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·Original Article·

Ephrin-B2/EphA4 forward signaling is required for regulation of radial migration of cortical neurons in the mouse

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ABSTRACT

Postmitotic neurons in the neocortex migrate to appropriate positions and form layered structures of nascent cortex during brain development. The migration of these neurons requires precise control and coordination of a large number of molecules such as axon guidance cues. The Eph–ephrin signaling pathway plays important roles in the development of the nervous system in a wide variety of ways, including cell segregation, axon pathfinding, and neuron migration. However, the role of ephrin-B2/EphA4 signaling in cortical neuron migration remains elusive. Here we demonstrated that ephrin-B2 and its receptor EphA4 were expressed in complementary and overlapping patterns in the developing neocortex. Deletion of the EphA4 gene in the embryonic cerebral cortex resulted in faster migration of cortical neurons, whereas knockdown or overexpression of ephrin-B2 did not alter the normal process of migration. These results suggest that ephrin-B2 forward signaling through EphA4 is required for the precise control of cortical neuron migration.

Keywords: neuron migration; Eph receptor A4; ephrin-B2; cerebral cortex development

INTRODUCTION

The mammalian neocortex is organized in a laminar structure. Each layer contains specific subtypes of neurons characterized by distinct projection patterns and gene expression profiles. During mouse cerebral corticogenesis, radial glial cells first generate neurons destined for lower layers, followed by upper-layer neurons, then cortical astrocytes[1]. It is well established that postmitotic neurons migrate along radial glial fibers from proliferative zones to their final position and this process is regulated by multiple factors, including extracellular guidance molecules and intrinsic transcriptional regulators in a spatially and temporally appropriate manner[2]. However, the exact molecular mechanisms underlying the migration and positioning of cortical neurons are not yet fully understood.

Ephrins are cell-surface tethered ligands that bind to Eph receptor tyrosine kinases[3]. Their interactions stimulate bi-directional signaling which controls critical processes in the developing and adult brain, such as cell segregation[4,5], neuronal migration[6,7], tissue pattern formation[8,9], axonal pathfinding[10-15], and synaptic transmission[16-18]. In vertebrates, Eph receptor tyrosine kinases are classified into two subgroups: EphA receptors, which preferentially bind glycosyl phosphatidyl inositol-anchored type-A ephrins; and EphB receptors, which typically bind to transmembrane type-B ephrins. In some cases, however, ephrin-B2 and -B3 may also interact with EphA4 and stimulate its downstream signaling[19,20]. The signaling cascade activated downstream of Eph receptors is known as forward signaling, whereas the signaling pathway activated downstream of ephrins is referred to as reverse signaling.

It has been reported that the repulsive signals mediated by ephrin-B2, together with its receptors EphA4 and EphB1, regulate the migration of branchial neural crest cells in various vertebrates[20]. In *Manduca sexta*, MsEphrin–MsEph receptor interaction prevents enteric neurons from migrating aberrantly across the enteric midline[21,22]. Ephrin-Bs can also regulate neuronal migration through their crosstalk with the CXCR4 receptor signaling pathway[23] or through their interaction with the reelin signal cascade. Compound mouse mutants (*Reln*$^{+/-}$*; Ephrin-B3*$^{-/-}$ or *Reln*$^{+/-}$*; Ephrin-B2*$^{-/-}$) and triple ephrin-B1, -B2, and -B3 knockouts recapitulate the neuronal migration defects observed in *reeler* mice[24]. It has been reported that ephrin-Bs and their receptors are expressed in different layers of the developing neocortex[25]. However, whether ephrin-Bs act through the classical Eph–ephrin pathway to control radial migration and lamination remains unclear.

In the present study, we set out to describe the expression pattern of ephrin-B2 and its receptors EphB1, -B2, -A4 in the neocortex at a selected embryonic stage. Using *in vivo utero* genetic gain- and loss-of-function approaches, we characterized the role of ephrin-B2/EphA4 signaling in cortical neuron migration.

