Supplementary Information

Supplementary Methods

Preparation of cortical tissue for PCR

The somatosensory cortex prepared from E14.5, P8 and P37 mice was collected in Trizol reagent (Invitrogen, Germany) and RNA was purified according to the manufacturer´s instructions. After DNase treatment (Thermo scientific, USA), limited reverse transcription was performed using the RevertAidTM H Minus M-MuLV Reverse Transkriptase (Thermo scientific, USA). PCR with sequence specific primer for *Efna5* and *β-actin* (*Actb*) was performed (*β-Actin* forw AGA GGG AAA TCG TGC G, *β-Actin* rev CAA TAG TGA TGA CCT GGC CGT, *Efna5* forw ATG TTG CAC GTG GAG ATG TTG AC, *Efna5* rev GCT ATA ATG TCA AAA GCA TCG CC).

DAB TUNEL based apoptosis detection assay

E14.5 cortical cells were either grown on ephrin A5-Fc-coated or Fc-control protein-coated coverslips in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.4 mM L-glutamine at 37°C and 5% CO_2 in a humid atmosphere. Coating of coverslips with recombinant 8 µg/ml ephrin A5-Fc or Fc was performed according to Zimmer et al. (2007). Cells were fixed after 5 hours and a DAB TUNEL based apoptosis detection assay (R&D systems, USA) was performed according to the manufacturer´s instructions. The percentage of DAB-stained cells was determined.

Histochemistry

For primary antibodies we applied: rabbit anti Aspm (gift from Prof. W.Huttner, MPI Dresden, Germany) 1:1000; rat anti BrdU (abcam, UK) 1:300, rabbit anti Calbindin (Swant, Switzerland) 1:1000, rabbit anti CDP (Cux2, Santa Cruz Biotechnology, USA) 1:200, rabbit anti EphA4 (Santa Cruz Biotechnology, USA) 1:100, rabbit anti Ki67 (Leica, Germany) 1:200, rabbit anti L1 (DSHB, USA) 1:100, mouse anti nestin (Millipore, USA) 1:500, mouse anti NeuN (Millipore, USA) 1:100, mouse anti Otx 1,2 (Chemicon, USA) 1:300, mouse anti Pax6 (Millipore, USA) 1:100, rabbit anti pTyr (PY350, Santa Cruz Biotechnology, USA) 1:50, rabbit anti Tbr1 (Abcam, UK) 1:400, rabbit anti Tbr2 (Abcam, UK) 1:500, and rabbit anti βIII-tubulin (Sigma, Germany) 1:300. Secondary antibodies: Cy2-conjugated anti-mouse IgG (Jackson,

USA) 1:500, Cy2-conjugated anti-rabbit IgG (Jackson, USA) 1:500, Cy3-conjugated anti rabbit IgG (Jackson, USA) 1:500 and Cy5-conjugated anti-mouse IgG (molecular probes, USA) 1:1000. For the Tbr2 immunostaining of organotypic slice cultures all washing steps were performed in PBS/0.2% Triton-X-100 for five times 20 minutes under orbital shaking (50 rpm). Sections were fixed in 4% PFA in PBS for 1.5 hours and blocked for 2 hours in 10% normal goat serum, 4% bovine serum albumine in PBS/0.2% Triton-X-100 prior to primary antibody treatment overnight and secondary antibody for 5 hours.

