Supporting Information

Ciani et al. 10.1073/pnas.1018132108

SI Materials and Methods

Neuronal Cultures and Transfections. Primary hippocampal cultures were prepared from embryonic day 18 Sprague–Dawley rat embryos and cultured in N2/B27 medium for 12–15 days in vitro (DIV) or as otherwise stated. Medium-density cultures (100 cells per mm^2) were treated with purified recombinant Wnt7a (50–100 ng/mL was used) (R&D Systems). Wnt7a was applied to the neurons acutely (short time: 15 min, 1 h, or 3 h) or for longer time (overnight: 16 h) depending on the experiment. High-density hippocampal neurons (250 cells per mm^2) were transfected at 7–9 DIV with EGFP-actin and Dvl1-HA constructs or with PSD-Vimentin-CFP by using Lipofectamine 2000 (Invitrogen) or calcium phosphate. After appropriate treatment, neurons were fixed at 12–14 DIV or used for electrophysiological experiments. According to the type of transfection method used, Lipofectamine or calcium phosphate, spine density varied in control cultures. Therefore, spine density was normalized to control in Fig. 3*C*. For CaMKII inhibition, neurons were treated with 1 μM of myristoylated AIP (ENZO Life Science) or with 5 μM of the inhibitor KN93 (Tocris) during Wnt7a treatment. For neurons expressing Dvl-HA, inhibitors were applied overnight between 12 and 13 DIV before fixation. To inhibit calcium signaling, 10 μM BAPTA-AM (Tocris), 50 μM 2-APB (Tocris), and 3 μM SKF96365 were used during Wnt7a treatment.

Mutant Mice. C57BL/6J *Dvl-1* null mice were obtained from heterozygous crosses. All mutant mice were maintained on a C57BL/6 background. *Wnt7a; Dvl1* double mutant mice were obtained from crosses of $Wnt7a^{-/+}$; $Dvl1^{-/-}$ mutant mice. Control mice were age-matched *wild-type* C57BL/6 animals. Genotypes were determined by three-primer PCR using ear clipping. For Wnt7a, the primers used were forward, 5′-TTCTCTTCGCTGGTACTCTGGGTG-3′, reverse, 5′-CAGCGCTGAGCAGTTCCAACGG-3′, and the Neo primer 5′-AGGCCTACCCGCTTCCATTGCTCA-3′. For Dvl1, the primers used were forward 5′-TCTGCCCAATTCCACCTGCTTCTT-3′, reverse 5′-CGCCGCCGATCCCTCTC-3′, and the Neo primer 5′-AGGCCTACCCGCTTCCATTGCTCA-3′.

Organotypic Brain Slices and Biolistic Particle Delivery System. Hippocampal slices were prepared as described (1) from postnatal day (P)8 *wild-type* or *Wnt7a; Dvl1* mutant mice. After dissection, slices were maintained in culture for 8–10 d, changing the medium every 2 d. After this period, cells were transfected by using a biolistic particle delivery system (Bio-Rad) with EGFP-actin and left to recover for an additional 2 d before fixation. EGFP signal was amplified by labeling with an anti-GFP antibody (Upstate Biotechnology) as described (2).

Golgi Labeling. P21 mouse brains from wild-type and *Wnt7a; Dvl1* mutant mice were quickly removed and dissected in phosphate saline buffer and incubated in Golgi solution (FD Rapid GolgiStain Kit from FD Neuro Technologies) for 7 d. The impregnation steps were followed according to the manufacturer. The specimens were then quickly frozen and cut on a Leica CM3050 cryostat. Serial sagital sections of 100 μm thickness were immediately collected on slides (Superfrost Plus–VWR) and left to dry in the dark at room temperature for 48 h. Staining procedures were followed according to the manufacturer, and slides were then dehydrated in graded ethanol, cleared with Xylene, and mounted with Permount (TAAB Laboratories) for microscopy.

