Transient expression of c-kit receptor in the immature projection neurons of the olfactory bulb

Hitoshi Yamatani

Doctor of Philosophy

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies

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CONTENTS

Abstract

In mammalian olfactory system, the olfactory sensory neurons make a stereotyped spatial map on the olfactory bulb (OB). Mitral/tufted cells, the olfactory second-order neurons relay the olfactory information from the OB to the olfactory cortex through the axonal bundle, the lateral olfactory tract (LOT) in the ventrolateral telencephalon. There is not a clear point-to-point topographic relationship between the spatial map on the OB to any of the olfactory cortices, as other sensory systems. Furthermore, the spatial representation of the OB is already lost in the LOT, in which mitral/tufted cell axons are intermingled randomly regardless of the position of their cell bodies.

In the present study, I demonstrated that c-kit receptor tyrosine kinase is expressed on a fasciculated subset of axons in the LOT at each developmental stage. The c-kit-expressing cell bodies were distributed in the intermediate zone in the whole OB. BrdU-labeling experiment showed that the newly-differentiated mitral/tufted cells radially migrating in the intermediate zone transiently expressed c-kit, so that the expression of c-kit was always fixed in the intermediate zone. These results indicate that mitral/tufted cell axons projecting at the same developmental stage are grouped together and constitute a special assembly within the LOT bundle, regardless of the positions of their cell bodies in the OB. The newly elongating c-kit-positive axons usually occupied the ventral surface area in the LOT. Therefore, there seems to be a developmental gradient in the organization of LOT axons from the dorsal depth to ventral surface. These results together with the previous axonal tracing studies suggest that arrangement of LOT axons is not based on the topographical position of the cell bodies but the developmental order of the axons.

Introduction

The topographic projection is one of the most striking features of processing in the nervous system. In the optic system, retinal ganglion cell axons form precisely ordered retinotopic projection from the retina to their target (Siminoff et al., 1966). Furthermore, in nonmammalian vertebrates, retinal axons are retinotopically ordered even in the optic tract (Scholes, 1979; Easter et al., 1981; Cima and Grant, 1982; Bunt and Horder, 1983; Thanos and Bonhoeffer, 1983; Stuemer and Easter, 1984; Thanos et al., 1984). This preordering of retinal axons in the optic pathway has been suggested to support the retinotopic projection. However, in mammals, there is slight tendency toward retinotopic order (Simon and O'Leary, 1991), but rather retinal axons are chronotopically ordered in the optic tract (Reese and Cowey, 1990; Reese, 1996). The axonal order in the optic tract therefore, indicates a chronological index of their arrival time during development in mammals.

In the mammalian olfactory system, each olfactory neuron expresses only one of about 1,000 different odorant receptor genes (Ressler et al., 1993; Vassar et al., 1993; Malnic et al., 1999). The olfactory neurons expressing the same receptor are scattered in the olfactory epithelium, but their axons converge into a few topographically fixed glomeruli, specialized synaptic structures in the olfactory bulb (OB). About 1,800 glomeruli are orderly arranged on the surface of mouse OB. The convergent projection of axons expressing the same olfactory receptor constructs a stereotyped spatial map on the OB (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998).

The odorant information converged in the glomerulus is subsequently transmitted to the second-order neurons, mitral and tufted cells in the OB. These neurons project axons caudally and construct the lateral olfactory tract (LOT) on the surface of the ventrolateral telencephalon (Fig. 2A; Hinds et al., 1972; Brunjes and Frazier, 1986). The LOT axons eventually sprout collateral branches invading the olfactory cortex and form connections with the third-order neurons (Fig. 2B). The olfactory cortex consists of morphologically distinct areas such as the anterior olfactory nucleus, the piriform cortex (PC), the olfactory tubercle (OT), the entorhinal cortex and the amygdala (Fig. 2A; Brunjes and Frazier, 1986). Previous axonal tracing studies show that each second-order neuron projects to the multiple target areas with massive sprouting of collateral branches (Ojima et al., 1984; Luskin and Price, 1982).

In contrast to the clear rule of the peripheral olfactory projection, the principal of central olfactory projection is obscure. Although a few studies suggest specific targeting of the central projections from second-order neurons in the same or neighboring glomeruli (Grafe and Leonard, 1982; Buonviso et al., 1991; Zou et al., 2001), it is hard to expect a point-to-point topographic relationship between the spatial map on the OB to any of the olfactory cortical target areas, like other sensory systems, such as the optic system. In fact, retrograde axonal labelings show that a single small cortical region receives inputs from the mitral/tufted cells scattering over the OB (Haberly and Price, 1977; Scott et al., 1980). Anterograde axonal tracings also show that the spatial relationships in the OB is not reflected in the olfactory cortical areas (Price, 1972, Devor et al, 1976). Furthermore, there is evidence that the spatial

representation of the OB is already lost at the level of the LOT, in which mitral/tufted cell axons are intermingled randomly regardless of the position of their cell bodies (Price and Sprich, 1975; Scott et al., 1980; Fukushima et al., 2002).

In the present study, we produced monoclonal antibody (mAb) H2C7 that recognizes a subset of LOT axons at each developmental stage. The H2C7 positive axons were grouped together and formed a fascicle within LOT, suggesting a special organization of LOT axons. I here show that mAb H2C7 recognizes c-kit receptor tyrosine kinase and that this protein is transiently expressed by immature mitral/tufted cells migrating in the intermediate zone on the OB. These findings suggest that the LOT axons are not randomly distributed but organized in a developmentally regulated manner.

