Ephrins guide migrating cortical interneurons in the basal telencephalon

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Cortical interneurons are born in the proliferative zones of the ganglionic eminences in the subpallium and migrate to the developing cortex along well-defined tangential routes. The mechanisms regulating interneuron migration are not completely understood. Here we examine the role of class-A members of the Eph/ephrin system in directing the migration of interneurons. In situ hybridizations demonstrated that ephrin-A3 is expressed in the developing striatum, an area that is strictly avoided by migrating cortical interneurons in vivo, which express the EphA4 receptor. We then examined interneuron migration in grafting experiments, where explants of the medial ganglionic eminence (MGE) from enhanced green fluorescent protein-expressing transgenic mice were homotopically grafted into host slices from wild-type littermate embryos. After blocking ephrin-A ligands, many interneurons invaded the striatal anlage. Moreover, stripe assay experiments revealed that ephrin-A3 acts as a repellent cue for neurons from the medial ganglionic eminence. Downregulation of the EphA4 receptor via siRNA transfection reduced the repulsive effect of ephrin-A3, indicating that EphA4 mediates at least in part the repulsive effect of ephrin-A3 on these cells. Together, these results suggest that ephrin-A3 acts as a repulsive cue that restricts cortical interneurons from entering inappropriate regions and thus contributes to define the migratory route of cortical interneurons.

Introduction

The correct function of the cerebral cortex requires a precise balance between excitatory glutamatergic projection neurons and inhibitory GABAergic interneurons. During development of the central nervous system, these two major cell classes are generated in different regions of the telencephalon and they then migrate from their remote sites of origin to their later destinations in the cerebral cortex, where they integrate in specific neuronal circuits. Cortical projection neurons arise in the proliferative ventricular zone (VZ) of the pallium[1] and migrate radially into the developing neocortex, forming its laminated structure.[2] In contrast, inhibitory cortical interneurons are born mainly in the medial and to some extend also in the caudal ganglionic eminence (MGE/CGE) of the subpallium and they then travel tangentially over rather long distances along defined routes towards their cortical target region.[3-6] Early in mouse development (E12-E13) interneurons migrate ventral of the striatal anlage (StrA), whereas at later stages (E13.5-E17) they preferentially take a dorsal route in the subventricular zone (SVZ) of the basal telencephalon.[7,8]

The cellular and molecular mechanisms regulating the migration of cortical interneurons from the subpallium to the developing cortex are beginning to be understood. Several factors, including brain-derived neurotrophic factor (BDNF), neurotrophins (NT4) as well as hepatocyte growth factor (HGF), posses a motogenic activity, thereby stimulating the undirected movement of the interneurons.[9-11] In addition, different groups of guidance molecules act as attractive and repulsive cues for interneurons, controlling their migratory pathway. For example, two isoforms of Neuregulin-1, membrane bound Nrg1 and secreted Nrg1, act as short-range and long-range attractants for interneurons, respectively.[12] In contrast, repulsive cues prevent cortical interneurons from entering inappropriate territories like the developing striatum or the preoptic area. Semaphorin/neuropilin interactions, for instance, repel cortical interneurons from the StrA.[13] Since Slit1 is expressed in the VZ of the subpallium and since interneurons express Robo proteins, it has been
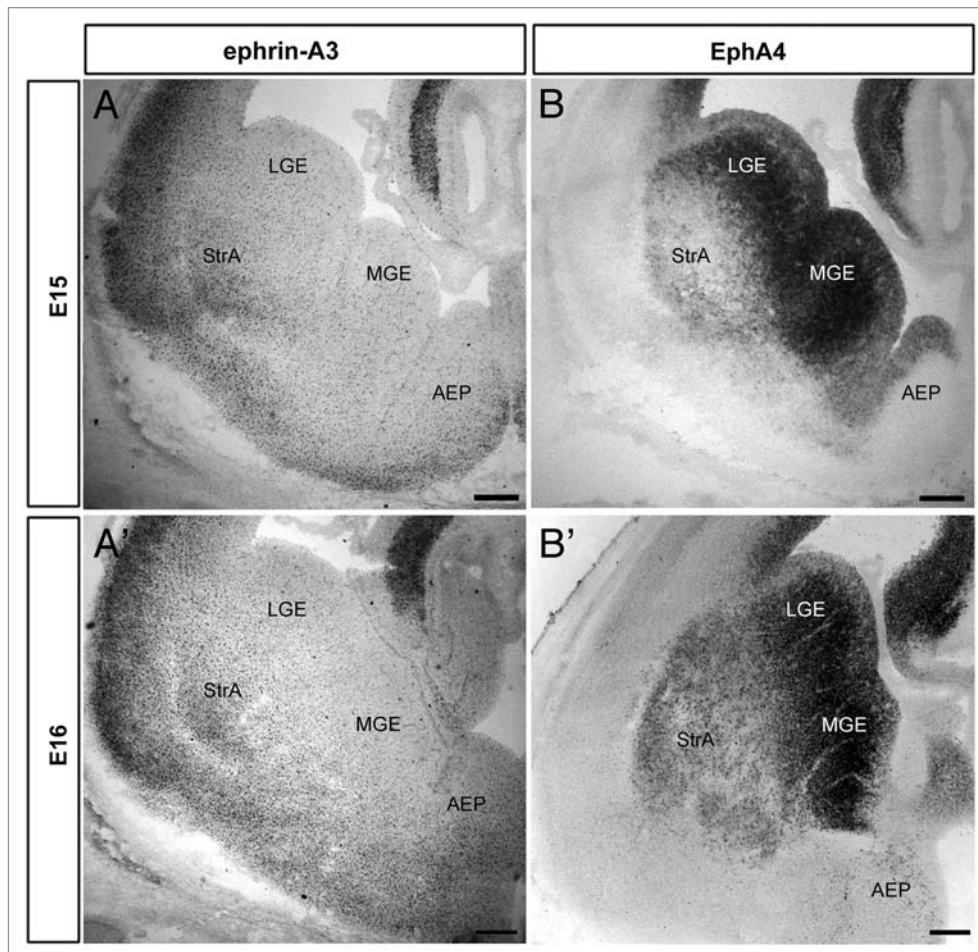
Figure 1. Ephrin-A3 and EphA4 are expressed complementary in the basal telencephalon. (A) In situ hybridization with ephrin-A3 riboprobes in coronal E15 (A) and E16 (A′) brain sections shows expression in the striatal anlage (StrA). (B) EphA4 is strongly expressed in the SVZ of the ganglionic eminences at E15 (B) and E16 (B′), while a weaker signal can be detected in the VZ and the StrA. Scale bars: 200 μm. Lateral is left and medial is right. AEP, area entopedunculare; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; StrA, striatal anlage; SVZ, subventricular zone; VZ, ventricular zone.

