Misrouting of mitral cell progenitors in the *Pax6/small eye* rat telencephalon

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Summary

The olfactory bulb is a protruding structure formed at the rostral end of the telencephalon. *Pax6*-mutant mice and rats lack the olfactory bulb and, instead, develop an olfactory bulb-like structure at the lateral part of the telencephalon. Here, we report that ectopic formation of the olfactory bulb-like structure in these mutants is caused by the abnormal migration of mitral cell progenitors, which first differentiate within the olfactory bulb. Cell-tracing experiments in whole embryos in culture indicate that, in the mutants, the mitral cell progenitors that originate from the rostral part of the telencephalon migrate caudally toward the lateral part of the telencephalon. Cell transplantation demonstrates that the abnormal cell migration is not autonomous to the mitral cell progenitors themselves. The mislocation of the olfactory bulb in the mutant is not caused by loss of olfactory nerve innervation. Furthermore, transfection of a *Pax6*-expression vector to the mutant telencephalon restores the normal migration of mitral cell progenitors. These results provide evidence that *Pax6* is required to position the mitral cell progenitors at the rostral end of the telencephalon.

Key words: *Pax6*, Olfactory bulb, Cell migration, Telencephalon, Whole embryo culture, Brain organ culture, Electroporation, Rats

Introduction

The olfactory bulbs (OBs) are structures that protrude from the rostral telencephalon and function as the primary processing center for odor information. The OB has a laminar structure, consisting of several types of neurons and glial cells (Hinds, 1968; Pinching and Powell, 1971). Mitral cells, which differentiate first among the OB neurons, receive direct axonal innervation from olfactory receptor neurons in the olfactory epithelium and their axons project to the olfactory cortex (Hinds and Ruffett, 1973). Several interneurons also have important roles in local circuits within the OB. However, it is unclear how the OB forms at the precise position of the rostral telencephalon.

Numerous lines of evidence demonstrate that establishment of regional identity and specification of cell types in the telencephalon is regulated by several transcription factors (Shimamura et al., 1995; Rubenstein et al., 1998; Campbell, 2003). *Pax6*, a member of the Pax family of transcription factors, has crucial roles in various developmental processes of the telencephalon including dorsal-ventral and anterior-posterior patterning, specification of neuronal subtypes, neuronal migration and axonal projection (Engelkamp et al., 1999; Kawano et al., 1999; Stoykova et al., 2000; Bishop et al., 2000; Bishop et al., 2002; Yamasaki et al., 2001; Yun et al., 2001; Pratt et al., 2002).

Pax6 homozygous-mutant mice (*Sey/Sey*) lack OB protrusion but still have olfactory bulb-like structures (OBLSs) at the lateral side of the telencephalon (Lopez-Mascaraque et al., 1998; Jimenez et al., 2000; Hirata et al., 2002). This may indicate that *Pax6* function is required to determine the position where the OBs should form within the telencephalon. However, the mechanisms by which *Pax6* determines OB position in the developing telencephalon and the primary cause of ectopic formation of the OBLS in the *Pax6* mutant are unknown.

In this study, we investigated how the OBLS develops ectopically in *Pax6*-mutant rats ($rSey^2/rSey^2$) using long-term culture systems of whole embryos and brain explants. Cell-tracing analyses revealed that misposition of the OBLS was not caused by ectopic generation of mitral cells, but by abnormal migration of mitral cell progenitors in the telencephalon of *Pax6* mutants. Transplantation of mitral cell progenitors showed clearly that abnormal cell migration was caused by non-cell autonomous defects of the mitral cell precursors. Removal of olfactory innervation did not affect the migration pattern of the mitral cell progenitors, indicating that the mutant phenotype is not caused by the impairment of olfactory nerve innervation. Furthermore, transfection of exogenous *Pax6* into the mutant telencephalon restored abnormal cell migration, implying that *Pax6* function is required within the telencephalon. These results demonstrate that loss of *Pax6* function in the telencephalon disrupts a positional cue that is required to direct the mitral cell progenitors to the rostral end.

Materials and methods

Animals

Pregnant Sprague-Dawley (SD) rats were purchased from Japan Charles River (Tokyo, Japan). Heterozygous *Pax6* mutants on SD

background rats (*rSey²*) (Osumi et al., 1997) were intercrossed in our laboratory to obtain homozygous embryos, which are distinguished from wild-type and heterozygous littermates by an eyeless phenotype. GFP-transgenic rats [*TgN(act-EGFP)Osb4*] (Ito et al., 2001) were provided by Dr Okabe and crossed with the *rSey²* strain. Midday on the day a vaginal plug was found was determined as embryonic day 0.5 (E0.5). The following experimental procedures were approved by the Committee for Animal Experiment of Tohoku University Graduate School of Medicine.

Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde and sectioned with a Cryostat (CM3050, LEICA). The sections were immersed in 5% skimmed milk in TBST (tris-buffered saline plus 0.01% tween20) for 30 minutes, and incubated with anti-Neuropilin 1 (rabbit polyclonal, a gift from Dr Hirata), anti-calretinin (mouse monoclonal, CHEMICON), anti-GAD67 (rabbit polyclonal, CHEMICON), anti-bromodeoxyuridine (BrdU, mouse monoclonal, Beckton-Dickinson) antibodies at 4°C overnight. Cy3-conjugated anti-rabbit or anti-mouse IgG antibody (Jackson) was used as the secondary antibody. After washing with TBST, the sections were examined under the fluorescent microscope (Axioplan-2, Zeiss) equipped with a cooled CCD camera (Roper). Confocal images of GFP/Cy3 double-positive cells were acquired using a Leica TCS NT confocal microscope and 3D images constructed with Leica confocal software. Whole-mount immunostaining of the telencephalon was performed according to previous procedures (Nomura et al., 1998). For detection of BrdU, sections were treated with 2M HCl at 37°C for 30 minutes before immunohistochemistry.

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were transcribed from neuropilin 1 (Kawakami et al., 1996), *Pax6* (Osumi et al., 1997), netrin G1 (Nakashiba et al., 2000) cDNAs that had been subcloned in pBluescript using the DIG RNA labeling kit (Roche). The hybridization procedures have been described previously (Osumi et al., 1997; Takahashi and Osumi, 2002). Hybridization signals were detected with AP-conjugated anti-DIG antibody (Roche) and nitroblue tetrazolium, 5-bromo-4-chloro-3-indol-phosphate (Roche).