Immunofluorescence, Image Acquisition, and Analyses. Dissociated neurons were fixed with 4% paraformaldehyde/4% sucrose or with cold 100% methanol, permeabilized with 0.025% Triton, blocked with 5% BSA and then incubated with primary antibodies overnight at 4 °C. Primary antibodies against tubulin (Tuj-1) (Chemicon), HA (Boehringer), GFP (Upstate Biotechnology), vGlut1 (Chemicon), vGat (Synaptic Systems), PSD-95 (Affinity Bioreagents), Gephyrin (Synaptic Systems), Phospho-Vimentin (Ser82, MBL), and Dvl1 (3) were used. Secondary antibodies Alexa 488, Alexa 568, and Alexa 647 were from Molecular Probes. Fluorescence images of neurons with a typical pyramidal morphology were captured with a Leica TCS SP1 Confocal microscope by using a 63× oil objective (NA = 1.32), producing images stacks of 157.8 × 157.8 μm with an average *z* depth of ≈5 μm. For Golgi and PSD-95-Vim-CFP analysis, single plane images were captured on an Olympus BX60 widefield microscope by using a 100× oil objective (NA = 1.3), producing images 82.5.8 × 65.5 μm. Images were acquired with either Metamorph or Leica software and analyzed by using Volocity software. Synaptic puncta, their colocalization, and MAP2 volume were detected by using custom Volocity protocols that use standard thresholding techniques, and punctum density was normalized to MAP2 volume. To measure spine morphology, ≈300 spines from 5 to 10 neurons were analyzed per condition per experiment. Spines, defined as dendritic protrusions with a definable head, were counted manually on 2–3 stretches of secondary or tertiary dendrite of ≈100 μm in length and normalized against dendritic length. Spine width was quantified by visual placement of a line tool across the maximum head width. 3D rendered reconstructions of transfected cells were obtained from confocal stacks and processed with Imaris Software. For quantification of Vimentin phosphorylation (pVim), regions of interest around spines were traced in PSD-Vim-CFP images and then transferred to pVim images. Area and fluorescence intensities were measured in both pVim and PSD-Vim-CFP images, and pVim signal was normalized to PSD-Vim-CFP signal. All imaging experiments were performed on 6–10 cells per condition within each experiment, and each experiment was repeated in at least three independent cultures (at least 3 mice per genotype for Golgi staining).

CaMKII Phosphorylation. Cell lysates were prepared from 12 DIV hippocampal neurons exposed to 50 ng/mL Wnt7a or BSA for 2, 20, or 60 min. Lysates were run on SDS/PAGE and Western blots probed with antibodies against CaMKII or phospho-Thr286 CaMKII (Cell Signaling). Band intensity was measured by using ImageJ software. The level of CaMKII activation was determined by calculating the ratio pCaMKII/total CaMKII.

Synaptosomal Preparation. Synaptosomes were prepared as described (4). All steps were performed at 4 °C. Adult mouse brains were homogenized in buffer A (0.32 M sucrose, 4 mM Hepes at pH 7.4, containing protease and phosphatase inhibitors) and spun at 800 × *g* for 10 min. The supernatant was centrifuged at 9,000 × *g* for 15 min, and the pellet was resuspended in buffer A. This sample was layered on top of a discontinuous sucrose gradient (0.8/1.0/1.2 M sucrose in 4 mM Hepes at pH 7.4) and centrifuged at 65,000 × *g* for 45 min. Synaptosomes were taken from the 1–1.2 M sucrose interface and resuspended in buffer B (0.32 M sucrose, 4 mM Hepes at pH 7.4, and 150 mM NaCl plus phosphatase inhibitors). The synaptosome fraction was then incubated with an equal volume of buffer C (1% Triton X-100, 0.32 M sucrose, 12 mM Tris at pH 8.0) for 15 min and centri-

fuged at 82,500 × *g* for 45 min. The supernatant, corresponding to the synaptosomal membrane fraction (SMF) was removed and the pellet, corresponding to the postsynaptic density fraction (PSD), was resuspended in buffer B. Equal amount of proteins (Lowry assay) were loaded onto an SDS/PAGE. The antibodies used were as follows: mouse anti-PSD-95 (Affinity Bioreagents), mouse anti-Synaptophysin (Chemicon), and rabbit anti-Dvl1 (3).

Electrophysiology. Hippocampal cultures plated at 250 cells per mm^2 were used for recording at 12–14 DIV. Neurons cotransfected with Dvl1-HA and EGFP-actin or with empty vector and EGFP-actin were recorded by patching the EGFP-actin labeled neurons. Cotransfection of EGFP-actin and Dvl1-HA (assessed by anti-GFP and anti-HA immunofluoresence) was essentially 100%. Recordings from CA3 cells were made in acutely prepared 300-μm transverse hippocampal slices from P14 mice. Coverslips or slices were placed in a chamber on an upright microscope and continuously perfused at room temperature with recording solution containing 125 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM $NaHPO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (1), and 25 mM D-glucose. TTX (100 nM) was included when recording mPSCs. Cells were voltage-clamped in the whole cell configuration by using borosilicate glass microelectrodes filled with a pipette solution containing 139 mM D-gluconic acid lactone, 10 mM Hepes, 10 mM EGTA, 10 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM GTP adjusted to pH 7.2 with CsOH. QX-314 (10 mM; Ascent Scientific) was included when recording evoked currents. EPSCs were recorded at −60 mV in the presence of 10 μM bicuculine, whereas IPSCs were recorded at 0 mV in the presence of 10 μM DNQX and 50 μM AP-5 (all from Ascent Scientific). Postsynaptic currents in CA3 cells were evoked by using a concentric bipolar stimulating electrode (FHC) placed in the stratum lucidium. Currents were recorded by using an Axopatch headstage connected to an Axopatch 200A amplifier, filtered at 1 kHz and digitized onto computer at 10 kHz by using WinEDR. Currents were analyzed by using a combination of WinEDR and WinWCP (freely available at http://spider.science.strath.ac.uk/sipbs/software_ses.htm).