proposed that Slit1/Robo interactions are involved in regulating the migration from the MGE by a repulsive activity. Indeed, Slit1 has been shown to repel subpallial GABAergic neurons in vitro.[14-16] However, analysis of double (Slit1/Slit2) knockout mice has shown that the migratory pattern of interneurons was normal, suggesting that these molecules are not required for the migration of these neurons from the subpallium to the cortex.[17]

Another class of guidance factors that is expressed in the basal telencephalon during the period of interneuron migration are Eph receptor tyrosine kinases and their ligands, the ephrins. The Eph/ephrin system plays important roles in directing axonal growth and cell migration as well as other processes related to nervous system development such as neural progenitor proliferation and neurogenesis.[18,19] Eph/ephrin interactions are often repulsive, preventing growing axons or migrating cells from entering inappropriate areas (reviewed in refs. 20–22). However, there is also evidence for attractive effects of the Eph/ephrin system on neuronal cells.[23-25] We have previously demonstrated that ephrin-A5 is expressed in the VZ of the ganglionic eminences and that it has a chemorepellent activity on MGE-derived neurons, channelling

migrating interneurons into the SVZ of the eminences.[26] In the present study we examine the effects of another member of the ephrin-A family, ephrin-A3, on the tangential migration of MGE-derived cortical interneurons. We show that ephrin-A3 is expressed in the developing striatum and that it interacts with the EphA4 receptor, which is expressed by migrating cortical interneurons. Together, these results provide evidence that ephrin-A3 acts as a repulsive cue preventing cortical interneurons from entering the striatum and thus contributes to regulate the tangential migration of cortical interneurons.

Results

Ephrin-A3 and EphA4 are expressed in the basal telencephalon during tangantial migration of cortical interneurons. Cortical interneurons arising from the MGE migrate along distinct routes towards the cortex, thereby avoiding the striatal anlage (StrA), which represents a non-target territory for cortical interneurons. We found that ephrin-A3, a member of the A-ephrins that acts as a repellent in other regions of the developing nervous system,[31-33]

is expressed in the StrA during the peak of tangential migration as revealed by in situ hybridization on coronal brain sections (**Fig. 1A and A'**). Moreover, the EphA4-receptor expressing cells follow a corridor dorsally of the StrA in the route of migrating interneurons (**Fig. 1B and B'**) and were already identified as cortical interneurons in a previous study.[26] Thus, the complementary expression patterns of ephrin-A3 and EphA4 suggest that ephrin-A3 might prevent migrating cortical interneurons expressing the EphA4 receptor from entering the striatum. The spatial and temporal expression patterns described here lead to some specific predictions how members of the ephrin-A system might contribute to specify the migratory pathway of cortical interneurons. To test this hypothesis we used functional in vitro assays, which are described in the following sections.

Blocking of ephrin-A ligands in the slice assay results in invasion of the striatum by cortical interneurons. Since cortical interneurons bypass the striatum during their tangential migration towards their target regions, we first performed grafting experiments in order to examine whether ephrin-A3 is involved in repelling interneurons from the striatal anlage. For this, MGE explants from E14 EGFP mice were homotopically transplanted in the MGE of E14 WT slices and after 2 days in vitro the migration pattern in the basal telencephalon was examined. To interfere with the Eph/ephrin system, we added EphA6-Fc to block endogeneous ephrin-A ligands[34] in some grafting experiments. As illustrated in **Figure 2B**, in control experiments the majority of EGFP neurons migrate through the VZ and SVZ of the eminences towards the cortex, while only few cells invade the StrA. In contrast, after blocking of ephrin-A3 in the striatal anlage by soluble EphA6-Fc, there is an increase in the number of EGFP-MGE neurons invading the striatal mantle (**Fig. 2C**). For a quantitative analysis of the migration pattern we measured the fluorescence intensities of the EGFP neurons in defined segments of a column of the LGE whose size and position was dependent on the dimensions of the slice according to Zimmer et al.[26] using the ImageJ software as described in more detail in the methods section. The segment with the highest fluorescence intensity was defined as 100%, while that with the lowest intensity was set to 0%. The relative fluorescence intensities of the remaining segments were then calculated in reference to these set values (**Fig. 2A**). As illustrated in **Figure 2D**, the fluorescent intensities of the segments representing the striatal anlage are increased after EphA6-Fc application compared to control values. Thus, blocking of endogeneous ephrin-A ligands results in a reduction of the repulsive potential of the striatal anlage.

Ephrin-A3 acts as a repellent cue for MGE-derived neurons. To directly test the effect of ephrin-A3 on cortical interneurons, we performed the stripe assay according to Vielmetter et al.[28] where MGE-derived neurons were plated on alternating stripes of labeled ephrin-A3-Fc and unlabeled control protein (**Fig. 3A**). After 2 days in vitro, dissociated MGE neurons strongly avoided the ephrin-A3-Fc containing stripes. As shown in **Figure 3A and C**, 68% of the cells avoided ephrin-A3 and grew on the control stripes (p < 0.001, paired t-test). In control experiments (alternating stripes of labeled and unlabeled control protein) neurons

showed no preference for one kind of stripes (**Fig. 3B and C**). Thus, ephrin-A3 acts as a repellent cue for neurons derived from the MGE.

