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Supplemental Data

Uncoupling Dendrite Growth and Patterning:

Single-Cell Knockout Analysis of NMDA Receptor 2B

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Supplemental Experimental Procedures

Genotyping of mice

All genotyping was done using polymerase chain reaction (PCR) amplification of genomic DNA prepared from mouse tissue. Tissue samples were incubated in NaOH (50 mM) at 95°C for 40 minutes, neutralized with 1 M Tris, and supernatant was added to PCR mixture for amplification. As described in (Zong et al., 2005), genotyping of MADM mice was performed by primer sequences: (1) 5′-CTCTGCTGCCTCCTGGCTTCT-3′ and 5′-TCAATGGGCGGGGGTCGTT-3′ to amplify a 250 bp fragment from targeted alleles, and (2) 5′-CTCTGCTGCCTCCTGGCTTCT-3′ and 5′-CGAGGCGGATCACAAGCAATA-3′ to amplify a 330 bp fragment from wild-type alleles. Genotyping of Cre-targeted mice was determined by primer sequences: (1) 5′-CACCCTGTTACGTATAGCCG-3′ and 5’-GAGTCATCCTTAGCGCCGTA-3’ to amplify a 300 bp fragment from targeted alleles, and (2) 5’-CCAATCTGCTCACACAGGATAGAGAGGGCAGG-3’ and 5’-CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG-3’ to amplify a 500 bp fragment from PCR positive control. Genotyping of NR2B-targeted mice was determined by primer sequences: (1) 5’-GGCTACCTGCCCATTCGACCACCAAGCGAAAC-3’ and 5’-ATGGAAGTCATCTTTCTCGTG-3’ to amplify a 551 bp fragment from targeted alleles, and (2) 5’-ATGAAGCCCAGCGCAGAGTG-3’ and 5’-ATGGAAGTCATCTTTCTCGTG-3’ to amplify a 180 bp fragment from wild-type alleles. The reaction solutions contained 16 μl ddH_2O, 2 μL 10x PCR reaction buffer (15

mM $MgCl_2$), 0.4 µL dNTPs (10 mM), 0.4 µL oligonucleotide primer mix (10 pmol/µl), 0.2 µL Qiagen Taq DNA polymerase (5 units/µl; Qiagen, Hilden, Germany), and 1 µl of solubilized genomic DNA (20 µl final volume). The cycling conditions for MADM- and Cre-targeted amplification: (1) 94°C 3', (2) 94°C 20″ (melting), 58°C 25″ (annealing), 72°C 45″ (extension) for 32 cycles, and 3x 72°C 5'. The cycling conditions for NR2B-targeted amplification: (1) 94°C 3', (2) 94°C 20″, 58°C 25″, 72°C 60″, for 32 cycles, and 3x 72°C 5'. Reaction products were run on 1.5% agarose gels and visualized using ethidium bromide.

Hippocampal culture

Hippocampi were dissected from postnatal day 0 *NR2B*$^{-}$ *GR/RG;nestin-Cre* mice and incubated for 10 minutes at 37°C in 10 mg/mL trypsin (Sigma, St. Louis, MO: Cat. T1005). Cells were then washed in Hank's balanced salt solution, dissociated by trituration with a fire-polished, siliconized Pasteur pipette, and plated on matrigel–coated glass coverslips in 24-well plates. Cells from one hippocampus were plated into ~6 coverslips. The cultures were maintained in 5% CO_2 at 37°C in MEM (Invitrogen, Carlsbad, CA: Cat. 51200) supplemented with B-27 (Gibco-Invitrogen: Cat. 17504-044) and 10% fetal bovine serum (Omega Scientific: Cat. FB-01). Cultures were fed 1 day after plating by replacing half the medium with medium containing the addition of 4 µM of cytosine arabinoside (Sigma: Cat. C6645). In all experiments, neurons were used between 4 and 6 days after plating.

