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Supplemental Information

Cadherin-9 Regulates Synapse-Specific Differentiation in the Developing Hippocampus

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Inventory of Supplemental Information

1. **Supplemental Figure 1:** This figure is linked to main figure 2 and provides additional information about the in vivo specificity of antigens used to identify hippocampal cell types in culture.

2. **Supplemental Figure 2:** This figure is linked to main figure 4, which describes the synaptoporin assay. Here we provide direct evidence that co-expression of synaptoporin and VGLUT1 selectively labels DG mossy fiber presynaptic sites in vivo and in culture.

3. **Supplemental Figure 3:** This figure shows low magnification images of DG neurons expressing epitope-tagged cadherin-9. A higher magnification image of a mossy fiber bouton expressing epitope-tagged cadherin-9 is shown in the main figure 5I.

4. **Supplemental Figure 4:** This figure is linked to main figure 6 and shows that the cadherin-9 shRNA significantly reduces endogenous cadherin-9 mRNA from cultured neurons.

5. **Supplemental Figure 5:** This figure is linked main figure 8 and shows a low magnification image of a CA3 neuron infected with a GFP-expressing lentivirus and then filled with Lucifer yellow dye.

6. **Supplemental Figure 6:** This figure is linked to main figure 8 and shows that neither spine density nor length is altered in DG and CA1 neurons expressing the cadherin-9 shRNA.