Downregulation of EphA4 diminishes the ephrinA3-induced repulsion of cortical interneurons. In our previous study we demonstrated that EphA4 is expressed by cortical interneurons.[26] To examine, whether the repulsive effect of ephrin-A3 on cortical interneurons is mediated by the EphA4 receptor, we transiently downregulated the EphA4 receptors by small interfering RNA (siRNA). For this, MGE-derived neurons growing on alternating stripes of ephrin-A3-Fc and control protein were transfected with siRNA against the mRNA of EphA4 using lipofection. Additonal application of fluorescence labeled Alexa555 siRNA allowed the visualization of the transfected neurons. This set of experiments allowed direct comparison of the effect of ephrin-A3 on transfected cells with repressed EphA4 receptors and on non-transfected neurons with normal EphA4 expression. To verify the knockdown efficacy of the EphA4 siRNA, MGE-derived cells transfected with EphA4 siRNA and control Alexa555 siRNA or control siRNA alone were stained with an antibody against the EphA4 receptor after 2 days in vitro. To quantify the amount of EphA4 expression in cells transfected with EphA4 siRNA compared to non-transfected neurons, images were taken using a confocal Laser Scanning Microscope. For each picture the fluorescence intensities of the EphA4 signal of transfected neurons were measured using ImageJ and calculated relative to these of non-transfected cells. As shown in **Figure 4A–A'''** application of EphA4 siRNA diminishes the relative fluorescence intensities of transfected cells. The decline is about 32% compared to neurons transfected with control Alexa555 siRNA (**Fig. 4B**; p < 0.001, Student's t-test), demonstrating that this siRNA approach significantly reduces the EphA4 expression in MGE derived neurons.

As illustrated in **Figure 4C and D**, downregulation of EphA4 receptors by siRNA transfection diminishes the repulsive effect of ephrin-A3. While 67% of the non-transfected cells avoided the ephrin-A3-Fc containing stripes, only 61% of the transfected cells (labeled red) with reduced EphA4 expression did so (p < 0.01, Student's t-test). Application of the control Alexa555 siRNA alone showed no effect; in this case transfected as well as non-transfected cells avoided the ephrin-A3-Fc stripes (**Fig. 4E**). Moreover, application of EphA4 siRNA had no impact on the distribution of the interneurons on Fc/control stripes (**Fig. 4F**). Taken together, these data suggest that EphA4 mediates the repulsive effect of ephrin-A3.

Discussion

In addition to intrinsic programs, extracellular guidance factors that define the route of migrating cells and identify target and non-target areas are also required. In the present study we demonstrated that ephrin-A3 is expressed in the developing striatum, a territory not invaded by cortical interneurons, and that EphA4, a possible receptor for the ephrin-A3 ligand, is present along their pathway. This complementary expression of ligand and receptor suggests that repulsive EphA4/ephrin-A3 interactions
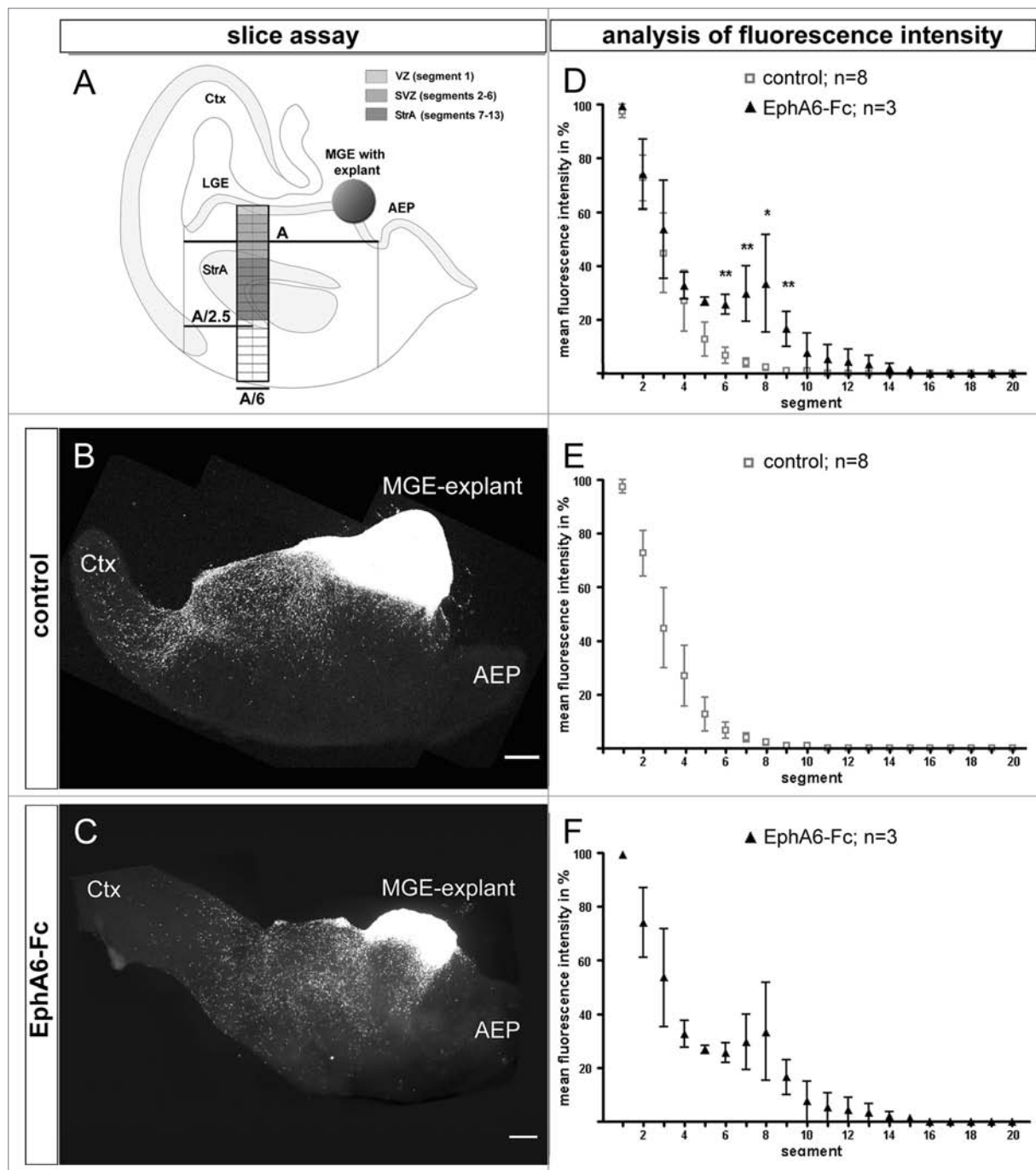
Figure 2. MGE-derived neurons invade the striatum after blocking of ephrin-A ligands in grafting experiments. The tangential migration of EGFP-MGE neurons in WT slices was investigated after blocking of ephrinA-function. (A) Schematic view of a coronal brain slice as used for the grafting experiments, illustrating how the size of each slice was measured to determine the dimensions of the analyzed area, which was horizontally divided into 20 segments, starting with segment 1 in the VZ. The segment with the highest fluorescence intensity in each slice was set to 100%, the lowest to 0%. The relative intensities of the remaining segments were calculated in relation to these two reference values (see Methods for details). (B) Grafted EGFP-MGE cells migrate into the cortex after 2 days in vitro, avoiding the mantle zone of the striatum in control conditions. (C) After blocking endogenous ephrin-A ligands with EphA6-Fc, grafted MGE neurons invade the striatal anlage. Quantification of the relative fluorescence intensities in control conditions (E) and after blocking ephrin-A ligands with EphA6-Fc (F). (D) Represents the direct comparison of fluorescence intensities obtained after blocking of ephrin-As and in control conditions. Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**). Scale bars: 200 μm; n = number of analyzed slices (mean ± SEM). Lateral is left and medial is right. AEP, area entopedunculare; Ctx, cortex; MGE, medial ganglionic eminence; VZ, ventricular zone.

