Supplementary Information:

Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning

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Supplementary Figure 1

Developmental expression of EB3 in MF axons is visualized by X-gal staining of coronal sections for the EB3-β-gal fusion protein (blue) in EB3lacZ mice from birth (PW0) to six weeks age (PW6). Structure of the hippocampus is visualized by eosin counterstaining (red). Arrowheads indicate the localization of EB3 protein in IPB axons. Scale bars, 300 µm (left panels) and 150 µm (right panels).
Developmental expression of EphB1 in the hippocampus is visualized by X-gal staining of coronal sections for cytoplasmic, unconjugated β-gal (blue) in EphB1\textsuperscript{lacZ} mice from birth (PW0) to six weeks age (PW6). Structure of the hippocampus is visualized by eosin counterstaining (red). Expression of EphB1 is restricted to the CA3 pyramidal cell layer (arrowheads) and the proliferative subgranular zone (SGZ) of the DG. Scale bars, 300 μm (left panels) and 150 μm (right panels).
Developmental expression of EphB2 in the hippocampus is visualized by X-gal staining of coronal sections for the EphB2-β-gal fusion protein (blue) in EphB2\textsuperscript{lacZ} mice from birth (PW0) to six weeks age (PW6). Structure of the hippocampus is visualized by eosin counterstaining (red). In the hippocampus EphB2 is initially strongly expressed in the CA3 pyramidal cell layer during early postnatal stages and then becomes more broadly expressed as the animal ages, including the CA3 area where IPB axons traverse (arrowheads). Scale bars, 300 μm (left panels) and 150 μm (right panels).
Expression of EphB1 and EphA4 in the DG-CA3 region of the adult hippocampus (10 weeks old) by IF with anti-β-gal antibodies. EphB1 is specifically expressed in CA3 pyramidal cells and SGZ, but is not localized in the granule cells or their MF axons which are visualized with anti-calbindin antibodies. EphA4 is expressed in both CA3 pyramidal neurons and DG granule cells and some labeling can be detected in MF axons. Scale bar, 300 µm.
Time course of MF axon pruning in $EB3^{+/−}$ null mice during postnatal development. In WT, anti-calbindin IF shows a longer IPB at PW3 that becomes pruned at later stages (distance between arrowheads). IPB shortening is not observed in $EB3^{+/−}$ mice as they age.
Defective MF axon shortening in $EB3^{lacZ/lacZ}$ adult mice at 8–10 weeks age.  

**a**, Anti-calbindin IF shows that the IPB in $EB3^{lacZ/lacZ}$ mice is longer than in WT mice.  

**b**, Timm stain also reveals a longer IPB in $EB3^{lacZ/lacZ}$ mice compare to that of WTs.
Characterization of \( EB3^{3F} \) and \( EB3^{5F} \) mice. **a**, Genotyping of \( EB3^{5F} \) mutants with Southern blot. The initial primary targeted \( EB3^{neo^{5F}} \) allele (left panel) and the \( EB3^{5F} \) allele after Cre mediated excision of the \( PGK-neo \) cassette to generate the secondary targeted allele (right panel) are indicated. NsiI is used to digest the genomic DNAs and a 5’ external probe as indicated in Fig. 3a was used in the hybridization. The mutant band in the secondary targeted allele is smaller than that in the primary targeted allele indicating that the \( PGK-neo \) cassette was deleted successfully. **b**, Analysis of EB3 phosphorylation in WT and \( EB3^{5F/5F} \) mutant primary dentate granule cells. Primary dentate granule cell neurons from WT and \( EB3^{5F/5F} \) mutants were exposed to pre-clustered EphB1-Fc, fixed, and then labeled for IF analysis with anti-calbindin (blue), anti-ephrin-B3 (red), and anti-phospho-ephrin-B (green) antibodies. Arrowheads in the high magnification image of WT cultures indicates EB3 protein becomes clustered and tyrosine phosphorylated (yellow). While granule cell neurons from \( EB3^{5F/5F} \) mutants show expression and clustering of the EB3-5F protein in response to pre-clustered EphB1-Fc, the signal for phospho-ephrin-B is weak and likely reflects co-expression of lower levels of endogenous ephrin-B1 and/or ephrin-B2. Scale bars, 20 \( \mu m \) (left panels) and 10\( \mu m \) (right panels).
Time course of hippocampal granule cell axon pruning *in vitro* that is initiated by co-culture with Cos-1 cells. Scale bar, 20 µm.
Reverse signaling induces neurite retraction in live transfected NG108 cells expressing EB3. 

**a**, Co-transfected f-EGFP identifies long neurites in NG108 cells differentiated for 36 hours with dibutyrylCAMP. In the control Fc only stimulations, expression of EB3-WT, EB3-3F, and EB3-5F proteins (red) are readily detected in the neurites. Exposure to pre-clustered EphB1-Fc for 20 min results in tyrosine phosphorylation of EB3-WT protein as visualized with anti-phospho-ephrin-B antibodies (indicated by arrows in the bottom panels of EB3-WT group), but not in f-EGFP control transfected cells or in those expressing EB3-3F or EB3-5F mutant proteins. 