Single cell RT-PCR and RT-PCR of thalamic axons

Individual cells and thalamic axons were manually isolated under visual control using a micromanipulator and fire-polished borosilicate glass capillaries (outside-Ø 1 mm, wall thickness 0.13-0.22 mm; tip diameter 40 µm, Hilgenberg or Science products, Germany). Each cell was isolated using a fresh capillary and washed twice in fresh buffer (HBSS, free of Ca^{2+} and Mg^{2+}; 1% FBS) before transferred to a PCR vial containing 4.5 µl lysis & first strand buffer (50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM $MgCl_2$; 1 mM DTT; 0.5% (v/v) Igepal CA-630; 100 µg/ml acetylated BSA (Sigma-Aldrich, Germany); 10 µM each of dATP, dCTP, dGTP und dTTP; 3.5 nM SR-T_{24}-Primer (5'-GTTAACTCGAGAATTCT$_{24}$-3'); 0.04 U/µl *RiboLock™* RNase inhibitor (Fermentas, Germany); 0.03 U/µl *SuperaseIn™* RNase inhibitor (Ambion, USA) followed by subsequent freezing in liquid nitrogen. The protocol for global cDNA synthesis we applied here was adopted from Iscove et al. (2002) with modifications, including a limited reverse transcription prior to exponential amplification, leading to size-restricted and 3´enriched cDNA fragments. Qualitative evaluation of amplified cDNAs from single-cells and thalamic axons was done by PCR amplification using specific primers for relevant transcripts and *housekeeping* genes (*ActB* forward CAGCATTGCTTCTGTGTAAATTATG, *ActB* reverse GCACTTTTATTGGTCTCAAGTCAGT, *Pax6* forward CGGATCTGTGTTGCTCATGT, *Pax6* reverse CAACCTTTGGAAAACCAACA, *Tbr2* forward CCTGGTGGTGTTTTGTTGTG, *Tbr2* reverse AATCCAGCACCTTGAACGAC, *HuD (ELAVL4)* forward TGCACATTGAAGAGGCAAAC , *HuD (ELAVL4)* reverse TCCAAAAACCGAAAAGAGGA, *EfnA5* forward CTTTTGAAAATCGCCTCCAC, *EfnA5* reverse AGACAGACCTGCCCATTCAC, *EphA4* forward AAATCAAGCCGTTTCACCAC, *EphA4* reverse CGTCCCCTTCACAGATGAAT). As negative controls we used reaction products of "picked" controls, whereby the whole manual cell isolation procedure was performed without taking up a cell. As positive control, we used

cDNA libraries generated from single cell equivalent dilutions (10 pg) of E14.5/E16.5 RNA isolated from whole brain tissue to validate the functionality of the respective primer in the limited 3´cDNA libraries.

Imaging and detailed description of analysis

Fluorescent pictures were captured with a confocal laser scanning microscope TCS SP5 (Leica Microsystems, Germany) or Axio Cellobserver Z1 (Zeiss, Germany). Digital photomicrographs of DAB-staining and *in situ* hybridizations were taken using a light transmission microscope BX40 (Olympus, Germany) and a digital camera DP70 (Olympus, Germany). Photoshop CS5 was applied for editing images, while ImageJ software was used for cell number counting and measurements of the radial extension of cortical structures. Analysis was performed blindly. For quantification of the relative radial extension of cortical layers, coronal sections of the somatosensory cortex (Bregma: 0, interaural: 3.8 mm) were used. Immunohistochemistry against Cux2/Otx 1,2 in combination with DAPI nuclear labeling served to clearly distinguish between the infragranular layers 5/6 and the superficial layers 1, 2-4.

For quantification of cell staining in embryonic brains, coronal sections of the dorso-lateral cortex from sections with evident ganglionic eminences in the basal telencephalon were analyzed. For Tbr1, the thickness of the cortical plate (CP) was measured in comparison to the radial extension of the cortex. Moreover, we counted the number of Tbr1 positive cells normalized to a defined area. The division angle of apical progenitors at E13.5 was determined using Aspm-staining labeling cell poles in combination with DAPI nuclear staining illustrating the division plane. The angles between the division plane and the lateral ventricle were determined and classified into three groups. Angles within 60-90° were defined as vertical, 30-60° as oblique and 0-30°C as horizontal divisions. For Ki67, cell numbers in the transient, proliferative layers (VZ and SVZ) were counted and the proportional distribution was calculated.

