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Pax6-dependent boundary defines alignment of migrating olfactory cortex neurons via the repulsive activity of ephrin A5

Tadashi Nomura[1], Johan Holmberg[2], Jonas Frisen[2] and Noriko Osumi[1,3,*]

Neuronal migration is a prerequisite event for the establishment of highly ordered neuronal circuits in the developing brain. Here, we report *Pax6*-dependent alignment of the olfactory cortex neurons in the developing telencephalon. These neurons were generated in the dorsal part of telencephalon, migrated ventrally and stopped at the pallium-subpallium boundary (PSB). In *Pax6* mutant rat embryos, however, these neurons invaded the ventral part of the telencephalon by crossing the PSB. Ephrin A5, one of the ligands for EphA receptors, was specifically expressed in the ventral part of the telencephalon, and its expression level was markedly reduced in the *Pax6* mutant. Gain- and loss-of-function studies of ephrin A5 indicated that ephrin A5 plays an important role in the alignment of olfactory cortex neurons at the PSB. Our results suggest that Pax6-regulated ephrin A5 acts as a repulsive molecule for olfactory cortex neurons in the developing telencephalon.

KEY WORDS: Neural patterning, Neuronal migration, Telencephalon, Pax6, Ephrin A5

INTRODUCTION

The mammalian telencephalon is one of the most intricate structures in the vertebrate brain, consisting of large numbers of neurons that form highly ordered and complex neuronal networks. At early embryonic stages, the telencephalon can be divided into two subdomains: pallium and subpallium. The pallium consists of four major subdivisions, including the medial, dorsal, lateral and ventral pallium, and each pallial region gives rise to specific cortical structures, such as the hippocampus, neocortex, olfactory cortex and amygdala/endopiriform nucleus (Puelles et al., 2000). Furthermore, specific sets of transcription factors are expressed in regionally restricted manners in the pallial or subpallial regions (Puelles et al., 2000). Appropriate patterning of the telencephalon is required for not only the production of specific sets of neurons, but also the establishment of stereotypic patterns of axonal projection and neuronal migration (reviewed by Wilson and Rubenstein, 2000).

The olfactory cortex is the three-layered simple cortex that occupies at the surface of the ventrolateral part of the telencephalon. The olfactory cortex consists of distinct areas such as the anterior olfactory nucleus, the piriform cortex and olfactory tubercle, and functions as the secondary processing center of odor information by receiving axons from the olfactory bulb. Previous studies have indicated that the most of the olfactory cortex neurons are derived from the lateral and ventral pallium (De Carlos et al., 1996; Yun et al., 2001). They are born at early embryonic stages (E12-15 in rat embryos) (Bayer, 1986; Valverde and Santacana, 1994) and radially migrate towards the surface of the ventrolateral telencephalon (De Carlos et al., 1996). However, recent studies demonstrated that a specific neuronal population that are born at the medial/dorsal pallium migrate ventrally towards the olfactory cortex (Tomioka et al., 2000; Jimenez et al., 2002). These neurons are identified by a specific antigen expression (lot antigen) and thought to be guide-post neurons for the lateral olfactory tract (Sato et al., 1998). Therefore, neurons derived from the various pallial regions contribute to the olfactory cortex via distinct migratory pathways.

Pax6 is a transcriptional factor that plays key roles in the developing central nervous system (reviewed by Osumi, 2001; Simpson and Price, 2002). In the developing telencephalon, *Pax6* is strongly expressed in the pallium and governs the anteroposterior and dorsoventral patterning of the telencephalon (Bishop et al., 2000; Bishop et al., 2002; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Muzio et al., 2002). Previous studies have indicated that *Pax6* function is important for establishment and/or maintenance of the pallial-subpallial boundary (PSB) (Stoykova et al., 2000; Toresson et al., 2000; Kim et al., 2001; Yun et al., 2001; Hirata et al., 2002). Impaired PSB formation in the *Pax6* mutant might result in increased tangential migration of subpallial cells into the cortex, as well as altered routing of the lateral cortical stream towards the amygdala and lateral cortex (Brunjes et al., 1998; Chapouton et al., 1999; Tole et al., 2005). We have reported previously that olfactory bulb neurons misrouted caudally in the *Pax6* mutant telencephalon (Nomura and Osumi, 2004). These lines of evidences suggest that *Pax6* controls neuronal migration patterns by direct and/or indirect regulation of expression of specific guidance molecule(s), or their signaling cascade components, in the developing telencephalon.

Here, we report that *Pax6* regulates the tangential migration of a subset of the olfactory cortex neurons. A population of olfactory cortex neurons is established at the dorsal part of the telencephalon at early embryonic stages. These neurons migrate ventrally on the telencephalon and align at the PSB, and they contribute in the formation of the future primary olfactory cortex. In the *Pax6* mutant, however, these neurons never stopped at the PSB but rather invaded the ventral part of the telencephalon. We found that ephrin A5 has a crucial role for the alignment of these neurons at the PSB. Gain- and loss-of-function studies of ephrin A5 revealed that it is necessary and sufficient for stopping these neurons at the PSB. The results indicate that *Pax6* controls the alignment of olfactory cortex neurons by regulating ephrin A5 expression in the developing telencephalon.

[1]Division of Developmental Neuroscience, Center for Translational and Advanced Animal Research (CTTAR), Tohoku University School of Medicine, 2-1, Seiryo-machi, Aoba-ku, 980-8575, Japan. [2]Department of Cell and Molecular Biology, Karolinska Institute, SE-171 77 Stockholm, Sweden. [3]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, 332-0012, Japan.

*Author for correspondence (e-mail: osumi@mail.tains.tohoku.ac.jp)

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MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley (SD) rats were purchased from Japan Charles River (Tokyo, Japan). Heterozygous *Pax6* mutants (*rSey*^{2}) (Osumi et al., 1997), GFP-transgenic rats [TgN(act-EGFP)Osb4] (Ito et al., 2001) on SD background rats and ephrin A5 targeting mice (Frisen et al., 1998) were intercrossed in our laboratories. The midday of finding a virginal plug was considered embryonic day 0.5 (E0.5). All experimental procedures described in this study were approved by the Committee for Animal Experiment of Tohoku University Graduate School of Medicine.

Isolation of rat ephrin A5 cDNA

Full-length rat ephrin A5 complementary DNA (cDNA) was amplified by PCR from cDNA of E12.5 rat embryonic heads using specific primers for mice ephrin A5 (5′-ATG TTG CAC GTG GAG ATG TTG ACG CTC G-3′ and 5′-GTT GTT GCT TAG AAA TCA GG).

Cell labeling and gene transfer in cultured embryos

The experimental procedures for whole-embryo culture and electroporation have been described in detail previously (Osumi and Inoue, 2001; Takahashi et al., 2002). A plasmid solution containing pCAX-ephrin A5, pCAX-GFP, or pCAX-Pax6 (Takahashi and Osumi, 2002) was microinjected into the lateral ventricle of cultured embryos and electroporated using an electroporator (CUY21, Neppa Gene). The telencephalon organ culture was performed as described previously (Nomura and Osumi, 2004).

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were transcribed using DIG RNA labeling kit (Roche Molecular Systems, NJ) from *Pax6, Dlx1,* ephrin A1 (I.M.A.G.E. 3484518)*, A2, A3* (I.M.A.G.E. 4397263)*, A5, Epha7* (I.M.A.G.E. 3991628) cDNAs that were subcloned into pBluescript. The hybridization procedures have been described previously (Osumi et al., 1997).

Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and sectioned (12 μm) with cryostat (CM 3050, LEICA). Sections were incubated overnight with anti-Pax6 (Inoue et al., 2000), anti-β-III tubulin, anti-calretinin, anti-glutamate (Chemicon), anti-calbindin (Chemicon), anti-reelin (Ogawa et al., 1995) or anti-EphA4 (Takemoto et al., 2002), anti-reticulon-1 (Hirata et al., 2002) antibodies. Cy3-conjugating secondary antibodies (Jackson) were applied to the sections. After rigorous washing with TBST, the sections were examined by fluorescent microscopy (Axioplan2, Zeiss) and images were captured with cooled CCD camera (Roper Scientific).