Statistical Analyses. Values given are mean ± SEM. All data presented is the pooled data from at least three independent cultures or mice per experimental condition, as indicated in figure legends. Normality of datasets was assessed by using the Kolmogorov-Smirnov test. For datasets with a normal distribution, means were compared by Student's *t* test or ANOVA as appropriate. For datasets with a nonnormal distribution, the Mann–Whitney test or Kruskal-Wallis test was used. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

1. De Simoni A, Yu LM (2006) Preparation of organotypic hippocampal slice cultures: Interface method. *Nat Protoc* 1:1439–1445.
2. Gogolla N, Galimberti I, DePaola V, Caroni P (2006) Preparation of organotypic hippocampal slice cultures for long-term live imaging. *Nat Protoc* 1:1165–1171.
3. Krylova O, Messenger MJ, Salinas PC (2000) Dishevelled-1 regulates microtubule stability: A new function mediated by glycogen synthase kinase-3beta. *J Cell Biol* 151:83–94.
4. Cohen RS, Blomberg F, Berzins K, Siekevitz P (1977) The structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. *J Cell Biol* 74:181–203.

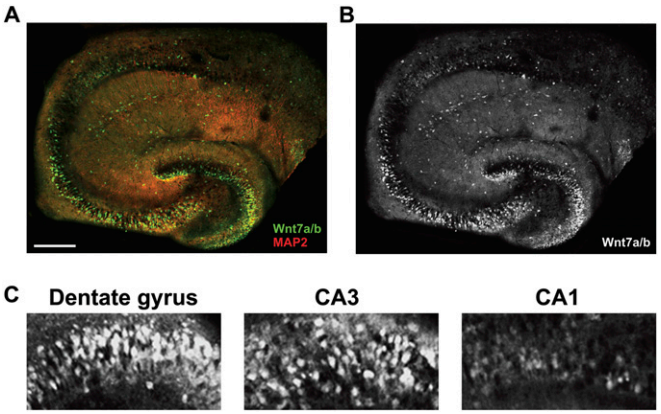


Fig. S1. Wnt7a/b is expressed in the principle cell layers of the hippocampus around the peak of synaptogenesis. (*A*) Transverse slice of the hippocampus of a P15 *wild-type* mouse, stained with an antibody that recognizes both Wnt7a and the closely related family member Wnt7b (green), and counter stained for the dendritic marker MAP2 (red). (Scale bar: 200 μm.) (*B*) Grayscale image of the Wnt7a/b signal. (*C*) Enlarged regions from *B* demonstrating high Wnt7a/b levels in neurons of the dentate gyrus (*Left*) and CA3 (*Center*), and relatively lower levels in the CA1 (*Right*).