might contribute to identify the striatum as a non-target region for cortical interneurons. To test this hypothesis, we first applied grafting experiments, which are used widely to study interneuron migration,[13,17,26,35] and found that a substantial portion of the transplanted MGE-neurons invaded the striatum after blocking endogenous ephrin-A ligands in the slices with recombinant EphA6-Fc. EphA6 can bind to various ephrin-A ligands.[34,36] Since ephrin-A3 is the only detectable ligand expressed in the StrA during these developmental stages (data not shown) we suggest ephrin-A3 as the effecting ligand. We next exploited the stripe assay to directly demonstrate that ephrin-A3 has a repulsive effect on MGE-derived neurons. In the same assay, after downregulation of the EphA4 via siRNA techniques, the repulsive effect of ephrin-A3 was significantly reduced, indicating that EphA4/ephrin-A3 interactions have a chemorepellent activity on cortical interneurons. However, EphA4 siRNA knockdown did not completely abolish the response of cortical interneurons to ephrin-A3. One possible explanation for this could be that EphA4 siRNA knockdown was not complete in the neurons and that remanent EphA4 receptors could still mediate a weak repulsive effect. Moreover, other EphA receptors could also be involved. One possible candidate is EphA3, since it is also expressed in the VZ and SVZ of the ganglionic eminences (data not shown). Further experiments with a combined downregulation of EphA3 and EphA4 are required to resolve this issue.

Several earlier reports demonstrated already that one major developmental role of Eph/ephrin signaling is to mediate contact-dependent repulsion that prevents migrating cells from crossing into ligand expressing regions and thereby confining them to an appropriate pathway.[19,37] Examples include the restriction of early migrating neuronal crest cells to a ventral path in the caudal part of somites[38,39] or to confine their migration to defined segments in the hindbrain.[40] Interestingly, at later stages neural crest cells differentiate to form melanoblasts that migrate along a dorsolateral route, and in this case ephrin ligands now act as an attractive signal for the migrating cells.[41] The mechanisms underlying this switch between repulsion and attraction are currently not known.

Previously, we have already demonstrated that ephrin-A5 is expressed in the VZ of the ganglionic eminences, the dorsal boundary of the migratory route of cortical interneurons, and that this molecule serves as an inhibitory border to channel these neurons into the SVZ.[26] Thus, as illustrated in **Figure 5**, the deep corridor of migrating cortical interneurons in the SVZ is at least in part defined by the concerted action of two different ephrin-A ligands, with ephrin-A5 flanking the dorsal portion and ephrin-A3 the ventral portion of this migratory pathway. In addition, ephrin-A3 might also prevent cortical interneurons migrating superficially from invading the striatal anlage, which forms the upper border of this pathway.