MATERIALS AND METHODS

Animals

Pregnant ICR mice were from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. *EphA4*-floxed mice in a C57BL/6 genetic background were generous gifts from Dr. Binhai Zheng and the homozygous colony was maintained by in-crossing homozygotes. Characterization and genotyping of these mice were as described previously[26]. Timed pregnant mice were maintained on a 12 h light/dark cycle at 23°C with food and water available *ad libitum*. All procedures were approved by the Ethics Committee for the Care and Use of Animals and were in accord with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

In situ Hybridization

In situ hybridization (ISH) was performed as described previously[27]. Briefly, embryos were fixed overnight in 4% paraformaldehyde at 4°C, and transferred into 30% sucrose before embedding in OCT. ISH was performed on cryosections (12 μm thick) with digoxigenin-labeled single-stranded RNA probes (300–400 bp). The primer sequences used for ISH were as follows: ephrin-B2 sense, 5'-AGTGCGCAGAACTGGGAG-3', antisense, 5'-GTTGTTGCCACCTCGCTT-3'; EphA4 sense, 5'-GGTATAAGGACAACTTCACGGC-3', antisense, 5'-CTTCTGTGGTATAAACCGAGCC-3'; EphB1 sense, 5'-CAGTCGCTCCCCTTCAGA-3', antisense, 5'-TGGCCACCAGAGACACAA-3'; EphB2 sense, 5'-TCATAAGGGAAGTGACGGTTCT-3', antisense, 5'-CCCTTGGTGTATTGCCTAAGTC-3'.

Expression Constructs and Transient Transfection of Cultured Cells

The pLVUbi-ephrin-B2-2A-GFP expression construct was generated by sub-cloning the mouse ephrin-B2 coding sequence (a gift from Dr. I Ethell) into the lentiviral expression vector pLVUbi-2A-GFP (a gift from Dr. ZL Qiu). A Cre recombinase expression construct was also generated (pLVUbi-Cre-2A-GFP) by sub-cloning the Cre recombinase coding sequence into the vector pLVUbi-2A-GFP. The cytomegalovirus promoter allows simultaneous expression of GFP and Cre recombinase. The short-hairpin RNA (shRNA) constructs designed to specifically knock down ephrin-B2 were gifts from Dr. MB Dalva[28].

To evaluate the knockdown efficacy of ephrin-B2 shRNA plasmids, two types of evaluation were carried out on either HEK293T or NIH3T3 cells. These cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 (1:1 *v*/*v*; Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum. HEK293T cells were transiently transfected with pLVUbi-ephrin-B2-2A-GFP in combination with ephrin-B2 shRNA plasmids in various ratios using the calcium phosphate method (Invitrogen). Seventy-two hours post-transfection, cells were harvested and subjected to Western blot analysis. NIH3T3 cells were transfected with either Ephrin-B2 shRNA or nonsense shRNA plasmids using Nucleofector™ (Amaxa, Cologne, Germany). Forty-eight hours after transfection, cultured cells were subjected to Western blot analysis.

Western Blot Analysis and Quantification

Western blotting was performed as described previously[29]. Briefly, cells were washed and lysed with ice-cold lysis buffer (20 mmol/L Tris–HCl, pH 7.4, 0.1% sodium dodecyl