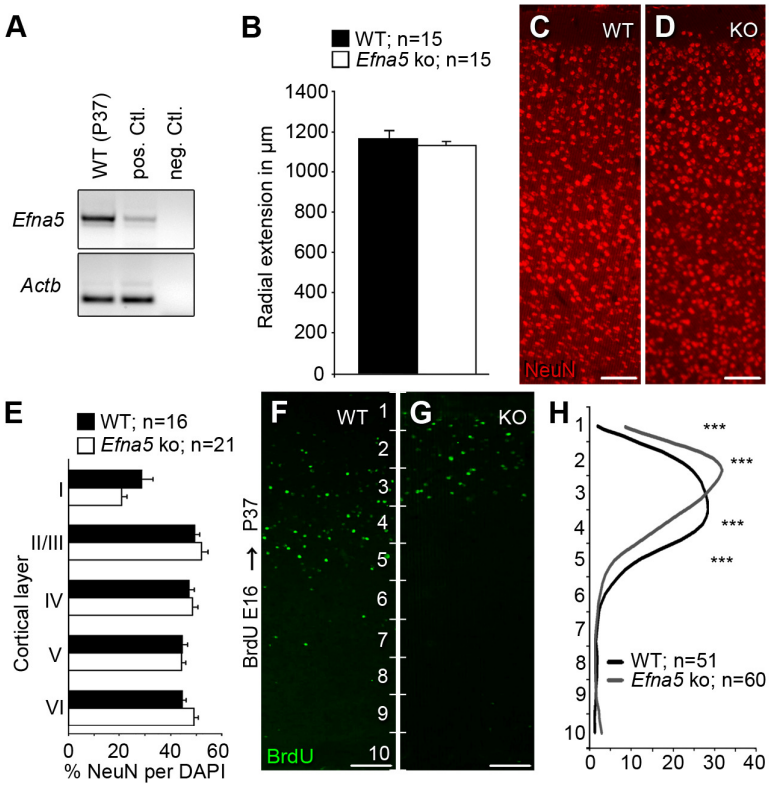
For quantification of cortical progenitors at E16.5 and E18.5, a radial section of the dorso-lateral cortex was taken, divided horizontally in 20 equal segments and cells per segments were analyzed. For the analysis at E13.5, we divided the cortical section into 10 segments.

For analyzing the *Insm1*-signal, coronal sections of the dorso-lateral cortex were taken, and grey values were measured with ImageJ. Thereby, the darkest position was defined as 100% and the brightest position as 0%. The grey values were calculated relatively in percentage.

For the analysis of cell pairs formed in co-cultures with thalamic explants, only pairs located clearly within the axons were taken into consideration. The number of cell tracker green-positive pairs was counted, which consisted either of two nestin or two βIII-tubulin labeled cells or mixed pairs. The proportional incidents are illustrated in percentages.

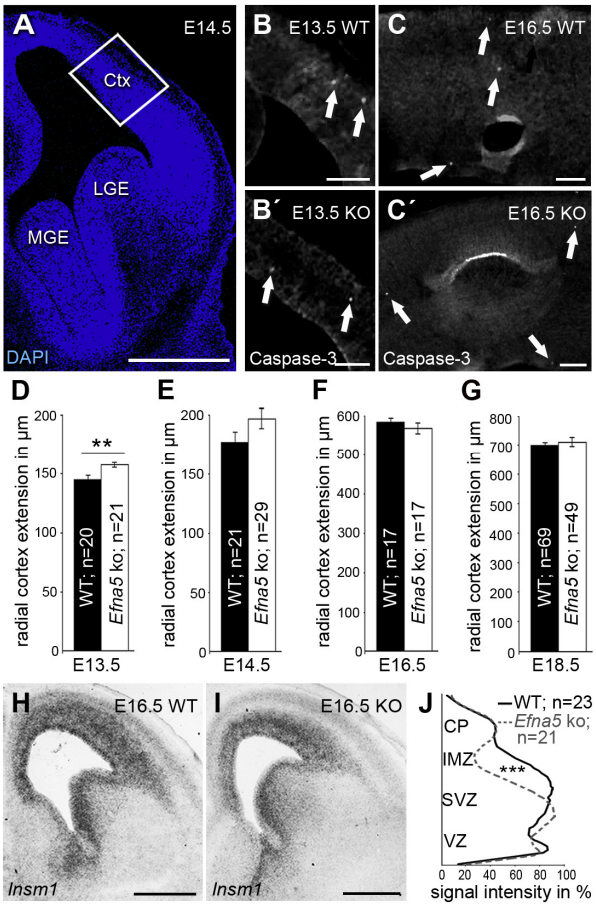
For analyzing the number of Tbr2 positive cells in cortices of organotypic slices treated with ephrin A5-Fc- or Fc-coated beads, cells per section within the width of the beads were counted and normalized with flanking lateral and medial regions. The mean for all samples for Fc-protein- and ephrin A5-Fc-coated beads was calculated and the change in percentage is illustrated. The number "n" refers to the number of analyzed cells (single cell RT-PCR), cell pairs (pair cell assay and co-culture of thalamic explants with cortical single cells), explants or sections of at least three independent experiments. *Student´s t-test*, *Chi-Square test, One-way* and *Two-way ANOVA* as well as *Bonferroni test* were used for statistical analysis (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Supplementary Figure Legends