Materials and Methods

Mice

ICR-+/+, WB-*W*/+, WB–*Sl*/+, and C57BL/6-*Sl*^{d}/+ mice were purchased from Japan SLC (Shizuoka, Japan) and maintained at the National Institute of Genetics. *W/W* and *Sl/Sl*^{d} neonatal mice were generated by the cross of the corresponding heterozygotes and identified by the coat color. C57BL/6-c-kit (151) Cre transgenic mice that have the cre recombinase gene under the c-kit promoter (Eriksson et al., 2000) were provided by Dr. Björn Eriksson of Umeå University. The mice were crossed with C57BL/6-CAG-CAT-Z transgenic mice having the lacZ gene inducible by Cre recombinase (Sakai and Miyazaki 1997), which were provided by Dr. Jun-Ichi Miyazaki of Osaka University. The resulting c-kit Cre/CAG-CAT-Z double transgenic mice were identified by PCR of the tail genomic DNA with specific primers. C57BL/6-green mice whose cells express EGFP (Okabe et al., 1997) were provided by Dr. Masaru Okabe of Osaka University. The green mice were crossed with wild-type female mice to generate green mouse embryos, which were selected under UV light (Model UVM-57, UVP, Upland, CA). Developmental stages were calculated by considering the day on which a vaginal plug was detected as embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0).

Production of mAb H2C7

Hybridoma clones were produced by fusion of mouse myeloma cells and lymphocytes of Armenian hamsters that had been immunized with mouse olfactory cortices

dissected from E14 mouse embryos, as described previously (Sato et al., 1998). MAb H2C7 was selected from the hybridoma clones through immunohistochemical screening on coronal section of E14.5 mouse telencephalon. For purification of the mAb, 1 x 10^8 hybridoma cells were cultured in the gas permeable bag (i-MAb Monoclonal Antibody Production Kit; Diagnostic Chemicals, Oxford, CT) with the serum-free medium (GIT medium; Wako, Osaka, Japan) for 2 weeks. The mAb was affinity purified from the culture supernatant by HiTrap protein A (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column chromatography with protein A MAPS2 kit (Bio-Rad Laboratories, Hercules, CA).

Histochemistory

Mouse embryonic and neonatal brains were fixed with 4% paraformaldehyde (PFA) in PBS or Zamboni's solution overnight at 4℃, immersed in 20% sucrose in PBS overnight at 4℃, and frozen in OCT compound (Tissue-Tek 4583; Sakura Finetechnical, Tokyo, Japan). Coronal sections 14 μm thick were cut on a cryostat and placed on glass slides coated with APS (Matsunami Glass, Osaka, Japan). The section were incubated with 10 mM Tris-HCl, pH 7.4, 130 mM NaCl, 0.1% Tween 20 (TBST) for 10 min to remove the OCT compound, and then with mAb H2C7 (1.2 μg/ml) and rabbit anti-neuropilin-1 antibody (0.5 μg/ml), rabbit anti-cholecystokinin-8 antibody (1:200; Affinity Research Product Ltd., Mamhead Castle, UK) or rabbit anti-glutamate decarboxylase (GAD) polyclonal antibody (1:1000; Chemicon international, Temecula, CA) overnight at 4℃. Binding of these antibodies was detected with Cy3-

labeled goat anti-hamster antibody (1:200; Jackson ImmunoResearch, West Grove, PA) and Cy2-labeled goat anti-rabbit antibody (1:200; Jackson). In some immunostaining, anti-c-kit mAb ACK2 (a generous gift from Dr. Takahiro Kunisada of Tottori University) and goat anti-c-kit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Fluorescent images were obtained with a fluorescence microscope (Axioplan 2; Carl Zeiss, Jena, Germany) or a confocal laser-scanning microscope (Olympus FV-500; Olympus, Tokyo, Japan).

For whole-mount immunostainning, telencephalons were dissected out and freed from the pia matter, and fixed with 4% PFA in PBS for 2 hr at room temperature. The specimens were treated with methanol containing 0.3% H_2O_2 for 10 min to quench the endogenous peroxidase activity and incubated in 5% skim milk / TBST for 1 hr to block nonspecific binding of antibodies. Subsequently, the specimens were incubated with mAb H2C7 overnight at 4℃, biotinylated goat anti-hamster IgG antibody (1:200; Jackson) for 2 hr and finally streptavidin-biotin-peroxidase complex (1:300; Vector Laboratories, Burlingame, CA) for 2 hr at room temperature. The immunolabeling was visualized with diaminobenzidine.

For lacZ staining, brains were fixed with 0.2% glutaraldehyde, 2% formaldehyde, 5 mM EGTA, 2 mM $MgCl_2$ / PBS for 30 min at room temperature, washed three times with PBS, immersed in 20% sucrose / PBS overnight and frozen in OCT compound. Cryosections 20 μm thick were prepared and incubated with 1 mg/ ml 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-gal), 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ / PBS at 37℃ overnight. After washed twice in PBS, the sections were

counterstained with hematoxylin (Matsunami).