As stated in the introduction, members from different families of guidance cues have been implicated to regulate the precise migration patterns of cortical interneurons during cortical development. In particular, it has been suggested that neuropilins mediate the sorting of migrating cortical and striatal interneurons. Neuropilins are co-receptors for class 3 semaphorins
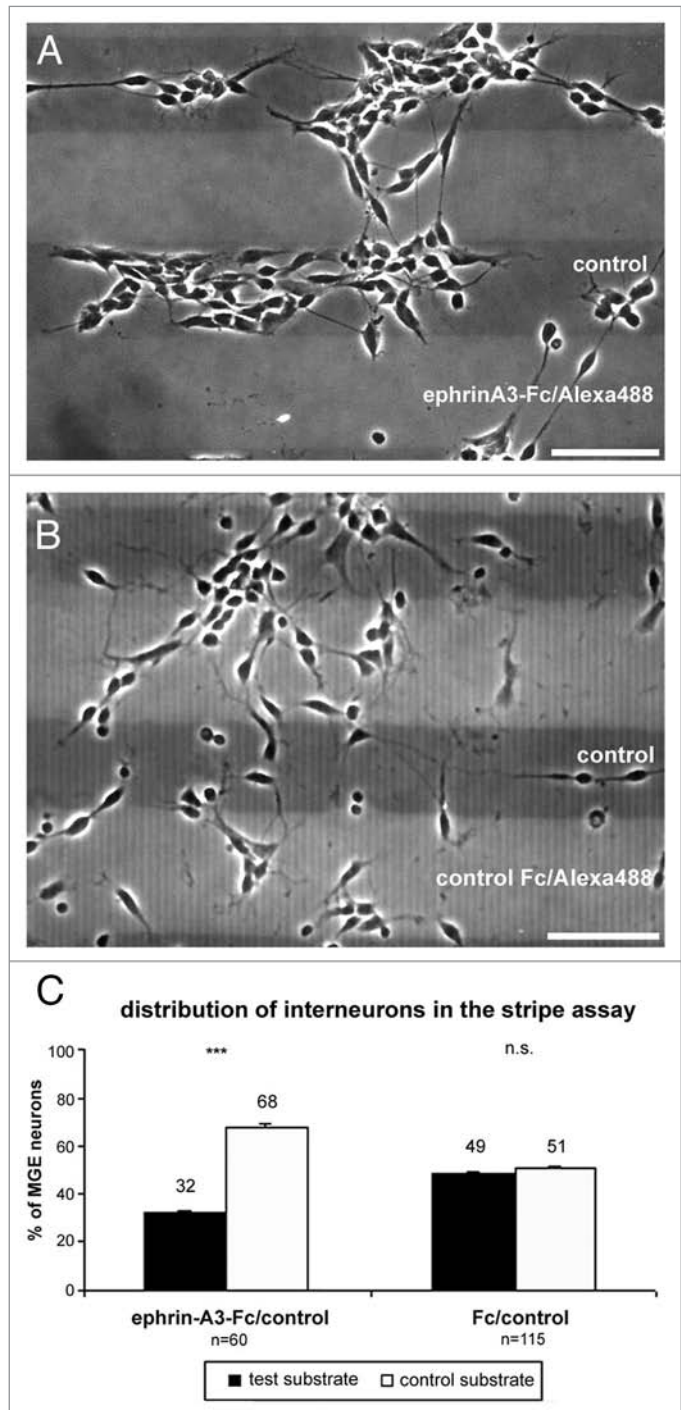

Figure 3. Ephrin-A3 has a repulsive effect on MGE-derived neurons. (A) Dissociated neurons from the MGE (E14) growing on alternating stripes of Alexa488-labeled ephrin-A3-Fc and unlabeled control protein clearly avoid ephrin-A3 stripes and preferentially grow on the control stripes after 2 days in vitro. (B) On alternating stripes of labeled and unlabeled control protein the cells show no preference. (C) Quantification (mean ± SEM) of the distribution of neurons from the MGE in the stripe assay with ephrin-A3-Fc or control after 2 days in vitro. Paired t-test; p < 0.001 (***). n = number of analyzed images. Scale bars: 50 μm.
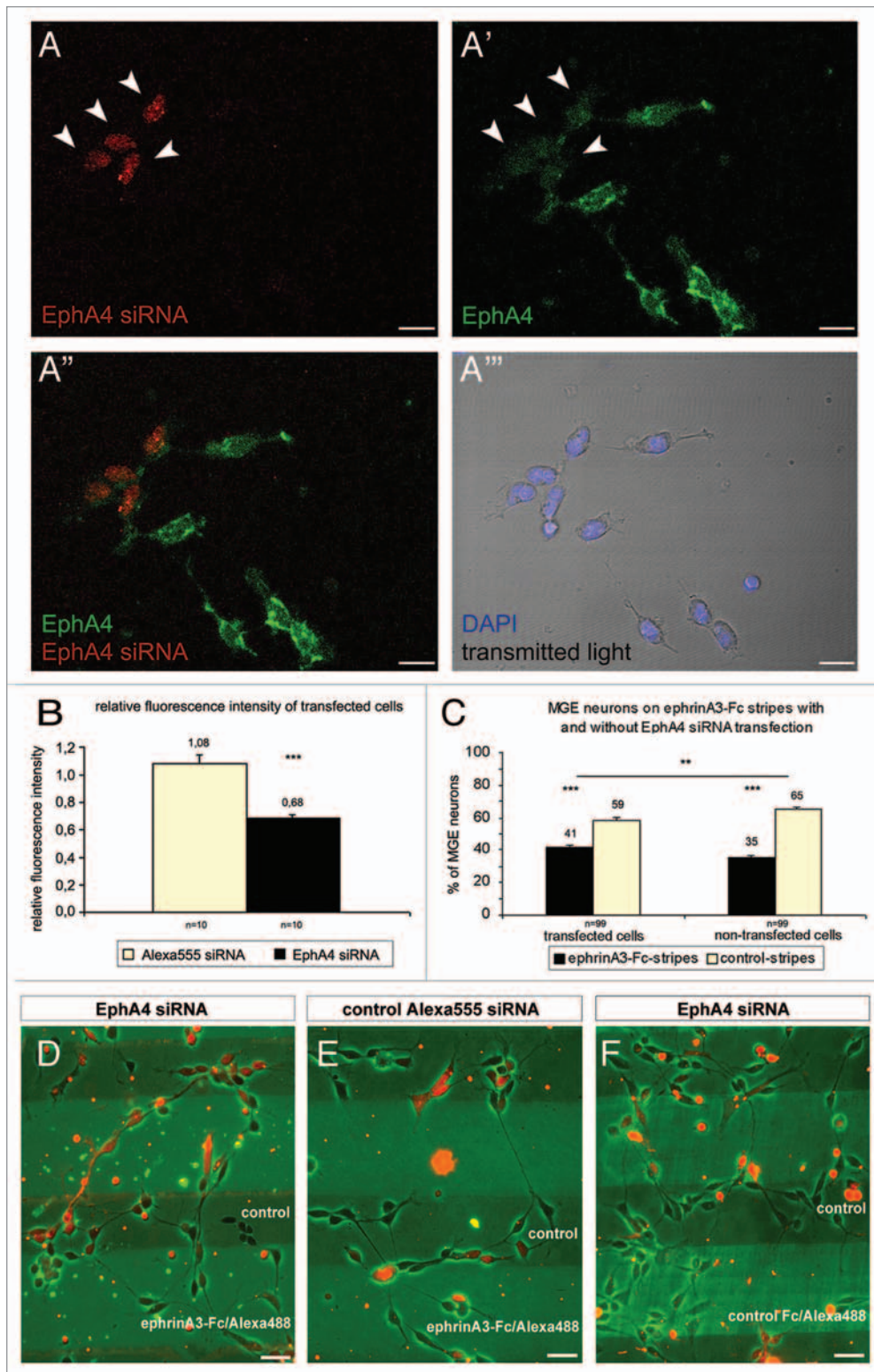
Figure 4. Downregulation of EphA4 diminishes the repulsion of cortical interneurons by ephrin-A3. (A) Verification of the knockdown efficacy. Immunocytochemistry against EphA4 was performed on MGE-derived neurons after 2 days in vitro. Neurons transfected with EphA4 siRNA (A) show less EphA4 expression than non-transfected cells (A'; arrowheads). (A'') Overlay of EphA4 siRNA transfection and EphA4 expression. (A''') Overlay of DAPI-stained nuclei and a transmitted light image to visualize the neurons. (B) Application of EphA4 siRNA results in reduced fluorescence intensity and thus less EphA4 expression compared to neurons transfected with control Alexa555 siRNA. Student's t-test; $p < 0.001$ (***). (C) Quantification (mean ± SEM) of the distribution of neurons in the stripe assay with and without EpA4 siRNA transfection. (D) Neurons transfected with EphA4 siRNA (red) showing a reduced repulsion induced by ephrin-A3-Fc in the stripes assay after 2 days in vitro compared to untransfected cells. (E) Application of Alexa555 labeled control siRNA has no effect as all cells still avoid the ephrin-A3-Fc containing stripes. (F) Application of EphA4 siRNA does not affect MGE-derived neurons growing on Fc/control stripes. Student's t-test; $p < 0.01$ (**). Scale bars: 20 μm (A) and 10 μm (F). n = number of analyzed images.