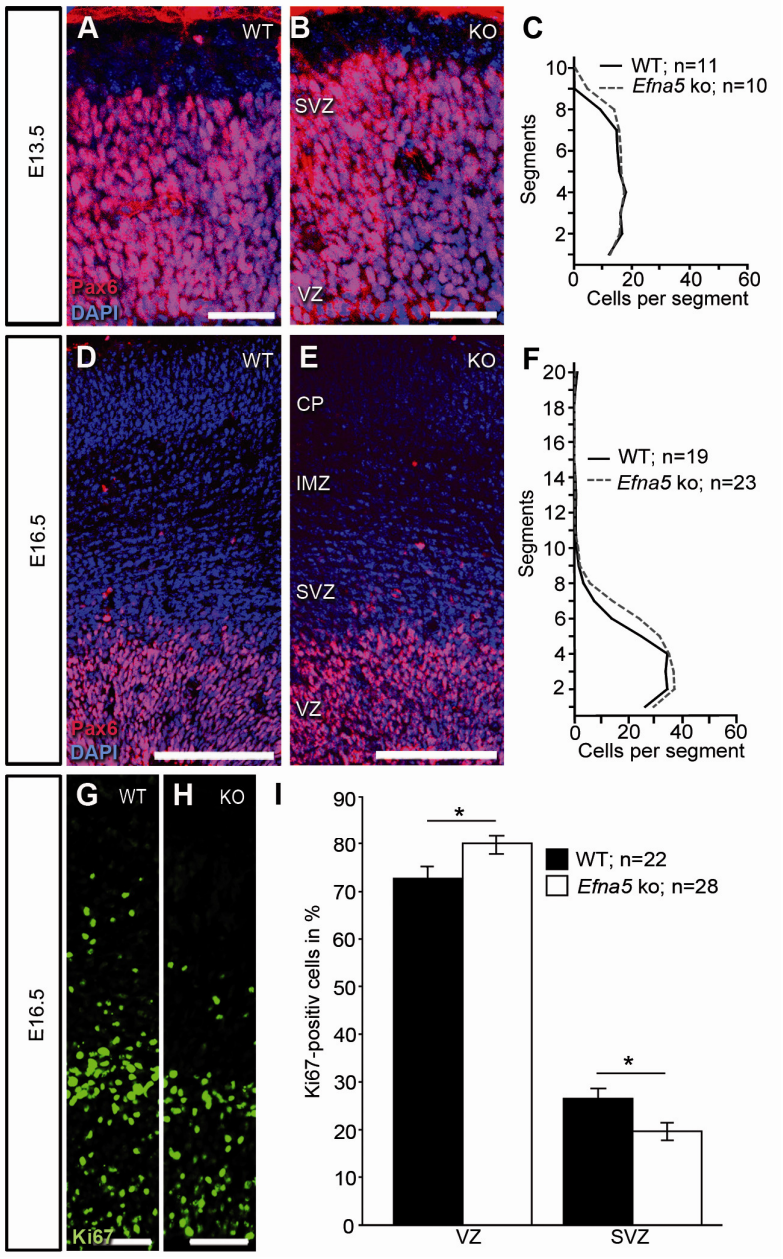
Supplementary Figure 1: Additional analysis of the adult *Efna5* and wildtype cortex.