Birth date analysis of neurons by BrdU pulse labeling

To perform 5-bromo-2′-deoxyuridine (BrdU) pulse chasing, 50 mg/kg of BrdU solution was intraperitoneally injected into pregnant rats/mice. Sections were treated with 2 N HCl at 37°C for 10 minutes, and incubated with anti-BrdU antibody (BD Biosciences).

Functional blocking of Eph/ephrin and detection of Eph by receptor-ligand binding system

To block endogenous EphA/ephrin A binding, 10 μg/ml of the soluble form EphA3-Fc fusion protein (R&D Systems) was applied to the telencephalon organotypic culture medium (Dufour et al., 2003). Detection of endogenous EphA receptors in the developing cortex was performed as previously described (Flenniken et al., 1996).

Cell counts and statistical analysis

To quantify the number of BrdU-positive cells in the LOT area and olfactory tubercle, we chose six serial sections rostral to the anterior commissure, and captured images with 10× objective lens. The average of total number of BrdU-labeled cells in LOT area was 158.0±10.5 (wild type rat, n=3), 77.0±11.53 (*Pax6* mutant rat, n=3), 206.6±15.5 (ephrin A5$^{+/-}$ mouse, n=3) and 117.3±19.2 (ephrin A5$^{-/-}$ mouse, n=3). The average of total number of BrdU-positive cells in the olfactory tubercle was 69.0±12.9 (wild type rat, n=3), 261.0±48.2 (*Pax6* mutant rat, n=3), 98.6±49.9 (ephrin A5$^{+/-}$ mouse, n=3), 184.0±20.0 (ephrin A5$^{-/-}$ mouse, n=3). Statistical analysis was performed in each experiment by unpaired Student's t-test.

RESULTS

Alteration of the normal dorsoventral migration pattern of olfactory cortex neurons in *Pax6* mutants

In the early stages of the developing telencephalon, neurons that originate from the dorsal part migrate ventrally and align at the future olfactory cortex (Tomioka et al., 2000; Jimenez et al., 2002). This pattern suggests the existence of guidance cue(s) in the early stages of developing telencephalon to direct these neurons to the specific position. To label these neurons specifically, we electroporated a *green fluorescent protein* (*GFP*)-expression vector into the developing telencephalon and examined the migration pattern of the labeled neurons in a whole-embryo culture system (Fig. 1A). The dorsal part of the telencephalon of E11.75 rat embryos (corresponding to E9.75 mouse embryos) was labeled and cultured for 48 hours in a whole-embryo culture system. A large number of GFP-labeled cells were found to have migrated from the electroporated area towards the ventral part of the telencephalon (n=10, Fig. 1B,C,C′). The migration process of these neurons stopped at the PSB, the area corresponding to the future olfactory cortex area (Fig. 1C′, Fig. 2C). To characterize the GFP-labeled migrating cells, we performed immunohistochemistry using several markers for different types of neurons. Pax6 was expressed in neuroepithelial cells found in the dorsal part of the telencephalon, including the *GFP*-plasmid introduced area, but not detected in the GFP-positive migrating neurons (Fig. 1C,C′). GFP-labeled cells expressed type III β-tubulin, which is an early born neuronal maker (Fig. 1D,D′′). As calbindin (CB), reelin (Rln) and glutamate (Glu) are expressed in different groups of early-born neurons (Meyer et al., 1998), we tested whether the GFP-positive neurons also expressed these markers. Immunostaining with specific antibodies revealed that the GFP-positive neurons expressed CB, Rln and Glu (Fig. 1E-F′′; see Fig. S1A-A′′ in the supplementary material). To identify the birth date of the GFP-positive neurons, we performed BrdU pulse labeling in whole-embryo culture. When we added BrdU into the culture medium at E11.75, the most of the GFP-positive neurons incorporated BrdU (over 75%; see Fig. S1B-B′′ in the supplementary material), indicating that these neurons are born at around E11.75.

Next, we compared the migration pattern of these neurons in wild-type and *Pax6* mutant embryos. When we introduced *GFP*-expression vector into the dorsal part of the telencephalon in wild-type embryos, labeled neurons migrated ventrally, and stopped at the PSB (n=10, Fig. 2A). However, when we labeled the dorsal part of the telencephalon in the *Pax6* mutant embryos, labeled neurons migrated ventrally, passing through the PSB, and they also migrated into the ventral part of the mutant telencephalon (n=10, Fig. 2B). In the wild-type embryo, the PSB corresponded to the boundary of *Dlx1* expression and high *Pax6* expression (Fig. 2D and E), as shown previously in the mouse embryo (Toresson et al., 2000; Yun et al., 2001). In the *Pax6* mutant rat embryo, a large number of the GFP-labeled neurons invaded the *Dlx1*-positive area (Fig. 2F,H), which was never observed in the wild-type embryo. Considered altogether, our results indicate impaired migration patterns of the olfactory cortex neurons in the *Pax6* mutant embryo; the neurons derived from the dorsal part of the telencephalon migrate ventrally, as in the case of the wild type, but these ventrally migrating neurons do not align at the PSB. The same defect was also observed in the *Pax6* mouse embryo (data not shown).

Abnormal migration of *Pax6* mutant neurons is due to non-cell-autonomous defects in migrating cells

Although Pax6 was not expressed in the migrating neurons, it was expressed in the entire part of the pallial neuroepithelial cells, including precursors of olfactory cortex neurons and their migratory

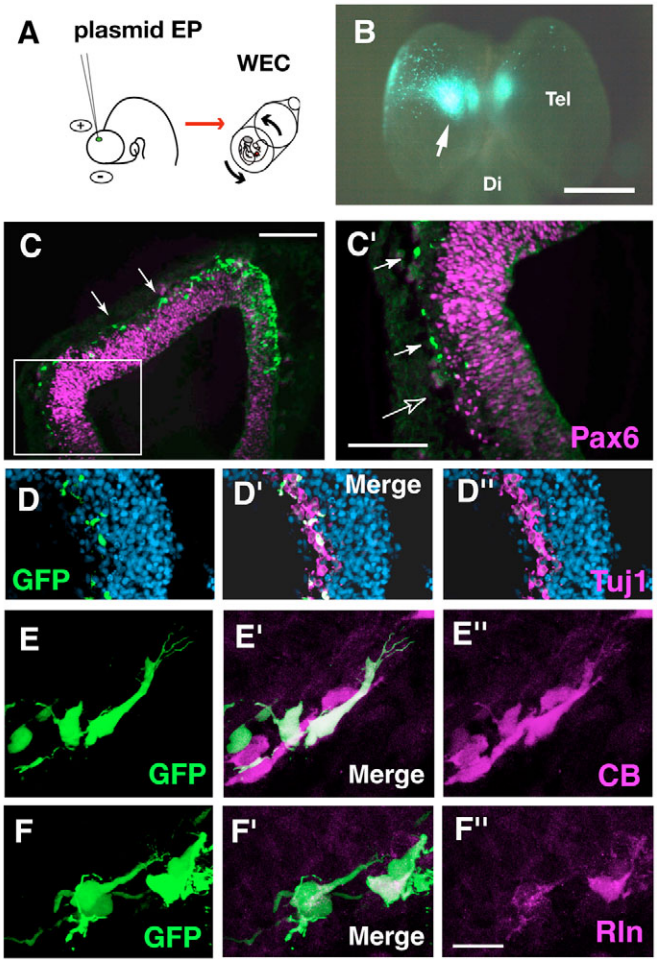

Fig. 1. Migration pattern and characteristics of olfactory cortex neurons. (**A**,**B**) The dorsal part of the telencephalon in E11.75 rat embryos is labeled by electroporation of *GFP*-expressing vector (arrow in B). After electroporation, the embryos were cultured for 48 hours in whole-embryo culture system. (**C**,C′) During WEC, GFP-labeled cells migrate dorsoventrally in the telencephalon (arrows in C,C′), and stop at the pallium-subpallium boundary (PSB, open arrow in C′). Immunostaining with anti-Pax6 antibody indicates that Pax6 is not detected in the GFP-positive migrating cells (arrows in C,C′). Inset in C is at higher magnification in C′. (**D-F**′′) Immunostaining with anti-type III β-tubulin (Tuj1, D-D′′), anti-calbindin (CB, E-E′′) and anti-reelin (Rln, F-F′′) antibodies of GFP-labeled telencephalon. GFP-positive migrating neurons (green cells in D-F) are positive for β-tubulin (D-D′′), CB (E-E′′) and Rln (F-F′′). Di, diencephalons; Tel, telencephalon. Scale bars: 500 μm in B; 100 μm in C′; 20 μm in F.