Immunofluorescence staining of cultured neurons

Hippocampal cells were fixed in ice-cold 4% paraformaldehyde (PFA; Sigma: Cat. 441244) in phosphate buffer supplemented with 4% sucrose. Fixed cells were then permeabilized in 0.1M phosphate buffer saline, pH 7.4 (PBS; Sigma: Cat. P3813) plus 0.3% Triton X-100 (PBST; Sigma: Cat. 234729), blocked with 10% normal donkey serum (NDS) in PBST for 30 minutes, and incubated for 1 hour at RT in primary antibodies in PBST: chicken anti-GFP (1:500; Aves Labs, Tigard, OR: Cat. GFP-1020) preabsorbed goat anti-MYC (1:200; Novus, Littleton, CO: Cat. NB600-338), and rabbit anti-NR2B (1:100; formerly Chemicon; Millipore Billerica, MA: Cat. AB1557P) or

mouse anti-NR1 (1:500; BD Biosciences: Cat. 556308). After three 10 minute PBST washes, cells were incubated for 1 hour at RT in secondary antibodies in PBST: donkey anti-chicken FITC (1:500; Jackson ImmunoResearch, West Grove, PA: Cat. 703-095-155) donkey anti-goat Cy5 (1:500; Jackson ImmunoResearch: Cat. 705-175-003) and donkey anti-rabbit Cy3 (1:500; Jackson ImmunoResearch: Cat. 711-165-152) or donkey anti-mouse Cy3 (1:500; Jackson ImmunoResearch: Cat. 715-165-150). After three 10 minute PBST washes, last wash with a fluorescent stain that binds strongly to DNA, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma: Cat. D8417), and final 10 minute washes in PBS, samples were mounted in Fluoro-Gel mounting medium (EMS, Hatfield, PA: Cat. 17985-10). Cells were imaged through a 40x (1.3 NA) oil objective by 1-μm optical sectioning using confocal microscopy (Carl Zeiss, Inc.; Oberkochen, Germany). Preabsorption of goat anti-Myc (see above) was conducted by postfixing three wild-type brains cut into small pieces, blocked at 4°C overnight with 10% NDS in PBST, and transferred into 30 ml of working solution goat anti-Myc in PBST for >2 days absorption.

Ca^{2+} imaging

Hippocampal neurons 4-6 days in vitro from postnatal day 0 *NR2B*$^{-}$ *GR/RG;nestin-Cre* (MADM-Green-KO) and *GR/RG;nestin-Cre* (MADM-WT) mice were used for calcium imaging. *NR2B*$^{+/-}$ and *NR2B*$^{-/-}$ cells originate from MADM-Green-KO mice whereas *NR2B*$^{+/+}$ cells originate from MADM-WT mice. Cells were loaded 30–60 min with 2 μM Fura-2, acetoxymethyl (AM) ester form (Invitrogen: Cat. 1221), and 0.02% Pluronic F-127 (Invitrogen: Cat. 3000MP) in conditioned hippocampal growth medium in a 37°C/5% CO_2 incubator. Fura-2 fluorescence, measured at 0.33 Hz, was elicited by excitation at 340 and 380 nm, detected at 510 nm, and imaged with a charge coupled device camera (C4742-95 Orca; Hamamatsu Photonics). The 340/380 ratio was quantified using regions of interest outlining the entire cell body. After background subtraction, ratios were converted to $[Ca^{2+}]_i$ based on an empirical fit of ratios obtained from a Fura-2 Ca^{2+} imaging calibration kit (Invitrogen: Cat. F-6774). Bath solution exchanges were performed via gravity-fed perfusion, with 50% volume exchange in <2 s. To induce NMDA-mediated responses, >30 sec of baseline recording in a Mg^{2+}-free