Expression cloning of the antigen recognized by mAb H2C7

The procedures of expression cloning were described previously (Hirata et al., 2002; Tozaki et al., 2002). In brief, pools of cDNA expression clones prepared from mRNA of E14.5 mouse olfactory bulbs were transfected into COS7 cells. After 48-hr culture, the cells were fixed with 4% PFA in PBS and immunostained with mAb H2C7. Among 400 pools containing about 40,000 cDNA clones screened, a pool generating H2C7-positive COS7 cells was identified. The pool was divided into smaller pools and assayed repeatedly until the single cDNA clone was isolated.

In situ Hybridization

The mouse c-kit cDNAs encoding 3710-4244 and 4244-5093 were subcloned into pBluescript KS (-). Using the plasmids linearized with Not Ⅰ and HindⅢ as templates, digoxigenin-labeled single strand RNA probes were transcribed with T3 and T7 RNA polymerase (DIG RNA Labeling Kit; Roche Diagnostics Gmbh, Mannheim, Germany). Following the previous procedures of Nomura et al. (1988) with minor modification, cryosections were post-fixed with 4% PFA, 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB) for 15 min, treated with 50 µg/ml proteinase K in 10 mM Tris and 1 mM EDTA (TE) for 10- 30 min, fixed again, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. The sections were pre-hybridized with the hybridization solution (50% deionized formamide, 10% dextran sulfate, 1 x Denhardt's

solution, 600 mM NaCl, 0.25% SDS, 200 µg/ml yeast total RNA in 10 mM Tris-HCl pH7.6) for 2 hr at room temperature. The hybridization solution containing 0.5 µg/ml RNA probe was applied on sections, which were covered with glass slips and incubated at 50℃ overnight in a moisture chamber. After hybridization, the sections were briefly rinsed with 5 x SSC and 2 x SSC for 30 min at 50℃, and treated with RNase A (10 µg/ml) at 37℃ for 30 min. Subsequently, the sections were washed with 2 x SSC for 20 min at 50℃ and 0.2 x SSC for 15 min twice at 50℃. Hybridization signals were detected by Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions. After the color reaction, the slides were rinsed with distilled water and mounted with crystal mount (Cosmo Bio, Tokyo, Japan).

Bromo-deoxyuridine labeling

Pregnant mice were injected intraperitoneally with 3 mg 5-Bromo-2'-deoxyuridine (BrdU; Sigma, St. Luis, MO) at various gestation periods. Brains were dissected out from the offsprings at certain developmental stages and processed for cryosections. The sections were first immunostained with mAb H2C7 and Cy3-labeled anti-hamster Ig antibody as described, then fixed again, and treated with 4 M HCl for 5 min. After neutralization with 0.1 M Tris-HCl, pH 9.0, the sections were incubated with mouse anti-BrdU antibody (1:100; BD Biosciences, Franklin Lakes, NJ) and with FITC-labeled anti-mouse Ig antibody (1:200; Amersham, Buckinghamshir, UK).

Culture

The olfactory bulbs were dissected out from E14.5 green mice embryos, freed from the pia matter, and cut into small fragments. The fragments were placed on 4-well or 2-well chamber slides (Nalge Nunc, Naperville, IL) coated with poly-L-lysine (Sigma), and were cultured at 37℃ for 3 days in Neurobasal medium (Life Technologies, Rockville, MD) with B27 supplement (Life Technologies) that had been conditioned with glial cells as described previously (Tozaki et al., 2002). Organotypic cultures were prepared essentially following the procedures reported previously (Sugisaki et al., 1996). The telencephalon hemispheres of E12.5 ICR mouse embryo was cultured on collagen-coated membrane filters (Transwell-col 3418; Costar, Cambridge, MA) in the above-mentioned culture media. In the co-culture, the olfactory bulbs isolated from E14.5 green mouse embryos were combined with E17.5 *W/W* mutant telencephalon hemispheres, and cultured as well as the whole-mount culture.

Results

mAb H2C7 recognizes a segregated fraction of LOT axons running in the LOT

mAb H2C7 was isolated because it recognized a subset of axons in the LOT during development. From E14.5 through E16.5, mAb H2C7 strongly labeled axons positioned in the ventral and superficial part of the LOT with gradually reduced staining in the deep part of the LOT. (Fig. 1B-E). The axons strongly labeled with mAb H2C7 were grouped together within the LOT and segregated from other LOT axons. At E18.5, the position of H2C7-positive axons seemed shifted more ventrally in the LOT with increase of H2C7-negative axons (Fig.1F,G). The ventral positioning of H2C7-positive axons was observed at all rostro-caudal levels of the LOT. Postnatally, the proportion of H2C7-positive to -negative axons was reduced, and the segregation of H2C7-positive axons became more prominent (Fig. 1H,I). After P10, mAb H2C7 did not recognize LOT axons any longer.

Whole-mount immunostaining of the telencephalon with mAb H2C7 clearly showed the spatial distribution of H2C7-positive axons. At E16.5, mAb H2C7 strongly stained axons on the ventral edge of the LOT (Fig. 2C, D). These axons seemed to emerge from widespread parts of the OB and subsequently take the ventral course in the LOT, although positions of H2C7-positive cell bodies were obscure in whole-mount staining. From this stage, LOT axons begin to sprout collateral branches over the various cortical areas, such as the anterior olfactory nucleus, the piriform cortex (PC), and the OT (Fig. 2A, B) (Hirata and Fujisawa, 1999). mAb H2C7, however, hardly recognized these collateral branches, but basically labeled the primary axons in the LOT (Fig.