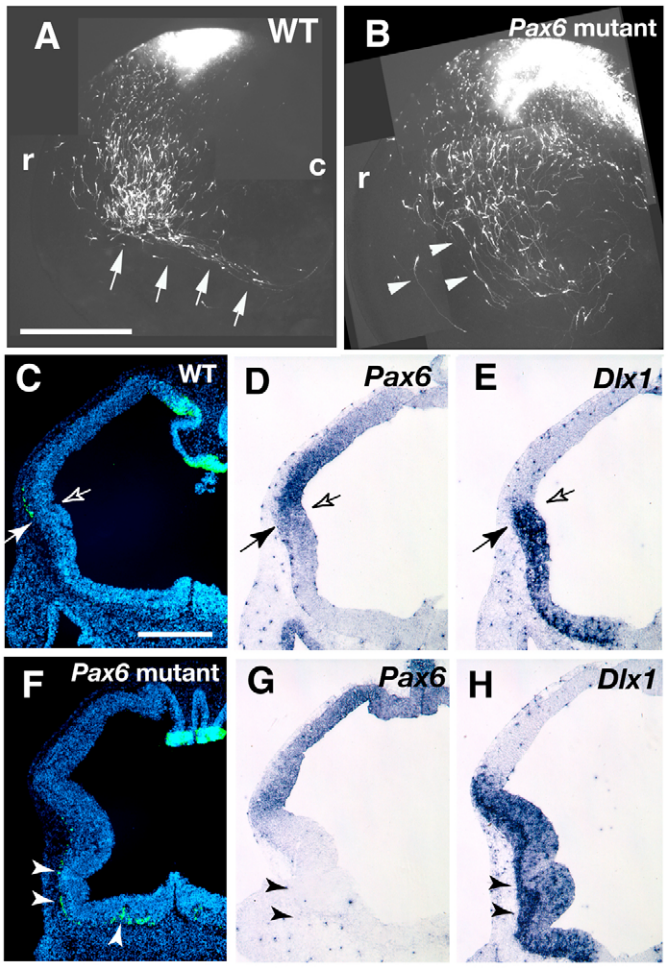

Fig. 2. Abnormal migration of olfactory cortex neurons in *Pax6* mutant telencephalon. Lateral views (**A**,**B**) and coronal sections (**C-H**) of the wild-type (A,C-E) and *Pax6* mutant (B,F-H) telencephalon with GFP-labeled olfactory cortex neurons. Olfactory cortex neurons stop and align at the PSB in the wild type (arrows in A,C-E), whereas these neurons invaded the *Dlx1*-positive more ventral part of the *Pax6* mutant telencephalon (arrowhead in B,F-H). r, rostral; c, caudal. Open arrows indicate the PSB. Scale bars: 500 μm.

pathway (Fig. 1C,C′). These findings suggest that the migratory defect in the mutant could be due to cell-autonomous or non-cell-autonomous defects of migrating cells. To investigate this issue further, we performed cell transplantation experiments between the wild-type and *Pax6* mutant telencephalons and examined the migratory behavior of the implanted cells in the cultured embryos (Fig. 3A). We prepared donor cells from *GFP*-transgenic rats using the method described previously (Nomura and Osumi, 2004). Transplantation of cells isolated from the dorsal part of the telencephalon of the *Pax6* mutant into the wild-type telencephalon, the GFP-positive transplanted cells migrated ventrally and stopped at the PSB (*n*=3), like wild-type cells in the wild-type background (Fig. 3B,B′,D; see Fig. S2A,B in the supplementary material; data not shown). In the opposite experiment, where the wild-type cells were transplanted into the *Pax6* mutant telencephalon, the GFP-positive donor cells migrated ventrally and further invaded the most ventral part of the telencephalon without stopping at the PSB (*n*=3), like mutant cells in the mutant background (Fig. 3C-D, see Fig. S2C,D in the supplementary material; data not shown). These findings indicate that the abnormal routing of the neurons in the *Pax6* mutant is due to non-cell-autonomous defects of the migrating neurons in the mutant telencephalon.

Reduced ephrin A5 expression in the ventral telencephalon in *Pax6* mutants

To identify responsible gene(s) that controls the ventral migration of the olfactory cortex neurons downstream to Pax6, we investigated expression patterns of a variety of genes that encode attractive/repulsive molecules for neuronal migration and axon guidance. Especially, we focused on Eph/ephrin signals because

previous studies indicate that Eph/ephrins act as repulsive molecules for axons via a contact-dependent manner (reviewed by Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001; Poliakov et al., 2004).

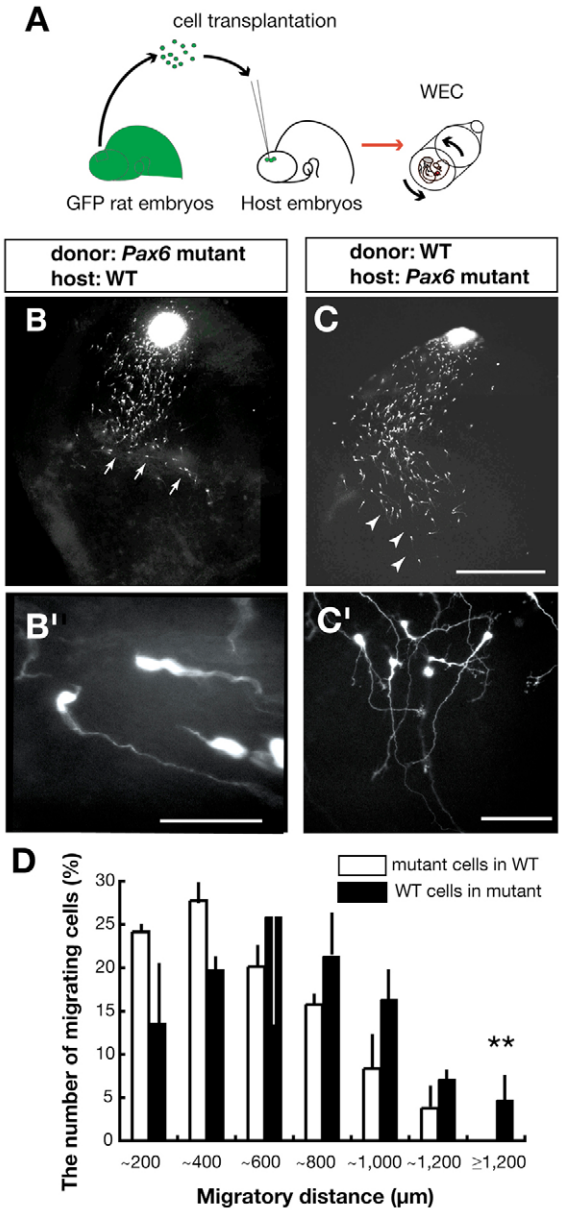

Fig. 3. Abnormal migration in *Pax6* mutants is due to non-cell autonomous defects of migrating cells. (**A**) Experimental procedures for the cell transplantation between wild type and mutants using whole-embryo culture. (**B**,**C**) Lateral views of the wild-type (B) and *Pax6* mutant telencephalon (C) after 48 hours in whole-embryo culture. The *Pax6* mutant-derived cells stopped at the PSB (arrows in B,B′), whereas wild-type-derived cells invaded the ventral part of the mutant telencephalon (arrowhead in C,C′). (**D**) Comparison of migratory distance of the GFP-positive cells in the wild type and *Pax6* mutant telencephalon. The number of GFP-positive cells was calculated at each distance and quantified as a percentage of total number of migrating cells. In the *Pax6* mutant telencephalon, the number of GFP-positive cells derived from wild type was significantly increased in the area over 1200 μm distant from the injection point. Data are presented as percentage of the labeled cells in each area against the total number of labeled cells (mean±s.d. of three samples in each group). $^{**}P<0.01$. Scale bars: 500 μm in B,C; 50 μm in B′,C′.