(reviewed in ref. 42), which are present on interneurons migrating to the cortex, but not to the striatum where Sema3A and Sema3F are expressed.[13] Expression of neuropilins allows cortical interneurons to bypass the striatum and migrate to the cortex, whereas striatal interneurons, which do not express neuropilin receptors, are able to invade the striatum. Loss of neuropilin function increases the number of interneurons that migrate into the striatum and decreases the number of cortical interneurons. Moreover, a recent study also showed that chondroitin sulfate proteoglycans (CSPGs) are coexpressed with Sema3A in the striatal anlage and act as additional repulsive cues for cortical interneurons.[43] This study also provided evidence that CSPGs bind Sema3A and increase the repulsive strength of the striatum to migrating cortical interneurons. Moreover, Shipp et al.[44] could demonstrate that chondroitin sulfate interacts with various axon guidance proteins, including ephrin-A1 and ephrin-A5. It would therefore be interesting to examine whether there is a functional interaction between ephrin-A3 and the CSPGs in the striatal anlage that potentiates the repellent activity of these different classes of signaling molecules.

Taken together, these studies indicate that the tangential migration of cortical interneurons is tightly controlled by a variety of guidance systems. These different guidance systems might be redundant, acting independently, or might act differently on distinct subsets of cortical interneurons and thereby increase the precision and fidelity of the assembly of cortical circuits that give rise to complex behaviour.

Materials and Methods

Animals. Enhanced green fluorescent protein (EGFP)-expressing[27] C57BL/6 and wildtype (WT) mice (from the C57BL/6 and NOR strains) were used in this study. For staging of the mouse embryos, the day of insemination was considered as embryonic day (E) 1. Mice were bred and maintained under standard conditions and were kept with access to food and water ad libitum on a 12 h light/dark cycle. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Friedrich Schiller University, Jena, Germany.

Preparation of dissociated neurons of the MGE. Time pregnant mice were deeply anesthetized using peritoneal injection of 10% chloralhydrate. The E14 mouse embryos were removed from the uterus and the brains were prepared. The MGEs of the embryos were dissected in Gey's balanced salt solution (GBSS) supplemented with 0.65% D-glucose and collected in ice-cold Hank's balanced salt solution (HBSS; Invitrogen, Germany) supplemented with 0.65% glucose. After incubation with 0.25% trypsine in HBSS for 17 min at 37°C, the tissue was dissociated into single cells by trituration and filtered through a nylon gauze to remove cell aggregates. Neurons were incubated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Germany) supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin, 10,000 μg/ml streptomycin, 95.4 μg/ml D-glucose and 200 mM L-glutamine at 37°C and 5% CO_2 in a humid atmosphere for 2 days.