7. **Supplemental Experimental Procedures**

Figure S1

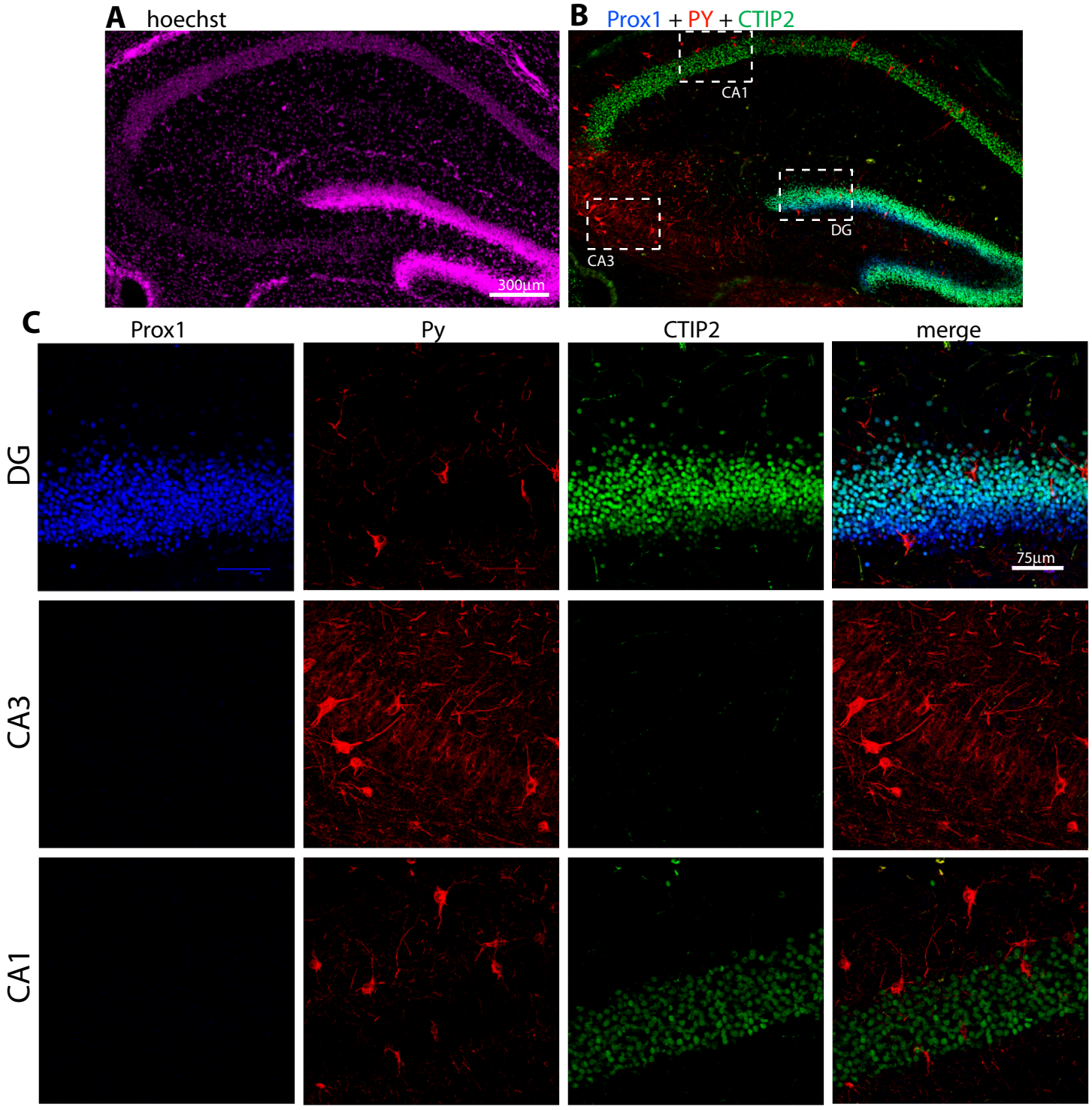

Figure S1. Identifying hippocampal cell types in vivo. (A) Image of a P12 mouse hippocampus stained with Hoechst. **(B)** The same view in (A) but immunostained with antibodies against Prox1, PY, and CTIP2 to identify DG, CA3, and CA1 neurons respectively. **(C)** Magnified images of the regions outlined in (B). Although both DG and CA1 neurons express CTIP2, they are unambiguously identified by co-staining for Prox1. All DG neurons express Prox1 but no CA1 neurons express Prox1. CA3 neurons express PY but not Prox1 nor CTIP2. PY is also expressed by scattered interneurons in each region.