2C-F). After E18.5, direct projection of H2C7-pisitive axons into the olfactory tubercle (OT), the ventral area of the olfactory cortex, manifested (Fig. 2E, F). These axons originated from the dorsomedial part of the OB, crossing other LOT axons and invading the OT (Fig. 2F). These ventrally projecting axons appeared to correspond with the ventrally segregated staining with mAb H2C7 on the section (Fig. 1H,I).

mAb H2C7 recognizes the c-kit receptor

To identify the antigen recognized by mAb H2C7, cDNA expression cloning was conducted. The screening yielded a cDNA clone that coded for mouse c-kit, a receptor tyrosine kinase containing five extracellular immunoglobulin-like repeats and an intracellular tyrosine kinase that is split into two domains by an insert sequence (Chabot et al., 1988; Yarden, 1987). Because mAb H2C7 binds the cell membrane of living cells, the epitope for mAb H2C7 is in the extracellular domain of c-kit protein.

Previous studies have reported that c-kit is expressed in a various subset of neurons (Hirota et al., 1992; Zhang and Fedoroff, 1997). Although the expression of c-kit mRNA in the adult OB is also reported, localization of c-kit protein in LOT axons has not been examined. Therefore, I analyzed expression of c-kit protein using anti-c-kit mAb ACK2 (Nishikawa et al., 1991) and polyclonal antibodies. These antibodies stained a fasciculated subset of axons in the LOT in the manner identical to that with mAb H2C7, confirming that mAb H2C7 certainly recognizes c-kit protein.

Transient expression of c-kit by the neurons in the intermediate zone of the OB

To examine which specific neurons project c-kit-positive axons into the LOT, I analyzed the expression of c-kit in the embryonic OB. Immunostainning with mAb H2C7 and in situ hybridization for c-kit mRNA brought similar staining patterns (Fig. 3A,B); the heavily labeled cell bodies were circularly distributed in the intermediate zone flanked between the ventricular zone and the mitral cell layer from E14.5 through P0. These cells had relatively small nuclei compared with those of mitral cells constituting the developing mitral cell layer (Fig. 3C). A small number of cells adjacent to the developing mitral cell layer were also c-kit-positive (Fig. 3C,D), and they occasionally had radially-oriented processes that were inserted into the mitral cell layer (Fig. 3C). In the immunostainning, axon-like processes intermingling with c-kit-positive cell bodies were also stained strongly on the caudal level of the OB. These axon-like processes elongated in the intermediate zone and eventually projected into the LOT (data not shown).

There are two possible cell types for the cells in the intermediate zone; one is the granule cells that eventually constitute the granule cell layer under the mitral cell layer (Hinds, 1968), and the other is newborn mitral/tufted cells that are migrating radially from the ventricular zone (Hinds, 1972; Hinds and Ruffett, 1973). To distinguish these two possibilities, I conducted BrdU-labeling and -tracing analyses. 5 –Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into DNA during the S phase of the cell cycle. When BrdU was injected at E11.5, many BrdU-labeled cells were detected in the c-kit-positive intermediate zone from E14.5 to E15.5

(Fig. 4A). The detailed examination confirmed that these cells were actually doubly positive for BrdU and c-kit (Fig. 4A). The majority of cells labeled at the same E11.5, however, were positioned more superficially in the mitral cell layer, when examined at E16.5 (Fig. 4B). BrdU/c-kit double positive cells were hardly detected anymore. Subsequent tracing of the BrdU-labeled cells showed that these cells that had completed the final mitosis at E11.5 mostly differentiated into mitral cells.

The BrdU injection at E12.5 brought a similar circumferential labeling of cells with a slightly shifted time course. At E15.5, BrdU-labeled cells were mainly detected in the ventricular zone (Fig. 4C), whereas at E16.5, many BrdU-labeled cells were in the intermediate zone, expressing c-kit (Fig. 4D). The c-kit-positive cells in the intermediate zone at E16.5 were not immunostained with anti-GAD67 antibody, a marker for granule cell (Fig. 4E). At the late stages, the BrdU-labeled cells were located in the mitral cell layer and the external plexiform layer (EPL) as differentiated mitral and tufted cells (Fig. 6). These observations argue that c-kit is transiently expressed by newly born mitral/tufted cells radially migrating from the ventricular zone toward the final positions, and thereby, the expression of c-kit is always fixed in the intermediate zone.

If this interpretation is correct, all mitral/tufted cells should once express c-kit in their early life. This assumption was tested with c-kit (151) Cre/CAG-CAT-Z double transgenic mice. In the mice, c-kit promoter drives the expression of Cre recombinase, which in turn excises the loxP sequence intervening between the basic promoter and lacZ gene. Therefore, once c-kit promoter is activated in some cells of the mice, the

cells will end up expressing lacZ permanently (Fig. 5A). The examination of the mice showed that almost all the mitral cells and tufted cells expressed lacZ in the adulthood (Fig. 5B), supporting that mitral/tufted cells once undergo the process of expressing c-kit receptor.