First we examined expression patterns of ephrin A and B ligands in the wild-type and *Pax6* mutant embryos. Among the ephrin ligands examined (ephrin A1, A2, A3, A5 and B1), only ephrin A5 was specifically expressed in the E11.5 wild-type telencephalon (Fig. 4A), as described previously in the mouse embryo (Mackarehtschian et al., 1999). At E11.5, ephrin A5 was broadly expressed in the telencephalon, and become restricted to the ventral telencephalon by E12.5 (Fig. 4A,C). Interestingly, the expression of ephrin A5 overlapped with that of *Pax6* at this stage, as *Pax6* was expressed in the entire part of the telencephalon at early embryonic stages (Fig. 4A,B) (Corbin et al., 2003). At E12.5, ephrin A5 was still expressed in the ventral part of the telencephalon (Fig. 4C). The ephrin A5-positive region corresponded to the *Dlx1*-positive region in which the ventrally migrating neurons did not invade (Fig. 4C,D; see Fig. S3A-C in the supplementary material). By contrast, the expression level of ephrin A5 in the ventral part of the telencephalon was markedly reduced in the *Pax6* mutant (Fig. 4E-G). The reduction of ephrin A5 expression in the mutant was evident at least from E11.5, and the low level of expression was persistently noted to E12.5 (Fig. 4E,G). GFP-positive cells distributed in the ventral part of the *Pax6* mutant telencephalon, in which ephrin A5 expression was decreased (Fig. 4H).

As the expression of *Pax6* and ephrin A5 overlapped at early embryonic stages, we speculated that *Pax6* function was required for the induction and/or maintenance of ephrin A5 expression at the ventrolateral telencephalon. To test this hypothesis, we introduced *Pax6*-expression vector into the ventrolateral part of the *Pax6* mutant telencephalon and then examined the expression of ephrin A5. After 24 hours of electroporation, strong ephrin A5 expression was induced in the ventrolateral telencephalon of *Pax6* mutant by *Pax6* overexpression (see Fig. S4A,B in the supplementary material). These results suggest that ephrin A5 expression in the ventrolateral telencephalon is induced and/or maintained by *Pax6* function at early embryonic stages.

Next, we examined the expression of putative receptors for ephrin A5 using ligand/receptor-detection system (Flennikin et al., 1996) and immunohistochemistry. Detection of receptors with ephrin A5-Fc proteins indicated the existence of putative receptors that can bind to ephrin A5 in the telencephalon at E12.5 (Fig. 4I). As it has been shown that EphA4 was expressed in the early stages of the telencephalon (Greferath et al., 2002), we examined the distribution of EphA4 protein with a specific antibody in embryos with GFP-labeled olfactory cortex neurons. EphA4 was expressed in the dorsal and ventral parts of the wild-type telencephalon at E12.5 (Fig. 4K). Furthermore, EphA4 was expressed in the GFP-positive neurons, which was revealed by immunostaining of dissociated cell cultures (Fig. 4L,M). Expression of EphA4 was not altered in the *Pax6* mutant telencephalon (data not shown). Another Eph member, *Epha7*, was also expressed at the dorsal part of the telencephalon, but not in the GFP-labeled neurons (data not shown). Taken together, the EphA/ephrin A-dependent signal might regulate the termination of olfactory cortex neurons at the PSB.

Blockade of EphA-ephrin-A interactions mimicked the *Pax6* mutant phenotype

To address whether EphA/ephrin A-dependent signal is responsible for the neuronal migration, we tested the effects of several functional blocking antibodies and Fc-fused proteins on the ventral migration of the neurons. After labeling the dorsal part of the telencephalon in the wild-type embryos by electroporation of the *GFP*-expression vector, and following culture for 24 hours in the WEC, the cerebral hemisphere was isolated to culture in a medium containing several

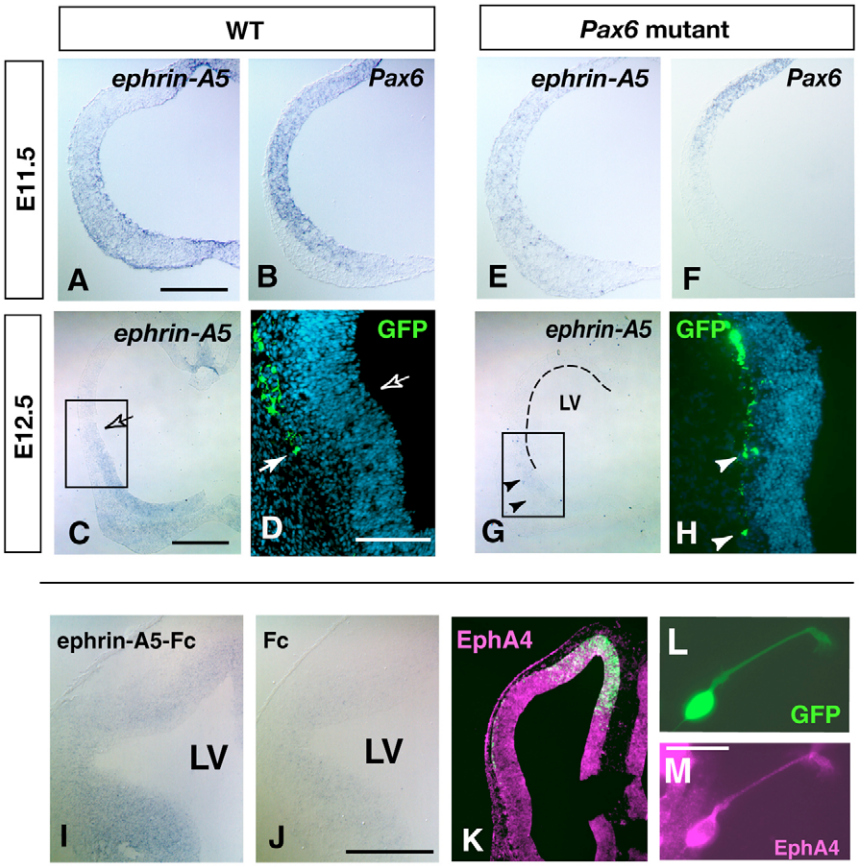

Fig. 4. Downregulation of ephrin A5 expression in the *Pax6* mutant telencephalon. (**A-H**) Expression patterns of ephrin A5 (A,C,E,G) and *Pax6* (B,F) in wild type (A-D) and *Pax6* mutant (E-H) telencephalon. *In situ* hybridization was performed at E11.5 (A,B,E,F) and E12.5 (C,D,G,H) telencephalon. In wild type, ephrin A5 is expressed at the ventral part of the telencephalon (A,C). GFP-labeled cells stop at the PSB, corresponding to the expression border of ephrin A5 (white arrow in D). Open arrows in C,D indicate the PBS. In the *Pax6* mutant, expression of ephrin A5 is reduced in the ventral part of the telencephalon (E,G), where the GFP-labeled cells invaded (arrowhead in **H**). (**I-M**) EphA receptor expression in the wild type E12.5 telencephalon. Expression of Eph receptors is detected by incubation with soluble-ephrin A5-Fc protein (I). No signal is detected with Fc control protein (J). (K-M) Immunohistochemistry with anti-EphA4 antibody in the embryo labeled by GFP plasmid electroporation. EphA4 is expressed in the dorsal and ventral parts of the telencephalon (K), including GFP-positive cells (L,M). LV, lateral ventricle. Scale bars: 500 μm in A,C,D,J; 200 μm in D; 20 μm in M.

