Supplemental Data

An RNAi-based Approach Identifies New Molecules Required for Glutamatergic and GABAergic Synapse Development

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Supplemental Experimental Procedures

Transcriptional Profiling

To identify developmentally-regulated genes, mRNA was isolated from whole mouse hippocampi at P1, P7, and P14 using RNeasy Midi kits (Qiagen). For each experimental condition, 10μg of total RNA was used to generate biotin-labeled cRNA following Affymetrix standard protocols, and 15μg of labeled cRNA was fragmented and hybridized to the Affymetrix mouse MG_U74Av2 array. To identify activity-regulated genes, cortical neurons were isolated from P0 mice and cultured in Neurobasal medium supplemented with B27 (Invitrogen) for 5 days before being depolarized with 55mM KCl. Total RNA was collected from matched cultures before depolarization and 1 hour and 6 hours after depolarization using Qiagen RNeasy Mini kits (Qiagen). 1μg of total RNA was used to generate biotin labeled cRNA following Affymetrix standard protocols. 10μg of the labeled cRNA was hybridized to the Affymetrix mouse MOE430 array. All microarray experiments were replicated independently three times for each condition. The analysis of the microarray data was performed using the DNA-Chip (dChip) software package (Li and Wong, 2001). Genes were considered to be developmentally regulated during the first two weeks of hippocamal maturation if: (1) there was at least a 1.5 fold difference between any of the two developmental stages (P1, P7, and P14) and (2) there was an absolute hybridization intensity difference of more than 100 (arbitrary units) in the expression level between any two of the developmental stages. Activity-regulated genes were chosen based on the following criteria: (1) there was at least a 1.5 fold difference between the depolarized (at either 1 hour or 6 hours) and non-depolarized samples and (2) there was an absolute difference of more than 100 in the expression level between the depolarized (at either 1 hour or 6 hours) and non-depolarized samples. Gene induction was confirmed by a semi-quantitative RT-PCR assay using mRNA independently

isolated from cortical neurons that had been depolarized in the manner described above. The following Rem2 primer sequences were used: 5' AAGACAGGGAAGAAATCCGTGC 3' and 5' AAGTGGACCAGGACAGAGGACAAG 3'.

Generation of diced siRNAs

mRNA was isolated from E18, P5, and P11 rat cortex and hippocampus using the Qiagen RNeasy Midi kit (Qiagen). mRNA was reverse transcribed using both random hexamers and oligo-dT primers (Superscript First Strand Synthesis System, Invitrogen). Primers were designed to amplify a 500-1000 bp region of the gene of interest, with a bias towards the C-terminus of the coding region of the gene. To generate the T7 RNA polymerase promoter-tagged double-stranded DNA template, two sets of primers were used per gene. An outer primer pair was used for the initial PCR reaction from the single-stranded DNA. A pair of nested primers that contained the T7 sequence (5' GCGTAATACGACTCACTATAGGGAGA 3') appended to the 5' end of both primers was used for a second PCR reaction using the product of the first PCR reaction as template (for primer sequences see Supplemental Table). The following additional primer pairs were used with the T7 sequence appended to the 5' end: PSD-95 N-terminal 5' ATGGACTGTCTCTGTATAGTG 3' and 5' TCCAATCTGCAACCTGCCATC 3'; PSD-95 C-terminal 5' GTCATGCACGAGGATGCC 3' and 5' GATCTCTTCAAAGCTGTCGCC 3'; GluR2 N-terminal 5' TGGTGTCTCTTCTAACAGC 3' and 5' CCCTGCATTTCCTCTCCG 3'; GluR2 C-terminal 5' GAGCTCAAAACAAATGGACCC 3' and 5' AGATGGGTTAATATTCTGTGG 3'. PCR conditions were optimized on a per gene basis to isolate a single band on an agarose gel, and the identity of the PCR product was confirmed by sequencing. The PCR products from the nested reactions were purified using QiaQuick PCR purification columns (Qiagen), and in vitro transcription was performed using a T7 MegaScript kit (Ambion). The double-stranded RNA was digested using recombinant Dicer enzyme (BLOCK-iT Dicer RNAi kit from Invitrogen or T7 epitope-tagged, purified recombinant Dicer (gift of J. Myers)) and siRNAs were purified as directed (BLOCK-iT kit). siRNA purity was assessed on a 2% agarose gel and siRNAs were diluted to a concentration of 50 ng/μl in 1x TE and stored at –70ºC.

Generation of shRNAs

Sequences for shRNA construction were chosen with the aid of the Dharmacon siDesign Center (design.dharmacon.com) and the GenScript siRNA Target Finder (www.genscript.com) and cloned into the pSuper shRNA expression vector as instructed (OligoEngine). The following 19 bp sequences

were used (the vector inserts 3' TT overhangs to make a 21 bp siRNA). Rem2 5' GACGGATCATGGTGGACAA 3', 5' GAACTCAGAGGACACCTAT 3', 5' GCACATAGAGACATCAGCC 3'; Sema4A 5' AGTACAAGGAGCTGAACAA 3', 5' GCCTCTTCTACTGTCTATA 3', 5' GCAGCCATGTGGTCCTTTA 3'; Sema4B 5' GAAGAAAGGCTGATTAGAA 3', 5' CCAAAGCGTGACTGTCAAA 3', 5' CAGATCCTGACAGGAAGCA 3'; Sema4D 5' AGTGGACCTCCTTCCTAAA 3', 5' AGCAGACGGAGTGCCTTAA 3', 5' CTCTGCTGCTATCATCAAA 3'; cadherin-11 5' GGAACCACCTTCAGAATTT 3', 5' GGTATTCAATTGATCGTCA 3', 5' AGACTTGTATGGCTCCAAA 3'; cadherin-13 5' GTCCTAAACTTGACCTTCA 3', 5' ACGATCGCTACTTATCAAC 3', 5' CTATCAGGTACTCTGTTTA 3'; Ube2d3 5' TGAAACGGATTAATAAGGA 3', 5' ATATCAAGGTGGTGTATTC 3', 5' GAGATTGCACGGATCTATA 3'; APC5 5' TGTCAAAGCTGTACAAACT 3', 5' TCATTACCTCAGCTACTTA 3', 5' GCCGTGCCATGTTCTTAGT 3'.