Tyrode's solution (containing 151 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and 0.5 μM TTX) preceded a 20 s application of the same Tyrode's solution with 100 μM NMDA (Tocris: Cat. 0114) and 10 μM Glycine (Tocris: Cat. 0219). To selectively block NR2B-containing NMDA receptors, we included 3 μM ifenprodil (IF, Sigma-RBI: Cat. I2892) in the Tyrode's solution before and during stimulation. To block all NMDA receptors we included 50 μM D-2-amino-5-phosphonopentanoate (D-AP5, Tocris: Cat. 0106) throughout the experiment. For each cell, the stimulation response was measured by taking the difference between the average of the first 15 s after stimulation onset and the average of the immediately preceding baseline 15 s before stimulation onset. In some experiments, after a wash in 5 mM K^+ Tyrode's, we stimulated cells with 90 mM K^+ to verify that MADM-labeled cells underwent normal Ca^{2+} elevation in response to activation of voltage-dependent Ca^{2+} channel.

Dendritic spine analysis

Using a 63x (1.3 NA) oil objective and 5x zoom, dendrite segments were imaged by 1-μm optical sectioning using confocal microscopy to visualize entire extent of dendrite process and to capture spine details in all focal planes. Segments were randomly selected from GFP+ *NR2B*$^{-/-}$ and *NR2B*$^{+/-}$ cells as follows: distal end segments of dentate gyrus granule cells, lateral and basal segments (50-μm from branch point) of CA1 pyramidal cells, and secondary segments of layer 4 spiny stellate cells in the barrel cortex. Images were uploaded into Imaris to visualize spines in 3D and to perform spine counts. Spine counts were per 10-μm segment in hippocampal cells and per 20-μm segment in barrel cortical cells. For each cell, >2 segments were quantified and averaged. All protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft. For each genotype and cell type, >20 cells were analyzed from >10 brains.