agents (Fig. 5A). These experiments identified EphA/ephrin A signaling as the possible guidance cue for the olfactory cortex neurons. Thus, when the wild-type brains were cultured with 10 μg/ml of Fc protein, the GFP-positive neurons derived from the dorsal part of the telencephalon migrated ventrally and stopped at the PSB (n=4, Fig. 5B,D). By contrast, when the wild-type brains were cultured in a medium containing 10 μg/ml of EphA3-Fc proteins, which inhibits EphA/ephrin A signal by competing with the endogenous EphA proteins (Ciossek et al., 1998), the olfactory cortex neurons did not stop at the PSB but rather continued to migrate into the ventral part of the telencephalon (n=4, Fig. 5C,D). The ventral migration of the olfactory cortex neurons was not altered by adding 10 μg/ml of EphB1-Fc protein into the medium (n=4, data not shown). As EphA3 receptor is known to bind specifically to ephrin A ligands (O'Leary and Wilkinson, 1999), we considered that blockade of EphA/ephrin A interactions resulted in alteration of the migratory pattern of olfactory cortex neurons in the wild-type telencephalon.

Alteration of migratory direction of olfactory cortex neurons by gain- and loss-of-function of ephrin A5 in the telencephalon

Together with the expression pattern of ephrin A5 in the developing telencephalon, the functional blocking analysis in the wild-type brains implies that ephrin A5 in the ventral part of the telencephalon might prevent the invasion of olfactory cortex neurons into the ventral part of the telencephalon, and that the impaired alignment of the neurons in the *Pax6* mutant is due to low expression of ephrin A5. To test the above possibility, we first examined the neuronal migration in embryos in which ephrin A5 was misexpressed in the migratory route of olfactory cortex neurons. We injected DiI solution into the dorsal part of the telencephalon to label the olfactory cortex neurons, and then we electroporated an ephrin A5 expression vector along with the *GFP* vector into the lateral part of the telencephalon, 12 hours after DiI injection (corresponding to E12.25) (Fig. 6A). As a control experiment, we electroporated *GFP* expression vector alone into the migratory pathway of the DiI-labeled ventrally migrating neurons. In this case, the olfactory cortex neurons crossed the GFP-positive area without any stopping (n=5, Fig. 6B and B′). However, when the ephrin A5 expression vector was co-electroporated with *GFP* vector in the lateral part of the wild-type telencephalon, the DiI-labeled neurons did not invade the ephrin A5-expressing area and stopped at the edge of ephrin A5-positive areas (n=4, Fig. 6C). Next, we tested whether electroporation of ephrin A5 expression vector restores the *Pax6* mutant phenotype of the olfactory cortex neurons. Misexpression of ephrin A5 specifically in the ventral part of the telencephalon resulted in termination of ventral migration of the neurons at the border of the ephrin A5-positive area and lack of invasion to the ventral part of the telencephalon (n=5, Fig. 6D,D′). Thus, the neuronal migration pattern of the olfactory cortex neurons in the *Pax6* mutant was rescued by misexpression of ephrin A5. Taken together, these results indicate that ephrin A5 can potentially terminate olfactory cortex neuron migration at its expression boundary, i.e., the PSB.

To investigate further the role of ephrin A5 as the molecule responsible for stopping olfactory cortex neurons at the PSB, we examined the migratory behavior of these neurons in ephrin A5-deficient mice (Frisen et al., 1998) and compared the phenotype of the mice with that of *Pax6* mutant rat embryos. For this purpose, we labeled the dorsal most of the telencephalon in wild-

type mice at E9.75 (corresponding to E11.75 of rat embryos) by *GFP* electroporation. The labeled neurons migrated ventrally, and stopped at the PSB in 48-hour whole embryo culture, as in the case of wild-type rat embryos (n=4, Fig. 7A,C). However, labeling the same region of ephrin A5-null mutant mice telencephalon showed crossing of olfactory cortex neurons through the PSB and their invasion of the ventral part of the telencephalon (n=4, Fig. 7B,C). This abnormal migration pattern was similar to that of the *Pax6* mutant rat and mouse embryos (Fig. 2B; data not shown). Together with the gain-of-function studies, these results indicate that ephrin A5 function is necessary and capable of altering the migration route, i.e. stopping PSB crossing, of olfactory cortex neurons.

Altered number of early-born olfactory cortex neurons in both *Pax6* and ephrin A5 mutants

As similar migratory defects of the olfactory cortex neurons were observed in both *Pax6* and ephrin A5 mutant embryos, we expected that these two mutants had similar abnormalities in olfactory cortex development at later stages. As most of GFP-positive ventrally migrating neurons are born at E11.75 (as shown in Fig. S1A-A′′ in the supplementary material), we traced these neurons by BrdU pulse chasing, and compared the distribution of these neurons at later embryonic stages. Surprisingly, when we performed BrdU pulse labeling in E11.75 wild-type by intraperitoneally injection, most of the BrdU incorporated cells specifically located at the olfactory cortex, especially accumulated

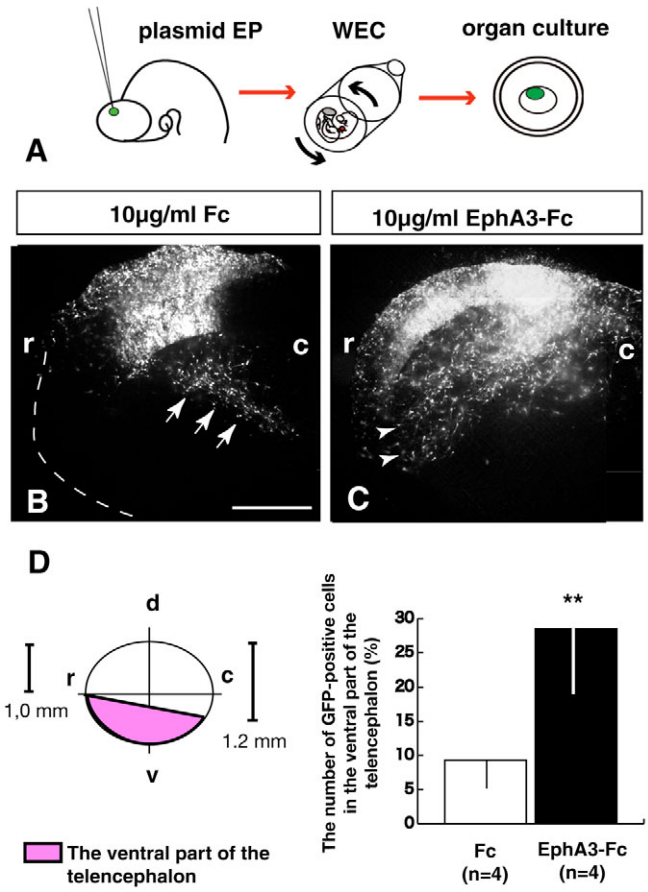

Fig. 5. Blockade of EphA/ephrin A interaction induced abnormal migration of olfactory cortex neurons. (**A**) Experimental procedures for EphA/ephrin A signaling blockade. (**B**,**C**) Migration patterns of GFP-positive cells in media containing Fc (B) or EphA3 (C). In Fc-containing media, the GFP-labeled neurons stopped at the PSB (arrows in B), whereas in the Eph-A3-Fc-containing media, the GFP-labeled neurons crossed the PSB and invaded the ventral part of the telencephalon (arrowheads in C). (**D**) Comparison of the number of GFP-positive cells in the ventral part of the telencephalon (the purple area) in Fc- and EphA3-treated telencephalon. Because in control (Fc treated) samples most of the GFP-positive cells aligned at 1.0-1.2 mm distant from the dorsal margin of the telencephalon, we marked this point as 'prospective PSB' and compared with the number of GFP-positive cells ventral of this point. Data are presented as percentage of the labeled cells in the ventral telencephalon against the total number of labeled cells (mean±s.d. of four samples in each group). $^{**}P$<0.01. r, rostral; c, caudal; d, dorsal; v, ventral. Scale bar: 500 μm.