Neuronal cell culture and transfection

Neurons were cultured at low density on a glial monolayer. Glia were isolated from P1-P2 rat cortex by plating dissociated cells at low density in DMEM+10%FBS on uncoated 10 cm tissue culture dishes. The media was exchanged one day after plating to remove non-adherent cells. By 7-10 days after plating, the glia had formed a confluent monolayer. Once confluent, the glia were trypsinized and plated at low density on 12 mm glass coverslips coated overnight at 37ºC with poly-D-lysine (20 μg/ml) and laminin (3.4 μg/ml) in 24 well plates. Dissociated hippocampal neurons from E18 rats were plated at a density of 65,000-100,000/well onto the monolayer of confluent glia grown on the 12 mm glass coverslips in Neurobasal media with B27 supplement (Invitrogen). AraC (Sigma) was added to a final concentration of 5 μM 24 hrs after neuronal plating. At 4DIV, neurons were transfected by the calcium phosphate method (Xia et al., 1996) with 500 ng GFP plasmid/well and diced siRNAs at the following concentrations: 30 nM siRNA per gene per well (i.e. 30 nM-60nM total siRNA) for pools containing 1 or 2 genes and 20 nM siRNA per gene (i.e. 60 nM-80nM total siRNA) for pools containing 3 or 4 genes. For experiments using diced siRNAs, the control condition is diced siRNAs targeting the DsRed gene at an equivalent concentration. shRNA pSuper constructs were transfected as pools of three constructs targeting an individual gene at a concentration of 33 ng for each construct (i.e. 100 ng total plasmid/well). For experiments using three shRNAs simultaneously, the control condition is empty pSuper vector at 100ng total plasmid. For rescue and single pSuper experiments, shRNAs

were transfected at a concentration of 33ng for each construct. For rescue and subcellular localization studies, myc expression plasmids were transfected at a concentration of 100ng.

293T cell culture and western blotting

HEK 293T cells grown in 12 well tissue culture plates were transfected by the calcium phosphate method with 200 ng GFP, 50 ng each myc-CMV construct, and either 1 μg each shRNA construct or 30 nM diced siRNAs. For the Sema4D HEK 293T experiments, 500ng of myc-pCS vector and 1ug shRNA construct were transfected by the calcium phosphate method. Antibodies used for Western blotting were as follows: mouse anti-myc (1:1000; Santa Cruz), mouse anti-cadherin-11 (1:500; Zymed), and mouse anti-β-actin (1:5000; Abcam). To make the cadherin-13 RNAi-resistant plasmid, the following changes (lower case letters) were introduced into the sequence targeted by shRNA_2_cdh13: ACcATaGCTACgTATCAAC using QuikChange Site Directed Mutagenesis (Stratagene).

Electrophysiology

Whole-cell voltage clamp recordings were obtained using an Axopatch 200B amplifier at room temperature. Extracellular perfusion medium contained (in mM): 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$ with 0.001 tetrodotoxin and 0. 02 bicuculline. Electrodes were back-filled with (in mM): 115 Cs methansulfonate, 20 CsCl, 10 Hepes, 2.5 $MgCl_2$, 4 MgATP, 0.4 Na_3GTP, 10 Na phosphocreatine, 0.6 EGTA pH 7.25; and tip-filled with (in mM): 115 $CsSO_4$, 15 CsCl, 10 Hepes, 6 $MgCl_2$, 0.2 EGTA pH 7.4. Electrode resistances were 3-5 MΩ. R_s and R_{in} were monitored throughout experiments using a pulse test and recordings where these parameters changed by more than 30% during the recording were discarded. To record mEPSCs, the membrane potential was held at -70mV, events were filtered at 1kHz, and series resistance was left uncompensated. Data was recorded in 20s epochs for a total duration of 200-300s per recording. mEPSC events were detected and analyzed off-line using IGOR PRO software (Wavemetrics, Lake Oswego, OR) and custom macros (C. Chen and B. Sabatini). mEPSC event detection criteria included an amplitude greater than 4pA, a minimum rise rate of 0.3pA/ms, and a decay time constant between 1-12ms. Membrane capacitance was measured by applying a 5mV hyperpolarization step to the cell for 10ms and calculating the area under the curve. Cumulative averages for each transfection condition were normalized to control values and compared using a two-sample t-test. See below for handling of normalization and error.

Immunocytochemistry

For NR2B staining, neurons were fixed at 14DIV with 2% formaldehyde/4% sucrose for 2 minutes at room temperature. Coverslips were then transferred to 100% methanol for 10 minutes at -20°C. Next, coverslips were washed three times with 1x PBS for 5 minutes each and incubated overnight at 4°C in a humidified chamber with rabbit anti-NR2B (1:150; gift of Dr. Morgan Sheng) antibodies in 1x GDB (0.1% gelatin, 0.3% TritonX-100, 4.2% 0.4 M phosphate buffer, 9% 5 M NaCl). After overnight incubation, coverslips were washed three times with 1x PBS for 5 minutes each and then incubated with Cy3- and Cy5-conjugated secondary antibodies (1:300 each; Jackson ImmunoResearch Laboratories) in 1x GDB for 1 hour at room temperature. Coverslips were then washed three times with 1x PBS for 10 minutes each, dipped in dH_2O, and mounted on glass slides with Aquamount (Lerner Laboratories). For synaptotagmin I, VGLUT, $GABA_AR\beta2/3$, $GABA_AR\gamma2$, VGAT, GAD65, GAD67, and myc staining, neurons at 14DIV were fixed with 4% paraformaldehye/4% sucrose for 8 minutes at room temperature. Coverslips were washed three times with 1x PBS for 5 minutes each and incubated overnight at 4°C in a humidified chamber with rabbit anti-synaptotagmin I (1:1000; Sigma), guinea pig anti-VGLUT (1:400; Chemicon), mouse anti-$GABA_AR\beta2/3$ (1:200; Chemicon), rabbit anti-$GABA_AR\gamma2$ (1:100; Chemicon), guinea pig anti-VGAT (1:200; Chemicon), rabbit anti-GAD65 (1:200; Chemicon), mouse anti-GAD67 (1:100; Chemicon), and/or mouse anti-myc (1:100; Santa Cruz Biotechnology) antibodies in 1x GDB. After overnight incubation, coverslips were processed as above. For GluR2 staining, neurons at 14DIV were incubated with 6μg mouse anti-GluR2 (Chemicon) antibody in 0.5ml media for 1 hour at 37°C. Coverslips were washed three times with 1x PBS and fixed with 4% paraformaldehye/4% sucrose in 1x PBS for 8 minutes at room temperature. Coverslips were washed three times with 1x PBS for 5 minutes each and incubated in 1x GDB overnight at 4°C in a humidified chamber. After overnight incubation, coverslips were processed as above. For PSD-95 (mouse anti-PSD-95; 1:200; Affinity BioReagents) and synapsin I (rabbit anti-synapsin I; 1:200; Chemicon) staining, neurons were fixed at 14DIV with either 2% formaldehyde/4%sucrose and 100% methanol or 4% paraformaldehyde/4% sucrose and processed as above.