Figure S2

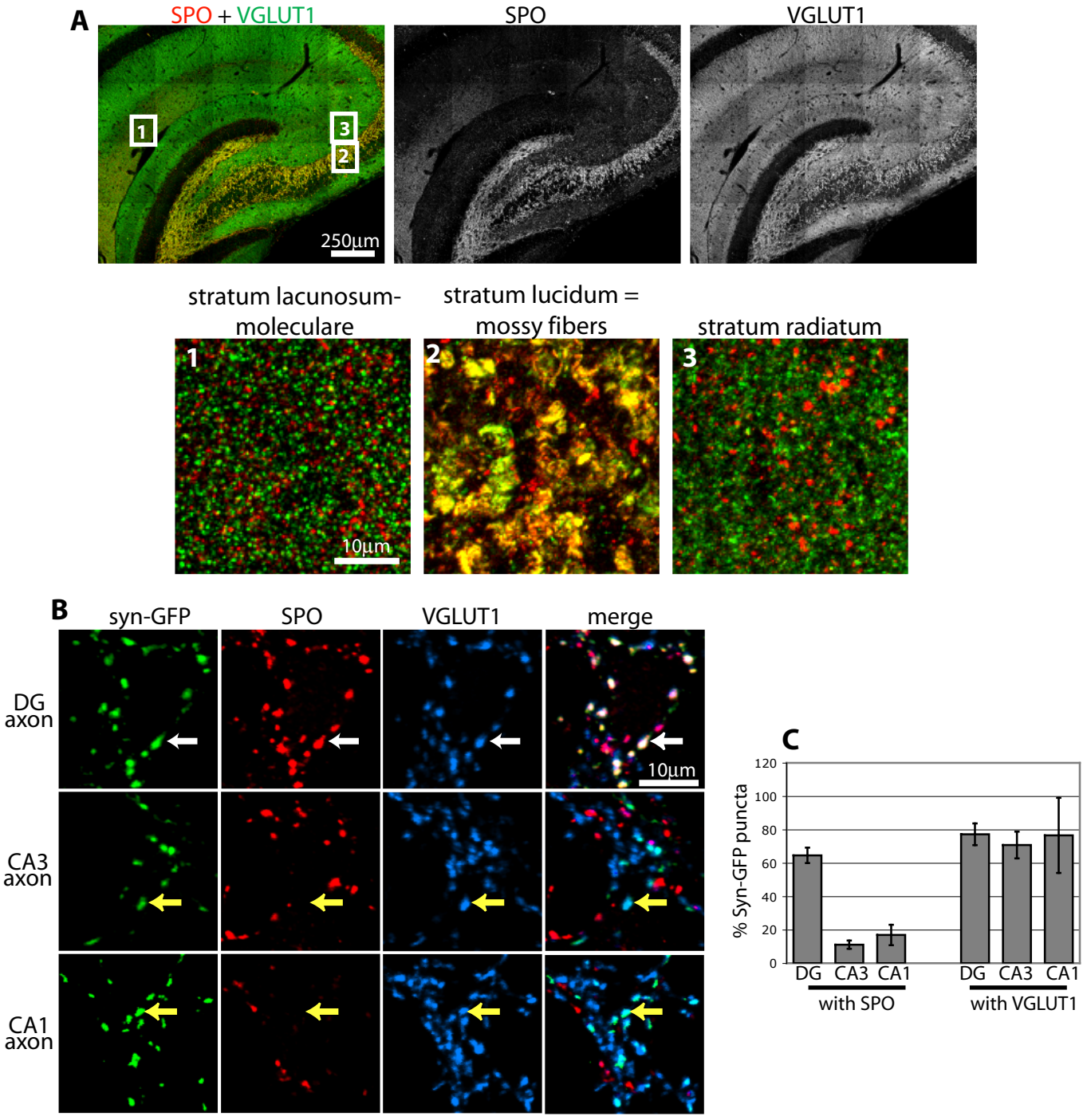

Figure S2. Colocalization of synaptoporin (SPO) and VGLUT1 uniquely labels DG mossy fiber synapses in vivo and in vitro. **(A)** SPO and VGLUT1 immunostaining in hippocampus. Higher magnification images from boxed regions are shown in the lower panel. SPO is expressed outside the stratum lucidum, but the only excitatory synapses in the hippocampus that express SPO are mossy fiber synapses (box 2, SPO + VGLUT1 merge appears yellow). **(B)** Microislands were transfected with synaptophysin-GFP (syn-GFP) and then immunostained for GFP, SPO, VGLUT1, and cell type markers Prox1, PY, or CTIP2 (not shown). DG neurons are the only excitatory neurons in which the syn-GFP labeled synapses express both SPO and VGLUT1. Triple labeled synapses appear white in merged panels (white arrows). In contrast, syn-GFP expressed in a CA3 or CA1 neuron is co-expressed with VGLUT1 but not SPO and synapses appear cyan in merged panels (yellow arrows). **(C)** Quantification of the percent of syn-GFP puncta that express synaptoporin or VGLUT1. All cell types are excitatory and express VGLUT1 at presynaptic sites but only DG neurons also express SPO.