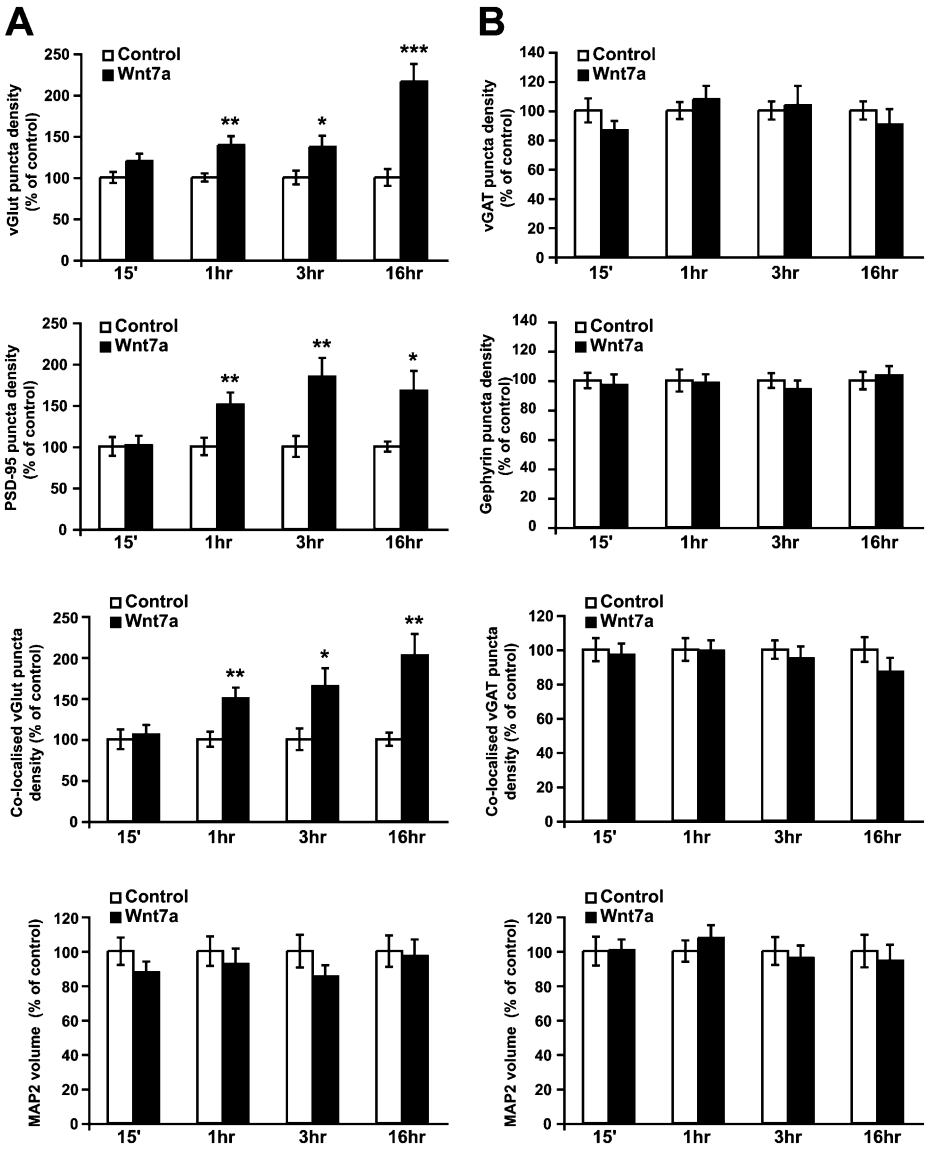


Fig. S2. Time-course of the effect of Wnt7a on excitatory and inhibitory synapses. Fourteen DIV hippocampal cultures were treated with 100 ng/mL Wnt7a for 15 min, 1 h, 3 h, or 16 h (overnight 13–14 DIV). (*A*) Wnt7a increases the density of vGlut and PSD-95 puncta and the density of vGlut puncta colocalized with PSD-95 (normalized to MAP2 volume) within 1 h. The strength of these effects increases with longer treatment times. (*B*) No changes are observed in the density of vGAT and Gephyrin puncta and the density of vGAT puncta colocalized with Gephyrin (normalized to MAP2 volume) at any of the treatment times. The mean volume of MAP2 (used to normalize puncta number to culture density) is not significantly altered by any of the Wnt7a treatment times used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Student's *t* test. All experiments were performed by using at least three independent cultures.

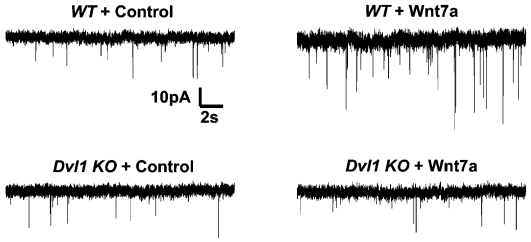


Fig. S3. Wnt7a is unable to increase mEPSC frequency and amplitude in cultured neurons from *Dvl1* knockout mice. Representative mEPSC traces from *wild-type* (*WT*) and *Dvl1* mutant cells treated for 3 h with control or Wnt7a. Quantification of mean mEPSC frequency and amplitude are shown in Fig. 2.