Cell labeling and cell transplantation in cultured embryos

The experimental procedures of whole embryo culture (WEC) and cell labeling have been described previously (Ishii et al., 2000; Takahashi and Osumi, 2002; Takahashi et al., 2002). To label the progenitors of the olfactory bulb neurons, DiI (D-282, Molecular probe) solution dissolved in dimethylfomamide was microinjected with a fine-tipped glass needle (Shutter Instrument Co) into the rostral end of the telencephalon. To analyze the birthdate of the labeled cells, embryos were cultured in medium containing 40 μM BrdU (Boehringer Mannheim) for 30 minutes. In whole brain culture (WBC), brain tubes including the forebrain, midbrain and hindbrain regions were isolated by removing mesenchymal tissues and cultured for 24 hours in rotating bottles filled with culture medium for WEC (100% rat serum containing 2 mg ml^{-1} glucose and 0.025% antibiotics) in 60% O_2. Telencephalic organ culture (TOC) was performed as described previously (Sugisaki et al., 1996). Briefly, after 48 hours in WEC or WBC, the telencephalic hemispheres were dissected out, treated with 0.2% collagenase IV to remove the pia matter, and cultured on a collagen-coated membrane filter (transwell-COL 3492, Coaster) for 2-3 days at 37°C, in 0.25% CO_2. To transplant OB neuronal progenitors, small fragments of the rostral telencephalon of *Pax6* homozygote embryos and their littermates on GFP-transgenic background were dissected out, and treated with 0.2% collagenase to remove the mesenchyme. The fragments were further dissociated into single cells with 0.25% trypsin, and ~50-100 cells microinjected into the rostral telencephalic wall of host embryos.

Gene transfer by electroporation

Details of electroporation in cultured embryos were described previously (Takahashi et al., 2002). After 2 hours of preculture in WEC, embryos were transferred into tyrode's solution, of 0.1 μl plasmid vector solution (either pCAX-GFP or pCAX-Pax6) (Takahashi et al., 2002) and microinjected into the telencephalic vesicle. Square pulses (70V, 5 Hz) were delivered into the embryos using an electroporator (CUY21, NEPPA GENE) and tweezer-type electrodes (CUY- 650).

Results

The OBLS is ectopically formed in the *Pax6* mutant rat telencephalon

Previous studies have shown that *Sey/Sey* embryos have OBLS at the lateral side of the cortex (Lopez-Mascaraque et al., 1998; Jimenez et al., 2000; Hirata et al., 2002). To test whether rat *rSey²/rSey²* embryos also exhibit a similar phenotype, we examined expression of several markers for OB neurons. Neuropilin 1 (Nrp) is strongly expressed in mitral cells (Kawakami et al., 1996; Sugisaki et al., 1996; Hirata and Fujisawa, 1997), one of the projection neurons that differentiated first, in the developing OB of wild-type rat embryos at E16.5 (roughly corresponding to E14.5 of mouse embryos) (Fig. 1A,E). In the OB, Nrp immunoreactivity was localized at the external plexiform layer, which consists of the neuropiles of mitral cells (Fig. 1A,I). In contrast, a Nrp-positive structure was observed on the lateral side of the *rSey²/rSey²* telencephalon (Fig. 1B,K), similar to that in murine *Sey/Sey* embryos (Hirata et al., 2002). This Nrp-positive structure in *rSey²/rSey²* telencephalon also expressed netrin G1 which marks mitral cells in the developing OB (Fig. 1G,H) (Nakashiba et al., 2000). In the OB and OBLS, Nrp or netrin G1-positive mitral cell did not express Pax6 (data not shown). Next we examined whether interneurons exist in the OBLS using several markers. Calretinin-immunopositive cells, which are corresponding to the periglomerular cells, are located at the glomerular layer of the developing OB (Kosaka et al., 1995) (Fig. 1I). Calretinin-positive cells were also observed in the superficial area of the OBLS (Fig. 1K). In the OB, GABAergic and dopaminergic interneurons express glutamic acid decarboxylase (GAD67) and tylosine hydroxylase (TH), respectively. (Esclapez et al., 1994; Kosaka et al., 1995). GAD67-immunopositive cells located at the glomerular and the external granule cell layers in the OB (Fig. 1J), while they distributed at the peripheral area of the OBLS, adjacent to the Nrp-positive area (Fig. 1L). We could not detect any TH-immunopositive cells in the OBLS, similar to the previous report of mouse *Pax6* mutant (Jimenez et al., 2000).

Taken together, these results indicate that the OBLS is formed at the lateral side of the cortex in *rSey²/rSey²* embryos as in the case of the mouse *Pax6* mutant. Because the mitral cells are first neurons to differentiate in the OB (Hinds, 1968), we focused on the mitral cell development in the following experiments.

Cell tracing of mitral cell progenitors in wild-type and *Pax6*-mutant rats

Because the OBLS developed at an ectopic position in the *Pax6* mutant telencephalon, we hypothesized two possibilities to account for the defect in OB formation. One is that the origin