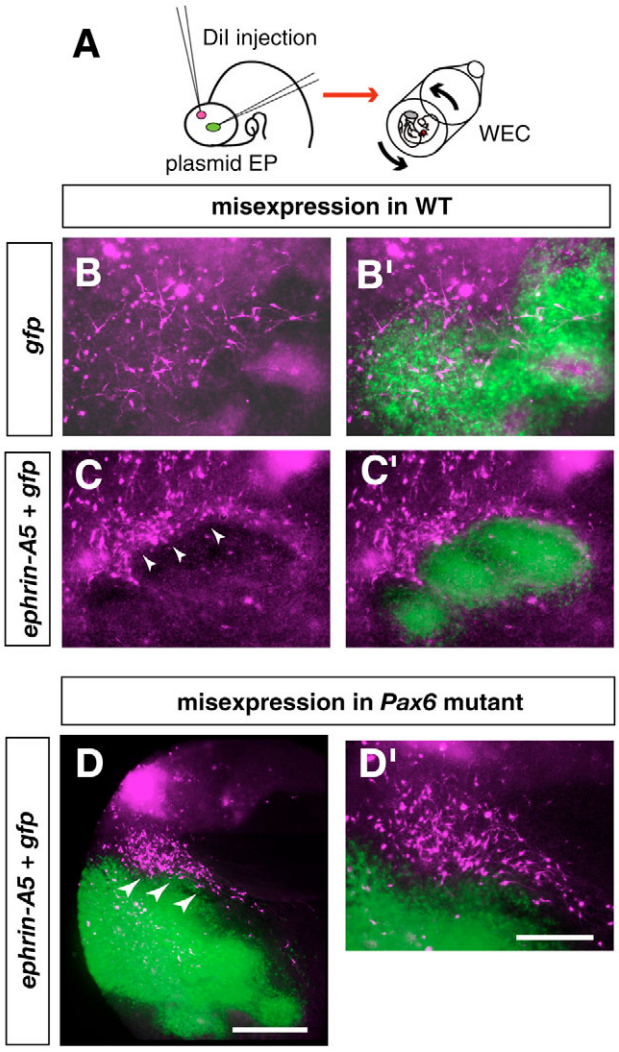

Fig. 6. Altered migration patterns of ventrally migrating neurons by overexpression of ephrin A5. (**A**) Experimental procedures for ephrin A5 misexpression in whole-embryo culture system. (**B-D**) Migration patterns of DiI-labeled neurons in the wild-type (B,C) and *Pax6* mutant (D) embryos, in which ephrin A5 and/or *GFP* expression vector were electroporated. DiI-labeled neurons pass through the area in which *GFP* expression vector was electroporated (B,B′), whereas these neurons stop at the border area with misexpressed ephrin A5 expression vector and *GFP* vector (arrowheads, C-C′). (D,D′) DiI-labeled neurons stop at the border area with misexpressed ephrin A5 in the *Pax6* mutant embryo (arrowheads in D). Scale bars: 500 μm in D; 100 μm in D′.

inside to the LOT (Fig. 8A,B). In the *Pax6* mutant rat at E18.5, however, fewer BrdU-positive neurons were noted in the LOT region (Fig. 8C,F). We also examined the distribution of BrdU-labeled neurons in the olfactory cortex in the wild-type and ephrin A5$^{-/-}$ mice at E18.5. In the wild-type and ephrin A5$^{+/-}$ mice, BrdU-positive cells accumulated in the LOT region of the olfactory cortex (Fig. 8D and data not shown). In the ephrin A5$^{-/-}$ mouse, fewer BrdU-positive cells were found in the LOT region, similar to the *Pax6* mutant rat (Fig. 8E,F).

Next, we examined the number of BrdU-positive cells in the olfactory tubercle region. In wild-type rats or mice, a few BrdU-positive cells distributed at the olfactory tubercle, which is ventral to the LOT area (Fig. 8G,I,J). By contrast, in both *Pax6* mutant rats and ephrin A5$^{-/-}$ mice, the number of BrdU-labeled cells was increased in the olfactory tubercle region, compared with that of the wild type (Fig. 8H,K,L). Although BrdU-incorporated cells were also observed in the preoptic area, the rostroventral region of the diencephalon, there was no difference in the cell number between the wild-type and *Pax6* or ephrin A5 mutant (data not shown). These results indicate similar defects in the number of early-born olfactory cortex neurons in both *Pax6* and ephrin A5-deficient embryos. Such a defect is probably due to impaired alignment of the olfactory cortex neurons at the PSB, and abnormal invasion to the ventral part of the telencephalon in early developmental stages.

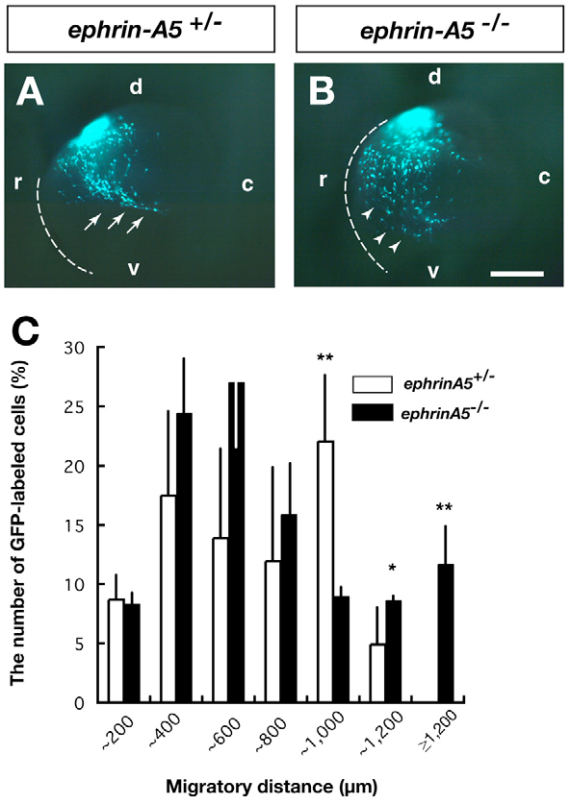

Fig. 7. Altered migration patterns of ventrally migrating neurons by loss of ephrin A5. (**A**,**B**) Altered migration pattern of olfactory cortex neurons in ephrin A5-deficient embryos. In the ephrin A5$^{+/-}$ mice, the dorsally derived GFP-labeled neurons stop at the PSB (arrows in A). However, in ephrin A5 homozygous mutant embryos, the GFP-labeled neurons invaded the ventral part of the telencephalon (arrowheads in B). (**C**) Comparison of migratory distance of GFP-positive cells in between and ephrin A5$^{+/-}$ and ephrin A5$^{-/-}$ mice. In ephrin A5$^{-/-}$ mice the number of GFP-positive cells was significantly increased in the area over 1200 μm distant from the injection point. Data are presented as percentage of the labeled cells in each area against the total number of labeled cells (mean±s.d. of three samples in each group). *P<0.05, **P<0.01. Scale bar: 500 μm.

DISCUSSION

In the present study, we found abnormal alignment of the olfactory cortex neurons at the PSB in the *Pax6* mutant, which was probably due to low expression of ephrin A5. Gain- and loss-of-function studies indicated that *ephirn A5* guides the ventrally migrating neurons and prevents their crossing of the PSB. Our results indicate that *Pax6* regulates the alignment of olfactory cortex neurons at the PSB by regulating ephrin A5 expression in the early developmental stages of the telencephalon (Fig. 9A).