A, Ephrin A5 (*EfnA5*) and β-actin (*ActB*) PCR of wildtype cortical tissue at P37. **B**, Quantitative analysis of the radial extension (from layer 6 to the pia) of the adult cortex at P37 reveals no differences between wildtype and *Efna5* knockout mice. **C-E**, Analysis of the neuronal density in wildtype (**C**) and *Efna5* mutant cortices (**D**) with NeuN (Rbfox3) immunohistochemistry. The percentage of NeuN (Rbfox3)-stained neurons per total cell number labeled with DAPI (not shown) was determined for the different cortical layers at P37 resulting in no significant differences neither by two-way *ANOVA* (n.s., p=0.93) nor by *Bonferroni Test* for the different layers (3 brains per genotype) (**E**). **F-H**, BrdU birthdating experiments were performed to follow the fate of late born neurons at E16.5. The distribution of BrdU-labeled layer 2-4 cells in the adult wildtype (**F**) and *Efna5* knockout (**G**) somatosensory cortex at P37 revealed a shift towards the pia and a reduction in the radial extension of BrdU labeled cells in the knockouts (**H**; *Bonferronie Test*; ***, p<0.001 for segment 1, 2, 4, 5). Scale bars: 100 µm in **C**, **D**, **F**, **G**.



Supplementary Figure 2: Additional analysis of the embryonic *Efna5* and wildtype cortex

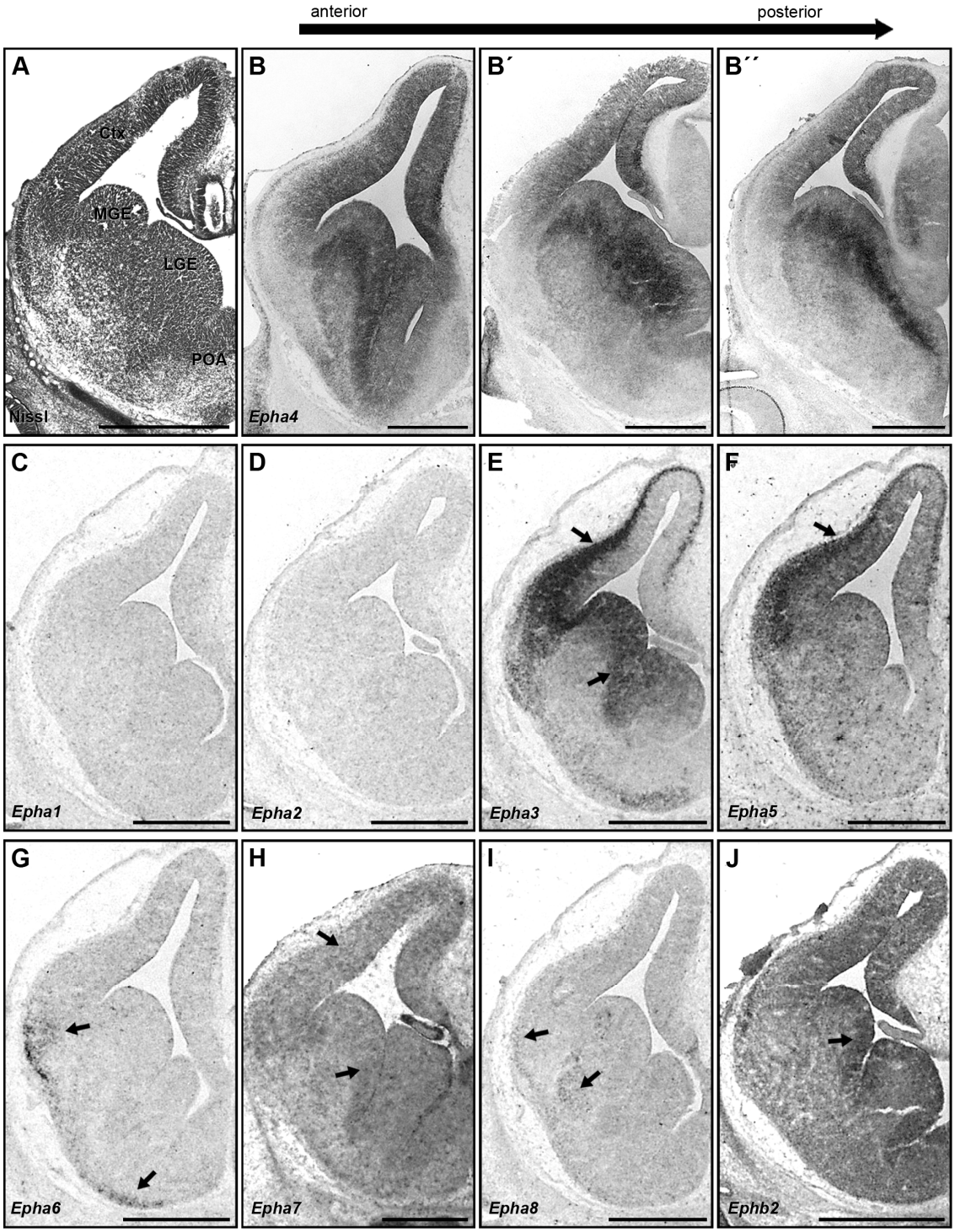
A, The white rectangle in the DAPI stained E14.5 coronal section illustrates the dorso-lateral part of the neocortex used for analysis at embryonic stages. **B-C´**, Immunostaining against Caspase-3 in coronal sections of E13.5 and E16.5 wildtype (**B**, **C**) and *Efna5* deficient embryos (**B´**, **C´**) revealed no apparent differences. **D-G**, The radial extension of the dorso-lateral cortex was measured in wildtype and *Efna5* deficient embryos at E13.5 (**D**; 2 brains per genotype), E14.5 (**E**; 3 brains per genotype), E16.5 (**F**; 2 brains per genotype) and E18.5 (**G**; 4 brains for wildtype and 2 brains for *Efna5* deficient embryos). At E13.5 the radial extension of *Efna5* deficient cortices was significantly increased (*Student´s t-test*; **, p≤0.01), while no differences were observed at E14.5-E18.5. **H-J**, *In situ* hybridization against *Insm1* in WT (**H**; 2 brains) and *Efna5* knockout (**I**; 3 brains) E16.5 coronal sections revealed significantly decreased signal intensities in the SVZ of mutant cortices (**J**; two-way *ANOVA*; ***, p≤0.001). In **D-G**, **J**, "n" refers to the number of analyzed sections. Ctx=cortex, *Insm1*=insulinoma associated 1, LGE=lateral ganglionic eminence, MGE=medial ganglionic eminence. Scale bar: 500 µm in **A**, **H, I**; 100 µm in **B -C´**.