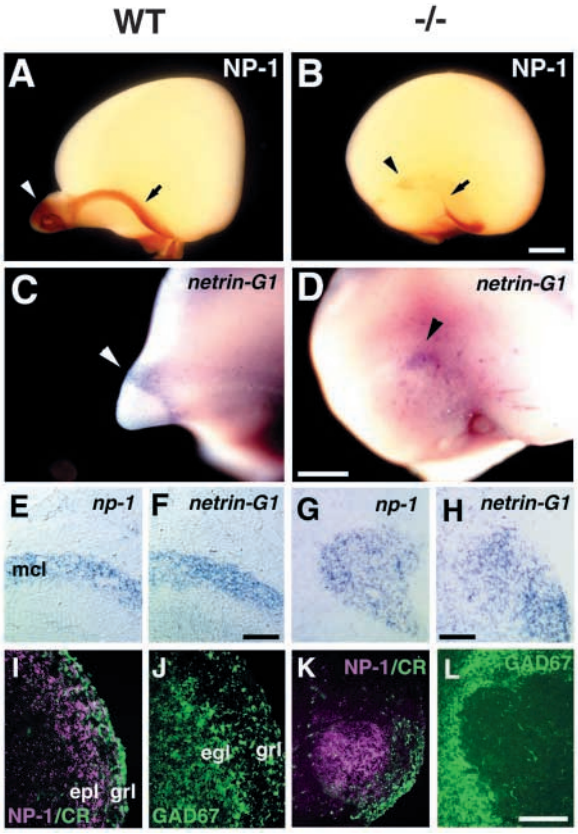

Fig. 1. The OBLS forms ectopically at the lateral part of the telencephalon of *Pax6* mutants. (A-D) Lateral views and (E-L) coronal sections of wild-type (WT, A,C,E,F,I,J) and *Pax6*-mutant telencephalons (–/–, B,D,G,H,K,L), stained with anti-neruopilin 1 (Nrp, A,B,I,K), anti-Calretinin (CR, I,K), anti-GAD67 antibodies (J,L) or hybridized with specific probes for neuropilin 1 (*Nrp*, E,G), netrin G1 (C,D,F,H**)**. A-H and I-L show E16.5 and E18.5 embryos, respectively. Nrp and netrin G1 are expressed in the protruding OB in the wild type (arrowheads in A,C), whereas these markers are observed at the lateral part of the mutant telencephalon (arrowheads in B,D). Note that Nrp protein is localized mostly at the neuropiles and axons of mitral cells (arrowheads and arrows in A,B). The distribution of *Nrp* and netrin G1 overlap in the OB and the OBLS (E-H). Nrp, CR and GAD67 are expressed in the different types of neurons of the OB and the OBLS (I-L). egl, external granule cell layer; epl, external plexiform layer; grl, glomerular layer; mcl, mitral cell layer. Scale bars: A-D, 1 mm; E-L, 50 μm.

of the mitral cells may be altered in the mutant. A previous study suggested that the mitral cells are derived from the dorsal part of the telencephalon (Bulfone et al., 1998; Puelles et al., 2000). Thus, the mitral cells of the *Pax6*-mutant OBLS differentiate in an ectopic position of the telencephalon compared to wild type. The other possibility is that the mitral cells of the mutant embryo migrate abnormally and colonize in an ectopic position to form the OBLS. To test these possibilities, we performed cell-tracing analyses in embryos in culture and compared the origin and migration patterns of the mitral cells in wild-type and *rSey*^{2}/*rSey*^{2} embryos (Fig. 2A). Because mitral cells are born at ~E12-E13 in rat embryos (Bayer, 1983), we labeled a part of the telencephalon at E12.5 and cultured the labeled embryos for 48 hours.

When the rostral-most telencephalon of wild-type embryos (*n*=4) was labeled by the lipophilic fluorescent dye DiI (Fig. 2C), most labeled cells stayed in the area close to the dye-injection point (Fig. 2B,E). Only a few of labeled cells were detected in the lateral part of the cortex. In contrast, when a similar area was labeled in *rSey*^{2}/*rSey*^{2} embryos (Fig. 2D, *n*=4), a large number of labeled cells were observed to migrate caudally toward the lateral part of the telencephalon (Fig. 2B,F). The area in which DiI-labeled cells accumulated seemed to correspond to the area where the OBLS would form later in development (Fig. 2F, arrowheads).

Differentiation of the mitral cells did not occur in embryos just after WEC (data not shown). To examine whether the labeled cells contribute to the OB or the OBLS and differentiate into mitral cells, we isolated telencephalons and cultured them as explants. After 48 hours of WEC followed by 3 days of telencephalic organ culture (TOC), a protruding OB, marked by calretinin, developed at the rostral end of the telencephalon of wild-type embryos (Fig. 3E″). Nrp and netrin G1 were also detected in this OB (Fig. 3F″ and data not shown). Therefore, this long-term culture system seemed to be suitable for examining the development of mitral cells from their origin to initial differentiation. Next, instead of DiI injection, we electroporated a *GFP*-expression vector to focally label the rostral telencephalon because GFP-fluorescence is stable after the immunostaining using detergents. When the GFP gene was introduced into the rostral part of the dorsal telencephalon (i.e. the *Pax6*-positive area) of wild-type embryos, GFP-positive cells stayed in the rostral part of the telencephalon, even 48 hours after electroporation (Fig. 3A-D). After 3 days of TOC, most GFP-positive cells contributed to the OB evaginated from the rostral tip of the telencephalic explants (Fig. 3E-E″). GFP-labeled cells within the protruding OB expressed Nrp, indicating that they differentiated into mitral cells (Fig. 3F-F″,G-G″). Of the GFP-positive cells, 69.8±6.2% were Nrp-positive in the OB (*n*=4). To further confirm that cells that express GFP were mitral cells, we examined their birthdate by labeling with BrdU. GFP-transfected embryos were cultured in BrdU-containing medium for 30 minutes, 6 hours after electroporation (*n*=3). This corresponds to E12.75. After a further 3 days of TOC, ~50% of GFP-positive cells in the OB were labeled with BrdU (Fig. 3H-H″). Because the peak of mitral cell neurogenesis occurs at ~E12-E13 in rat embryos (Bayer, 1983), these BrdU/GFP-positive cells are considered to be mitral cells. Taken together, we conclude that the mitral cells originate from the rostral-dorsal telencephalon in normal development.

Next, we asked whether the cells originating from the rostral telencephalon of *rSey*^{2}/*rSey*^{2} embryos could form the OBLS. When the rostral end of the dorsal telencephalon of E12.5 mutant embryos was labeled with GFP, a large number of labeled cells migrated caudally toward the lateral part of the telencephalon within 48 hours of WEC (data not shown), as observed in the DiI labeling (Fig. 2D). After 3 days of TOC, calretinin-positive and Nrp-positive OBLS developed at the lateral surface of the mutant telencephalon, which included the GFP-positive cells derived from the rostral part of the dorsal telencephalon (Fig. 4D-D″,E-E″). Of the GFP-positive cells, 64.7±1.5% expressed Nrp in the OBLS, indicating that they differentiated into mitral cells (*n*=4) (Fig. 4F-F″). We detected no GFP-positive cells in the OBLS when other areas of the