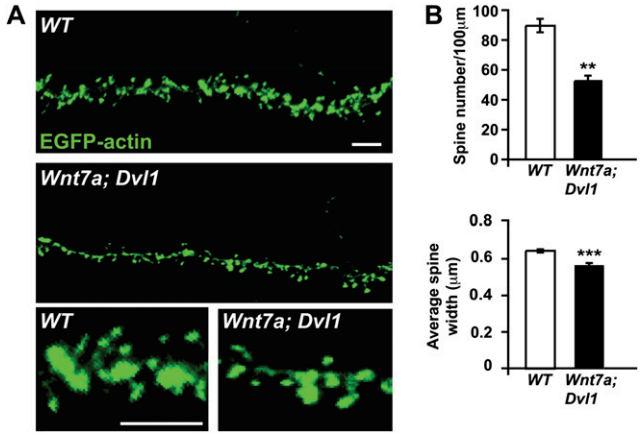


Fig. S4. Wnt signaling-deficient mutant mice exhibit defects in dendritic spine morphogenesis in organotypic brain slices. (*A*) Organotypic hippocampal brain slices from P8 *wild-type* (*WT*) and *Wnt7a; Dvl1* mutant mice were biolistically transfected with EGFP-actin to reveal dendritic spines. (Scale bars: 10 μm.) (*B*) Quantification shows a 30% reduction in spine number and a 15% reduction in spine width in *Wnt7a; Dvl1* mutant mice compared with *WT* control animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Student's *t* test for spine number and Mann–Whitney test for spine size.

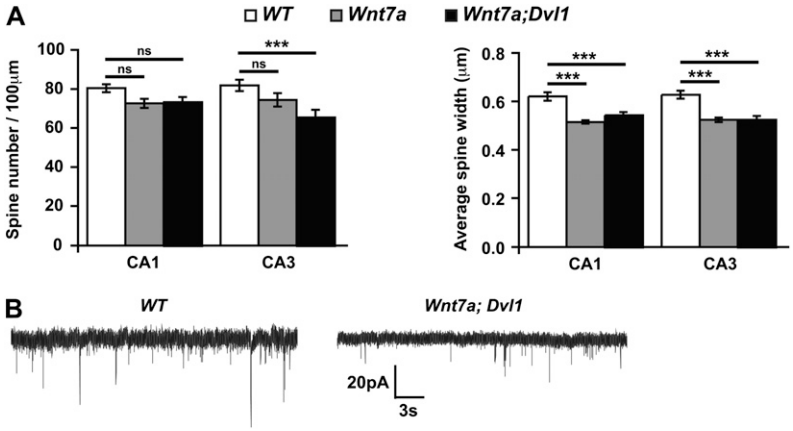


Fig. S5. Defects in spine morphogenesis and excitatory synaptic transmission in Wnt signaling-deficient mice. (*A*) Quantification of mean spine number and size in Hippocampi of *wild-type* (*WT*), *Wnt7a* single mutant, and *Wnt7a; Dvl1* double mutant mice, as assessed by Golgi staining. Note that the single knockout does not display a significant defect in spine number in the CA3, whereas the double knockout does. The *wild-type* and *Wnt7a; Dvl1* mutant data are also presented in Fig. 3. (*B*) Representative 30-s traces of mEPSCs recorded from CA3 cells in acute hippocampal slices from P14 *WT* or *Wnt7a; Dvl1* mice. Quantification of mean mEPSC frequency and amplitude are shown in Fig. 3. $***P < 0.001$ by ANOVA for spine number and Kruskal-Wallis test for spine size. ns, not significant.

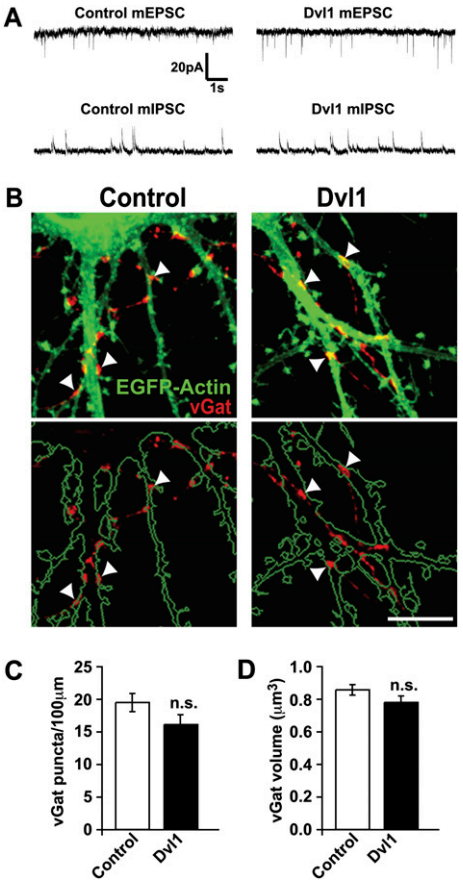


Fig. S6. Postsynaptic activation of the Wnt pathway enhances excitatory synaptic function without affecting the number of inhibitory inputs. (*A*) Representative 10-s traces of mEPSCs and mIPSCs in control and Dvl1-expressing cells. Quantification of mean mEPSC frequency and amplitude are shown in Fig. 4. (*B*) Control and Dvl1-transfected neurons cotransfected with EGFP-actin (green) were stained for the inhibitory presynaptic marker vGat (red). vGat puncta are mainly found on the shafts of proximal dendrites (arrowheads) and the cell body. (Scale bar: 10 μm.) (*C* and *D*) Quantification reveals no significant (ns) differences in the density (*C*) or volume (*D*) of vGat puncta contacting control or Dvl1-transfected cells, as assessed by Student's *t* test in *C* and Mann–Whitney test in *D*.

