Supporting Information

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SI Text

Expression Plasmids and Cell Culture. Full-length mouse ephrin-A3 and ephrin-A5 cDNA was ligated into pcDNA3 (Invitrogen) to generate pcDNA-ephrin-A3 and pcDNA-ephrin-A5. Mouse Ext1 cDNA (gift from Dr. Esko) was ligated into pcDNA3.1-mycHis (Invitrogen) to generate pcDNA-Ext1-mycHis. cDNAs encoding the ectodomains of ephrins and Eph receptors were ligated into a pcDNA3 (Invitrogen)-based vector that had been modified to fuse a FLAG tag to the 3′ end of the insert in-frame. The expressed ectodomains comprise the following regions: mouse ephrin-A1 (U26188, ^{1}Met–^{184}Ala); mouse ephrin-A2 (U14752, ^{1}Met–^{188}Ser); mouse ephrin-A3 (XM_892839, ^{1}Met–^{213}His); mouse ephrin-A5 (U90664, ^{1}Met–^{209}Arg); chicken ephrin-B1 (NM_205035, ^{1}Met-1–^{226}Lys); human ephrin-B2 (L38734, ^{1}Met–^{224}Glu); human EphA2 (M59371, 1Met–^{534}Asn); mouse EphA3 (M68513, 1Met–^{540}His); chicken EphA4 (D38174, ^{1}Met–^{547}Thr); chicken EphB2 (M62325, ^{1}Met–^{547}Lys). 293T cells were cultured in 10% FBS (FBS) in DMEM. Wild-type (CHO-K1) and CHO-*pgs*D-677 (3) cells were cultured in 10% FBS in αMEM. Primary neurons were prepared from the cortex of *Nestin-Cre;Ext1*$^{flox/flox}$ conditional knockout mice and their littermate controls at E15.5, and cultured as described (1).

Heparin-Sepharose Chromatography. FLAG-tagged recombinant ectodomains of ephrins and Eph receptors were transfected into 293T cells, and their culture supernatants were collected 3 days after transfection. After clarification, 0.8 ml of culture supernatants were supplemented with 0.05% Triton X-100 and incubated with 20 μl of heparin-Sepharose (Amersham) for 1 h at 4°C. After washing the gel three times with 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Triton X-100 (PBS-T), bound materials were eluted consecutively with PBS-T containing 0.2, 0.3, 0.4, 0.6, 0.8, or 1.2 M NaCl (final concentration). For each step, 45 μl of NaCl solution was added to heparin-Sepharose, incubated for 3 min on ice, and eluates were collected and analyzed by immunoblotting with monoclonal anti-FLAG antibody (clone M2, Sigma). Gels were then eluted once more before elution with the next NaCl solution. To analyze binding of full-length (cell surface-expressed) ephrin-A3 and ephrin-A5, 293T cells transfected with pcDNA-ephrin-A3 or pcDNA-ephrin-A5 were lysed 2 days after transfection with 1% Triton X-100 in PBS containing a protease inhibitor mixture (Sigma). Cell lysates were clarified by centrifugation and applied to heparin-Sepharose as described above. After washing with PBS-T containing 0.2 M NaCl (final concentration), bound materials were eluted with PBS-T containing 1.2 M NaCl (final concentration). The collected fractions were analyzed by immunoblotting with polyclonal anti-ephrin-A3 or anti-ephrin-A5 antibody (Invitrogen).

Heparan Sulfate-Binding Assay. Purified heparan sulfate (Sigma, H7640) was biotinylated with NHS-LC-biotin (Pierce) as described previously (2). Copper-coated 96 well plates (HisGrab Copper Coated, High Binding Capacity Plates, Pierce) were coated overnight at 4°C with 1 μg/ml of recombinant ephrin or Eph receptors ectodomains fused with human IgG Fc and a C-terminal hexahistidine tag (R&D Systems). After washing with PBS containing 0.05% Triton X-100 (washing buffer), biotinylated heparan sulfate in blocking buffer (Blocker Metal Chelate-Compatible Formulation, 1/1,000 dilution in PBS, Pierce) was added and incubated for 3 h at room temperature. Wells were then washed four times and incubated with horseradish peroxidase-conjugated streptavidin-biotin complex (Vector Laboratories) for 30 min at room temperature. Binding was detected by 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and the absorbance was measured at 405 nm.