**b**, Live cell images of NG108 cells transfected with f-EGFP and EB3-WT show rapid neurite retraction but not in f-EGFP only transfected control cells following exposure to pre-clustered EphB1-Fc. 

**c**, Quantitation of the data from 110 imaged neurites. The percentage of neurites exhibiting no retraction, partial retraction, and full retraction is shown following EphB1-Fc stimulations. Full neurite retraction is defined as a reduction in neurite length to at least 50% of the starting length. Scale bars: 20 µm.
Developmental expression of Grb4 and EB3 in hippocampal granule cells. Primary hippocampal neurons were dissected from PW1, PW2, PW3, PW4 wild-type mice and cultured overnight prior to labeling with anti-Grb4, anti-EB3, and anti-Calbindin antibodies. Expression of Grb4 and EB3 are undetectable in PW1 calbindin-positive neurons, but are readily detected in PW2 to PW4 neurons. Scale bar, 10 µm.
Cartoon depicting the mechanism by which EB3 transduces reverse signals to mediate axon retraction/pruning. Upon encountering EphB molecules expressed on the surface of target cells, EB3 becomes clustered and tyrosine phosphorylated. This results in Grb4 binding to EB3 via its SH2 domain and recruitment of both Dock180 and PAK via its second SH3 domain to increase Rac-GTP levels (by Dock180) and mediate signaling downstream of active Rac (by PAK). It is also likely that Grb4 recruitment to tyrosine phosphorylated ephrins modulates the subcellular localization of Grb4, its associated SH3 binding partners, and Rac.
Axon pruning of primary hippocampal neurons from WT and $EB3^{-/-}$ null mice initiated by either EphB2-Fc (5 µg/ml) or Sema3A-Fc (0.4 µg/ml) treatment. n = 21–37, * P < 0.0001. Mean ± s.e.m. Scale bar, 20 µm.
Expression of dominant-negative Grb4-SH3Mut-DsRed (red fluorescence) in hippocampal neurons had no effect on the axon pruning response of live cells initiated by Sema3A-Fc exposure for 36 h. Quantification showed that neurites from Grb4-DsRed transfected cells exhibited a reduction in length by 76.8% in response to Sema3A-Fc compared to Fc controls, while neurites from Grb4-SH3Mut-DsRed transfected cells showed 71.3% reduction in response to Sema3A-Fc compared to their Fc control (n = 31–38 neurons per group, P < 0.0001). Scale bar, 20 μm.
Supplementary Methods

Generation of EB3 tyrosine to phenylalanine cytoplasmic domain point mutant mice

The EB3 targeting vectors used incorporated loxP site-specific recombination sequences and were designed to generate two-step mutations from a single gene targeting events in murine embryonic stem cells (Fig. 3a). The initial insertions, which are called EB3neo<sup>3F</sup> or EB3neo<sup>5F</sup>, inserts into the EB3 fourth intron (171 bp upstream of the splice acceptor for the fifth exon) a neomycin resistance (neo) cassette that was flanked with loxP sequences. As designed, this replaces wild-type EB3 exon 5 encoding amino acids 205–340 including the cytoplasmic domain with engineered mutations 3F or 5F in which tyrosine codons at positions 311, 318, 323 (for 3F) or 311, 318, 323, 337, and 338 (for 5F) are changed to phenylalanines. The 5’ arm of the targeting vector contained 7.2 kb of homology, the 3’ arm 7.5, that were flanked by either a diphtheria toxin (DT-A in Fig. 3a) or thymidine kinase (TK in Fig. 3a) expression cassette, respectively, for negative selection. The targeting vectors were electroporated into the ES cell line R1, selected with G418 and ganciclovir, and individual colonies were expanded and screened by Southern blot to detect for homologous recombination. Chimeric mice were made from two lines of each mutation and germline transmission was obtained, leading to recovery of the initial targeted EB3neo<sup>3F</sup> or EB3neo<sup>5F</sup> lines. Because this strategy was based on our initial gene targeting of EB3 that showed insertion of a neo cassette into the fourth intron disrupts the gene and leads to the null phenotype of a hopping locomotion, the initial EB3neo<sup>3F</sup> or EB3neo<sup>5F</sup> lines when made homozygous also resulted in a loss-of-function hopping phenotype as expected. These mice were then crossed to a germline Cre-expressing transgenic mouse to remove the loxP-flanked neo cassette and convert the locus to generate the intended EB3<sup>3F</sup> and EB3<sup>5F</sup> alleles that express the intended EB3 protein with select point mutations in the cytoplasmic domain. Animals were genotyped by Southern blot with the
probes as indicated (Fig. 3a and Supplementary Fig. 7a) and PCR with forward primer TCCCATCTTCAGGTCCCCGAG and reverse primer TGGAAATCCAGGTGTCCGGCC for wild-type (380 bp), and forward primer GGTGCTTCTGCGAGTGG and reverse primer GCATACTTATACGAAAGTTATATATAAGGG for mutant (530 bp). The 3F and 5F mutations in the EB3 exon 5 were further confirmed by sequencing PCR amplified products from EB3<sup>3F/3F</sup> and EB3<sup>5F/5F</sup> mice.