Quantification of protein levels

Image acquisition and quantification were performed in a blinded manner. 10 cells per condition from two separate coverslips were analyzed for each of 3 experiments. Twelve-bit images

were acquired on a Zeiss LSM5 Pascal confocal microscope using a 63x objective at 1024x1024 pixel resolution. For each experiment, all images were acquired with identical settings for laser power, detector gain, and amplifier offset with a pinhole diameter equivalent to one Airy unit for the 633 nm laser; pinhole diameters for the 543nm and the 488nm lasers were set such that optical slice thickness was conserved. Laser power and detector gain were set so that pixel intensities remained within the dynamic range. Images were acquired as a z-stack (4-5 optical sections, 0.75 μm step size). Maximum intensity projections were created from the z-stacks and analyzed using MetaMorph image analysis software (Molecular Devices). For each experiment, background for each channel was calculated by determining average pixel intensity in a non-neuronal region for three randomly chosen images and then averaging the values obtained from the three images. This value plus one standard deviation was subtracted from each channel before analysis. For quantification of protein levels, a region of dendrite approximately 50 μm in length was chosen at random from a transfected cell and carefully traced using the GFP signal. The average pixel intensity for this region of interest was then determined for the Cy3 and Cy5 channels. The average pixel intensity for each condition in the experiment was obtained by averaging these values from each image. For normalization and statistical analysis, see below.

Quantification of synapse density and criteria for positive pools in screen

Image acquisition and quantification were performed in a blinded manner. For PSD-95/synapsin I experiments, 8-bit images of neurons co-transfected with GFP and diced siRNAs were acquired on a Zeiss LSM510 or Zeiss LSM5 Pascal confocal microscope using a 63x objective and 1024x1024 pixel resolution. Within each experiment, images were acquired with identical settings for laser power, detector gain, and amplifier offset with pinhole diameters set as above. Images were acquired as a z-stack (4-8 optical sections, 0.48 or 0.75 μm step size) and maximum intensity projections were created from the stacks. Synapse density was quantified as the overlap of GFP, anti-PSD-95, and anti-synapsin I staining using Openlab software (Improvision) and custom macros. Threshold for each channel in each image was calculated as the mean pixel intensity for the entire image plus two standard deviations above the mean. A binary mask that included all pixels above threshold was created for each channel. Using the GFP mask as a guide, the cell body was manually excluded from further analyses. Then, using the mask for each channel, regions of triple co-localization greater than 2 pixels in size were defined as objects. To calculate synapse density, the number of objects was divided by the area of the neuron as measured using the GFP mask minus the cell body. Approximately 16 images from at least two separate coverslips were acquired and analyzed for each

condition within an experiment. The synapse density for each condition within an experiment was calculated by averaging the synapse density from each of the images corresponding to that condition. The average synapse density was compared between experimental conditions and control, and a pool was considered "positive" if it was significantly different ($p<0.05$) from the control by a two-sample t-test. All positive pools were repeated in a second experiment, and only pools that were positive by the above criteria in at least two experiments were analyzed further. PSD-95/synapsin I synapse density for neurons transfected with shRNAs targeting individual genes was determined in the same manner and statistical significance was assessed using a multi-factorial ANOVA on the combined raw data. See below for a description of data normalization and statistical analysis from combined experiments.

MetaMorph image analysis software was used to determine the density of PSD-95 puncta independent of a requirement for co-localization with synapsin I using the same images that had been used to quantify the density of PSD-95/synapsin I co-localized puncta in neurons transfected with shRNAs. For each experiment, the threshold for the PDS-95 channel was determined by visual inspection using an image of a neuron transfected with control shRNAs. The threshold was chosen such that all punctate structures were included in the analysis. This threshold was applied uniformly across all images within an experiment. A binary mask including all pixels above threshold was created for the GFP and PSD-95 channels for each image. The cell body was deleted from the GFP mask by excluding all regions of continuous pixel intensity greater than 50 pixels in diameter. The "logical and*"* function was used to determine regions of overlap between the GFP mask and the PSD-95 mask greater than 2 pixels in diameter. The number of sites of co-localization of GFP and PSD-95 was divided by the area of the GFP mask to determine the density of postsynaptic puncta on the transfected cell. The density of synapsin I puncta independent of a requirement for colocalization with PSD-95 was determined in the same manner. The density of PSD-95 puncta or synapsin I puncta for each condition within an experiment was calculated by averaging the puncta density from each of the images. Statistical significance was assessed using a multi-factorial ANOVA on the combined raw data.

For synapse density experiments using GluR2/synaptotagmin I, $GABA_AR\beta2/3$/VGAT and $GABA_AR\gamma2$/GAD67 immunostaining and for myc localization experiments, 12-bit images of neurons co-transfected with GFP and shRNAs or myc-tagged cDNA constructs were acquired on a Zeiss LSM5 Pascal confocal microscope as described above. Synapse density was quantified as the overlap of GFP, anti-GluR2 and anti-synaptotagmin I, GFP, anti-$GABA_AR\beta2/3$ and anti-VGAT, or GFP, anti-$GABA_AR\gamma2$ and anti-GAD67 staining using MetaMorph image analysis software. For each experiment,

thresholds for the GluR2, synaptotagmin I, $GABA_AR\beta2/3$, $GABA_AR\gamma2$, VGAT, and GAD67 channels were determined by visual inspection using an image of a neuron transfected with control shRNAs. A mask of the presynaptic marker (synaptotagminI, VGAT, or GAD67) and a mask of the postsynaptic marker (GluR2, $GABA_AR\beta2/3$, $GABA_AR\gamma2$) were made as described above. The "logical and" function was used to determine regions of overlap between the GFP mask and the regions of overlap of the pre- and postsynaptic masks. The number of sites of triple co-localization was divided by the area of the GFP mask to determine the density of synaptic puncta on the transfected cell. Normalized synapse density was calculated as described above. Statistical significance was assessed using a multi-factorial ANOVA on the combined raw data.