After P7, c-kit was expressed in a slightly different type of cells that were located in the EPL (Fig. 4F). These c-kit-positive cells did not contain cholecystokinin-8, a marker for tufted cells (Seroogy et al., 1985; Matsunami et al., 1988) and morphologically smaller than the typical tufted cells situated in the EPL. Similar expression of c-kit mRNA was reported in the adult OB in mice. According to the reports, the c-kit-positive cells are categorized into tufted cells (Hirota et al., 1992), short axon cells (Schneider and Macrides, 1978) or glial cells (Motro et al., 1991). To examine whether these c-kit-positive cells in the EPL contributed for the c-kit positive LOT projection, I injected a retrograde fluorescent tracer into the LOT. The c-kit-positive cells in the EPL were, however, never retrogradely labeled (data not shown) although many mitral cells and tufted cells incorporated the tracer. Therefore, these c-kit-positive cells in the EPL appear to be interneurons in the OB and not to contribute for the c-kit-positive axons in the LOT.

The elongation of c-kit-positive neurites in culture

The transient expression of c-kit in newly born mitral/tufted cells suggests that this protein is expressed in growing axons *in vivo*. Therefore, I investigated how the expression of c-kit protein is regulated in cultured neurons. To readily detect neurites,

the OB explants from E14.5 green mouse embryos whose cells expressed enhanced green fluorescent protein (GFP) were cultured on poly-L-lysine-coated dish (Fig. 7A, B). mAb H2C7 recognized only a fraction of the GFP-expressing neurites in culture. The c-kit-negative and –positive neurites were clearly distinguished. The proportion of c-kit-positive to total neurites was about 40%, which was constant during the culture period of 1- 7 days. Furthermore, there was no correlation between the length of neurites and the expression of c-kit protein (data not shown). These results suggest that the neurite outgrowth itself is not linked with the expression of c-kit.

In the LOT, c-kit-positive axons were always fasciculated and segregated from c-kit-negative axons (Fig. 1). In the explant culture, however, c-kit-positive neurites were not fasciculated and elongated randomly on the dish (Fig. 7A, B), indicating that the selective fasciculation of c-kit-positive axons was not reproduced in the explant culture. I further examined how c-kit-positive axons behaved in a more natural environment under two different organotypic cultures.

First, the whole telencephalon of E12.5 mouse embryo was cultured for 3 days (Fig. 7). At the starting point of culture, OB axons hardly grew out into the telencephalon, yet. After 3-day cultivation, OB axons formed LOT-like fiber bundles (Fig. 7D, E). C-kit-positive axons grouped together within the LOT and elongated in the superficial part of the LOT bundle (Fig. 7C, E, F). The c-kit-positive axons were, however, positioned in the dorsal part of the LOT, instead of the ventral part.

When the embryonic OB is co-cultured with the LOT position of E14.5 telencephalon strip, mitral cell axons selectively grow into the LOT (Hirata and

Fujisawa, 1999). Thus, the OB of E14.5 green mouse was combined with the LOT position of the E14.5 telencephalon strip and co-cultured for 3 days to examine the projection of c-kit-positive axons (Fig. 8). The GFP-expressing axons preferentially elongated into the LOT (Fig. 8D, E). A fraction of these axons expressed c-kit. Although these c-kit-positive axons preferentially grew in the LOT pathway as well, they did not choose the superficial or ventral part of the LOT and were randomly distributed within the developed LOT bundle (Fig. 8E, F). Consequently, the c-kit-positive axons were intermingled with other c-kit-negative axons.

The LOT projection in *W* and *Sl* mutant mice

The c-kit is encoded by the *W* locus in mice (Chabot et al., 1988; Geissler et al., 1988). The ligand for c-kit is stem cell factor (SCF), which is encoded by the *Sl* locus in mice (Zsebo et al., 1990; Flanagan and Leder, 1990). The interaction between c-kit and SCF is considered to be essential for the development of melanocytes, erythrocytes, mast cells and germ cells, because mutations in either the *W* or *Sl* locus result in serious defects in differentiation of these cells. I examined whether c-kit or SCF was involved in the projection of LOT axons.

The *W* mutation results in a deletion of the transmembrane domain of c-kit (Hayashi et al., 1991). Accordingly, in the *W/W* mutant telencephalon, the staining of LOT axons with mAb H2C7 was completely abolished (Fig. 9B), although some cell bodies were still immunoreactive to the mAb because of accumulation of the truncated c-kit protein.

The *Sl/Sl*^{d} mutant mice that have deletions in SCF genes (Flanagan et al. 1991) provided an ideal material to address the involvement of SCF/c-kit interaction in the LOT projection. In the mutant mice, mAb H2C7 gave indistinguishable patterns of staining from the wild type at each developmental stage. For example, at P2, the selective fasciculation of c-kit-positive axons in the ventral superficial part of the LOT was obvious in the mutant mice (Fig. 9C,D). The direct projection of c-kit-positive axons over the OT was also indistinguishable from that of wild-type mice (Fig. 9E-H). Furthermore, visualization of all the LOT axons with anti-neuropilin-1 antibody did not reveal a defect in the LOT projection (Fig. 9A-D). Therefore, the interaction between c-kit and SCF does not seem to be essential for the projection of the c-kit-positive axons.

Discussion

In contrast to the clear rule of the peripheral olfactory projection, the principal of central olfactory projection is obscure. Mitral/tufted cell axons are reported to be intermingled randomly in the LOT regardless of the position of their cell bodies. I addressed the issue of the OB axon organization in the LOT.