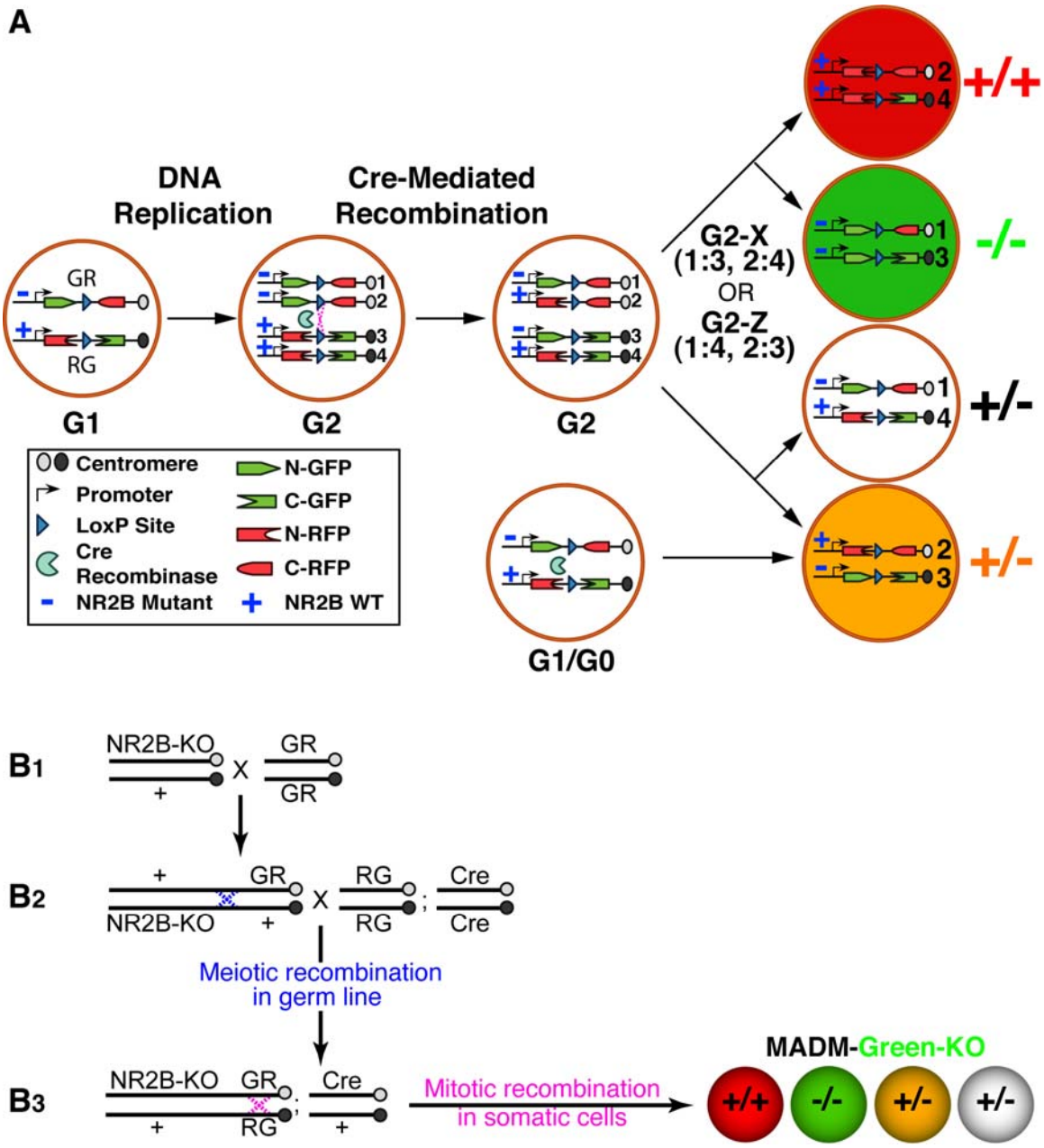

Figure S1. Schematic of the MADM strategy and crossing schemes

(A) Schematic of the MADM method illustrating Cre-mediated inter-chromosomal recombination that results in reconstitution of two fluorescent markers, GFP and RFP (dsRed2-Myc). If recombination occurs in G2 phase, chromatids can segregate to generate two differentially labeled cells with altered genotypes (G2-X segregation), or one doubly labeled cell and one unlabeled cell without changing the genotype (G2-Z

segregation). Recombination in G1 or postmitotic cells (G0) generates a doubly labeled cell without changing the genotype. This scheme generates MADM-Green-KO mice (Figure 1A); to generate MADM-Red-KO mice, simply place *NR2B* mutant allele on the RG chromosome instead of the GR chromosome.

(B) Crossing scheme to generate MADM *NR2B* knockout mice. (B_1) Mice carrying one MADM cassette (*GR*) are crossed to mice heterozygous for a null mutation of *NR2B*. (B_2) Trans-heterozygous progeny are then crossed to mice carrying the reciprocal MADM cassette (*RG*) and Cre transgene of choice. (B_3) Mice that inherit a chromosome that has undergone meiotic recombination between *GR* and *NR2B-KO* allele, as well as *RG* and *Cre,* can produce mutant cells labeled in green only (MADM-Green-KO; Figure 1A) through Cre/loxP-directed mitotic recombination in somatic cells. MADM-Red-KO can be generated by crossing mice heterozygous for a null mutation of *NR2B* with mice carrying *RG* in (B_1); the trans-heterozygous progeny can then be crossed to GR/GR; Cre/Cre in (B_2).