Pax6-dependent brain patterning is important for alignment of the olfactory cortex neurons

Numerous studies have reported that the patterning and regional specification of the brain are essential for guidance of axonal projection and neuronal routing (Logan et al., 1996; Mastick et al., 1997; Shigentani et al., 1997; Kawano et al., 1999; Jones et al., 2002; Marin et al., 2002; Shinozaki et al., 2002). In the present study, we demonstrated that the border for alignment of the olfactory cortex neurons is the PSB, which is defined by the expression of region-specific transcriptional factors such as *Pax6* and *Dlx1* (Puelles et al., 2000; Toresson et al., 2000; Yun et al., 2001). Furthermore, the olfactory cortex neurons do not stop in the *Pax6* mutant telencephalon, in which the PSB formation is severely disorganized (Toresson et al., 2000; Yun et al., 2001). These results suggest that appropriate formation of the PSB during early stages of embryogenesis is required for the olfactory cortex neurons to stop/align at the presumptive olfactory cortex.

In normal development, the olfactory cortex neurons stop at the PSB and never invade the ventral part of the telencephalon. This implies the existence of repulsive/non-permissive cues at the PSB and/or the ventral part of the telencephalon. We identified ephrin A5 as the routing signal that terminates olfactory cortex neuron migration. In support of the above conclusion, our results showed that ephrin A5 was specifically expressed in the ventral part of the telencephalon and exhibited repulsive/non-permissive activities towards the ventrally migrating neurons. Although ephrin A5 expression is severely reduced in the *Pax6* mutant, it is still not clear how *Pax6* regulates ephrin A5 expression at the ventral part of the telencephalon. As the expression domains of *Pax6* and ephrin A5 overlapped in the E11.5 rat telencephalon, and electroporation of *Pax6* expression vector induced ephrin A5 expression, we favor the scenario in which *Pax6* directly activates/maintains the ventral ephrin A5 expression at early stages. However, it is also possible that the ventralization of the *Pax6* mutant telencephalon alters the identity of the progenitor cells, and that such mis-specification of the ventral part of the telencephalon could secondarily affect ephrin A5 expression.

In the ephrin A5-deficient mice, the olfactory cortex neurons do not stop at the PSB, and the number of the early-born olfactory cortex neurons is reduced like in the *Pax6* mutant rats/mice. However, we could not detect any abnormalities in the dorsoventral patterning or in formation of PSB radial glial bundle formation in the ephrin A5 mutant telencephalon (see Fig. S5 in the supplementary material). Previous studies have shown that the establishment of the PSB was governed by distinct regulatory systems including the mutual interaction of specific transcription factors such as Pax6 and Gsh1/2 (Toresson et al., 2000; Yun et al.,

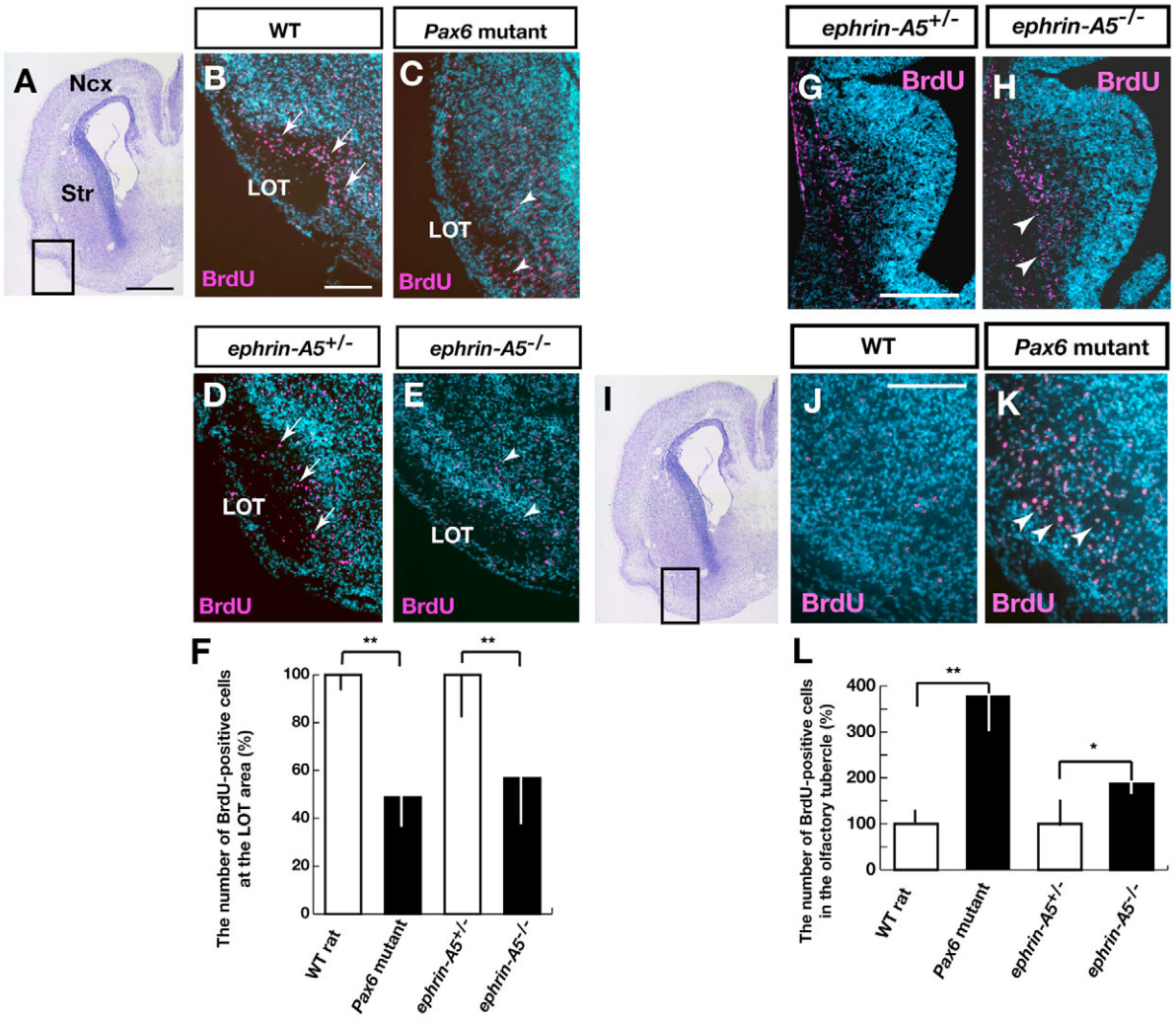

Fig. 8. Altered distributions of early-born olfactory cortex neurons in the *Pax6* and ephrin A5 mutants. (**A**) Nissl staining of the E18.5 rat telencephalon. (**B-E**) Immunostaining with anti-BrdU antibody in E18.5 wild-type rat (B), *Pax6* mutant rat (C), E18.5 ephrin A5$^{+/-}$ mouse (D) and ephrin A5$^{-/-}$ mouse (E) telencephalon. BrdU was injected at E11.75 (rat) or E9.75 (mice). (B-E) LOT area of the olfactory cortex. In the *Pax6* mutant and ephrin A5$^{-/-}$, fewer BrdU-labeled cells are present in the LOT area (arrowheads in C,E) compared with the wild type or ephrin A5$^{+/-}$ (arrows in B,D). (**F**) The number of BrdU-labeled cells in the LOT area of the wild-type, *Pax6* mutant rats and ephrin A5-deficient mice. BrdU-labeled cells are decreased in *Pax6* mutant rats and ephrin A5$^{-/-}$ mice, compared with wild-type rats and ephrin A5$^{+/-}$ mice. Data are presented as percentage of the BrdU-labeled cells in the *Pax6* mutant rat or ephrin A5$^{-/-}$ mouse against the total number of labeled cells in the wild-type rat or ephrin A5$^{+/-}$ mouse (mean±s.d. of four animals in each group). $^{**}P<0.01$. (**G**,**H**) Distribution of BrdU-labeled cells in E12.5 ephrin A5$^{+/-}$ and ephrin A5$^{-/-}$ mice, in which BrdU was injected at E9.75. In ephrin A5$^{-/-}$ mice, the number of BrdU-labeled cells was increased at the ventral part of the telencephalon (arrowheads in H). (**I-K**) Distribution of BrdU-labeled cells in the olfactory tubercle (inset in I) of wild-type rats and *Pax6* mutant rat embryos at E18.5, in which BrdU was injected at E11.75. The number of BrdU-positive cells was increased in the *Pax6* mutant olfactory tubercle (arrowheads in K). (**L**) Comparison of the number of BrdU-labeled cells in the olfactory tubercle of wild type, *Pax6* mutant rats and ephrin A5-deficient mice. BrdU-labeled cells are increased in *Pax6* mutant rats and ephrin A5$^{-/-}$ mice, compared with wild-type rats and ephrin A5$^{+/-}$ mice. Data are presented as percentage of the BrdU-labeled cells in the *Pax6* mutant rat or ephrin A5$^{-/-}$ mouse against the total number of labeled cells in the wild-type or ephrin A5$^{+/-}$ mouse (mean±s.d. of three animals in each group). $^{*}P<0.05$, $^{**}P<0.01$. Scale bars: 500 μm in B,C,G; 100 μm in J. LOT, lateral olfactory tract; Ncx, neocortex; Str, striatum.