In the present study, I demonstrated that c-kit protein is expressed on a segregated subset of axons in the LOT at each developmental stage, and that this restricted expression of c-kit is brought by newly-differentiated mitral/tufted cells migrating in the intermediate zone. These results indicated that mitral/tufted cell axons projecting at same developmental stage are grouped together and constitute a special assembly within the LOT bundle, regardless of the position of their cell bodies in the OB. Because the newly elongating c-kit-positive axons usually occupied the ventral surface area in the LOT, there seemed to be a developmental gradient in the organization of LOT axons from the dorsal depth to ventral surface. Previous axonal tracing studies have failed to show a point-to-point topographic relationship between the position of cell bodies in the OB and the distribution of their axons within the LOT, which leads to the confusing concept of random diffuse arrangement of LOT axons (Price, 1972; Devor, 1976). These results are, however, reasonable because arrangement of LOT axons is not based on the topographical position of the cell bodies but the developmental status of the axons as the present study demonstrated. This developmental organization of LOT axons might have some special role as the foundation to provide the collateral projection toward the complex olfactory target

areas.

When the E14.5 OB explants were cultured in a dish, c-kit-positive neurites did not fasciculate and randomly mixed with c-kit-negative neurites. The whole-mount culture of the E12.5 telencephalon reproduced the segregated projection of c-kit-positive axons in the superficial part of the LOT although the axons chose the dorsal position but not the ventral part. By contrast, in the organotypic co-culture, c-kit-positive axons did not choose the superficial part but randomly elongated within the LOT, intermingling with c-kit-negative axons. In the whole-mount culture, OB axons sequentially elongate on the previously projected axons. One major difference of the condition between co-culture and whole-mount culture is that both c-kit-positive and –negative OB axons simultaneously start elongating in co-culture. Therefore the developmentally ordered projection of LOT axons might have some role for the selective fasciculation of c-kit-positive axons.

This developmental arrangement of OB axons in the LOT is similar to the axonal arrangement of the optic tract. In the optic pathway, retinal ganglion cell axons become reordered chronotopically as they pass through the chiasmatic region. Later arriving axons grow only within the superficial region of the optic tract and are segregated from the older retinal axons, which are found at deeper location within the pathway. Furthermore, the segregation of axons is also characterized with the difference in the range of fiber diameters; larger–sized axons positioned in the deeper part of the optic tract, and smaller axons in the superficial part (Colello and Guillery, 1992; Reese, 1996). This caliber difference is considered to reflect the difference in

classes of retinal ganglion cells and their birth dates. In the LOT, axonal sizes are reported to be different between the dorsal part and the ventral part of the LOT (Price and Sprich., 1975). Therefore it is possible that the chronological order of OB projection reflects different subtypes of mitral/tufted cells.

A phosphorylated form of MAP1B protein is reported to be localized in a subset of LOT axons (Harrison et al., 1993). Although the OB cells expressing this phosphorylated form are not clarified, its spatio-temporal distribution in the LOT is similar to that of c-kit, suggesting that some common regulations are operating for the expression of c-kit and the phosphorylation of MAP1B. Generally, this specific form of MAP1B is enriched in growing axons (Calvert et al., 1987), supporting the expression of this form in newly born projection neurons in the OB. Thy-1 protein, which is usually expressed by mature neurons, is conversely enriched in the deeper axons of the LOT (Xue et al., 1990), showing the complementary distribution of c-kit-positive axons. These observations support the idea that the LOT axons are organized in a developmentally-regulated manner.

Hinds, using Golgi and electron microscopic techniques, has outlined sequential steps of mitral cell differentiation (Hinds, 1972; Hinds and Ruffett, 1973). (1) First, mitral cells migrate radially from the ventricular zone into the deep part of the intermediate zone, having the primitive radial processes. (2) The cells then withdraw the radial processes and are reoriented horizontally in the intermediate zone, extending the axons tangentially. (3) The cells change the orientation again into the radial direction and migrate to the mitral cell layer to differentiate into mature mitral

cells. Although this scheme is solely based on the discontinuous morphological observations on fixed specimens, the developmental process of mitral/tufted cells observed in the present study fits well with this scenario. According to this scheme, the expression of c-kit could be attributed to the horizontally oriented cells on the second stage and the cells that resumed the radial migration on an early phase of the third stage.

Mitral/tufted cells are continuously generated between E12 and E18 and elongate their axon toward the LOT. In later developmental stages when the LOT bundle has almost been completed, many c-kit-positive axons were observed to project into the OT selectively and directly. The OT is the olfactory target area that receives the latest innervation by OB axons (Schwob and Price, 1984). Furthermore, the OT receives a heavier projection from tufted cells (Skeen and Hall, 1977; Haberly and Price, 1977), the late-born projection neurons in the OB (Hinds, 1968; Bayer, 1983). These facts suggest that the c-kit expression marks the selective projection to the OT at the late stages, because these axons were projected from late-born neurons.

In this study, I demonstrated that the c-kit expression of mitral/tufted cells is regulated in a time-dependent manner. A previous study on the mouse c-kit promoter reports that the essential cis-acting elements required for the cell type specific expression are localized within 105 bp of the transcription initiation site. This region contains consensus binding sites for Sp1, Ap-2 and several short GA-rich elements which resemble binding sites for the ETS-domain protein (Yasuda et al., 1993). These factors regulate the c-kit expression in hematopoietic cells and erythroid cells.