Preparation of slice cultures. Brains of E14 embryos of EGFP-expressing and WT mice were dissected coronally into 225 μm slices using a tissue chopper and transferred in GBSS supplemented with 0.65% D-glucose. WT slices, including the lateral and medial ganglionic eminence (LGE, MGE) and the area entopedunculare (AEP), were placed on glass coverslips coated with poly-l-lysine (10 μg/ml). Explants from the VZ of the MGE of corresponding green fluorescent slices were transplanted homotopically on WT slices. Slices were cultured in medium composed of 60% DMEM/F12 (Sigma, Germany), 30% HBSS, 10% FBS, 10,000 U/ml penicillin, 10,000 μg/ml streptomycin and 0.65% D-glucose for 2 days at 37°C and 5% CO_2 in a humid atmosphere. Depending on the culture condition, 10 μg/ml mouse EphA6-Fc (R&D systems, Germany) or, as a control, 5 μg/ml Fc protein (human IgG F(c) fragment; Rockland Immunochemicals; USA), clustered with 30 μg/ml anti-human IgG (Vector, USA) were added to the medium. After 1 day in vitro, a fraction of the culture medium with the applied substances was interchanged. Slices were fixed after 2 days in vitro using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h.
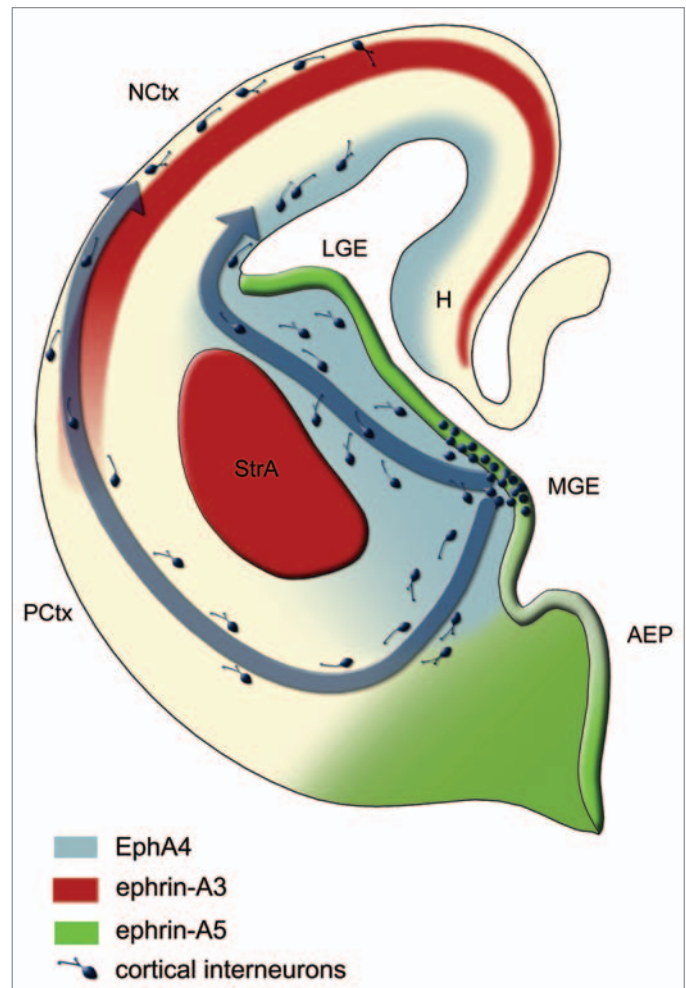

Figure 5. Different ephrin-A ligands act in concert to channel migrating MGE-derived neurons towards the cortex. Schematic drawing of a coronal brain slice from the left hemisphere; medial is left, dorsal is top. Ephrin-A5 expels interneurons from their place of origin in the ventricular zone of the MGE into the EphA4-expressing subventricular zone. It also prevents ventral migration and thus invasion of the AEP, directing these neurons towards the cortex. In the SVZ migrating interneurons are channeled to the deep corridor towards the cortex at least in part defined by the chemorepulsive activity of ephrin-A5 and ephrin-A3. Ephrin-A5 serves as a dorsal border in the VZ of the eminences and ephrin-A3 flanks the ventral portion as it is expressed in the striatal anlage. Ephrin-A3 is also expressed in the cortical plate, suggesting that it might prevent migrating interneurons from entering this structure and thus pushes them into their migratory pathway in the marginal zone of the neocortex.

Stripe assay. Stripe assays were performed according to Vielmetter et al.[28] using silicone matrices, obtained from the Max-Planck Institute for Developmental Biology (Tübingen, Germany), for stripe formation. Glass coverslips were placed on the silicone matrix and 30 μl of a 5 μg/ml human ephrin-A3-Fc (R&D systems, Germany) solution preclustered with 30 μg/ml anti-human IgG-Alexa488 (Invitrogen, Germany) in PBS were injected into the matrix channels. After incubation at 37°C for 30 min, the coverslips were washed with PBS and coated with 19.5 μg/ml laminin and 5 μg/ml poly-l-lysine (Invitrogen, Germany)

for 30 min, to obtain alternating stripes of labeled ephrin-A3-Fc and unlabeled control protein (laminin-poly-l-lysine). As a control experiment, alternating stripes of 3 μg/ml Fc (human IgG F(c) fragment; Rockland Immunochemicals) clustered with 30 μg/ml anti-human IgG-Alexa488 in PBS, and laminin-poly-l-lysine were used. Dissociated neurons were added at a density of 300 cells/mm^2.

siRNA transfection. MGE neurons growing on alternating stripes of ephrin-A3-Fc and control protein were transfected with 10 nM mouse EphA4 siRNA (Santa Cruz, Germany), that is a pool of 3 target-specific 20–25 nt siRNAs to knockdown gene expression, in combination with 10 nM Alexa555-labeled RNA dublex (BLOCK-iT Alexa Fluor red fluorescent oligo; Invitrogen, Germany) to enable the visualization of the transfected interneurons. For transfection of MGE-neurons reverse lipofection with Lipofectamine RNAiMAX (Invitrogen, Germany) was used according the protocol. The transfection occured for 5 h in antibiotics-free DMEM (Invitrogen, Germany) with 10% FBS, 95.4 μg/ml D-glucose and 200 mM L-glutamine at 37°C and 5% CO$_2$ in a humid atmosphere. Then the medium was substituted by culture medium containing 10,000 U/ml penicillin and 10,000 μg/ml streptomycin. Transfected neurons were incubated for 2 days in vitro at 37°C and 5% CO$_2$.

Immunocytochemistry. For immunocytochemistry E15 MGE cells were either co-transfected with 10 nM mouse EphA4 siRNA (Santa Cruz, Germany) and 10 nM Alexa555-labeled RNA dublex (BLOCK-iT Alexa Fluor red fluorescent oligo; Invitrogen, Germany) or with 20 nM Alexa555 control siRNA alone. After 2 days in vitro at 37°C and 5% CO$_2$ cells were fixed with 4% PFA in PBS for 20 min, followed by washing with PBS/0.1% Triton X-100 and blocked with 10% goat normal serum/5% bovine serum albumin (BSA)/0.1% Triton X-100 in PBS. Cells were then treated with a rabbit anti-EphA4 polyclonal antibody 1:800 (Santa Cruz Biotechnology, Germany) in blocking solution for 1 h at room temperature and then washed with PBS/0.1% Triton X-100 several times. As secondary antibody a goat anti-rabbit IgG Cy2 1:200 (Jackson ImmunoResearch Europe, UK) was applied in blocking solution for 1 h at room temperature. Cells were washed 3 times with PBS/0.1% Triton X-100 and in a final step nuclei were stained using DAPI (100 ng/ml; Sigma). Negative controls without using the primary antibody resulted only in background signals.