Analysis of GAD67 staining in hippocampus

P20-P23 wild-type and *Sema4D-/-* littermates were treated in parallel for all experiments. Mice were anesthetized and perfused intracardially with cold PBS followed by freshly prepared 4% paraformaldehyde in PBS. Brains were removed and post-fixed in the same solution overnight at 4°C. They were then equilibrated in 20% sucrose in PBS at 4°C. Once equilibrated, brains were mounted in Tissue-Tek O.C.T. compound and cut into 35-45μm-thick coronal sections on a cryostat (Leica) and stored in PBS at 4°C. For immunohistochemistry experiments, matched coronal sections were incubated in blocking solution (3% BSA, 0.3% Triton X-100, 3% Goat Serum, 0.2% Tween in PBS) for two hours at room temperature and incubated overnight at 4°C with mouse anti-GAD67 (1:1000; Chemicon) and rabbit anti-bHLHb5 (1:2000; gift of Dr. Sarah Ross). After overnight incubation, sections were washed four times with 1x PBS at room temperature and then incubated with Cy2- and Cy3-conjugated secondary antibodies (1:300 each; Jackson ImmunoResearch Laboratories) in blocking solution for 2 hours at room temperature. Following secondary incubation, sections were washed twice with 1x PBS, incubated with Hoechst 33342 (1:5000) for one minute, washed twice with 1x PBS, and mounted.

Image acquisition and quantification were performed in a blinded manner. The granule cell layer and polymorphic cell layer of the dentate gyrus, the pyramidal cell layer of CA1 and CA2, and stratum oriens and stratum radiatum of CA1 and CA3 from two pairs of hippocampal sections from three different sets of *Sema4D+/+* and *Sema4D-/-* littermates were imaged and analyzed for bHLHb5 and GAD67 staining. 12-bit images were acquired on a Zeiss LSM5 Pascal confocal microscope using a 25x oil-immersion objective at 1024x1024 pixel resolution. For each region of the hippocampus within a pair of sections, images were acquired with identical settings for laser power, detector gain,

amplifier offset, and pinhole diameter. The settings were such that all pixel intensities fell within the dynamic range. Images were acquired as a z-stack (12-24 optical sections, 2.0 μm step size), and individual images within the stack were analyzed using MetaMorph image analysis software. The average pixel intensity of the bHLHb5 and GAD67 signals were determined for a 400μm^2 area for multiple images from each region of the hippocampus. The average pixel intensity of the GAD67 signal was normalized to the average pixel intensity of the bHLHb5 signal for each image. The normalized pixel intensity for each region for each condition was obtained by averaging the values from the individual images within that region. For normalization and statistical analysis across experiments, see below. The granule cell layer of the dentate gyrus, the pyramidal cell layer of CA2, and stratum radiatum and stratum lacunosum-moleculare of CA1 and CA3 were also imaged and analyzed for Hoescht staining. Eight-bit images were acquired on a Zeiss LSM510 using a 25x oil-immersion objective, a Coherent Chameleon laser with an excitation wavelength of 790nm, and 1024x1024 pixel resolution. For each region of the hippocampus within a pair of sections, images were acquired with identical settings for laser power, detector gain, amplifier offset, and pinhole diameter. The settings were such that all pixel intensities fell within the dynamic range. A single image at the center of the section was acquired for each region of the hippocampus. Nuclei in the dentate gyrus were counted by hand. Nuclei in stratum radiatum and stratum lacunosum-moleculare were counted using Metamorph image analysis software. A threshold was determined for each pair of images by visual inspection and applied uniformly across both images. The 'dilate' function was used to fill in black pixels within nuclei, and a binary mask including all pixels above threshold was created for each image. The number of nuclei for each region of the hippocampus was determined by averaging the counts from the individual images. The average number of nuclei was compared between the *Sema4D+/+* and *Sema4D-/-* condition and considered significantly different if $p<0.05$ as determined by a two-sample t-test.

Sholl analysis

Image acquisition and quantification were performed in a blinded manner. 8-bit images were acquired on a Zeiss LSM5 Pascal confocal microscope using a 25x oil objective and 1024x1024 pixel resolution. All images were acquired with identical settings for laser power, detector gain, and amplifier offset with a pinhole diameter equivalent to one Airy unit for the 488 nm laser. Images were acquired as a z-stack (10-20 optical sections, 1.0 μm step size). Maximum intensity projections were created from the images and analyzed using Zeiss LSM Image Browser software. A series of concentric

circles of increasing radii (10 μm intervals) was drawn around the center of the cell body, and the number of dendrite crossings at each circle was counted. For each experiment, 20 images from two separate coverslips were acquired and analyzed for each condition, and each condition was analyzed in two independent experiments. The number of dendrite crossings at each radius for each condition was calculated by averaging the number of crossings from every image. The average number of crossings was compared between the experimental condition and control condition and considered significantly different if $p<0.05$ as determined by a two-sample t-test.

Data normalization and statistics

Data for synapse density measurements, quantification of protein levels, and quantification of GAD67 immunoreactivity in the hippocampus was processed in the following manner. Because synapse density and immunostaining varies from experiment to experiment, it was necessary to normalize values within each experiment in order to combine data from separate experiments. Within an experiment, the average value (i.e. synapse density, average pixel intensity, etc.) was obtained for the control and for experimental conditions. The normalized value of each experiment is the experimental average value divided by the control average value. The standard error of this ratio is determined as follows:

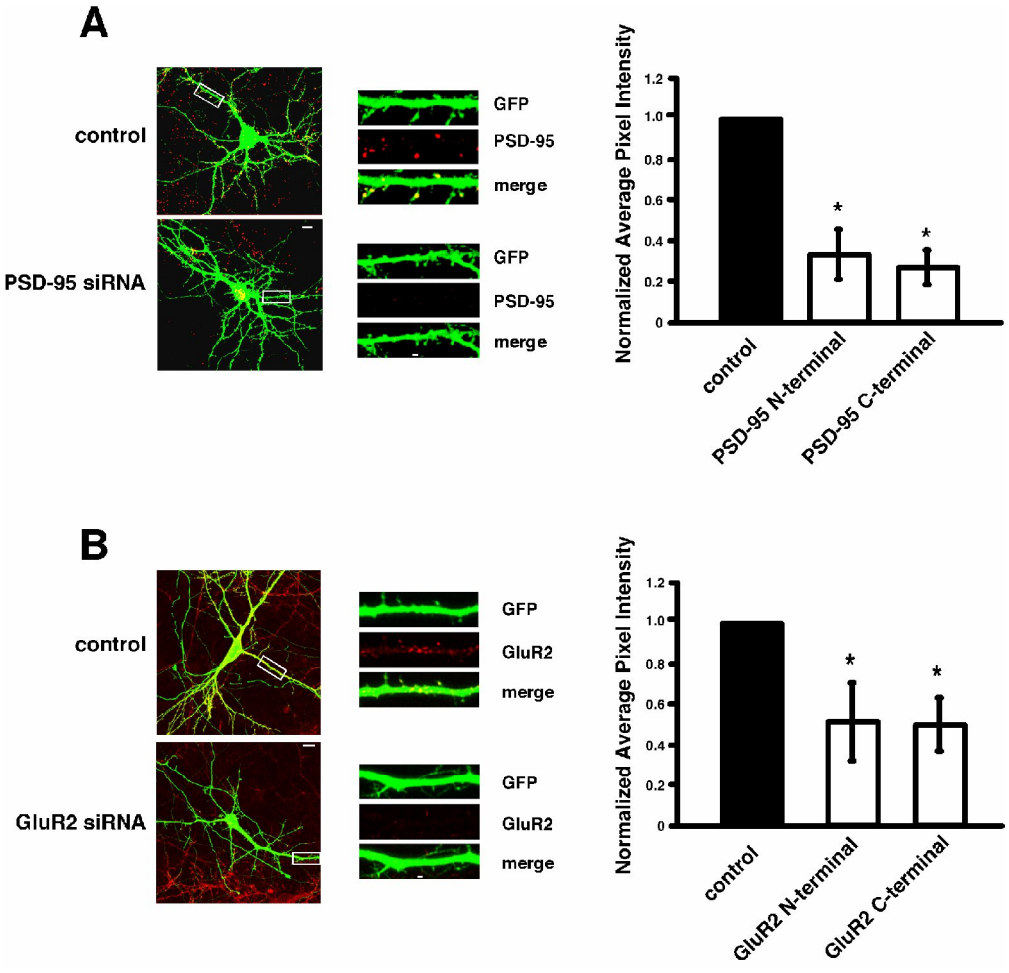
$$\text{std error of the ratio} = \text{ratio} * \sqrt{[(\text{std error experimental/mean experimental})^2 + (\text{std error control/mean control})^2]}$$

The normalized values from each separate experiment (at least 3 unless otherwise indicated) are averaged for each condition, and the error is determined as follows:

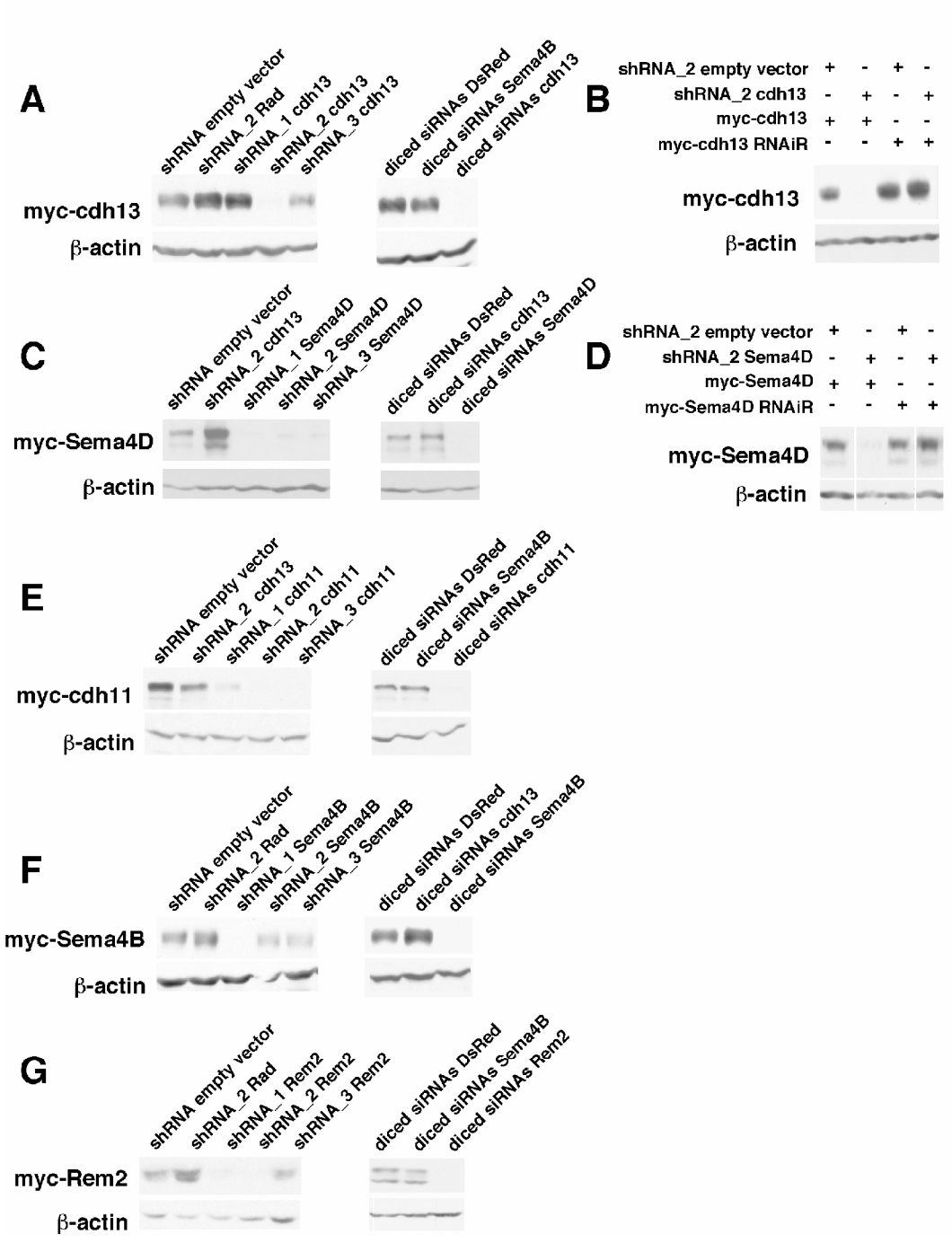
$$\text{std error of the average ratio} = \sqrt{[(\text{std error of the ratio})_1^2 + (\text{std error of the ratio})_2^2 + \ldots (\text{std error of the ratio})_n^2]}/n$$

Statistical analysis was performed comparing each experimental condition to control on the combined raw data from all experiments using the ANOVA factorial function in StatView 4.5 (Abacus Concepts).