Because these factors are also expressed in the nervous system (Pugh and Tjian, 1990; Shimada et al., 1999), they might regulate c-kit expression in the mitral/tufted cells.

In spite of the expression of c-kit in the nervous system, the function of c-kit/SCF signaling system in the nervous system is uncertain. Although there are studies reporting the impairment of learning and reduction of synaptic transmission in the hippocampus of *Sl/Sl*^{d} mice (Motoro et al. 1996; Katafuchi et al., 2000), morphological defects have not been detected in the nervous system of *Sl* or *W* mutant animals (Motro et al., 1991). In this study, I did not detect any abnormality in the LOT projection of either *W/W* or *Sl/Sl*^{d} mutant mice. Nevertheless, it is still possible that more detailed analyses involving ultrastructual and physiological techniques reveal some function of this signaling. It is also possible that some redundant molecules have compensated for the function of c-kit/SCF system in the LOT.

Acknowledgement

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Figure. 1

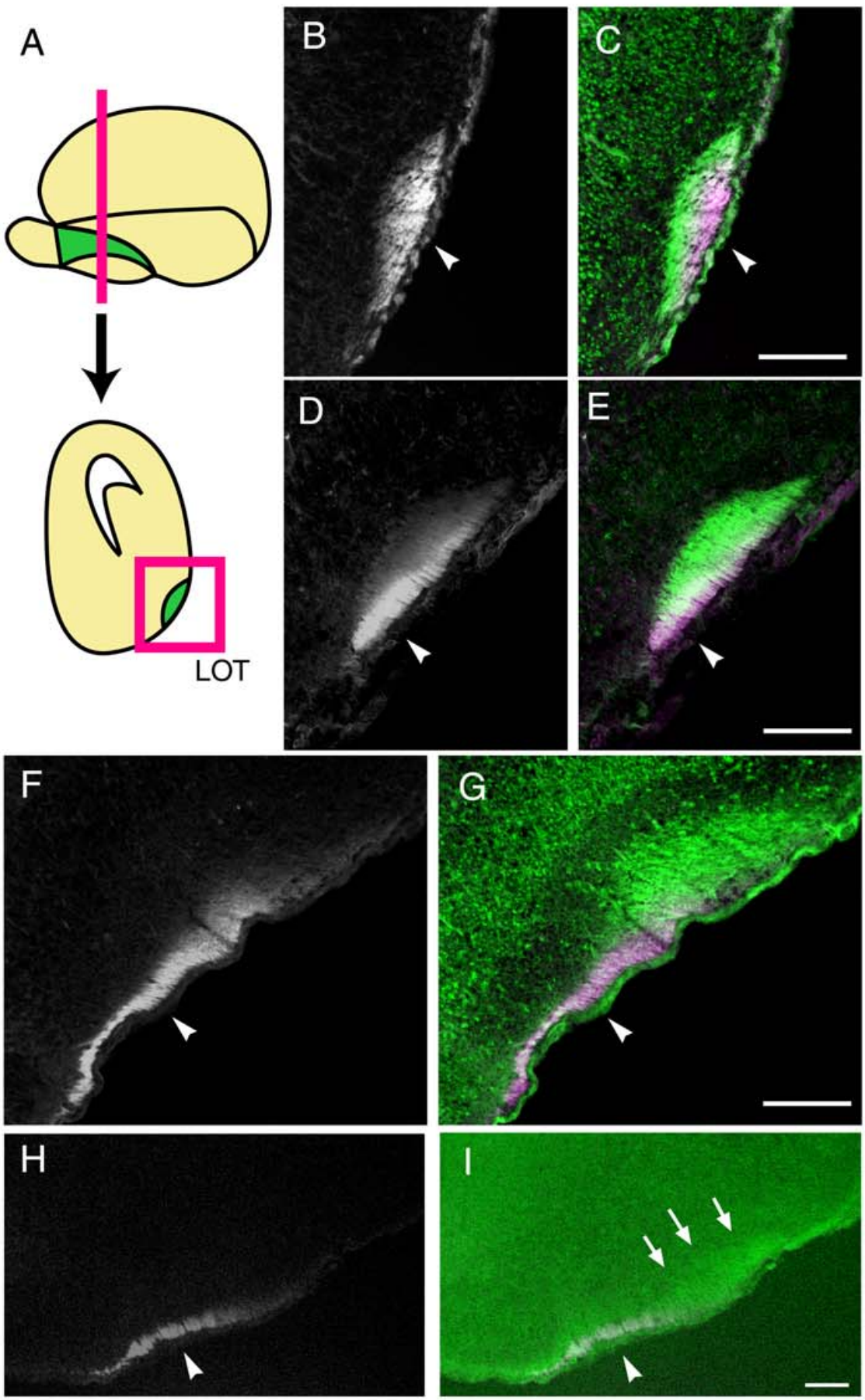

Figure. 1 Immunostaining of the LOT with mAb H2C7 at various stages. *A,* A schema of a coronal section of the mouse embryonic telencephalon. The OB axons form the bundle, the LOT (green) on the lateral surface of the telencephalon. *B,D,F,H,* Immunostaining of coronal sections of the LOT with mAb H2C7 at E14.5 (B), E16.5 (D), E18.5 (F) and P2 (H). *C,E,G,I,* Marged views of the immunostaining with mAb H2C7 (magenta) on the left panel and anti-neuropilin-1 antibody (green), a marker for OB axons. MAb H2C7 labels the axons in the ventral superficial part of the LOT (Arrowheads). In (I), the deep edge of the LOT is indicated by arrows, because anti-neuropilin-1 antibody does not clearly label the LOT at this stage. Dorsal is at the top, and lateral is to the right in all the panels. Scale bars = 100 μm