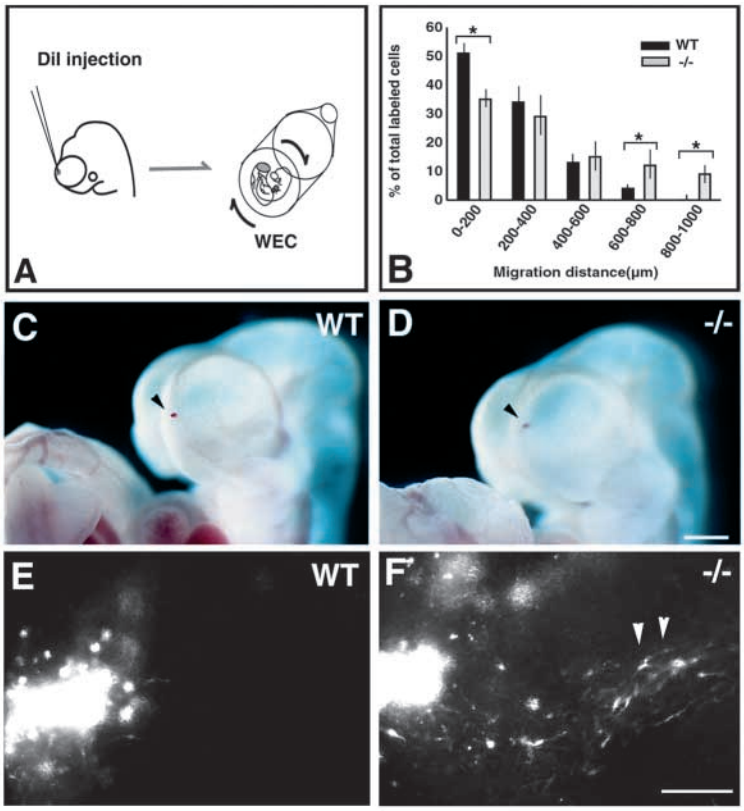

Fig. 2. The pattern of cell migration is abnormal in the telencephalon of *Pax6* mutants. (A) Experimental procedures for cell tracing in the telencephalon. DiI solution was injected into the rostral-most telencephalic wall of E12.5 embryos and whole embryos cultured for 48 hours. (B) Migration of the DiI-labeled cells in the telencephalon of wild type (WT, *n*=4) and *Pax6* mutants (–/–, *n*=4). Compared to wild type, the mutant cells migrate caudally from the injection point. * $P<0.05$, *t*-test. (C,D) Wild-type (C) and mutant (D) embryos just after injection of DiI into the rostral-most telencephalon. Arrowhead indicates the injection points. (E,F) Migration patterns of labeled cells in the wild-type (E) and mutant (F) telencephalon. In the wild type, the majority of labeled cells are distributed at the rostral part of the telencephalon (C). In the *Pax6* mutant, many labeled cells migrate caudally into the lateral part of the telencephalon (arrowheads in F). Scale bars: C,D, 1 mm; E,F, 200 μm.

telencephalon were labeled (data not shown). We also performed pulse labeling with BrdU to confirm the birthdate of the GFP-positive cells (*n*=3). After 30 minutes of pulse labeling, ~50% of GFP-expressing cells within the Nrp-positive OBLS were also labeled with BrdU (Fig. 4G-G″), indicating that they are mitral cells. Taken together, these data indicate that the origin of mitral cells is not altered in *rSey²/rSey²* mutants. However, in these mutants, the mitral cell progenitors migrated caudally toward the lateral part of the telencephalon and formed the OBLS in an ectopic position.

Abnormal migration of mitral cell progenitors results from non-cell autonomous defects of mitral cells themselves

Pax6 is expressed in the neuroepithelium of the dorsal telencephalon (Stoykova and Grüss, 1994), including the origin of the mitral cells. *Pax6* also regulates the development of the lateral part of the cortex (Hirata et al., 2002) and there are severe defects in formation of the olfactory epithelium and subsequent differentiation of neurons in *Pax6*-mutant mice and rats (Hogan et al., 1986; Hill et al., 1991; Fujiwara et al., 1994; Grindley et al., 1995). These lines of evidence led us to question whether the impaired migration in *rSey²/rSey²* mutants results from a defect that is cell-autonomous or non cell-autonomous to the mitral cell progenitors. To test these possibilities, we isolated neuroepithelial cells containing the mitral cell progenitors from the rostral telencephalon of E12.5 *rSey²/rSey²* embryos on a GFP-transgenic background (Ito et al., 2001), and transplanted them into either wild-type or heterozygous littermates, and vice versa. After transplantation, whole embryos were cultured for 48 hours followed by 3 days of TOC (Fig. 5A). The transplanted cells were easily distinguished in the hosts by their GFP-fluorescence.

When the rostral-most telencephalic cells of E12.5 homozygous embryos were transplanted into the same region of either wild-type or heterozygous (*rSey²*) embryos, most of the donor cells localized at the rostral part of the telencephalon and contributed to the host OB (Fig. 5B,C). Some of the donor cells expressed Nrp, indicating that they differentiated into mitral cells (Fig. 5C′). In these transplantation experiments we did not observe mutant donor cells that migrated toward the caudal part of the telencephalon (Fig. 5F). In contrast, when cells isolated from the rostral part of the telencephalon of E12.5 wild-type and heterozygous embryos were transplanted into the corresponding region of the telencephalon of homozygous mutants, many GFP-positive cells migrated caudally (Fig. 5D,E). These donor cells expressed Nrp and contributed the OBLS that formed in the lateral part of the mutant telencephalon (Fig. 5E′). This migration pattern of the donor cells resembled that of homozygous mitral cell progenitors labeled with either DiI or the GFP gene (Figs 2, 4). These results indicate that abnormal migration of the mitral cell progenitors is not caused by cell-autonomous defects of the mitral cells themselves, but by an impaired migratory environment in the mutant telencephalon.

Impaired migration of the mitral cell progenitors is not caused by lack of olfactory nerve innervation

We next examined whether penetration of the olfactory nerve into the brain affects mitral cell movement because development of the olfactory epithelium is also impaired in *Pax6* mutants (Hogan et al., 1986; Hill et al., 1991; Fujiwara et al., 1994; Grindley et al., 1995). To address this, we isolated brain tubes, by removing surrounding tissues containing the olfactory epithelium and nerve, and cultured them to access the localization of the mitral cells. Primary innervation of the olfactory nerve commences at E13 in rat embryos (Gong and Shipley, 1995). Thus, we isolated brain tubes from E12.5, and cultured them for 24 hours in rotating bottles (WBC) and, subsequently, as a TOC for 2 days (Fig. 6A). After a total 3 days of culture, specimens were stained for netrin G1.