**DNA Constructs**

To generate the vectors to co-express EB3 constructs and f-EGFP, the f-EGFP coding sequences were amplified from pEGFP-F vector (BD Bioscience) and ligated in the StuI and AgeI sites in pCMS-EGFP. Wild-type EB3 and EB3 mutant coding sequences were generated from pEXPRmELF3 (provided by Andrew Bergemann) and ligated in the NheI and XhoI sites of pCMS-f-EGFP. For DsRed expressing constructs, Grb4 or Grb4-SH3Mut (Kpn1/BamH1) and Pak1DN (EcoR1/BamH1) fragments were produced by PCR and ligated into pDsRed2-N1 (Clonetech).

**Primary cell culture of hippocampal neurons**

Dentate granule cell neurons from 2 week old mice were dissociated from micro-dissected DGs that were cut at 500 µm using a MacIlwain tissue chopper. The triturated dentate granule cells (1×10<sup>4</sup> cells per well) were grown on glass cover-slips (Fisher) coated with 10 µM polylysine overnight in 24 well dishes. Then the granule culture was grown in a medium of Neurobasal media (GIBCO) supplemented with B27 and 2 mM glutamine for 5–7 days. For transfections, postnatal day 1 (P1) mice were used and whole hippocampi were dissected for neuronal culture (1×10<sup>5</sup> cells per well). Transfections were performed with lipofectamine 2000...
at day 4 and CellTracker probe (Molecular Probe) treated Cos-1 cells (5×10^4 cells per well) were added in the neuron culture 48 h after transfection.

**Live cell analysis**

NG108 cells were transfected with f-EGFP or DsRed constructs using FuGene 6 (Roche) in 24 well dishes and were induced to extend neurites by the addition of 1 mM dibutyryl cAMP. Images were collected 48 h later using a digital camera which was connected with an Olympus IX70 fluorescence microscope. Primary hippocampal neurons were grown on glass coverslips. Images were also collected with digital camera before and after co-cultured with Cos-1 cells. The cells were then fixed and then processed for IF according to standard procedures. Primary antibodies to ephrin-B3 (R&D Systems), phospho-tyrosine (Upstate Biotechnology), phospho-ephrin-B (Cell Signaling), tau (Chemicon), calbindin (Swant) and Grb4 (Upstate Biotechnology) were detected with fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch) and images were collected using a Zeiss LSM510 confocal microscope.

For time-lapse live cell imaging, f-EGFP/EB3 transfected NG108 cells were grown in 35-mm coverslip culture dishes (MatTek) and induced to extend neurites by the addition of dibutyryl cAMP for 36 hours. Dishes were equilibrated on a 37 °C heated microscope stage incubator (Carl Zeiss microimaging) with an objective warmer and transfected cells/neurites were identified by f-EGFP expression before preclustered EphB-Fc reagents were added to a final concentration of 5 µg/ml. Images of neurites at least 3 times the cell diameter were acquired for 1 hour using a Zeiss LSM510 confocal microscope and retraction was classified as full if the neurite retracted at least 50% of its original length, partial if retraction was less than 50%, and none if no retraction was observed.
**Cell stimulations and immunoprecipitation**

NG108 cells were transfected with Flag-EB3 or Flag-EB3-3F and treated with G418 (1 mg/ml) for 7 days to obtain stable expressing cell lines. After serum starving for 6 h, the NG108 cells were stimulated with 5 µg/ml pre-clustered EphB2-Fc (R&D Systems) for 30 min and then lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM MgCl2, 1% NP-40, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitor mixture; Roche Molecular Biochemicals). Following immunoprecipitation with indicated antibodies for 1 h and incubation with protein G beads overnight at 4 °C, bound proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted with indicated antibodies (anti-Flag and anti-Flag-HRP from Sigma, anti-dock180 from Santa Cruz).

**Rac and Cdc42 activity assays**

Serum-starved Flag-EB3 and Flag-EB3-3F stably transfected NG108 cells were stimulated with 5 µg/ml pre-clustered EphB2-Fc (R&D Systems) for indicated time-points, lysed with ice-cold cell lysis buffer, diluted in P21-binding domain (PBD) binding buffer (25 mM Tris-HCl, pH 7.5, 40 mM NaCl, 30 mM MgCl2, 1% NP-40, 1 mM DTT, 1 mM PMSF and protease inhibitor mixture), and then incubated with 10 µg of PAK-GST protein beads (Cytoskeleton) for 1 h at 4 °C. Beads were washed three times with PBD binding buffer, bound proteins separated by SDS-PAGE, transferred, and immunoblotted with antibodies to Rac1 (Upstate) and Cdc42 (Santa Cruz).