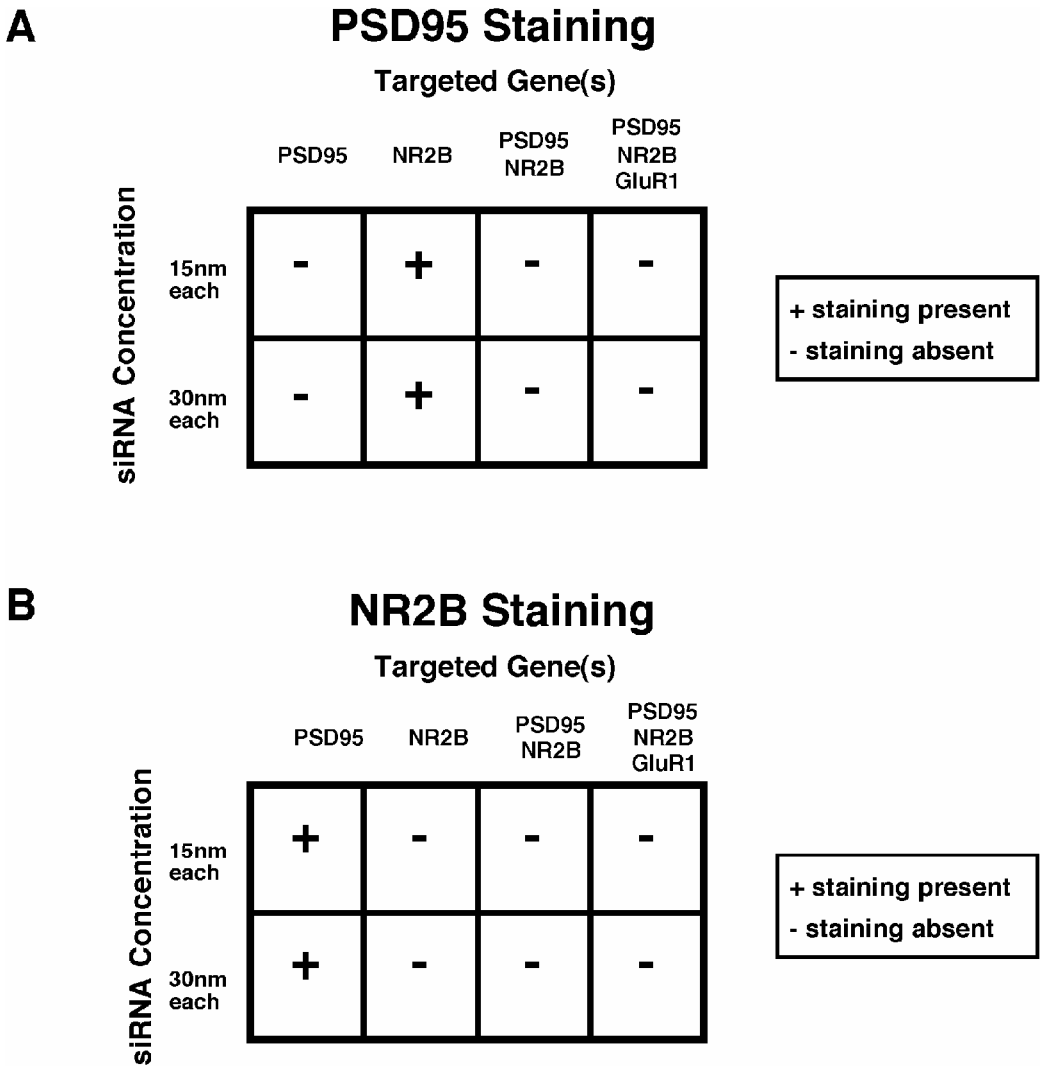
Supplemental Figures



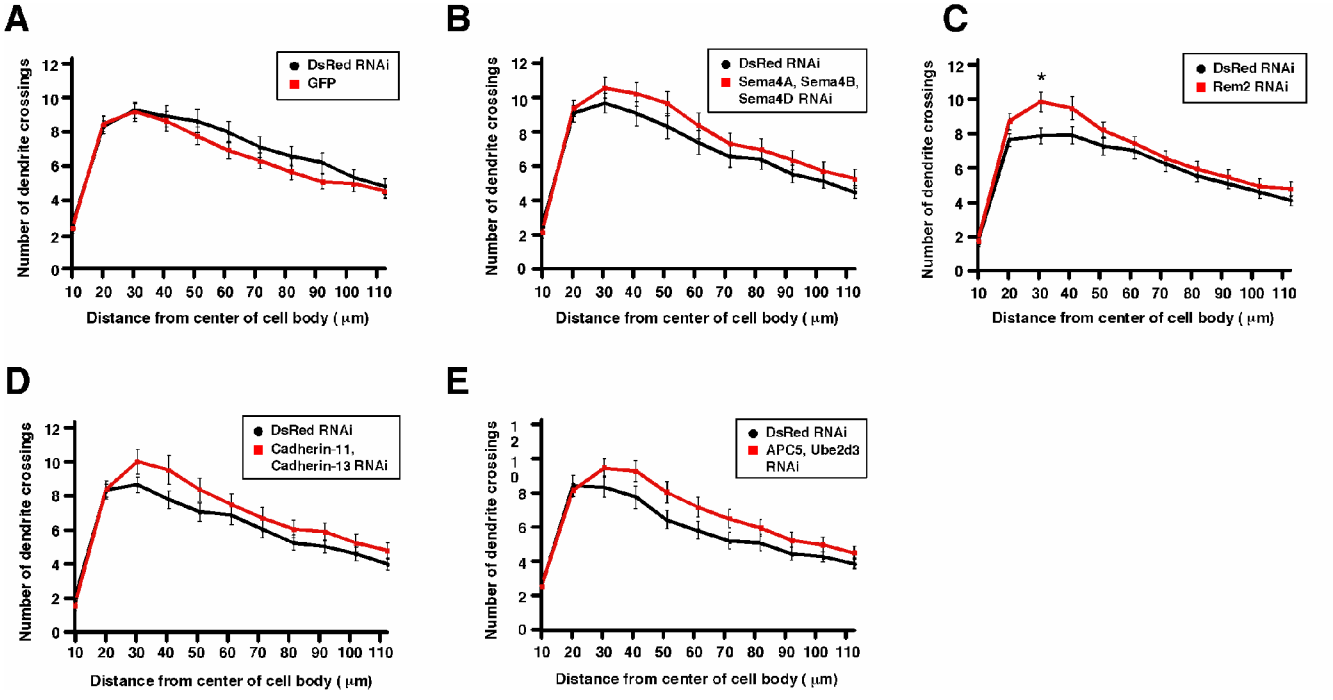
Supplemental Figure S1. Diced siRNAs targeting the PSD-95 and GluR2 gene products reduce protein levels for 10 days in cultured hippocampal neurons. **A)** Hippocampal neurons were co-transfected at 4DIV with GFP and diced siRNAs targeting DsRed (control) or PSD-95. Cells were stained for PSD-95 (red) at 14DIV. The white box indicates the region of dendrite magnified at right. PSD-95 protein levels as assessed by quantitative immunocytochemistry are shown at far right. **B)** Hippocampal neurons were co-transfected at 4DIV with GFP and diced siRNAs targeting DsRed (control) or GluR2. Cells were stained for surface GluR2 (red) at 14DIV. The white box indicates the region of dendrite magnified at right. Quantification of GluR2 protein levels is shown at far right. n=20 for each condition. The normalized values shown are the average of two independent experiments. Scale bars are 10 μm for whole cell images and 1 μm for dendrite images. Significance of $p<0.0005$ by multi-factorial ANOVA is indicated by asterisk.



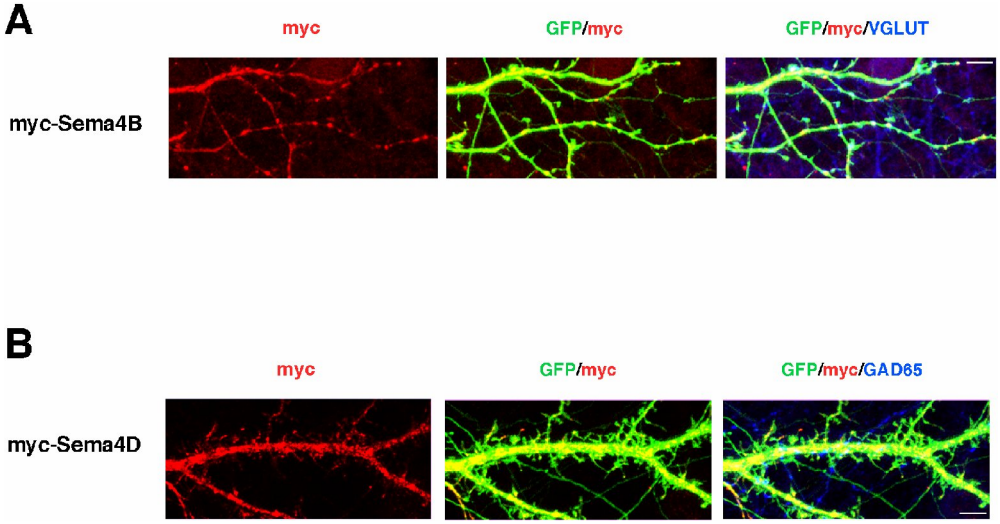