When wild-type brains (*n*=7) were cultured without olfactory nerve innervation, netrin G1 expression was observed

at the rostral part of the telencephalic explants (Fig. 6B, arrowhead). There were no netrin G1-positive cells at the lateral part of the telencephalon. This strongly supports a recent report that the specification of mitral cells occurs without olfactory nerve innervation (Long et al., 2003). In mutant explants (n=5), a cluster of netrin G1-positive cells was observed at the lateral part of the telencephalon, indicating that abnormal migration of mitral cells was reproduced during these culture conditions (Fig. 6C). Taken together, these results indicate that abnormal migration of mitral cell progenitors in the *Pax6* mutant is not caused by a lack of olfactory nerve innervation, but is intrinsic to the telencephalon.

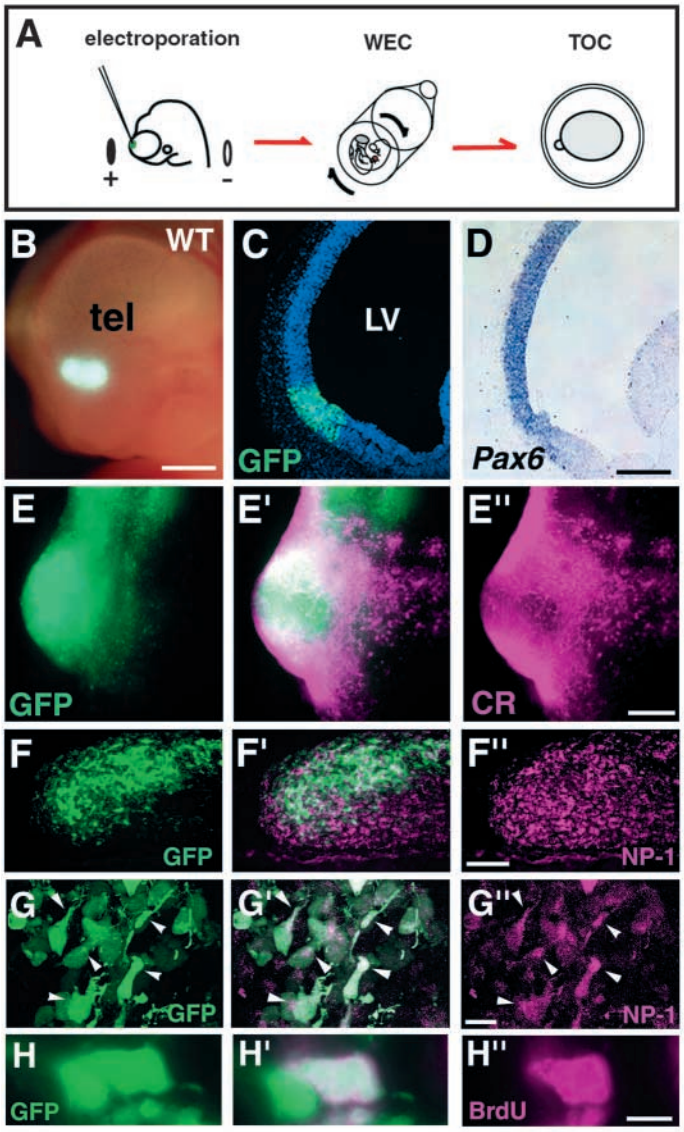

Fig. 3. Mitral cells are derived from the rostral part of the wild-type telencephalon. (A) Experimental procedures for cell tracing of the mitral cell progenitors by electroporation of *GFP*-expression vector. (B-D) A rostral-lateral view (B) and parasagittal sections (C,D) of the head of electroporated, wild-type embryos. At 24 hours after electroporation, strong GFP-fluorescence is seen at the rostral area of the telencephalon that expresses *Pax6* (C,D). (E-E″,F-F″) Distribution of GFP-positive cells in the telencephalon after 2 days of TOC. The OB evaginates at the rostral-most area of the telencephalic explants. F-F″ are transverse sections of a TOC sample. GFP-positive cells contribute to the OB marked by CR and Nrp staining (E′,F′). (G-G″) Confocal images of GFP-positive cells in the OB. Nrp is expressed at the neuropiles of the GFP-labeled cells (arrowheads). (H-H″) BrdU incorporation in the GFP-positive cells in the OB. tel, telencephalon; LV, lateral ventricle. Scale bars: B-D, 500 μm; E-F″, 100 μm; G-G″, 20 μm; H-H″, 10 μm.

Introduction of exogenous *Pax6* into the mutant telencephalon restores the normal migration of mitral cell progenitors

To further confirm that *Pax6* function is required to control migration of mitral cell progenitors in the telencephalon, we examined whether the mismigration of the mitral cell progenitors is rescued by transfection of an exogenous *Pax6*-expression vector into the mutant telencephalon.

Either *GFP* alone or *GFP* plus *Pax6* expression vectors were introduced into the E12.5 mutant telencephalon by electroporation. After WEC followed by TOC, we examined the localization of netrin G1-positive cells in the mutant telencephalon. GFP fluorescence showed that exogenous genes were expressed throughout the rostral-half of the telencephalic neuroepithelium, including the origin and the migratory pathway of the mitral cell progenitors (Fig. 7A). As expected, when we introduced the *GFP* gene alone into the mutant telencephalon, accumulation of netrin G1-positive cells was observed at the lateral surface of the telencephalon (8/8 cases) (Fig. 7B, Fig. 1D, arrowheads). However, when the *Pax6*-expression vector was electroporated into the mutant hemisphere, netrin G1-positive cells accumulated at the rostral-most telencephalon (4/10 cases; Fig. 7C, arrowhead). Nrp was also expressed in the area containing netrin G1-positive cells (Fig. 7E). Nrp-positive cells were sorted from those expressing exogenous Pax6 as is seen in wild-type embryos (Fig. 7E,F, and data not shown). In 3/10 cases, clusters of netrin G1-positive cells were observed in the rostral-most part of the cortex, but there were also some cells at the lateral side of the cortex (Fig. 7G). However, the localization of netrin G1-positive cells was unchanged (Fig. 7G) in three other cases, probably because of limited expression of the *Pax6* gene. These results demonstrate that transfection of exogenous *Pax6* restored normal migration to mitral cell precursors in the mutant telencephalon. Taken together, *Pax6* function in the telencephalon is necessary to retain mitral cells in the rostral-most part of the telencephalon.