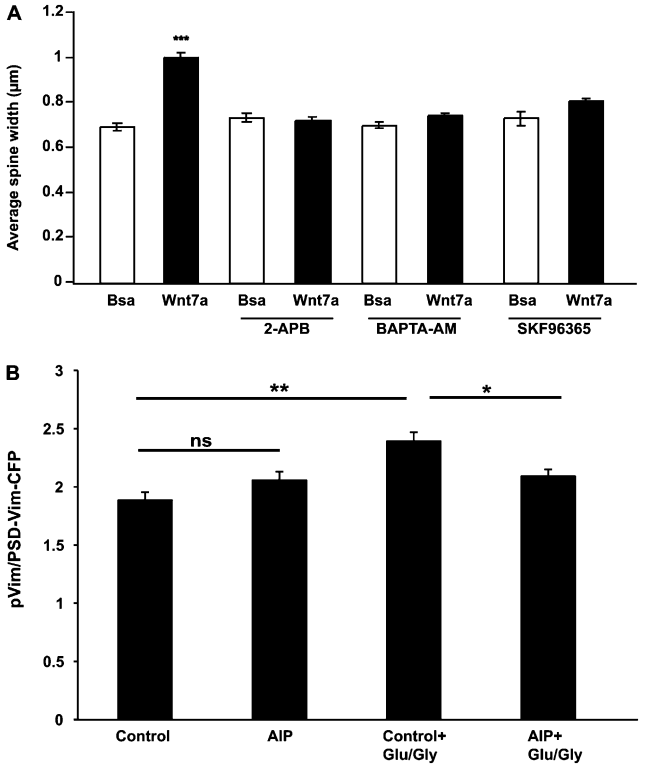


Fig. S7. Inhibition of calcium signaling blocks the Wnt7a effect on spine size and CaMKII phosphorylates the Vimentin domain within spines. (*A*) 12 DIV hippocampal neurons were treated with BSA or Wnt7a for 3 h in the presence of three different compounds that inhibit intracellular calcium rises: 2-APB, BAPTA-AM, and SKF36965. All three inhibitors block the effect of Wnt7a on spine growth. (*B*) Neurons stimulated with glutamate and glycine exhibit an increase in the phosphorylation of the Vimentin domain in PSD-95-Vim-CFP by CaMKII, an effect that is blocked by the myristoylated autocamide-2 related inhibitory peptide (AIP) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Mann–Whitney test in *A* and ANOVA in *B*. ns, not significant.