2001; Corbin et al., 2003) and complementary expression of R-cadherin and cadherin 6 (Inoue et al., 2001). These multiple regulatory systems for PSB formation are severely disrupted in the *Pax6* mutant telencephalon (Stoykova et al., 1997; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Therefore, although a part of the *Pax6* mutant phenotypes (i.e. impaired alignment of olfactory cortex neurons) is probably due to loss of ephrin A5 function, other downstream genes may also be involved in the PSB defect of the *Pax6* mutant.

Role of EphA/ephrin A signaling in olfactory cortex neuron alignment

Eph receptor tyrosine kinases and ephrin ligands play key roles in regulation of cell migration and axon guidance (reviewed by Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001; Poliakov et al., 2004). One major developmental role of Eph/ephrin signaling system is to mediate contact-dependent repulsion that prevents migrating cells or neuronal growth cones from crossing over a ligand-expressing territory, thus confining them to an appropriate

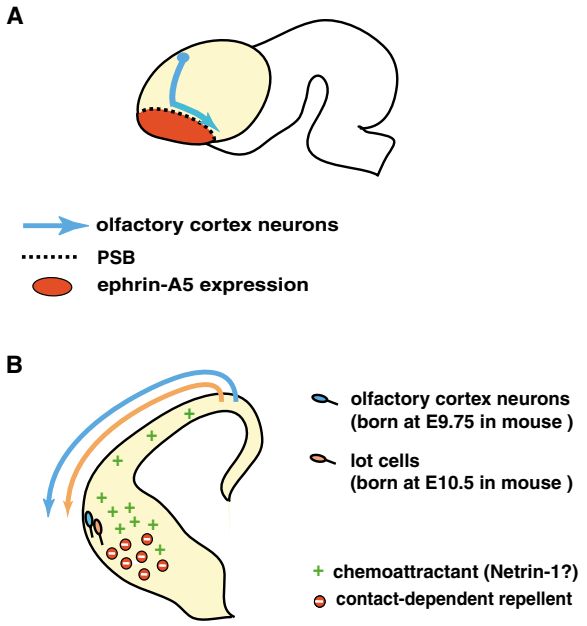

Fig. 9. Schematic diagram of *Pax6*/ephrin A5-dependent mechanisms for alignment of olfactory cortex neurons at PSB. (**A**) Olfactory cortex neurons are generated in the dorsal part of the rat telencephalon at around E11.75 (corresponding to E9.75 in mouse embryos). These neurons migrate ventrally and align at the PSB through ephrin A5 repulsive activity. In the *Pax6* mutant, reduced ephrin A5 expression allows these neurons to invade the ventral part of the telencephalon. (**B**) The ventrally migrating neurons include several distinct subtypes such as lot cells and olfactory cortex neurons. They migrate ventrally, probably owing to some attractant distributed in the dorsal part of the telencephalon and/or secreted from the ventral part of the telencephalon. Netrin 1 acts as an attractant for lot cells (Kawasaki et al., 2006). Ephrin A5 and other factors act as contact-dependent repellents for these neurons.

pathway (Krull et al., 1997; Wang and Anderson, 1997; Frisen et al., 1998; Feldheim et al., 2000; Swartz et al., 2001; Yokoyama et al., 2001; Dufour et al., 2003). We showed that the olfactory cortex neurons did not invade the region where the ephrin A5 was highly expressed in the telencephalon. This finding suggests that ephrin A5 acts as a contact-dependent repellent for the ventrally migrating neurons. However, a previous report showed that ephrin A5 is also involved in the cell-cell adhesion through heterophilic interaction with a specific splicing variant of *Epha7* (Holmberg et al., 2000). Although *Epha7* was not detected in the olfactory cortex neurons, it is possible that these migrating neurons attached to cells located at the PSB by ephrin A5-dependent cell adhesion. Stripe assays or other experiments should be performed to determine whether ephrin A5 functions as a repellent for the olfactory cortex neurons.

Multiple guidance mechanisms for olfactory cortex neurons

In general, directional guidance of neuronal migration requires various guidance cues such as chemoattractants and chemorepellents (Marin and Rubenstein, 2003). The ventrodorsal migration of GABAergic interneurons is regulated by several guidance molecules and motogenic factors such as BDNF, NT3, HGF, neuregulins and semaphorins (Marin et al., 2001; Powell et al., 2001; Polleux et al., 2002; Tanaka et al., 2003; Flames et al., 2004). A previous study has indicated that lot cells guide post neurons to the lateral olfactory tract, migrate dorsoventrally in the cortex and align at the PSB (Sato et al., 1998; Tomioka et al., 2000). A recent study has indicated that lot cells are attracted by diffusible factor(s) that are distributed in the dorsal and ventral parts of the telencephalon, and that netrin 1 acts as an attractant for these cells (Kawasaki et al., 2006). As the dorsoventral migration pattern of the olfactory cortex neurons is very similar to that of lot cells, it is possible that common mechanisms regulate the dorsoventral migration of these neurons in the cortex (Fig. 9B). In contrast to our present results, however, most lot cells are distributed at the PSB in *Pax6* and ephirn A5 mutants (Hirata et al., 2002) (T.N. and N.O., unpublished). This result indicates that the olfactory cortex neurons examined in the present study are a distinct population from lot cells, and at least the alignment of lot cells at the PSB is regulated by other mechanisms independent of *Pax6*/ephrin A5. The olfactory cortex neurons in the present study were generated at E11.75 in the rat embryo (corresponding to E9.75 mouse embryos), which is slightly earlier than that of lot cells (E10.5 mouse embryos) (Sato et al., 1998), or other olfactory cortex neurons that are previously reported to be derived from the lateral/ventral pallium by radial migration (Bayer, 1986; Valverde and Santacana, 1994; De Carlos et al., 1996; Yun et al., 2001). BrdU-pulse labeling data (Fig. 8) implies that the dorsally derived neurons migrate to the olfactory cortex and located at the layer I, the most superficial layer of the olfactory cortex. This is basically consistent with the previous idea by Valverde and Santacana (Valverde and Santacana, 1994) that the most superficial cells in the primary olfactory cortex are generated earlier than deeper cells (layer II and III). Therefore, it is suggested that neurons constitute the most superficial and deeper layer of the olfactory cortex have distinct origins, as we have previously suggested (Hirata et al., 2002). It has been reported that reelin-positive neurons originate from the cortical hem region at around E10.5-11.5 mouse embryos (Takiguchi-Hayashi et al., 2004). A recent study using *Dbx1*-Cre transgenic line also indicated specific population of the Cajal-Retzius cells are generated at the septum and the PSB, and they tangentially migrate ventrolaterally to spread out on the surface of the neocortex (Bielle et al., 2005). Therefore, several types of cortical neurons are sequentially generated during early embryonic stages, which ultimately migrate via specific routes to cover the entire part of the telencephalon. Elucidating the mechanisms that govern the spatiotemporally controlled generation and migration of the different cortical neurons should enhance our understanding of the coordinated organization of the mammalian cerebral cortex.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/7/1335/DC1

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