Discussion

In this study, we revealed that ectopic formation of the OBLS in the *Pax6* mutants is caused by abnormal migration of mitral cell progenitors. The migration defect was further proved to be caused by lack of *Pax6*-function in the mutant telencephalon (i.e. in the environment in which mitral cells migrate) rather than a defect in mitral cell progenitors. Furthermore, removal of olfactory innervation and introduction of exogenous *Pax6* to the mutant telencephalon demonstrated that *Pax6* functions are required in the telencephalon to regulate the migration pattern of the mitral cell precursors. These findings support a model in which *Pax6* controls positional information that is responsible for positioning the mitral cell progenitors in the telencephalon.

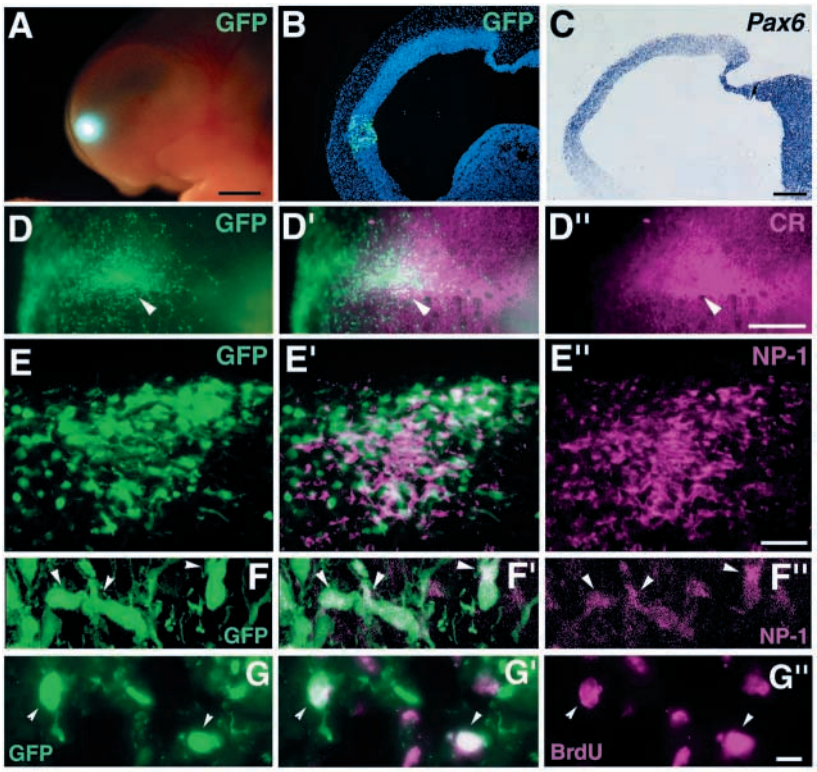

Fig. 4. Misrouting of mitral cell-precursors in the telencephalon of *Pax6* mutants. (A-C) Lateral view (A) and parasagittal sections (B,C) of the head of electroporated, mutant embryos. At 24 hours after electroporation, there is GFP fluorescence in the rostral-most telencephalon. (D-D″,E-E″) Distribution of GFP-positive cells following TOC for 3 days. E-E″ are transverse sections of a TOC sample. GFP-positive cells migrate caudally (arrowheads in D-D″), and constitute the OBLS, which is marked by CR and Nrp immunostaining (arrowheads in D′,E′). (F-F″) Confocal images of GFP-positive cells in the OBLS. Nrp protein is localized at the neuropiles of GFP-positive cells (arrowheads). (G-G″) GFP-positive cells are also labeled with BrdU (arrowheads). tel, telencephalon; LV, lateral ventricle. Scale bars: A-C, 500 μm; D-E″, 100 μm; F-G″, 10 μm.

Pax6 controls the positioning of mitral cell progenitors in the telencephalon

The most striking result in the present study is the abnormal migration pattern of mitral cell progenitors in the *Pax6* mutant. In these mutants, the mitral cell progenitors derived from the rostral region migrated caudally and clustered at the lateral surface of the telencephalon. This indicates that the ectopic formation of the OBLS in the mutants is not caused by an alteration of the mitral cell origin, but by the abnormal migration of mitral cell progenitors. It has been reported that *Pax6* regulates migration of cerebellar granule cells, which strongly express *Pax6*, in a cell-autonomous manner (Engelkamp et al., 1999; Yamasaki et al., 2001). By contrast, mitral cells originate from a *Pax6*-positive, dorsal part of the telencephalon, but the migrating precursors and differentiated mitral cells do not express *Pax6* (Fig. 2 and data not shown). It is, thus, unexpected that *Pax6* is required for the mitral cell progenitors to autonomously control their migration patterns, an effect that was proved by cell transplantation experiments. Such a cell non-autonomous function for *Pax6* in the control of neuronal migration has also been observed for the migration of neural crest cells from the midbrain towards the frontonasal region (Osumi-Yamashita et al., 1997; Nagase et al., 2001). Therefore, *Pax6* functions controls the migration of neuronal cells in both cell-autonomous and non-autonomous ways, depending on the developmental context.

It has long been believed that projection of the primary olfactory nerve to the rostral telencephalon affects development of the OB. In frog and chick embryos, removal of the olfactory epithelium abolishes OB development (Graziadei and Monti-Graziadei, 1991; Wang et al., 2001). In our study, however, ablation of olfactory innervation did not alter the location of the mitral cells within the telencephalon of wild-type rat embryos. This excludes the possibility that penetration of the olfactory nerve directs the migration of the mitral cell progenitors. However, the OB in the wild-type cultured brain and the OBLS in the *Pax6* mutant do not evaginate from the telencephalon. It may, thus, be possible that olfactory innervation controls further protrusion of the OB in later development. It has been reported that the frontonasal region may play an essential role for OB morphogenesis (LaMantia et al., 1993). Several types of secreted molecules such as retinoic acid (RA), FGF8 and BMP4 are expressed at the frontonasal region (LaMantia et al., 1993; LaMantia et al., 2000), and disruption of RA- and FGF signaling leads to severely compromised OBs (LaMantia et al., 1993; Anchan et al., 1997; Hebert et al., 2003; Garel et al., 2003). Therefore, interaction between the frontonasal region and the rostral part of the telencephalon, through the activities of these molecules, could be essential for OB protrusion.