sulfate (SDS), 1% Nonidet P-40, 1% sodium deoxycholate) supplemented with a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Whole-cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, and polypeptides were transferred to nitrocellulose membranes (Invitrogen). Blots were blocked with 5% fat-free milk in 10 mmol/L Tris-buffered saline (pH 8.0) for 30 min and incubated for 2 h at room temperature with the following antibodies: (a) goat anti-ephrin-B2 polyclonal antibodies (pAb, 1:1 000 dilution, R&D System, Minneapolis, MN; Cat. No. AF496); (b) horseradish peroxidase (HRP)-conjugated mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (mAb, 1:2 000 dilution, KangChen Biotechnology Inc., Shanghai, China; Cat. No. KC-5G5); then washed and incubated for 1 h at room temperature with the corresponding secondary antibodies: (a) HRP-conjugated bovine anti-goat IgG (1:10 000, Jackson ImmunoResearch Lab, West Grove, PA; Cat. No. 805-035-180); (b) HRP-conjugated goat anti-mouse IgG (1:10 000, Jackson ImmunoResearch Laboratories, Cat. No. 115-035-146). Peroxidase activity was detected using the SuperSignal WestPico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and visualized and digitized with ImageQuant (LAS-4000, Fujifilm, Tokyo, Japan).

Immunofluorescence and Image Analysis

Cryosections (14 μm thick) were incubated with blocking solution containing 4% goat serum and 0.5% Triton X-100 in phosphate-buffered saline (pH 7.4) for 1 h, followed by overnight incubation with rabbit anti-GFP pAb (1:500, Invitrogen, Cat. No. A11122) at 4°C. Incubation with goat anti-rabbit IgG conjugated with Alex488 (1:5 000, Jackson ImmunoResearch Laboratories) was performed next. Sections were imaged using a light microscope (BX51; Olympus Optical, Tokyo, Japan) equipped with a cooled CCD camera (DP72, Olympus). Data were obtained and analyzed using Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD).

In utero Electroporation

In utero electroporation was carried out as described previously[30]. In brief, timed pregnant *EphA4*-floxed homozygotes and their wild-type counterparts (C57BL/6 strain) were anesthetized with pentobarbital sodium (Nembutal, Sigma) on embryonic day (E) 13.5, 14.5, or 15.5. DNA solution [(~3.0 μg/mL plasmids mixed with 0.03% Fast Green (Sigma-Aldrich)] was loaded into a glass micropipette, and ~1.0 μL of the solution was carefully injected into the lateral ventricle of embryos. Five 50-ms square-wave pulses (35 V in E13.5 and 45 V in E14.5/E15.5 embryos) were then delivered at 950-ms intervals using a CUY21 electroporator (Nepa Gene, Chiba, Japan). After electroporation, the peritoneum and skin were closed in separate layers with surgical sutures and the embryos were allowed to grow for 4 or 5 days in the uterus before paraformaldehyde fixation.

Statistical Analysis

Statistical analysis was performed using PRISM v4.0 (GraphPad Software, Inc.). Data are presented as mean ± SEM and were analyzed with two-way ANOVA followed by either Dunnett's test or Student-Newman-Keul's test (as a *post-hoc* test). Differences were considered significant when P values were <0.05.

RESULTS

Ephrin-B2 and Its Cognate Receptor EphA4 Are Expressed in Complementary and Overlapping Patterns in the Developing Neocortex

To determine whether ephrin-Bs and receptors play roles during neocortical development, we characterized their expression at a key stage of corticogenesis using ISH. This revealed that at E15.5, ephrin-B2 and EphA4 mRNA expression overlapped exclusively in the ventricular zone (VZ) and subventricular zone (SVZ). Interestingly, they were expressed in a complimentary pattern in a vast region of the neocortex: ephrin-B2 in the intermediate zone, and EphA4 in the cortical plate and subplate zone (Fig. 1A, B). In contrast, intense EphB1 hybridization signals were seen primarily in the cortical plate. This observation is similar to previous reports[25]. However, EphB2 hybridization signals were very low in the neocortex at this stage (Fig. 1). These results suggested that ephrin-B2/EphA4 signaling is involved in the regulation of corticogenesis.