Supplementary Figure 3: Pax6 and Ki67 immunostaining in the embryonic *Efna5* and wildtype cortex.

A-F, Overlay of Pax6 immunostaining (red) and DAPI labeling (blue) in coronal sections of the E13.5 (**A**, **B**) and E16.5 (**D**, **E**) cortices. At E13.5, two-way *ANOVA* analysis revealed no differences between wildtype (**A**) and *Efna5* knockout embryos (**B**) as quantified in **C** (n.s., p=0.168; 2 brains per genotype). The cell number in segments 8 and 9 were increased as

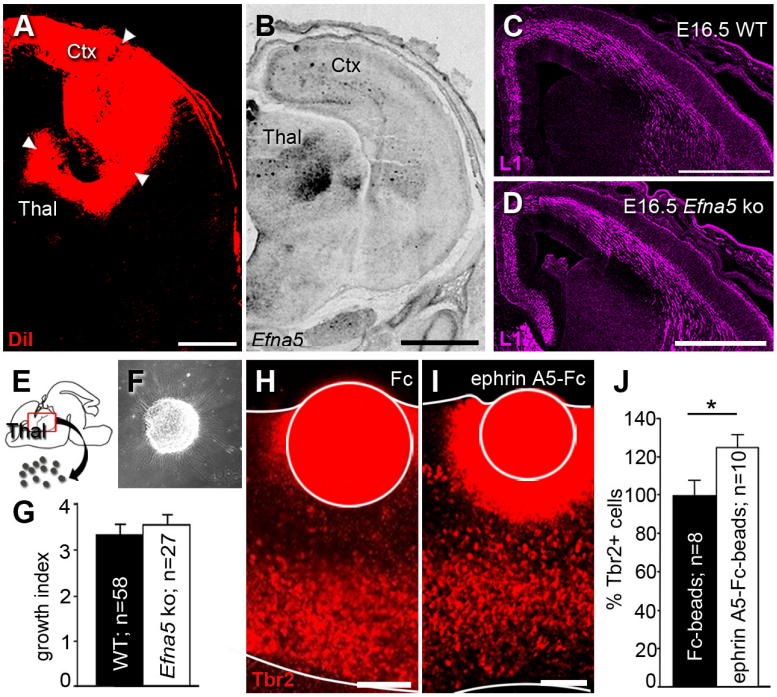
revealed by *Bonferroni Test* (**, p≤0.01). At E16.5, two-way *ANOVA* analysis also revealed no significant changes in *Efna5* knockouts (**E**) compared to wildtypes (**D**), as quantified in **F** (n.s., p=0.093; 3 brains per genotype). Yet, by applying *Bonferronie Test* increased number in segments 5-7 (**, p<0.01 for segments 5 and 7; ***, p≤0.001 for segment 6) were observed in *Efna5* knockout brains. **G-I**, Immunostaining in sections of E16.5 cortices against Ki67 revealed a decreased proportion of basally dividing cells in the SVZ of *Efna5* deficient mice (**H**) compared to wildtypes (**G**) as quantified in **I** (*, p<0.05, *Student´s t-test,* 3 brains per genotype). Number "n" refers to the number of analyzed sections. VZ=ventricular zone, SVZ=subventricular zone, IMZ=intermediate zone, CP=cortical plate. Scale bars: 100 µm in **D**, **E**, 50 µm in **A**, **B**, **G** and **H**.



