kinase C assay kit (Promega, Madison, WI, USA). Briefly, cortexes were dissected, and homogenized in TBS buffer (2ml buffer per one hemi-cortex) supplemented with protease inhibitor cocktail (Roche) by 20-30 strokes with a Teflon Dounce homogenizer. To isolate crude membrane fractions homogenates were centrifuged at 100,000 X g for 1h. The membrane-containing pellet was resuspended in lysis buffer containing 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 0.5% Triton-X100 and protease inhibitors. Protein concentration was determined by BCA assay (Pierce) and equalized by dilutions in lysis buffer. Assays were then performed at 30°C in a total volume of 25 µl containing 5 µl 5X PKC reaction buffer, 5 µl PLSRTLSVAAK peptide, 5 µl PKC activator, 1 µl peptide protection solution, and 9 µl sample (6-8 µg protein). Reactions were terminated after 30 min by incubation of the reaction mixture at 95C for 10 min. After adding 5 µl of 50% glycerol, each 10 µl of each sample was electrophoretically separated on a 0.8 % agarose gel at 100 V for 15 min. The gel was photographed and bands corresponding to the phosphorylated peptide were quantified using Image/J software. Values are represented as percent of control values at each age. For measurement of isoform-specific PKC activities, PKC-α, γ, or δ were immunoprecipitated with specific antibodies (Santa Cruz). Immunoprecipitates were resuspended in 30 µl of lysis buffer and PKC activity measured as before.

Image collection and analysis: Images were collected directly into Photoshop (Adobe; San Jose, CA) using 10X, 20X, or 63X PlanApo objectives on a Leica DM5000B epifluorescence microscope or into

Leica LCS software using a 20X or 63X PlanApo objective on a Leica SP2 AOBS laser-scanning confocal microscope.

Layer I thickness measurements: Images collected at 10X from *Emx1-Cre; Pcdh-*$\gamma^{fcon3/fcon3}$ or *Emx1-Cre; Pcdh-*$\gamma^{fcon3/+}$ brains from the S1 region of the cortex were imported into NIH Image/J. A line was drawn perpendicular to the surface of the brain from the top of the cell body-rich layer II to the meninges and measured in microns at multiple mediolateral points throughout the dorsal cortex.

Reconstructions: Z-stacks collected at 20X from 100 μm vibratome sections of *Thy1-YFPH* animals were imported into Neuromantic software (Developed by Darren Myatt, University of Reading, Berkshire, England. Available at: http://www.reading.ac.uk/neuromantic/). Single neurons were reconstructed in Neuromantic by carefully tracing dendritic branches in each confocal plane in the stack, and then either analyzed for numbers of bifurcations and cables (for apical tufts) or exported to Image/J (full neurons) for Sholl analysis. Images of cultured neurons were collected at 20X on the epifluorescence microscope and reconstructed using the Neuron/J plugin of Image/J. Sholl analyses of reconstructions from Neuromantic or Neuron/J were performed with the ShollAnalysis plugin for Image/J (developed by the Anirvan Ghosh Laboratory, University of California, San Diego). The area under the curve for each Sholl plot was calculated and the data were compared with 2-way ANOVA with Bonferroni posttests or Student's t-tests.

Spine and synapse quantification: High-resolution z-stacks of dendrites from Thy1-YFP mice were collected at 63X with a 2X digital zoom factor. Each stack was divided into 8-10 μm maximum z-projections with the Grouped ZProjector Image/J plugin. Spines were hand-counted from 40-50 10 μm long dendrite segments per genotype, per region. Images from 12 μm cryostat sections stained for synaptic markers were thresholded and quantified in Image/J using the PunctaAnalyzer plugin (developed by the Ben Barres Laboratory, Stanford University; Christopherson et al., 2005; Garrett and Weiner, 2009). Means were compared with Student's t-tests.

Tamoxifen injections: *Cre-ER;Pcdh-*$\gamma^{fcon3/fcon3}$*; Thy1-YFPH* mice and controls were injected intraperitoneally with 175 mg/kg tamoxifen (Sigma Aldrich; St. Louis, MO) diluted in corn oil to a final volume of 250μl. Five daily injections were performed beginning when the animals were one month of age. When mice reached two months of age, they were perfused transcardially with 4% PFA and processed by Vibratome sectioning and dendrite arborization analysis as detailed above. One set of animals in each experiment was used to verify tamoxifen-induction of Cre excision by cutting fresh-frozen brain sections on a cryostat, fixing with 100% methanol for 10 minutes at -20°C, and staining with antibodies against the γ-Pcdh-constant domain or against GFP. As found in our previous tamoxifen experiments using this *Cre-ER* line (Garrett and Weiner, 2009), excision at this dose was essentially complete, with only a small number of scattered astrocytes escaping excision in some sections.

Supplemental References

Cary, L.A., Han, D.C., Polte, T.R., Hanks, S.K., and Guan, J.L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J. Cell Biol. 140, 211-221.

Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28, 41-51.

Ferland, R.J., Cherry, T.J., Preware, P.O., Morrisey, E.E., and Walsh, C.A. (2003). Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. J. Comp. Neurol. 460, 266-279.

Garrett, A.M., and Weiner, J.A. (2009). Control of CNS synapse development by {gamma}-protocadherin-mediated astrocyte-neuron contact. J. Neurosci. 29, 11723-11731.

Ghosh, A., and Greenberg, M.E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 15, 89-103.

Gorski, J.A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J.L., and Jones, K.R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. J Neurosci 22, 6309-14.

Guo, C., Yang, W., and Lobe, C.G. (2002). A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. Genesis 32, 8-18.

Lobas , M.A., Helsper, L., Vernon, C.G., Schreiner D,. Zhang Y., Holtzman, M.J., Thedens, D.R., Weiner, J.A. (2011). Molecular heterogeneity in the choroid plexus epithelium: the 22-member γ-protocadherin family is differentially expressed, apically localized, and implicated in CSF regulation. J. Neurochem. doi: 10.1111/j.1471-4159.2011.07587.x

Nieto, M., Monuki, E.S., Tang, H., Imitola, J., Haubst, N., Khoury, S.J., Cunningham, J., Gotz, M., and Walsh, C.A. (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. J. Comp. Neurol. 479, 168-180.

Prasad, T., Wang, X., Gray, P.A., and Weiner, J.A. (2008). A differential developmental pattern of spinal interneuron apoptosis during synaptogenesis: insights from genetic analyses of the protocadherin-gamma gene cluster. Development 135, 4153-4164.

Schreiner, D., and Weiner, J.A. (2010). Combinatorial homophilic interaction between gamma-protocadherin multimers greatly expands the molecular diversity of cell adhesion. Proc. Natl. Acad. Sci. U. S. A. 107, 14893-14898.

Wang, X., Weiner, J.A., Levi, S., Craig, A.M., Bradley, A., and Sanes, J.R. (2002b). Gamma protocadherins are required for survival of spinal interneurons. Neuron 36, 843-54.

Weiner, J.A., Wang, X., Tapia, J.C., and Sanes, J.R. (2005). Gamma protocadherins are required for synaptic development in the spinal cord. Proc Natl Acad Sci U S A 102, 8-14.