Deletion of EphA4 Promotes Cortical Neuron Migration

To determine whether ephrin-B2/EphA4 interaction contributes to cortical neuron migration, the ephrin-B2 gene

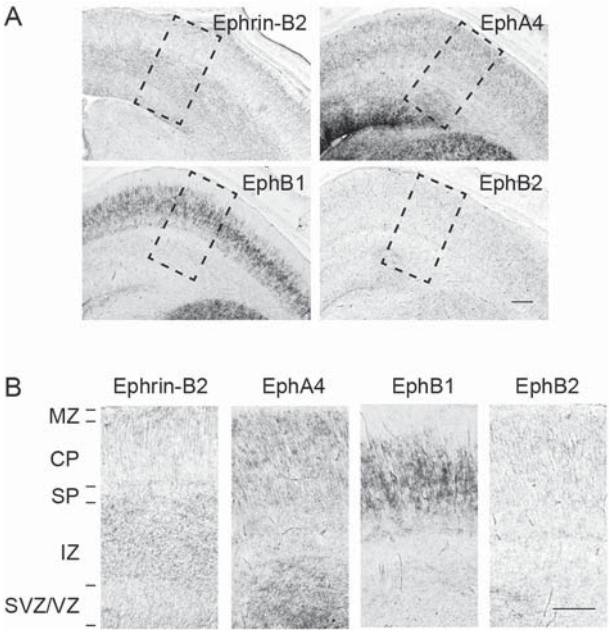

Fig. 1. Expression of ephrin-B2, EphA4, EphB1, and EphB2 in the developing neocortex. A: *In situ* hybridization in coronal sections of mouse brains at embryonic day 15.5. B: Enlarged view from each image in panel A. Scale bars, 100 μm. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

was knocked out by electroporating the vector pLVUbi-Cre-2A-GFP encoding Cre recombinase into the VZ of *EphA4*$^{flox/flox}$ mice, in which exon 3 of the *EphA4* gene was flanked by the corresponding recombination sequence[26]. Cre-mediated excision of exon 3 of the *EphA4* gene causes a frame-shift of the downstream coding sequence, resulting in a null allele[26]. Mouse embryos were electroporated at E15.5. The morphology and localization of GFP-marked cells in the dorsolateral neocortex (containing mainly the somatosensory cortex) were examined 4 days post-electroporation. The numbers of GFP-marked neurons were quantitated in the upper (II–III) and lower layers (IV–VI) of the neocortex, and the VZ/SVZ/white matter (WM) (Fig. 2B). We observed that *EphA4*-deficient cells had a morphology similar to normal cells, with one leading process extending toward the pia, typical of migrating neurons (Fig. 2A). However, deletion of the *EphA4* gene in *EphA4*$^{flox/flox}$ embryos by electroporation of Cre recombinase resulted in aberrantly faster migration of cortical neurons. Cell counts showed that the percentage of GFP-labeled cells in the upper layers in the *EphA4*-deficient neocortex (50%) was double of that of the control (25%). Consequently, GFP-labeled progenitor cells localized in the VZ/SVZ/WM of the *EphA4*-knockout (35%) mouse were fewer than those in the wild-type control (up to 50%) (Fig. 2). These results suggested that EphA4 is normally a repulsive molecule for the radial migration of cortical neurons.

Knockdown of Ephrin-B2 Has No Impact on Cortical Neuron Migration

Given that disruption of ephrin-B2/EphA4 forward signaling by EphA4 knockout promoted cortical neuron migration, we then investigated whether inhibition of ephrin-B2 reverse

