**Supplementary Methods:**
"GRIP1 controls dendrite morphogenesis by regulating EphB2 receptor trafficking" by Hoogenraad C. et al

**Antibodies and reagents**
Rabbit GRIP antibody (1756)1,2 and rabbit EphB2 antiserum3 were previously described. The following antibodies were obtained from commercial sources: mouse anti-GRIP1, mouse anti-p150Glued, mouse anti-GM130, mouse anti-NR1 (BD Transduction Laboratories), mouse-anti-GABAA receptor β-chain, mouse anti-kinesin heavy chain, mouse anti-GluR2 (Chemicon), rabbit anti-Flag, mouse anti-Flag, mouse anti-MAP2 (Sigma), mouse anti-Myc (Oncogene), goat anti-EphB2 (R&B Systems), mouse anti-β-tubulinIII (Covance), rabbit anti-Cortactin, rabbit anti-HA (Santa Cruz), rabbit anti-GFP (Medical and Biological Laboratories), mouse anti-GFP (Quantum), Alexa488-, Alexa568- conjugated secondary antibodies (Molecular probes) and CY5-conjugated secondary and anti-human Fc antibodies (Jackson ImmunoResearch Labs). EphrinB2-Fc, EphB2-Fc and control Fc were from R&B Systems. Doxycycline and Brefeldin A were obtained from Sigma.

**DNA Constructs**
The following mammalian expression plasmids have been described: myc-Cortactin and pSupercort3004, myc-PSD-955, HA-GluR26, HA-ephrinB1, Flag-EphB2, Flag-EphA4, Flag-EphA73, myc-liprinα1, myc-LAR7, CD88, pSuper vector9.

To generate βactin-GFP (GFP driven by β-actin promoter), GFP was ligated in the SalI and NotI sites in a modified pβactin-16-pl vector10. GRIP1-HA, GRIP1-HA and GRIP2-HA were generated by cloning rat GRIP1, pGRIP111 and GRIP21,2 cDNA in frame with a N-terminal HA epitope tag (YPYDVPDYA). GRIP1*-HA which is resistant to RNA interference (GRIP1*) was generated by mutating the GRIP siRNA#1 target site at four different nucleotides without changing the amino acid sequence. Deletion mutants of GRIP1 were cloned by PCR. Deletion mutants of Flag-EphB2 and fusion constructs of CD8-EphB2 were generated by cloning different parts of EphB2 by PCR in pβactin vector. Kinase-dead EphB2 was generated by site-directed mutagenesis (substituting Lys-
660 to Ala\(^3\)). To generate HA-KIF5GBD, the KIF5B sequence (amino acid 808-935) was amplified by PCR from rat SMART cDNA library (Clontech) with added restriction sites and subcloned in GW1-HA expression vector. The linker 2 (L2) region of GRIP1 (amino acids 754–986) was PCR amplified and subcloned in-frame downstream of the HA-tag/myristoylation modification sequence (MGQSLTTHA) to create Myr-HA-L2. Two oligonucleotides (TCCCTATCAGTGATAGAGA) encoding tetracycline-responsive element (TRE) sequences were inserted flanking the TATA box in the H1 promoter of pSuper, to generate pSuper-TRE. pTET-tTS containing the tetracycline-controlled transcription silencer (ptTS, Clontech) was used to regulate transcription of pSuper-TRE-based plasmids\(^{12}\). GRIP1-siRNA sequences were targeted against rat GRIP1 mRNA (NM_032069) corresponding to nucleotides 417-435 (siRNA#1), or 3165-3183 (siRNA#2). GRIP2-siRNA sequence was targeted against rat GRIP2 (AF205193; nucleotides 1388-1406). The complementary oligonucleotides were annealed and inserted into pSuper vector\(^9\).

**Primary Hippocampal neuron Cultures and Transfection**

Primary hippocampal cultures were prepared from embryonic day 18 (E18) rat brains\(^{13}\). Cells were plated on coverslips coated with poly-D-lysine (30 µg/ml) and laminin (2 µg/ml) at a density of 75,000/well. Hippocampal cultures were grown in Neurobasal medium supplemented with B27, 0.5 mM glutamine, 12.5 µM glutamate and penicillin/streptomycin. At DIV1, DIV4 or DIV13 hippocampal neurons were transfected using the calcium phosphate method\(^4\). Briefly, DNA (10 µg /well) was mixed with 250 mM CaCl\(_2\) and added to the same volume of 2X HEPES-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na\(_2\)HPO\(_4\), 15 mM D-glucose, 42 mM HEPES, pH 7.06). The DNA mix was incubated for 20 min and then added to the neurons in DMEM without glutamine at 37°C in 5% CO\(_2\) for 20 min. Next, neurons were washed 2 times with DMEM, incubated in DMEM for one hour and transferred in the original medium at 37°C in 5% CO\(_2\) for 4 days.