Figure S3

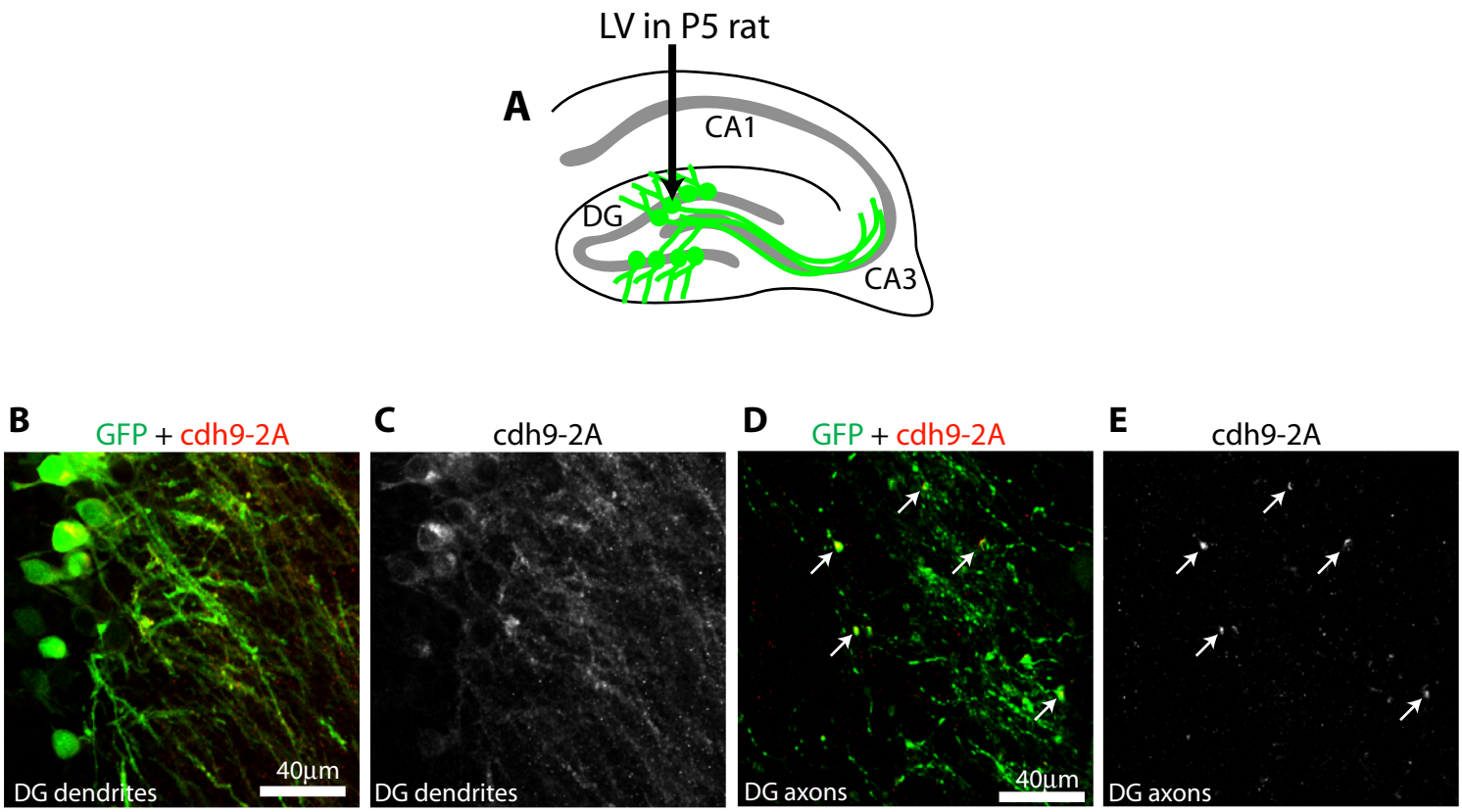

Figure S3. Cadherin-9 localizes to DG axons and dendrites. (A) Diagram of DG lentivirus injection strategy used for B-E and main Fig 5I. (B-E) Low magnification images of DG neurons from rats infected with lentivirus expressing cdh9-2A-GFP. Sections were immunostained for the 2A epitope, which remains attached to cdh9 (red), and GFP (green). Cdh9 localizes diffusely throughout the dendrites of DG neurons (B,C) but is found in discrete puntca at mossy fiber boutons along the axon (arrows in D,E). A magnified view of a mossy fiber bouton expressing cdh9-2A-GFP is shown in the main text (Figure 5I).

Figure S4

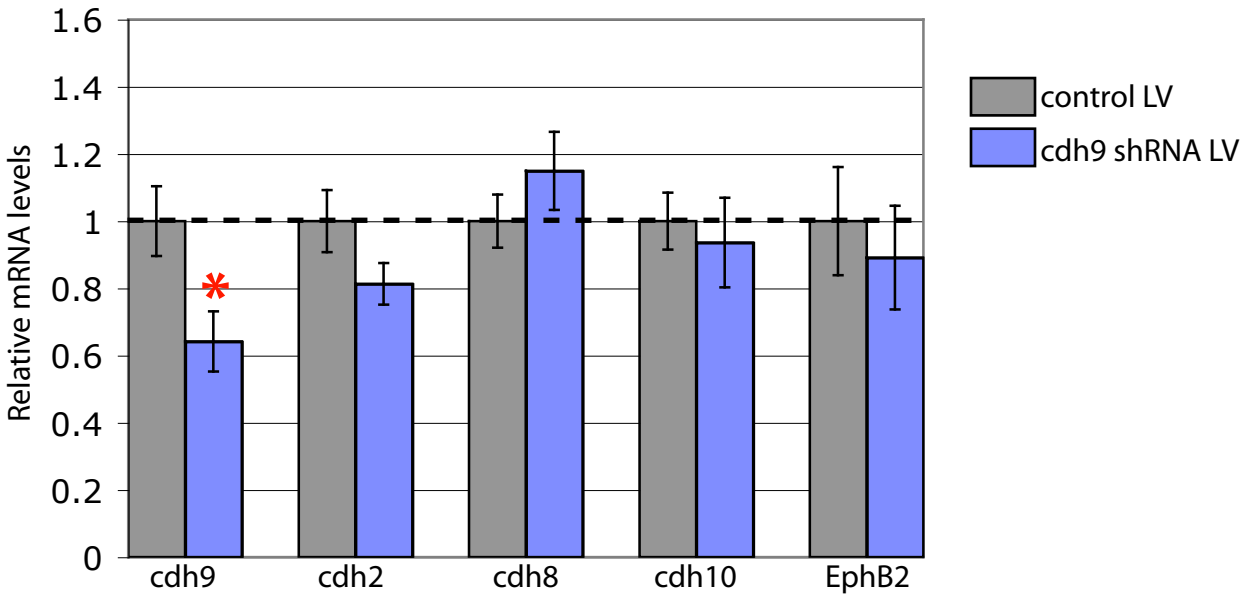

Figure S4. Cadherin-9 shRNA specifically decreases cadherin-9 expression. Relative mRNA levels in neurons infected with control or cdh9 shRNA lentivirus were determined by quantitative PCR. Only cdh9 mRNA is significantly decreased after infection with cdh9 shRNA. n=3. Error bars = SEM, * indicates $p<0.05$ by students t-test compared to control.