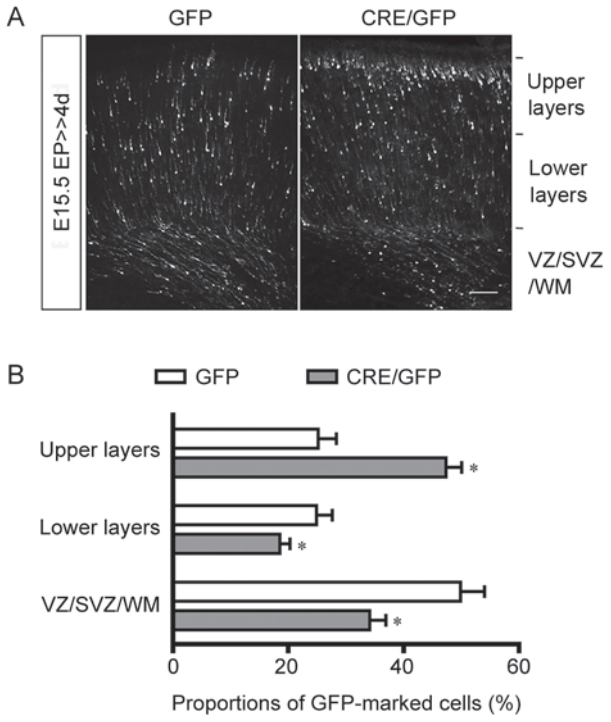

Fig. 2. Deletion of EphA4 receptors promotes cortical neuron migration. A: Photomicrographs showing the distribution of GFP-labeled cells in the neocortex of *EphA4*$^{flox/flox}$ mouse brain electroporated with a vector encoding GFP or Cre recombinase. The embryos were electroporated at E15.5 and maintained for 4 days. Scale bar, 100 μm. B: Quantification of the data shown in A. Upper layers represent II–III; lower layers represent IV–VI. VZ, ventricular zone; SVZ, subventricular zone; WM, white matter. Results are expressed as mean ± SEM of at least nine sections (three per embryo).

signaling also affects this process. We first evaluated the knockdown efficacy of ephrin-B2 shRNA by transfection of HEK293T cells with ephrin-B2 shRNA in combination with ephrin-B2 cDNA construct in ratios of 0:1, 1:1, and 2:1. Western blot analysis showed that the expression levels of exogenous ephrin-B2 were reduced significantly in a dose-dependent manner in the presence of ephrin-B2 shRNA (Fig. 3A). To further confirm the RNAi efficacy, the NIH3T3 cell line was transfected with either ephrin-B2 shRNA or nonsense shRNA. When shRNA transfection efficiency reached 80–90%, the endogenous ephrin-B2 mRNA level was reduced by ~80% (data not shown), accompanied by a significant reduction in the ephrin-B2 protein level (Fig. 3B). To unravel its role in the radial migration of cortical neurons, we evaluated the impact of reducing ephrin-B2 expression. Knockdown of ephrin-B2 in embryos by shRNA electroporation did not alter the morphology and migration of GFP-marked cells, as evidenced by the quantitative data, which showed no significant difference in the distribution of transfected cortical neurons between control and ephrin-B2 shRNA-transfected brains across each layer at different developmental stages (Fig. 3C, D). These results suggested that ephrin-B2 reverse signaling does not contribute to cortical neuron migration.

Ephrin-B2 Overexpression Does Not Perturb Cortical Neuron Migration

To further investigate whether ephrin-B2 plays a role in the regulation of cortical cell migration, we assessed the effect of its overexpression. The neocortex of embryos was electroporated with either ephrin-B2 or control at E15.5 and examined at P1. The percentage of GFP-labeled cells in each layer did not significantly differ between ephrin-B2-treated and control animals (Fig. 4), suggesting that ephrin-B2 overexpression does not perturb cortical neuron migration. Taken together, these data suggested that ephrin-B2/EphA4 reverse signaling is not involved in the radial migration of cortical neurons.