Analysis of EphA2/EphA4 Phosphorylation. Wild-type CHO cells (CHO-K1) and the *pgs*D-677 heparan sulfate-deficient CHO cells (3) were plated on 6 cm dishes and used for this experiment on the next day, when cells reached semiconfluency. Cortical neurons from E15.5 *Nestin-Cre;Ext1*$^{flox/flox}$ embryos and control littermates were plated at 2×10^{6} cells/6 cm dish and used at 2 days *in vitro* (DIV). Both CHO cells and cortical neurons were incubated with 1 μg/ml of ephrin-A3–Fc, ephrin-A5–Fc (R&D Systems), or human IgG Fc (Cappel) for 15 min at 37°C and lysed with PBS containing 1% Triton X-100, 1 mM Na_3VO_4 and protease inhibitor mixture (lysis buffer). Clarified cell lysates were incubated for 2 h at 4°C with protein G-agarose coupled with rabbit polyclonal anti-EphA2 antibody (Chemicon) or with anti-EphA4 antibody (Invitrogen) for CHO cells and cortical neurons, respectively. After washing with lysis buffer, immunoprecipitates were eluted by boiling in SDS/PAGE sample buffer, and total and tyrosine-phosphorylated EphA2 or EphA4 were detected by immunoblotting with polyclonal anti-EphA2 or EphA4 antibody or monoclonal anti-phosphotyrosine antibody (clone PY-20, BD Bioscience), respectively. Phosphorylation levels were measured by densitometric analysis using Image-J software.

Conditional Knockout Mice. For the analysis of endogenous EphA4 phosphorylation in the adult hippocampus, conditional *Ext1* knockout mice were generated from *Ext1*^{flox} (1) and CaMKII-Cre2834 transgenic mice (4). This Cre line drives neuron-specific recombination in the postnatal forebrain after postnatal day 17. To ascertain the pattern of Cre-mediated recombination, CaMKII-Cre mice were crossed with *R26R* reporter mice (5), and hippocampal sections were double-stained with anti-β-galactosidase antibody (Promega) and TO-PRO-3 (Invitrogen). To examine the ablation of heparan sulfate expression, hippocampal sections from *CaMKII-Cre;Ext1*$^{flox/flox}$ mice were stained with HepSS-1 anti-heparan sulfate antibody (Seikagaku America) and TO-PRO-3. Hippocampi were dissected from adult *CaMKII-Cre;Ext1*$^{flox/flox}$ and *Ext1*$^{flox/flox}$ (control) littermates and lysed with 1% Triton X-100. EphA4 phosphorylation was analyzed as described above in these hippocampal lysates.

Cell Rounding Assay. Morphological changes induced by ephrin-A treatment (6) were examined using wild-type and *pgs*D-677 CHO cells cultured on 13 mm glass coverslips coated with 0.5 mg/ml of polyethyleneimine (Sigma). Three hours before ephrin-A treatment, cells were replenished with low-FBS (0.5%) culture medium with or without 1 unit/ml of heparitinase (Sigma). Ephrin-A3-Fc, ephrin-A5-Fc, or human Fc was added to the medium at the final concentration of 1 μg/ml and incubated for 45 min at 37°C. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized in 0.2% Triton X-100 for 10 min at 4°C, followed by staining with rhodamine-phalloidin. For Ext1 restoration experiments, *pgs*D-677 cells were transfected with pcDNA-Ext1-mycHis and stimulated with ephrin-A–Fc proteins 36 h after transfection. Successfully transfected cells were identified by immunostaining with polyclonal anti-myc antibody (Sigma) and Cy2-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch). For quantification of cell

rounding, 10 optical fields under x60 magnification were randomly chosen in each coverslip, and cells were scored as rounded or not rounded by a blinded observer. Rounded cells were defined as those that lost polygonal morphology and lacked discernible stress fibers or focal contacts. A total of 50–60 cells were scored from each coverslip and the percentage of rounded cells relative to total cells was calculated from 4 coverslips per condition.