Figure S5

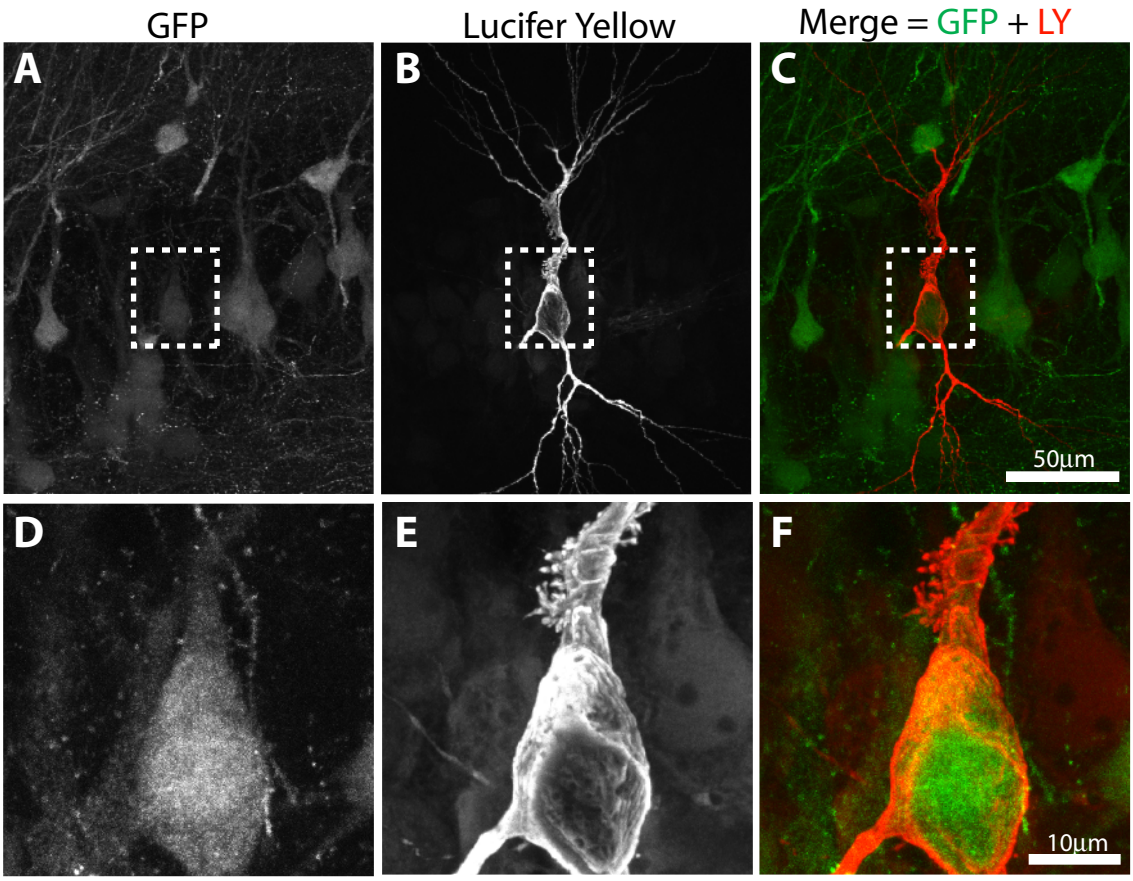

Figure S5. In vivo lentivirus injections. Example of a CA3 neuron infected with a GFP-expressing lentivirus (A) that was also filled with Lucifer yellow dye (B). Magnified images of the boxed regions (A-C) are shown below (D-F).