DISCUSSION

In the present study, we described the complementary expression patterns of ephrin-B2, EphA4, and EphB1 in

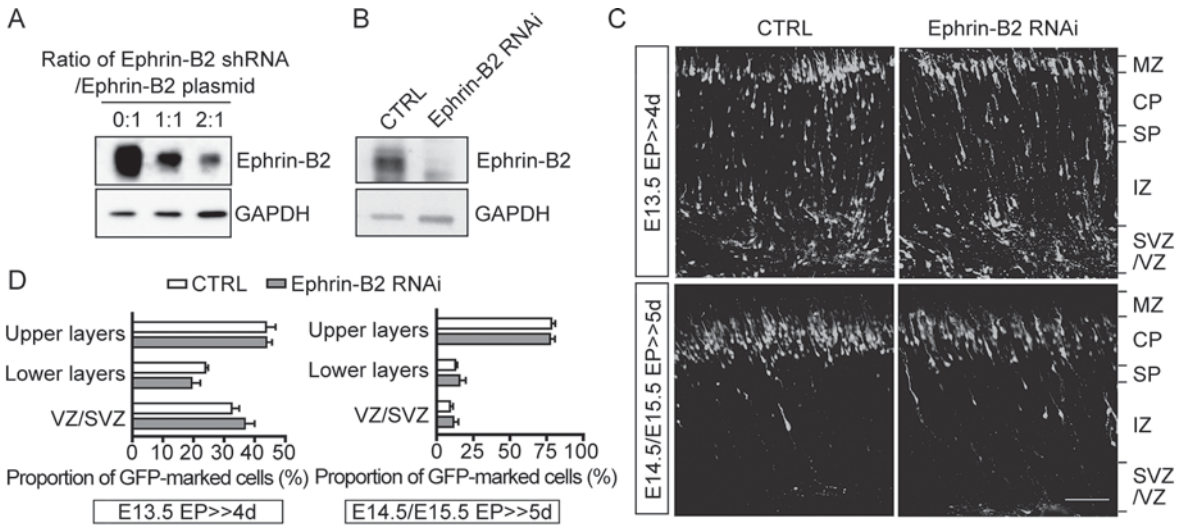

Fig. 3. Knockdown of ephrin-B2 does not impact cortical neuron migration. A: Verification of the knockdown efficacy of ephrin-B2 shRNA. HEK293 cells were co-transfected with ephrin-B2 specific shRNA and cDNA encoding ephrin-B2, followed by immunoblot analysis using ephrin-B2 antibody 72 h after transfection. B: Western blot analysis of cell lysates from cultured NIH3T3 cells transfected with either ephrin-B2 shRNA or control for 48 h. C: Photomicrographs showing GFP-labeled cells in coronal sections of mouse brain electroporated with ephrin-B2 RNAi or control plasmids at E13.5 or E14.5/E15.5. The embryos were allowed to develop for 4 or 5 days. Cortical laminar structures are indicated on the right. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Scale bar, 100 μm. D: Histograms showing the distribution of transfected cortical neurons in electroporated brains across different cortical zones at different developmental stages (upper layers contain MZ, CP, and SP; lower layers indicate intermediate zone). Results are expressed as mean ± SEM from at least nine sections from three mice.