For Fc-fusion proteins treatments, recombinant extracellular domains of EphB2 receptor and ephrinB1 ligand fused to the Fc fragment of human immunoglobulins and control human immunoglobulins were clustered using anti-Fc antibodies at 2:1 molar
ratio in sterile PBS with 1 µg/ul BSA for 1h at room temperature. For long treatments, at day 1 and day 3 after transfection unclustered and clustered Fc-recombinant or control Fc proteins were added to the neuron cultures at 5 µg/ml.

Cultures of mouse hippocampal neurons from triple homozygotes EphB1, EphB2, EphB3 mice were prepared from mouse E15-16 embryos (WT or mutants) as described previously. The hippocampal neuron cultures were transiently transfected with GFP at 7 DIV using Ca-phosphate method. The GFP-transfected cultures of hippocampal neurons at 21 DIV were fixed in 2% PFA.

**Live imaging of Hippocampal Neurons**

Time-lapse images were acquired by using LSM510 confocal microscope and software (Zeiss) and an Axiovert microscope (Zeiss). Neurons were plated on custom-made live imaging dishes. Glass coverslips (19 mm) were treated as normal, and affixed using Sylgard 184 silicon elastomer (Dow Corning) to the underside of a plastic culture dish with a circular hole drilled in the center. Neurons were kept on a 37°C microscope stage incubator with 5 % CO2 during confocal scanning. The intensity of the argon laser at 488 nm was set as low as feasible and the images were taken as fast as possible to avoid any toxicity to the neurons. After imaging the neurons were placed back in the incubator and 4 and 7 days later the same neurons were analyzed again. The position of the neurons on the coverslip was “remembered” by LSM software.

**Surface labeling and Immunocytochemistry of Hippocampal Neurons**

Live neurons were incubated with GluR2 and EphB2 N-terminal antibodies (10 µg/ml) at 37°C for 15 min. After washing in DMEM medium, the neurons were fixed for 5 minutes with 4% formaldehyde/4% sucrose in phosphate-buffered saline (PBS). The primary antibodies were detected by Alexa568 secondary antibodies in GDB buffer without TritonX-100.

For immunohistochemistry, neurons were fixed for 10 minutes with 4% formaldehyde/4% sucrose in phosphate-buffered saline (PBS) or ice-cold 100% methanol at –20°C. After fixation cells were washed two times for 30 min at room temperature, and incubated with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton
X-100, 30 mM phosphate buffer, pH 7.4) overnight at 4°C. Neurons were then washed three times in PBS for 30 min at room temperature and incubated with secondary antibody conjugated to Alexa488, Alexa568 or Cy5 in GDB for 2 hr at room temperature and washed three times in PBS for 30 min.

**Hippocampal Slice Culture and Transfection**
Organotypic hippocampal slice cultures were prepared from postnatal 7 day old rats as described previously 15. Slices were grown in an incubator set at 5% CO2, 35°C, and transfected after 36-48 hours in vitro using a biolistic gene gun (Bio-Rad) 16. Slices were fixed after 6 days in vitro in 4% PFA, 4% sucrose in PBS overnight. Confocal images were acquired using a LSM510 confocal microscope (Zeiss) with a 40x oil objective.

**Image analysis and quantification**
Confocal images of transfected neurons were obtained with sequential acquisition settings at the maximal resolution of the microscope (1024 x 1024 pixels). Each image was a z-series of 6-10 images each averaged 2 times. The resulting z-stack was 'flattened' into a single image using maximum projection. The confocal settings were kept the same for all scans when fluorescence intensity was compared. Morphometric analysis and quantification were performed using MetaMorph software (Universal Imaging Corporation).

Quantification of the number of primary dendrites, total dendrite length and Sholl analysis 17 was done with use of the images acquired with 63x objective with 0.7x electronic zoom. For dendrite length all the dendrites of the single neuron were traced in MetaMorph and the number of pixels was automatically converted to μm. For Sholl analysis concentric circles with 7 μm differences in diameter were drawn around the cell body, and the number of dendrites crossing each circle were manually counted. For total length of axons measurements, scans acquired with the use of a 10x objective with 1x electronic zoom were used and all axon segments of a single neuron were traced in MetaMorph. For the quantification of antibody staining, images were acquired with use of 63x objective with 0.7x electronic zoom and the average intensity of the soma, dendrites and Golgi region, by costaining with GM130, was measured in MethaMorph.
The axon and dendrites of GFP expressing neurons were identified based on their morphology and by immunostaining for the neuronal marker tau and dendritic marker MAP2. Acquisition of the images as well as morphometric quantification was performed under “blinded” conditions. Statistical analysis was performed with Student’s t test assuming a two-tailed and unequal variation. N defined as the number of transfected neurons.

REFERENCES