Figure S6

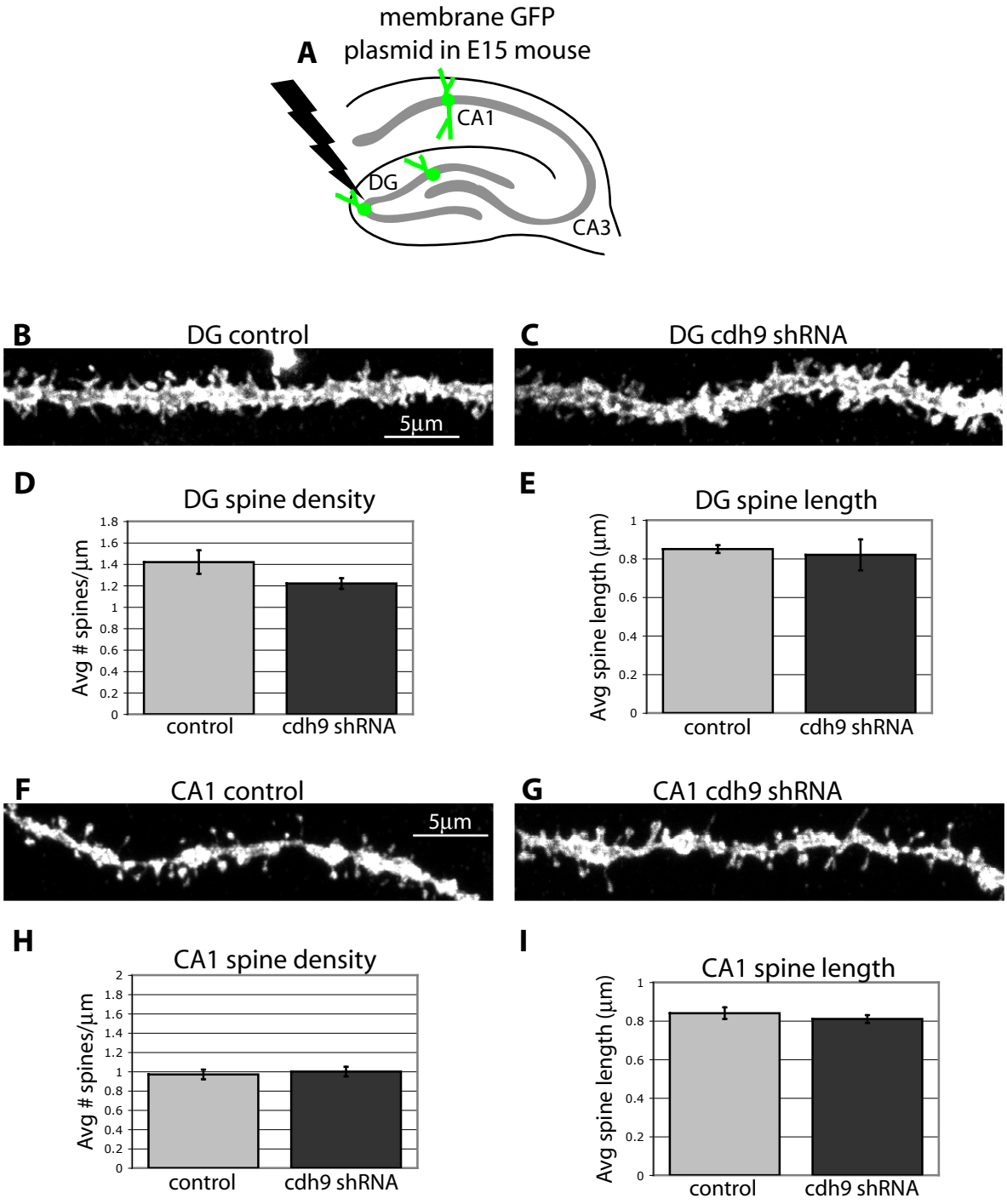

Figure S6. Cadherin-9 knockdown does not affect spine formation in DG and CA1 neurons. (A) Diagram of in utero electroporation transfection used in B-I. (B,C) Dendrites from P14 mouse DG neurons expressing membraneGFP and scramble shRNA or cdh9 shRNA. (D,E) Analysis of the average spine density or spine length reveal no significant differences between the conditions. (F,G) Secondary dendrites from P14 mouse CA1 neurons expressing membraneGFP and scramble shRNA or cdh9 shRNA. (H,I) Analysis of the average spine density or spine length reveal no significant differences between the conditions. Statistics are based on two-tailed t-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Microisland culture – Coverslips were prepared as described with modifications (Segal and Furshpan, 1990). P0 cortical glia were cultured on poly-D-lysine/collagen sprayed agarose-coated coverslips until confluent (glia media – DMEM plus penicillin/streptomycin, 10% FBS, and 20mM glucose). Next, P0 hippocampi were dissected in cold HEPES buffered saline solution (HBSS), incubated in papain enzyme for 30 min, dissociated, electroporated with plasmid as needed, and plated to microislands at $4x10^4$ cells/mL. After 2 hours, media was changed completely and every 3-4 days half the media was replaced. Neurons were cultured in Neurobasal A (Invitrogen) media supplemented with B27, penicillin/streptomycin, and 12mM glucose. Alternatively, neurons were transfected using the calcium-phosphate method.

Immunostaining and Antibodies – Cells were immunostained using standard procedures. Briefly, cells were fixed in 4% paraformaldehyde (PFA), washed with phosphate buffered saline (PBS), and incubated in blocking solution (PBS plus 3% bovine albumin and 0.1% TritonX 100) for 30 min. Then, cells were incubated in primary antibody diluted in blocking solution for 2 hours, washed, incubated in secondary antibody for 45 min, washed, and mounted for imaging. Primary antibodies were used as follows: chicken anti-MAP2 1:5000 (Abcam), rabbit anti-Prox1 1:5000 (Covance), mouse IgM anti-PY conditioned media 1:25 (gift of M. Webb(Woodhams et al., 1989)), rat anti-CTIP2 1:1000 (Abcam), goat anti-GFP 1:3000 (Abcam), mouse anti-αCAMKII 1:2000 (Chemicon), rabbit anti-SPO 1:5000 (Synaptic Systems), guinea pig anti-VGLUT1 1:5000 (Chemicon), rabbit anti-β-catenin 1:1000 (Upstate), rabbit anti-LY; 1:1000 (Abcam), rabbit anti-2A 1:2000 (Millipore), rabbit anti-cadherin-9 1:2000 (Thedieck et al., 2007). All secondary antibodies were from Jackson ImmunoResearch and were used at 1:1000.