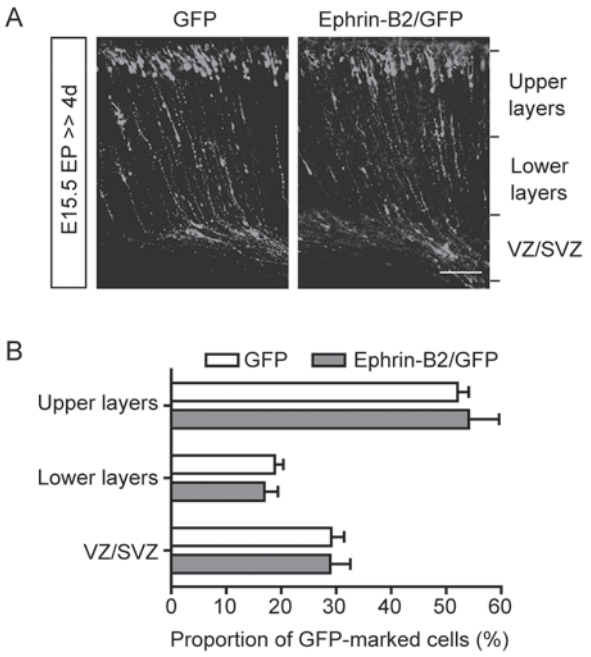

Fig. 4. Overexpression of ephrin-B2 does not alter cortical neuron migration. A: Immunofluorescent histochemical staining for GFP in coronal sections of mouse brains electroporated at E15.5 with either pLVUbi-2A-GFP or pLVUbi-ephrin-B2-2A-GFP constructs. Scale bar, 100 μm. B: Quantification of the data shown in A. Results are expressed as mean ± SEM. of at least nine sections from three animals.

the developing cerebral cortex. Their spatial expression patterns strongly suggested that cortical neuron migration is a highly complex but coordinated process. Deficiency of the neocortex-enriched gene *EphA4* resulted in aberrant migration of cortical neurons. This highlights the importance of this class of molecules and their interactions.

The migration of newborn neurons in the developing cerebral cortex is instructed by extracellular cues through activation of their guidance receptors and downstream signaling pathways[1]. Recent studies have demonstrated that Eph receptor tyrosine kinases and their ephrin ligands are present in the neocortex and have implied that their bi-directional signals regulate various aspects of progenitor maintenance and neuron migration[24,25,31,32]. However, the role of ephrin-B2/EphA4 signaling had not yet been studied. Here, we found that ephrin-B2/EphA4 forward signaling exerts repulsive effects on cortical neuron migration. Thus we identified new important players involved in the regulation of radial migration of cortical neurons during corticogenesis.

Our study provided evidence that EphA4-mediated forward signaling is required for cortical neuron migration. How might EphA4 function in modulating this migration? A likely scenario is that during cortical neurogenesis, ephrin-B2, which is predominantly expressed in the intermediate zone (Fig. 1), may act as a migration stop signal to the EphA4-expressing neural progenitor cells on their way from the VZ/SVZ to the cortical plate. Thus, those neurons that express lower levels of EphA4 have higher priority to pass through the “ephrin-B2-enriched barrier” over neurons with higher levels of EphA4. These mechanisms could ensure that neurons migrate in an orderly fashion. Indeed, we found that loss of EphA4 forward signaling in the neocortex disrupted the barrier function of ephrin-B2, resulting in faster neuron migration. Our functional assays of ephrin-B2/EphA4 signaling showed that, rather than its reverse signaling, the forward signaling has repulsive effects on migration, thus demonstrating novel molecular mechanisms regulating the radial migration of cortical neurons during corticogenesis.

We showed that alterations in ephrin-B2 expression levels did not affect cortical neuron migration. This may be attributed to several factors. Multiple ephrin members may work synergistically to regulate migration. It has been reported that *ephrin-B* gene depletion has a profound impact on the axonal arborization of Purkinje cells in the cerebellum[24]. Moreover, heterozygous *Reln*$^{+/-}$ does not have any evident defects, whereas the compound mouse mutant *Reln*$^{+/-}$*; ephrin-B2*$^{-/-}$ and triple *ephrin-B1, -B2, -B3* knockouts show neuronal migration defects in the neocortex, hippocampus, and cerebellum. These data suggested an intrafamily compensatory mechanism among different ephrin-Bs. Thus, it is likely that ephrin-B2 works synergistically with other regulators in cortical neuron migration, and changing its expression alone is not sufficient to disrupt this process.

In conclusion, we delineated the expression pattern of EphA4, EphB1, EphB2, and ephrin-B2 in the developing neocortex. Our functional study suggested that EphA4 plays an important role in the regulation of cortical neuron migration. Further characterization of the molecular mechanisms underlying EphA4-controlled cell migration may expand our understanding of the organization of the developing cerebral cortex. These studies also provide

insights into how disrupted cortical neuron migration may contribute to brain malfunctions.

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