Abnormal migration of mitral cell progenitors in *Pax6* mutants was rescued by transfection of exogenous *Pax6* into the mutant telencephalon. This indicates that mislocation of the OBLS in *Pax6* mutants is caused by loss of *Pax6* function in the mutant telencephalon. Recent studies have reported that Pax6 regulates rostral-caudal patterning of the telencephalon (Bishop et al., 2000; Bishop et al., 2002; Muzio et al., 2002). Because the rostral-lateral area of the telencephalon, which expresses *Pax6* strongly, is reduced in *Sey/Sey* embryos, *Pax6* is thought to coordinate the rostral-lateral identity of the telencephalon (Bishop et al., 2002; Toresson et al., 2000; Yun et al., 2001). It has also been demonstrated that the rostral-caudal patterning of the telencephalon is influenced by FGF signaling (Fukuchi-Shimogori and Grove, 2001; Storm et al., 2003; Garel et al., 2003). We also identified that expression of *fgfrs* is reduced severely in the telencephalon of *Pax6* mutants, especially in the rostral area (T.N. and N.O., unpublished). FGF signaling is, thus, likely to be disturbed in the telencephalon of *Pax6* mutants. These data support the idea that transfection of exogenous *Pax6* restores an impairment in regional identity in the rostral area of the mutant telencephalon and re-establishes the positional information that regulates the migration patterns of mitral cell progenitors. However, we cannot rule out the possibility that restoration of the cell migratory pattern in *Pax6*-transfected embryos is unrelated to the initial defect in

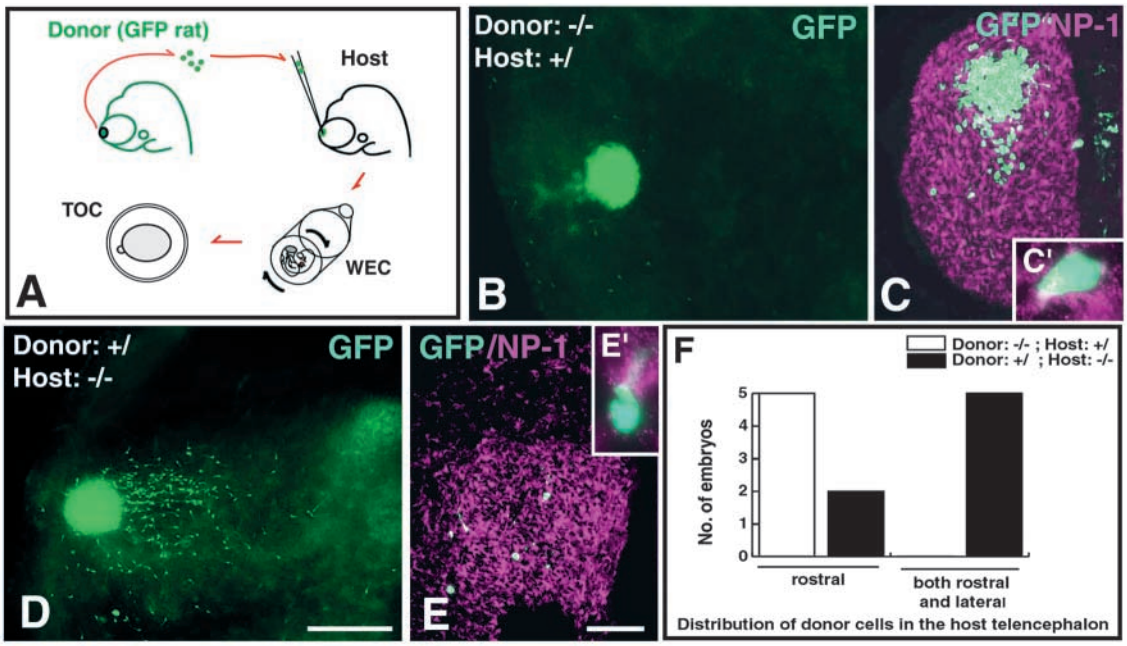

Fig. 5. Misrouting is caused by non-cell autonomous defects for the mitral cell precursors. (A) Experimental procedures for cell transplantation. The rostral part of the telencephalon of donor embryos was isolated, and neuroepithelial cells dissociated with enzymes and transplanted into the rostral part of the host telencephalon. Donor cells were isolated from GFP-transgenic rat embryos. (B,C) Distribution pattern of cells derived from homozygous mutants (–/–) transplanted into heterozygous (+/) embryos after 48 hours of WEC followed by TOC. Donor cells contribute to the host OB and express Nrp (C,C′). (D,E) Distribution pattern of +/– derived cells in –/– embryos after 48 hours in WEC (D) and 2 days in TOC (E). Donor cells migrate caudally toward the lateral telencephalon and invade the OBLS, which is marked by Nrp immunostaining (E). A donor-derived cell in the OBLS expresses Nrp (E′). (F) The distribution of donor cells after transplantation. The white bar indicates that donor cells are located only at the rostral part of the telencephalon. Black bars indicate that donor cells are located in both rostral and lateral parts of the host telencephalon. Scale bars: B,D, 500 μm; C,E, 100 μm; C′,E′, 10 μm.

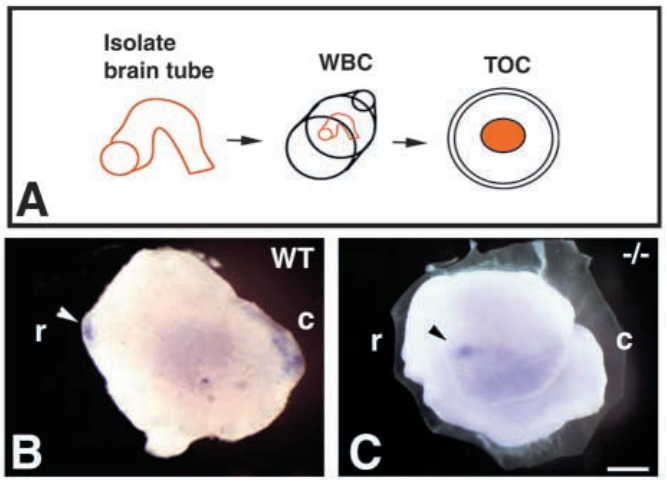

Fig. 6. Misrouting of the mitral cell precursors in *Pax6* mutants is not caused by lack of olfactory nerve innervation. (A) Experimental procedures for brain culture. After removal of the olfactory epithelium and head mesenchyme, brain tubes were cultured in rotating bottles (whole brain culture, WBC) and in TOC for 2 days. (B,C) Distribution of netrin G1-positive cells in the wild-type (WT; B) and the mutant (–/–; C) telencephalon after WBC. In the wild-type telencephalon, netrin G1-positive cells are located in the rostral-most telencephalon (arrowhead in B), whereas in the mutant they are in the lateral part of the telencephalon (arrowhead in C). Scale bar: 500 μm.