Synaptoporin assay- Cultures were immunostained with the following primary and secondary antibodies: GFP or CAMKII (Cy2), Prox1 (Cy3), SPO (Cy3), VGLUT1 (405), and CTIP2 (Cy5). GFP or CAMKII label the dendrites of neurons. Anti-Prox1 and anti-SPO are both rabbit polyclonal antibodies but since the proteins are expressed in non-overlapping cell compartments (Prox1 is nuclear while SPO is synaptic) the antibodies were used simultaneously with no ambiguity.

Microisland and synaptoporin assay analysis and statistics – For island analysis, confocal stacks were collected on a Leica SP2 confocal using a 40X objective and z-projections were analyzed. For islands requiring multiple fields of view, images were aligned and saved as a single image using Photoshop (Adobe). For SPO analysis, confocal stacks were collected on a Zeiss LSM 510 at 63x with 1.2X optical zoom. Images were corrected for background by adjusting intensity levels and settings were saved so that the same confocal and analysis settings were used for every image from an experiment. All data were collected and analyzed blind to the experimental condition. Image quantification (dendrite length, synapse number, etc) was also performed blind to condition using Openlab (Improvision) or ImageJ (NIH) and statistics were performed using Excel (Microsoft) and InStat (GraphPad). To accurately assign each presynaptic punctum to the correct target neuron, only synapses residing on proximal dendrites (<120μm from soma) were analyzed.

Electrophysiology – 10-14DIV microisland cultures were superfused with artificial cerebrospinal fluid (ACSF – in mM: NaCl 124, KCl 5, $NaHCO_3$ 26, NaH_2PO_4 1.25, Glucose 10, $MgCl_2$ 1, $CaCl_2$ 2) saturated with 95% O_2 and 5% CO_2. Recordings were performed at room temperature using Multiclamp700B (Axon Instruments). The

presynaptic cell was held in current clamp at -55mV and the postsynaptic cell was held in voltage clamp at -65mV. The presynaptic cell was stimulated for 20ms each at the minimum threshold to produce an action potential and repeated 10 times at a rate of 0.66Hz. EPSCs from post-synaptic cells were recorded at a sampling rate of 100kHz. Neurons with a leak current greater than -500pA or a resting membrane potential above -30mV, or an access resistance above 25MΩ were excluded from analysis. Electrode resistance was 3-6MΩ. Electrode solutions were cesium-based for the postsynaptic cell (in mM: CsCl 10, $CsMeSO_3$ 105, NaCl 8, ATP 0.5, GTP 0.3, HEPES 10, TEA 5, $MgCl_2$ 2, EGTA 1, QX314 2) or potassium gluconate-based for the presynaptic cell (in mM: K gluconate 125, NaCl 8, D-glucose 5, HEPES 5, ATP 0.5, GTP 0.3, $MgCl_2$ 2, EGTA 1). After recording, coverslips were immunostained to identify cell types.

shRNA and Lentivirus production and injections: Cadherin-9 shRNA was made by annealing oligos into the pSRretro.neo system (OligoEngine). The cadherin-9 target sequence is GATGTCAACAACAACCCTC. To make shRNA expressing lentivirus, a cassette encoding the H1promoter and shRNA was removed from pSR using HindIII and StuI and blunt ligated into the PacI site of pFsy1.1GW (Dittgen et al., 2004). Lentiviruses were generated by the Salk Institute Viral Vector Core. For in vivo injections, P5 rat pups were anesthetized using an isofluorane vaporizer and immobilized in a stereotaxic device. Following craniotomy, a Hamilton syringe was used to inject 1μL of virus. All procedures with animals were performed in accordance with National Institutes of Health and the UCSD animal care program.

In utero electroporations and spine/mossy fiber bouton analysis: Timed pregnant E15 CD-1 (Charles River) mice were in utero electroporated using standard methods. Briefly, mice were anesthetized using an isofluorane vaporizer and the uterine horns

containing embryos were exposed through a midline incision. Plasmid DNA (~2μg/μL + 0.01% Fast green dye) was injected unilaterally into the lateral ventricle using a pulled glass pipet. Electroporation was targeted to the hippocampus by positioning the positive electrode (Platinum Tweezertrode, Harvard Apparatus) on the medial side of the injection. 5 pulses of 36mV for 50 ms duration with 950 ms intervals were delivered. Embryos were replaced, the dam sutured, allowed to recover, and returned to her home cage. Electroporated pups were perfused with 4%PFA at P14, brains were sectioned to 100μm, and immunostained with anti-GFP. Confocal stacks of spines or individual mossy fiber boutons were collected on an Olympus Fluoview 300 and stacks were analyzed using ImageJ, Excel, and Instat. No distinctions were made between types of spines. All protrusions were considered spines and to examine possible changes in morphology the length from spine tip to dendritic shaft was also measured.