Supplemental Figure S2. Specificity of shRNA constructs and diced siRNAs. **A-G)** HEK293T cells were co-transfected with shRNA constructs or diced siRNAs (indicated at top) and (A) myc-cdh13, (B) myc-cdh13 or myc-cdh13 RNAi resistant (RNAiR), (C) myc-Sema4D, (D) myc-Sema4D or myc-Sema4D RNAiR, (E) myc-cdh11, (F) myc-Sema4B, or (G) myc-Rem2 and harvested for Western blotting after 48 hours. Anti-myc antibody was used to detect all proteins except myc-cdh11, in which case anti-cadherin-11 antibody was used; anti-β-actin antibody was used to assess protein loading.



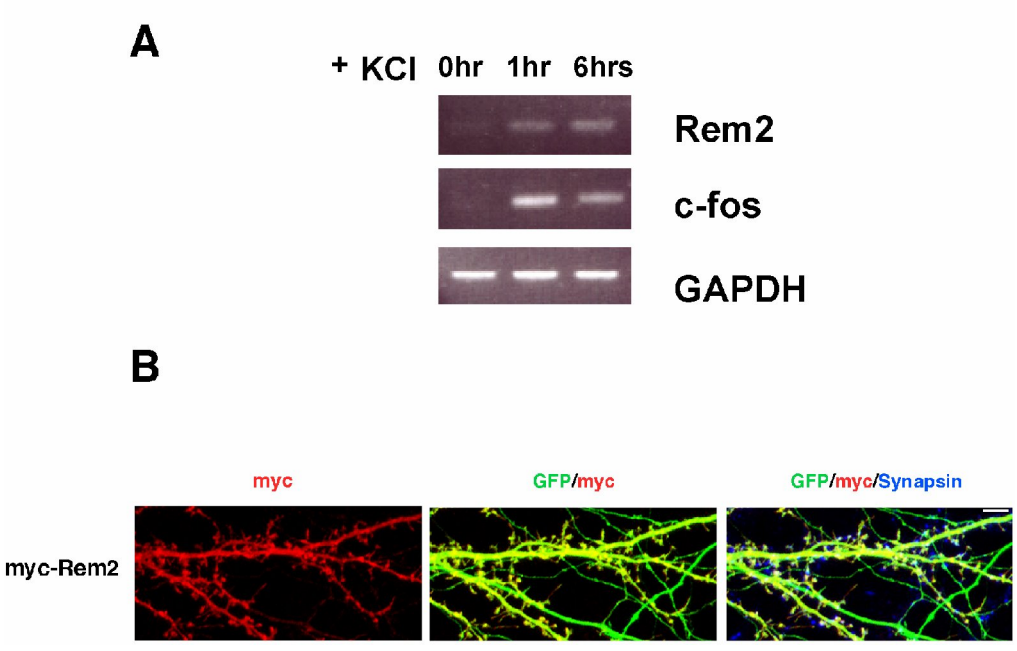
Supplemental Figure S3. Diced siRNAs reduce the protein levels of multiple genes simultaneously. Hippocampal neurons were co-transfected at 4DIV with GFP and diced siRNAs targeting PSD-95, NR2B, PSD-95/NR2B, or PSD-95/NR2B/GluR1 at a concentration of 15 nM each or 30 nM each as indicated. At 14DIV, neurons were fixed and stained for **(A**) PSD-95 and **(B)** NR2B. The vertical columns in the table represent the targeted gene(s), and the horizontal rows represent the siRNA concentration used to target each of the genes. A '+' sign indicates the presence of robust PSD-95 or NR2B staining in transfected cells as assessed by visual inspection. A '-' sign indicates the absence of PSD-95 or NR2B staining in transfected cells. At least 10 cells were analyzed per condition within an experiment, and each experiment was repeated three times.