Pax6 mutants, but is caused by a secondary event that is induced by transfection of exogenous *Pax6*. To solve this problem, it will be necessary to identify molecules downstream of *Pax6* that directly control the migration pattern of mitral cell precursors.

Mechanisms controlling migration patterns of mitral cell progenitors

What mechanisms could be responsible for controlling cell migration patterns of mitral cell precursors? Because mitral cell precursors migrate caudally towards the lateral part of the mutant telencephalon, it might be possible that migratory cue(s) that inhibit or attract migration of mitral cell precursors are absent and/or appear ectopically in the lateral region of the telencephalon of *Pax6* mutants. *Pax6* regulates the expression of cell adhesion molecules including R-cadherin, which is responsible for maintaining the compartment boundary between the dorsal and ventral parts of the telencephalon (Stoykova et al., 1997; Inoue et al., 2001; Tyas et al., 2003). Thus, it might be possible that contact-dependent, cell-cell interaction between mitral cell precursors and cortical neuroepithelial cells is important for positioning the olfactory bulb in the telencephalon. However, we could not restore the position of the OBLS using an expression vector to overexpress R-cadherin in the mutant telencephalon (data not shown). Also, we could not identify altered expression of neuronal guidance molecules, such as netrin 1, slit 2 and semaphorin 3A (T.N. and

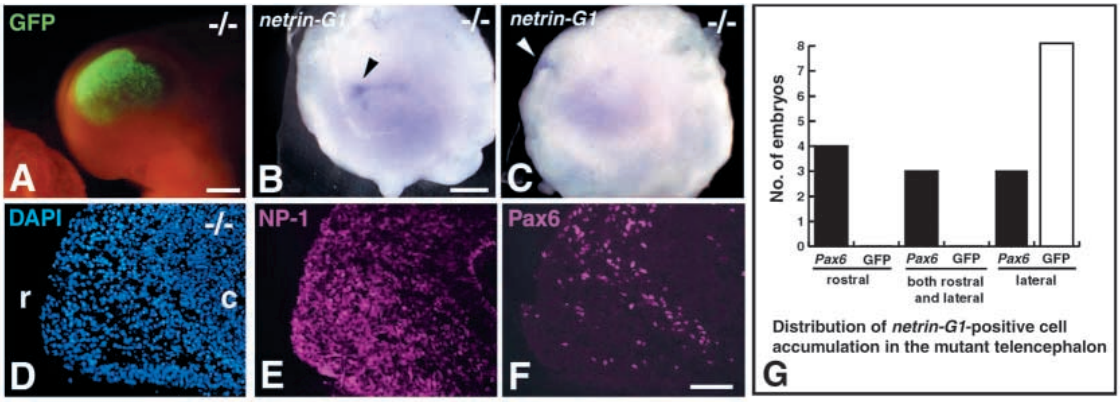

Fig. 7. Misrouting of mitral cells is restored by transfection of *Pax6* into the telencephalon of *Pax6* mutants. (A) Expression vectors were electroporated into the rostral half of the mutant telencephalon. (B-F) *Pax6*-mutant embryos/brains transfected with either *GFP* alone (B) or *GFP* plus *Pax6*-expression vector (C-F). (B,C) lateral views of cultured brains. After 48 hours of WEC followed by 2 days of TOC, netrin G1-positive cells are distributed at the lateral surface of the telencephalon in the mutant introduced *GFP*-gene alone (arrowhead in B). By contrast, netrin G1-positive cells (arrowhead in C) are localized in the rostral-most telencephalon in the embryo electroporated with *Pax6*. (D-F) horizontal sections of the rostral part of the brains transfected with *Pax6*-expression vector. Nrp-positive cells are localized at the rostral-most telencephalon, which corresponds to the netrin G1-positive area (E). Pax6-immunopositive cells are distributed at the rostral part of the telencephalon after 2 days of TOC (F). (G) The location of the accumulation of netrin G1-positive cells in the mutant telencephalon after electroporation. tel, telencephalon; r, rostral; c, caudal. Scale bars: A, 500 μm; B,C, 1 mm; D-F, 50 μm.

N.O., unpublished), implying that other molecules are responsible for controlling mitral cell migration.

The mitral cells originate from the rostral-dorsal telencephalon

Many researchers have investigated the developmental origin of the OB, but it is still ambiguous whether the OB is derived from the dorsal or ventral telencephalon. Several morphological analyses have classified the OB as a ventral telencephalon-derived structure (Källen, 1962; Nieuwenhuys, 1998). By contrast, molecular analyses and genetic approaches indicate that mitral cells originate from the dorsal telencephalon (Bulfone et al., 1998; Puelles et al., 2000). In the present study we performed cell tracing in WEC and TOC, which revealed directly that the mitral cells are derived from the rostral part of the dorsal telencephalon. However, several cell tracing and genetic analyses indicate that interneurons of the OB are derived from the ventral telencephalon (Anderson et al., 1999; Wichterle et al., 2001; Nery et al., 2002). Thus, it is likely that the OB is a mosaic structure consisting of several types of neurons that have different origins within the telencephalon, as in the case of the neocortex (Wilson and Rubenstein, 2000) and the olfactory cortex (Hirata et al., 2002). In *Pax6* mutants, the OBLS consists of the projection neurons and different types of interneurons probably corresponding to the perigromerular cells and the granule cells (Fig. 1). The question of whether the origins and migratory pathways of these interneurons are impaired in the mutants remains to be clarified. Precise fate mapping of the OBLS interneurons in *Pax6* mutants is required to further understand the roles of *Pax6* in positioning the different types of OB neurons in the telencephalon.

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