Analysis of in vitro assays and immunocytochemistry. For the stripe assay, pictures were taken with a digital camera (Spot, Diagnostic Instruments) in combination with the *Spot*-software and a Zeiss Axiovert S100 inverted microscope (Zeiss, Germany) using a 20x phase contrast objective (Zeiss PlanNeofluar, numerical aperture [NA] = 0.5) and 1.6 optovar in combination with fluorescence excitation to visualize the stripes. The distribution of the neurons on the stripes was determined using the cell counter plug-in of ImageJ, whereas only the location of the soma was taken into account. Total numbers of neurons on the alternating stripes were corrected according to the varying widths of the stripes, and a paired t-test was used for statistical comparison. Results (mean ± SEM) are presented as a percentage; "n" refers to the number of analyzed pictures.

For the stripe assay combined with siRNA transfection, phase-contrast pictures, taken with a 20x phase contrast objective (Zeiss PlanNeofluar, NA 0.5) and 1.6 optovar, were merged with photographs taken with fluorescence excitation, using the Spot-software, to visualize the transfected and non-transfected neurons per frame. The distribution of the neurons on the stripes was determined separately for transfected and non-transfected interneurons.

To verify the knockdown of gene expression by siRNA transfection pictures of transfected and non-transfected neurons were taken using a confocal Zeiss Laser Scanning Microscope LSM 510 and LSM 510 software with a 40x C-Apochromat waterimmersion objective (NA = 1.2). Transfected neurons visualized by Alexa555 siRNA were excited using a helium-neon laser (543 nm) and their emissions passed a 560-nm longpass filter set, whereas the EphA4-Cy2 signal was scanned using an argon laser (488 nm) and a 500–550-nm bandpass filter set. The DAPI-stained nuclei were visualized using a 2-photon titanium-sapphire laser (Mai-Tai, SpectraPhysics, Mountain View, CA) by a wavelength of 780 nm and a bandpass of 435–485-nm. For quantitative analysis of knockdown of EphA4 expression the shapes of transfected and non-transfected neurons on pictures showing only the EphA4-Cy2 signal were circumscribed and fluorescence intensities were measured using ImageJ. Fluorescence intensities of transfected cells were calculated relative to these of non-transfected cells for each image.

Pictures of migrating cells in the slice assay were taken either with a Zeiss Axiovert S100 inverted microscope (Zeiss, Germany) using a 10x objective (Zeiss PlanNeofluar, NA 0.6) under fluorescence excitation or with a Zeiss Laser Scanning Microscope LSM 510 and LSM 510 software using a 10x objective (Zeiss, NA 0.6) and an argon laser with 488 nm wavelength. A bandpass of 500–550 nm was applied as a filter for emission. Individual images were assembled in Adobe Photoshop 6.0 to a montage of the entire slice. For the quantitative analysis of the migration pattern a region in the LGE was chosen for measuring the distribution of fluorescence intensity across a column from the ventricular zone (VZ) to the ventral border of the slice using ImageJ. To ensure the sector represented the same region in each slice, we measured the distance from the cortical-striatal notch to the AEP (marked as A in **Fig. 2A**). The middle of the column analyzed was located at a distance of A/2.5 and the width of the sector was determined by A/6. The emerging area was then divided vertically into 20 equal segments, with segment 1 representing the VZ, segments 2–6 the subventricular zone (SVZ), and segments 7–13 the striatal anlage, according to Jacobowitz[29] and Kaufmann.[30] The segment with the highest absolute fluorescence intensity was defined as 100% and the one with the lowest fluorescence intensity was set to 0%. In relation to these values the relative fluorescence intensities in the remaining segments were determined. Results (mean ± SEM) are presented as a percentage; Student's t-test was used for statistical comparison.

In situ hybridization. Pregnant NOR mice were killed and the embryos were rapidly removed and decapitated. The E15 and E16 brains were frozen in 2-methylbutan at -40°C. Digoxigenin-labeled RNA-probes for EphA4 (75-761 of mouse

Epha4, GenBank accession number NM_007936) and ephrin-A3 (290-1156 of mouse Ephrina3, GenBank accession number NM_010108) were used for in situ hybridization. For this 18 μm coronal cryostat sections were dried 2–3 h at 56°C and fixed for 10 min in freshly prepared 4% PFA in Diethyl pyrocarbonate (DEPC)-treated 1M PBS (pH 7.4). After washing in DEPC-PBS and permeabilisation in 0.2 M HCL for 10 min, the slides were acetylated in 0.1 M triethanolamine with 5 mM acetanhydrid for 15 min. After washing in DEPC-PBS for 5 min, the sections were hybridized overnight at 68°C with 3 ng/μl Digoxigenin (DIG)-labeled riboprobe in 50% formamide, 5x Denhardt's solution, 5x SSC, 100 μg/ml bakers yeast tRNA and 400 μg/ml Torula RNA. The next day, sections were washed 5 min in 5x Saline sodium citrate buffer solution (SSC) at room temperature, 30 min at 68°C in 2x SSC/50% formamide, 60 min at 68°C in 0.2x SSC and 15 min in 0.2x SSC at room temperature. After short washing in 1x MaBS, the blocking reaction was performed with 2% blocking reagent (Roche, Germany) in 1x MaBS for 2–3 h. To detect the DIG-labeled riboprobes, slides were incubated overnight at 4°C with an anti-DIG Fab fragment conjugated with alkaline phosphatase (1:750; Roche, Germany). The next day, sections were washed 3 times for 10 min in 1x MaBS. After blocking the endogenous phosphatase activity with levamisole (Sigma, Germany), slides were covered with a mixture of 3.75 μl 5-Bromo-4-chloro-3-indolyl phosphate (Roche, Germany) and 5 μl Nitro blue tetrazolium chloride (Roche, Germany) in 1 ml reaction buffer 6 hours for EphA4 and overnight for EphrinA3 at room temperature for color reaction. Finally, slides were washed in 1 M PBS and embedded in Mowiol.

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