Western Blot: 293T cells were transfected using Fugene (Roche) and subject to electrophoresis by SDS-PAGE. Antibodies used for western blotting were: mouse anti-Flag 1:2000 and mouse-anti-GAPDH 1:5000 (Millipore).

Mouse cadherin-9 cloning: P7 mouse hippocampal cDNA library was made using oligo dT primers and the SuperScript kit (Invitrogen). Primers for the 5' (GAAGGTGAAGATAATGAGGACT) and 3' (GACAACACAGGCCACTCAATC) ends of cadherin-9 were used to amplify a 2500bp PCR product and cloned into pCRII TOPO TA (Invitrogen). The gene was fully sequenced and found to encode the predicted full-length cadherin-9 cDNA as described in genbank NM_009869.1. This clone was used to generate an in situ probe and PCR subcloning was used to generate all other constructs.

Quantitative PCR: Cultured hippocampal neurons were infected with control or cadherin-9 shRNA lentiviruses at 5DIV. After 4 days, mRNA was isolated using Trizol (Invitrogen) and cDNA was synthesized using iScript cDNA synthesis kit (BioRad). Quantitative PCR was performed in an Applied Biosystems (Foster City, CA) PRISM 7900HT Fast Real-Time PCR system with SYBR green PCR master mix. For analysis of cDNA levels, primers were designed around exon/intron boundaries using NCBI Primer Blast. PCR was run using the following cycling conditions: 95°C/10 min; 95°C/15 s and 57°C/1 min for 40 cycles. The relative abundance of each cDNA was determined by using a standard curve generated from 10-fold serial dilutions of cDNA from rat hippocampal neurons that were infected with control lentivirus. These values were normalized to GAPDH cDNA levels.

Intracellular injection of Lucifer yellow (LY) and TE analysis: Mice were anesthetized and transcardially perfused with PBS followed by 4% PFA at pH 7.4 and post-fixed in 4% PFA for 1 hour on ice. 100μm thick coronal sections were cut using a vibratome and stored in PBS on ice. Penetrating microelectrodes were pulled from standard borosilicate capillary glass with filament (1.0mm outer /0.58mm inner diameter) and back-filled with LY dye (5%). Slices were mounted on coverslips under PBS and virally infected CA3 neurons were filled via iontophoresis under visual guidance. Sections were post-fixed 15 minutes before immunohistochemistry. For each filled CA3 neuron, viral infection was confirmed based on GFP expression at the cell body by immunostaining after filling with anti-LY (555) and anti-GFP (647). Thorny excrescences were imaged on a Leica confocal microscope under 63X magnification with 3X optical zoom. Images were collected as z-stacks with 0.5 μm steps and analyzed using ImageJ. A protrusion was considered a filopodia if it was longer than the thickness of the primary dendrite.

Imaging and analysis of lentivirus infected mossy fiber boutons: 100μm vibratome sections were immunostained with antibodies against GFP, SPO, and VGLUT1. Images of infected DG axons in the CA3 stratum lucidum were collected (10μm stacks with 0.5μm sections) and each z plane was thresholded for triple overlapping pixels. Thresholded stacks were then collapsed and the number and size of puncta were quantified using Openlab (Improvision) software. Synapse density is defined as the number of thresholded boutons per 100μm^2 of infected axon area.

Electron Microscopy: Slices containing LY-filled neurons were photoconverted as previously described (Bushong et al., 2002). Briefly, slices were post-fixed in 2% glutaraldehyde for 10 minutes, blocked with glycine, and then converted in diaminobenzidine solution. Slices were washed in PBS and incubated in 2% OsO_4/1.5% $C_6N_6FeK_4$ in PBS for 1 hour at room temperature. Slices were washed and placed in 1% aq. thiocarbohydrazide for 20 min, 2% aq. OsO_4 for 1 hour, 2% aq. uranyl acetate at 4°C overnight, lead aspartate solution at 60°C for 30 min (Walton, 1979). The slices were washed, dehydrated with a series of ethanol and acetone, infiltrated and embedded in Durcupan resin. Specimens were imaged on an FEI Quanta FEG SEM equipped with a Gatan 3View SBFSEM system. SBFSEM volumes were collected as mosaics and mosaics were stitched, aligned, and segmented using IMOD (http://bio3d.colorado.edu/imod/). Mossy fiber boutons, evident by clusters of synaptic vesicles, were identified in individual sections and their area was analyzed using ImageJ.

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