Supplementary Figure 4: Eph-receptor expression analysis in the E14.5 wildtype cortex

A, Nissl stained E14.5 coronal section of the embryonic brain. **B-B´´**, *Epha4* is expressed in the proliferative zones of the developing cortex (E14.5) in anterior, medial as well as

posterior sections. The ephrin A5-affine receptors *Epha1* (**C**), *Epha2* (**D**), *Epha6* (**G**), *Epha7* (**H**), *Epha8* (**I**), and *Ephb2* (**J**) are not expressed in the developing neocortex (E14.5). *Epha3* (**E**) and *Epha5* (**F**) are expressed in the cortical plate, but not in the transient proliferative zones of the dorsal telencephalon (E14.5). Ctx=cortex, LGE=lateral ganglionic eminence, MGE=medial ganglionic eminence, POA=preoptic area. Scale bars: 500 µm in **A-J**.



Supplementary Figure 5: Thalamic ephrin A5 influences the generation of IPCs.

A, **B**, DiI crystal placed in the dorso-lateral embryonic cortex at E16.5 (**A**) retrogradely tags the thalamic region which expresses *Efna5* as shown with *in situ* hybridization (**B**). **C**, **D**, L1-immunostaining was performed in coronal sections of E16.5 wildtype (**C**) and *Efna5* mutant brains (**D**) to label thalamo-cortical projections in the developing cortex, which did not reveal alterations in *Efna5* deficient brains. **E-G**, thalamic explants (E14.5+1div) prepared from wildtype and *Efna5* knockout embryos did not show significant differences in the axonal outgrowth index (2 independent experiments for wildtype and knockout tissue). **H-J**, Tbr2 immunostaining of organotypic slices of *Efna5*-deficient brains (E14.5 + 2 days *in vitro*). Fc-protein-coated (**H**) or ephrin A5-Fc-coated protein-A-agarose beads (**I**) were placed in the CP/IMZ of the cortex, revealing an increase in Tbr2-positive cells in response to exogenous ephrin A5 (**J**; *Student´s t-test*; *, $p<0.05$; 3 independent experiments). For quantification, Tbr2-positive cells in slices treated with Fc-protein-coated beads were set to 100%. Number "n" refers to the number of analyzed explants in **G** and analyzed sections in **J**. Ctx=cortex, Thal=thalamus, CP=cortical plate, IMZ=intermediate zone. Scale bars: 500 µm in **A-D**; 50 µm in **H**, **I**.

Supplementary References

Iscove, N. N., Barbara, M., Gu, M., Gibson, M., Modi, C. and Winegarden, N. (2002). Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. *Nature biotechnology* **20**, 940-943.

Zimmer, G., Kastner, B., Weth, F. and Bolz, J. (2007). Multiple effects of ephrin-A5 on cortical neurons are mediated by SRC family kinases. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 5643-5653.