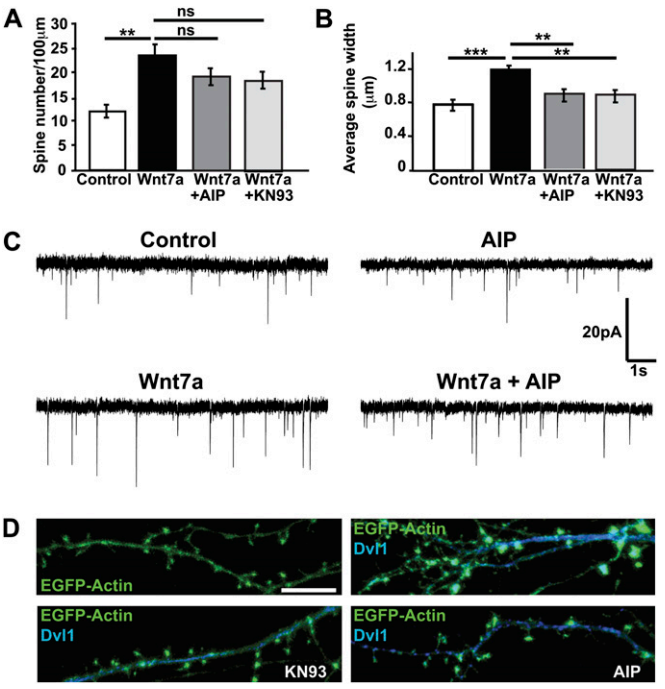
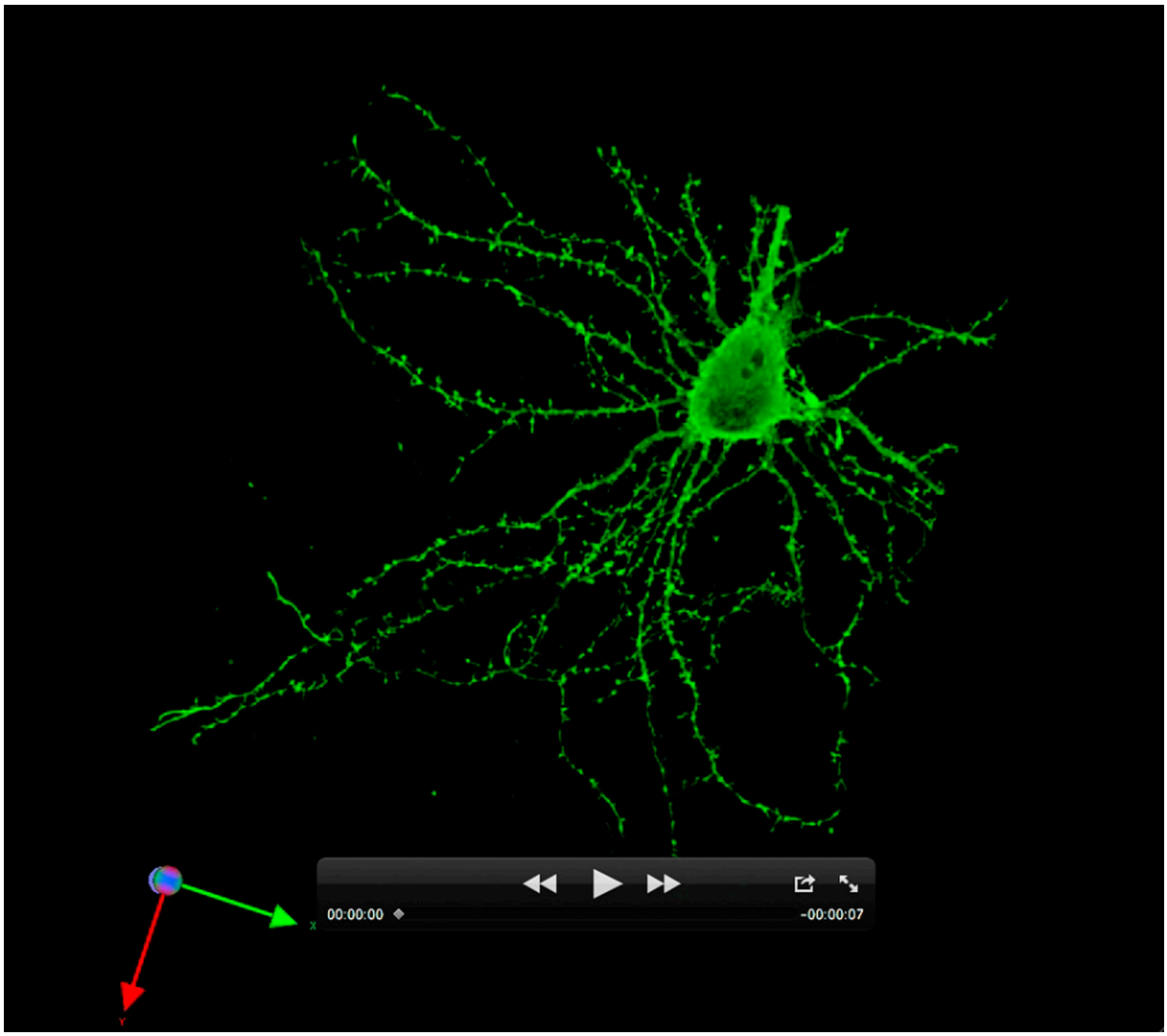
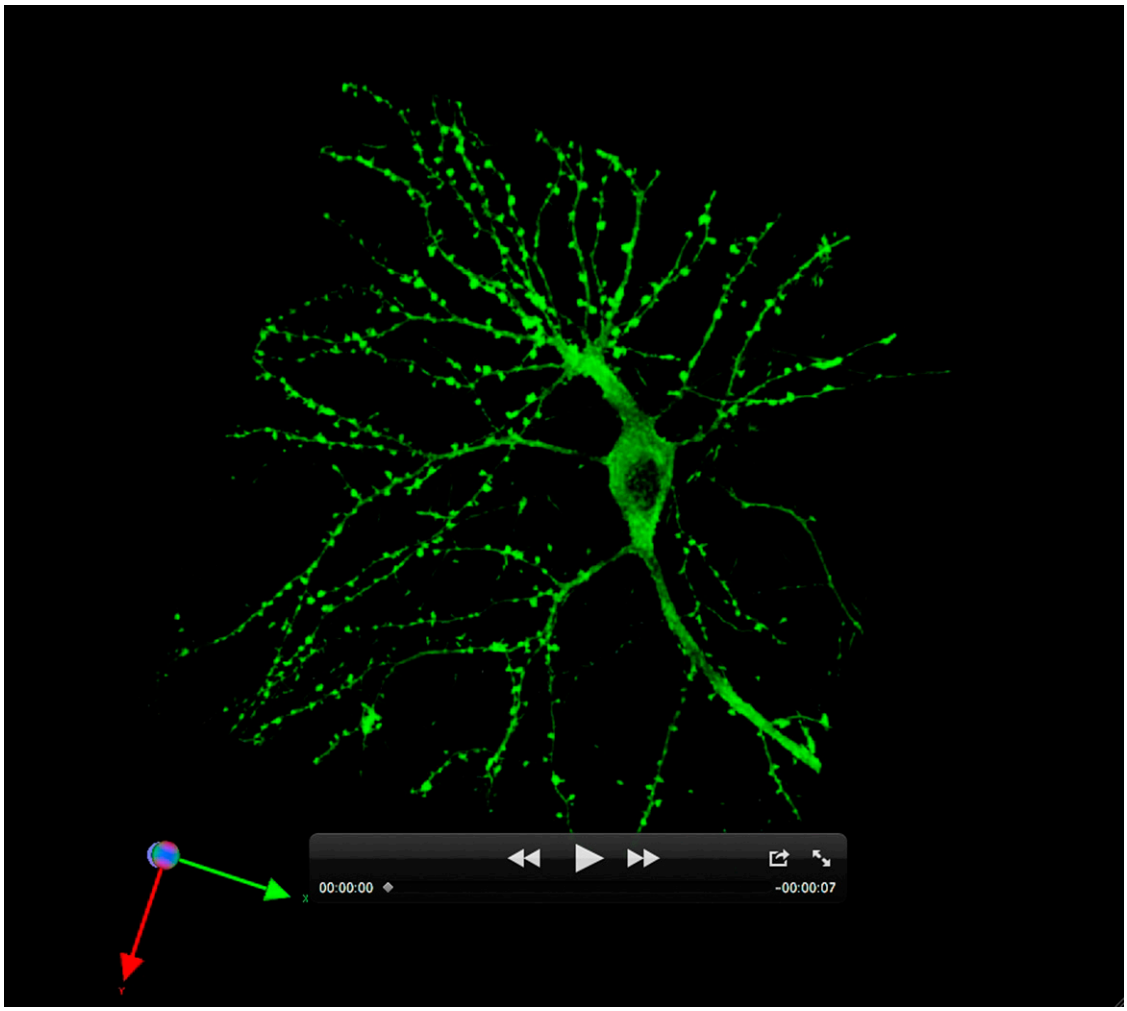