Growth Cone Collapse Assay. Cortical neurons were plated on coverslips coated with polyethyleneimine and laminin at 4×10^4 cells. At 2 DIV, neurons were treated with 1 μg/ml of ephrin-A3–Fc, ephrin-A5–Fc, or control Fc for 30 min at 37°C and then fixed in 4% paraformaldehyde at room temperature. After permeabilization, neurons were stained with rhodamine-phalloidin and fluorescent images of growth cones were photographed on a confocal microscopy. For quantitative analysis, growth cones with thin or tapered tips without discernible lamellipodia or filopodia were scored as collapsed (7). Partially shrunk growth cones with lamellipodia or filopodia were not scored as collapsed. For quantitative analysis, 20–30 optical fields under x60 magnification were randomly chosen in each coverslip, and the number of collapsed growth cones and the number of total growth cones were determined by a blinded observer. Four coverslips per condition were scored. For Ext1 restoration experiments, *Ext1* null neurons from *Nestin-Cre;Ext1*$^{flox/flox}$ embryos were transfected with pcDNA-Ext1-mycHis. Successfully transfected neurons were identified by double-labeling with anti-myc antibody, as described for the CHO cell rounding experiments. In this case, only the growth cones derived from Myc-immunoreactive cell bodies (i.e., expressing transfected Ext1) were scored. Statistical significance was evaluated by ANOVA.

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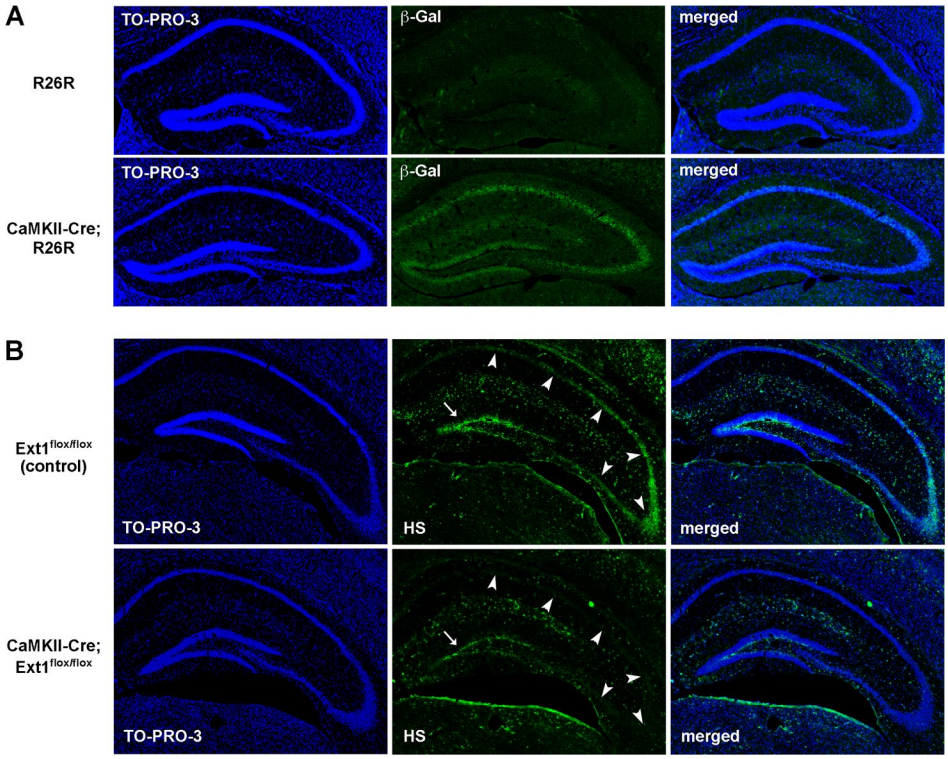

Fig. S1. Characterization of *CaMKII-Cre;Ext1*$^{flox/flox}$ conditional knockout mice. (A) Analysis of recombination patterns using *R26R* reporter mice. Hippocampal sections from adult *CaMKII-Cre;R26R* and control *R26R* mice were double-stained with TO-PRO-3 (for nuclei) and anti-β-galactosidase (for Cre-mediated recombination). (B) Analysis of the ablation of heparan sulfate expression. Hippocampal sections from adult *CaMKII-Cre;Ext1*$^{flox/flox}$ and control (Ext1$^{flox/flox}$) mice were double-stained with TO-PRO-3 and the HepSS-1 anti-heparan sulfate monoclonal antibody (HS). Note that heparan sulfate expression in the hippocampus is largely abolished in conditional mutant mice (arrowheads). Remaining immunoreactivities, including those in the hilus of the dentate gyrus (arrow) are derived mainly from glial and non-neural cells, in which CaMKII-Cre–mediated recombination does not occur.