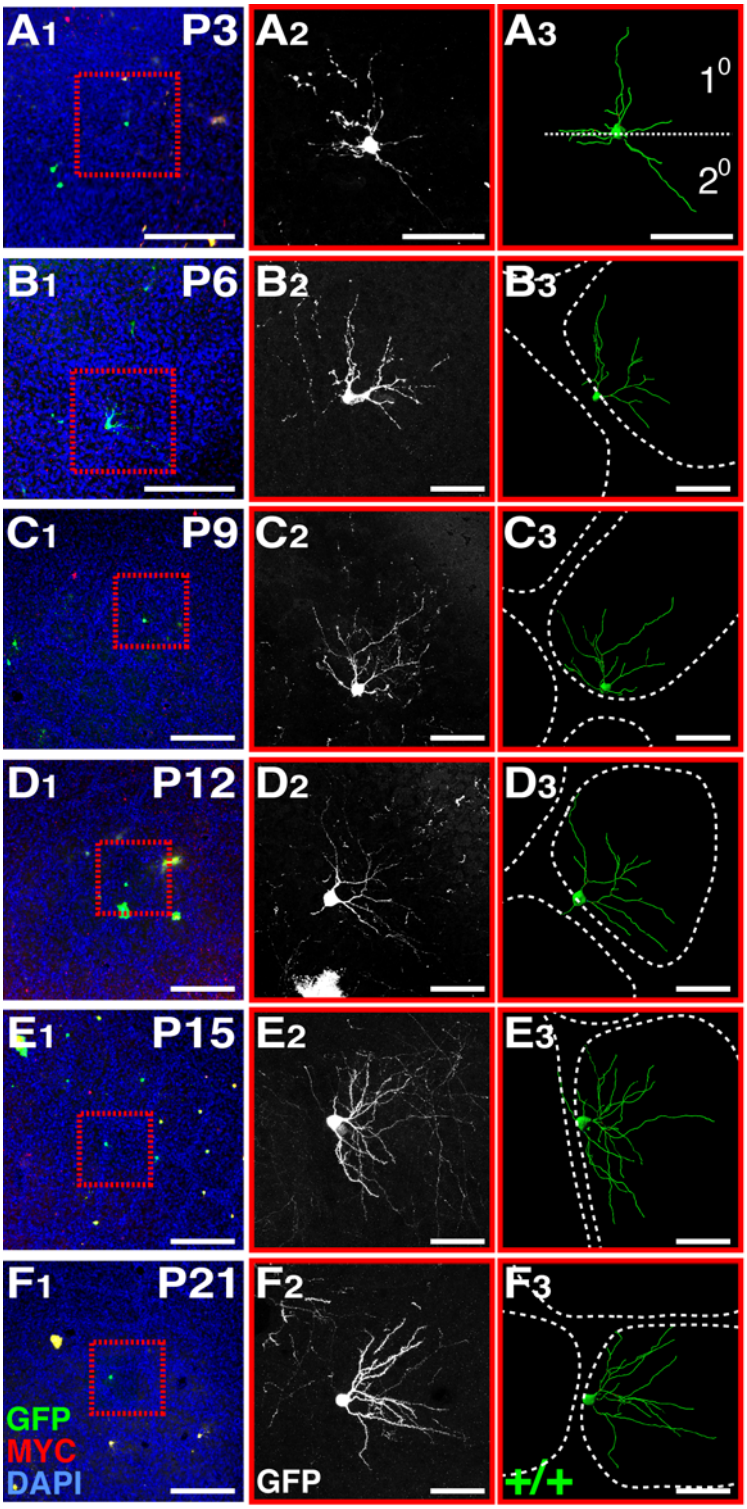

Figure S2. Dendrite development and distribution of *NR2B*$^{+/+}$ of layer 4 spiny stellate cells

Representative images and 3D reconstructions of sparse MADM labeled *NR2B*$^{+/+}$ bSCs at

postnatal days as indicated. Left panels are low magnification images showing labeled cells with DAPI staining. Middle panels are confocal images of MADM-labeled cells. Right panels are corresponding 3D tracings (superimposed over barrel boundaries for P6-P21). Scale bars, 100 μm for (A_1-F_1); 50 μm for the remainder. Qualitatively, dendrite distribution of *NR2B*$^{+/+}$ cells is indistinguishable from *NR2B*$^{+/-}$ cells (see Figures 5 and 6). These green-labeled *NR2B*$^{+/+}$ cells were generated by the MADM-Red-KO scheme (Figure 1A middle).

Supplemental References

Zong, H., Espinosa, J.S., Su, H.H., Muzumdar, M.D., and Luo, L. (2005). Mosaic analysis with double markers in mice. Cell *121*, 479-492.