Fig. S8. Wnt7a regulates spine size and excitatory synaptic function through Dvl1 and CaMKII. (*A* and *B*) Quantification of spine number (*A*) and size (*B*) in neurons exposed to Wnt7a or Wnt7a plus CaMKII blockers for 16 h. Inhibition of CaMKII with AIP or KN93 blocks the ability of Wnt7a to increase spine size, but not number. (*C*) Representative 10-s traces of mEPSCs in cultures treated with Wnt7a and AIP. Quantification of mean mEPSC frequency and amplitude are shown in Fig. 7. (*D*) Representative images show that inhibition of CaMKII with KN93 or AIP blocks dendritic spine growth in neurons expressing Dvl1. (Scale bar: 10 μm.) Quantification of mean spine number and size are shown in Fig. 7. $**P < 0.01$, $***P < 0.001$ by ANOVA for spine number and mEPSC amplitude and Kruskal-Wallis test for spine size and mEPSC frequency on pooled data from at least three independent cultures. ns, not significant.



Movie S1. Morphology of control hippocampal neuron. 3D projection of a confocal stack of a control hippocampal neuron expressing EGFP-actin cultured for 14 d and exposed to control media for 16 h. The pyramidal neuron exhibits a typical morphology with clear dendritic spines.

Movie S1



Movie S2. Wnt7a induces profound changes in spine morphogenesis. 3D projection of a confocal stack of a hippocampal neuron expressing EGFP-actin cultured for 14 d and exposed to Wnt7a for 16 h. Wnt7a increases the number and size of dendritic spines compared with control (Movie S1).

Movie S2