Supplemental Figure S4. Diced siRNAs targeting genes from the positive pools do not affect dendritic complexity. Hippocampal neurons were transfected at 4DIV with GFP and diced siRNAs as indicated below. Neurons were fixed at 14DIV and imaged by confocal microscopy. The number of dendrites crossing concentric circles of increasing radii centered on the cell body was counted from at least two independent experiments. **A)** Dendritic complexity of cells transfected with GFP alone or GFP and siRNAs targeting DsRed (60 nM). **B)** Dendritic complexity of cells transfected with GFP and siRNAs targeting DsRed (60 nM) or Sema4A/Sema4B/Sema4D (20 nM each). **C)** Dendritic complexity of cells transfected with GFP and siRNAs targeting DsRed (60 nM) or Rem2 (30 nM). **D)** Dendritic complexity of cells transfected with GFP and siRNAs targeting DsRed (60 nM) or Cadherin-11/Cadherin-13 (30nM each). **E)** Dendritic complexity of cells transfected with GFP and siRNAs targeting DsRed (60 nM) or Ube2d3/APC5 (30nM each). n=40 at each point for each condition. Error bars are $\pm$ standard error of the average number of dendrite crossings. Significance of $p<0.05$ by two sample t-test is indicated by asterisk.



Supplemental Figure S5. Subcellular localization of myc-tagged Sema4B and Sema4D in neurons. **A)** Immunostaining at 14DIV for myc (red) in the dendrites of cells co-transfected with GFP and myc-tagged Sema4B. **B)** Immunostaining at 21DIV for myc (red) in the dendrites of cells co-transfected with GFP and myc-tagged Sema4D. Note that the neuron in (B) is 7 days older than the neuron in (A), resulting in a more mature-appearing dendrite with an increased number of dendritic spines.



Supplemental Figure S6. Rem2 transcriptional regulation and subcellular localization in neurons. **A)** mRNA was isolated from cultured cortical neurons treated with high extracellular potassium for 0, 1, or 6 hours, and semi-quantitative RT-PCR was performed to assess mRNA levels. c-fos mRNA levels demonstrate gene induction while GAPDH, a gene not induced by membrane depolarization, mRNA levels were unchanged. **B)** Immunostaining at 21DIV for myc (red) in cells co-transfected with GFP and myc-tagged Rem2.

Supplemental Table S1

Candidate Gene List

For candidate gene list, including accession numbers and primer sequences, see Supplemental Table S1.

Supplemental Table S2

Electrophysiological Parameters

Condition	Capacitance (pF)	R_{in} (MΩ)	n
Rem2 RNAi	45.54±3.31*	941±133*	15
control	61.42±2.27	581±48	12
cadherin-11 RNAi	52.03±2.91*	687±72 *	15
control	64.79±2.96	390±34	16
cadherin-13 RNAi	59.19±2.98	498±64	14
control	64.79±2.96	390±34	16
Sema4B RNAi	45.59±2.80*	564±53*	15
control	66.69±3.68	417±24	15
Sema4D RNAi	48.78±2.90*	631±69*	13
control	66.69±3.68	417±24	15

* indicates different from control $p<0.05$ two sample t-test

Supplemental Table S3

GAD67 Staining Intensity and Nuclear Density for Wild-Type and Sema4D-/- Sections

Region of Hippocampus	Normalized GAD67 Intensity for Sema4D-/- Sections	n (images)
Dentate Gyrus – Granule Cell Layer	0.831 +/- 0.034***	42
Dentate Gyrus – Polymorphic Cell Layer	1.125 +/- 0.029	42
CA3 – Stratum Radiatum	0.984 +/- 0.027**	61
CA3 – Stratum Oriens	0.887 +/- 0.022***	48
CA2 – Pyramidal Cell Layer	0.939 +/- 0.036*	60
CA1 – Stratum Radiatum	0.934 +/- 0.016**	52
CA1 – Stratum Oriens	0.987 +/- 0.019**	55
CA1 – Pyramidal Cell Layer	0.953 +/- 0.019**	54

* indicates different from Sema4D+/+ $p<0.05$ by multi-factorial ANOVA
** indicates different from Sema4D+/+ $p<0.005$ by multi-factorial ANOVA
*** indicates different from Sema4D+/+ $p<0.0005$ by multi-factorial ANOVA

Region of Hippocampus	Normalized Number of Nuclei for Sema4D-/- Sections	n (images)
Dentate Gyrus – Granule Cell Layer	1.042 +/- 0.298	6
CA2 – Pyramidal Cell Layer	1.046 +/- 0.163	6
CA3 & CA1 – Stratum Radiatum & Stratum Lacunosum-Moleculare	1.018 +/- 0.063	12

Supplemental References

Li, C., and Wong, W. H. (2001). Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A *98*, 31-36.

Xia, Z., Dudek, H., Miranti, C. K., and Greenberg, M. E. (1996). Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci *16*, 5425-5436.