Figure. 2

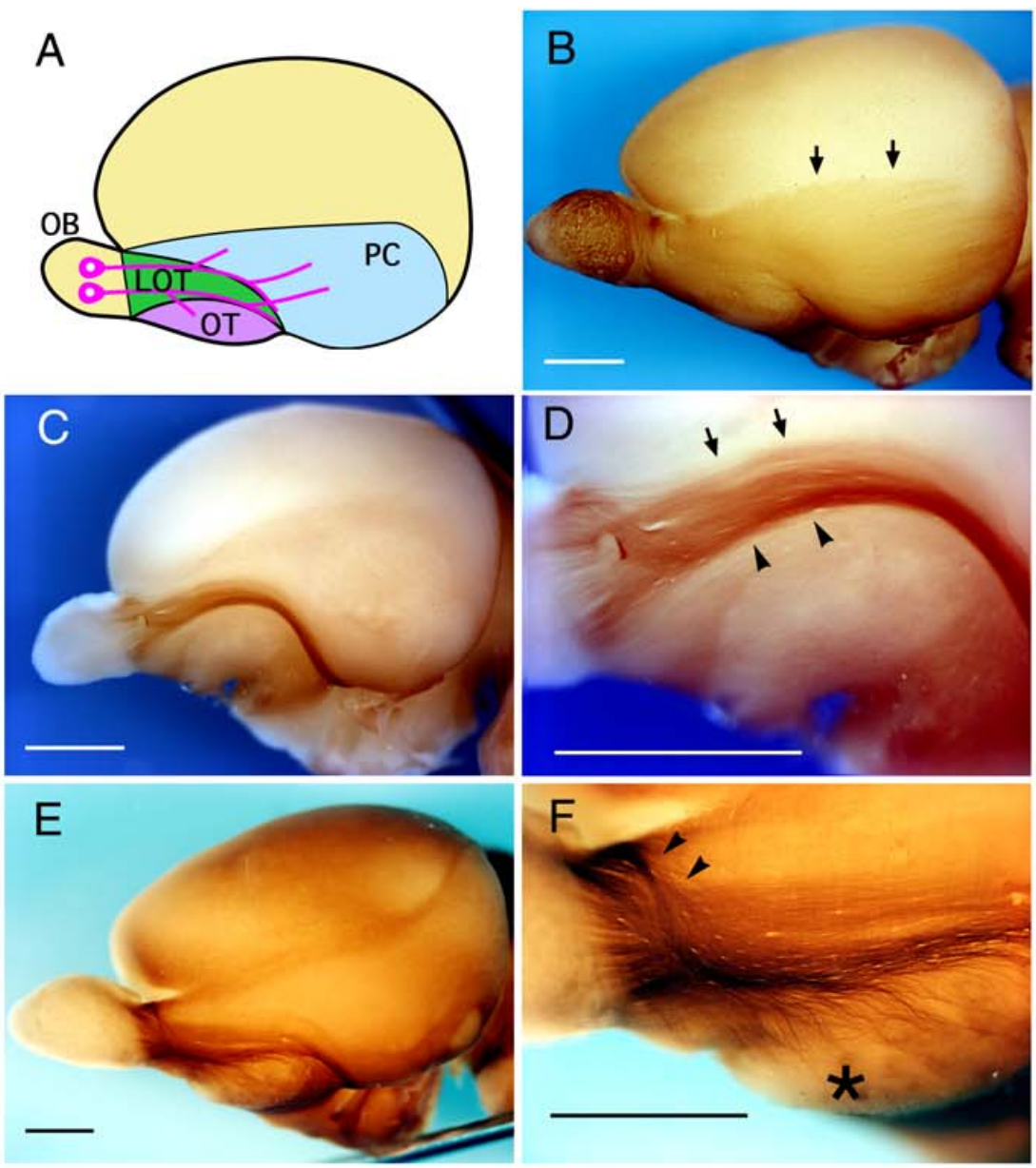

Figure. 2 Spatial distribution of H2C7-positive axons in the developing telencephalon. *A,* Schematic drawing of a ventrolateral view of the telencephalon. The OB primary axons form the LOT on the ventrolateral surface of the telencephalon and sprout collateral branches innervating the olfactory cortical areas such as the piriform cortex (PC) and the olfactory tubercle (OT). *B,* The E18.5 telencephalon immunostained with anti-neuropilin-1 antibody. Note the massive projection of collateral branches over the olfactory cortical areas (arrows). *C-F,* Whole-mount immunostaining of E16.5 (C,D) and P2 (E,F) telencephalon with mAb H2C7. *D,F,* Higher magnification views of (C) and (E), respectively. In (D), H2C7-positive axons (arrowheads) occupy the ventral part of the LOT. The dorsal edge of the LOT is indicated by arrows. In (F), H2C7-positive axons from the dorsal medial OB (arrowhead) cross the primary axons and directly invade the OT (asterisk). Rostral is to the left, and dorsal is at the top in all the panels. Scale bars = 1.0 mm

Figure. 3

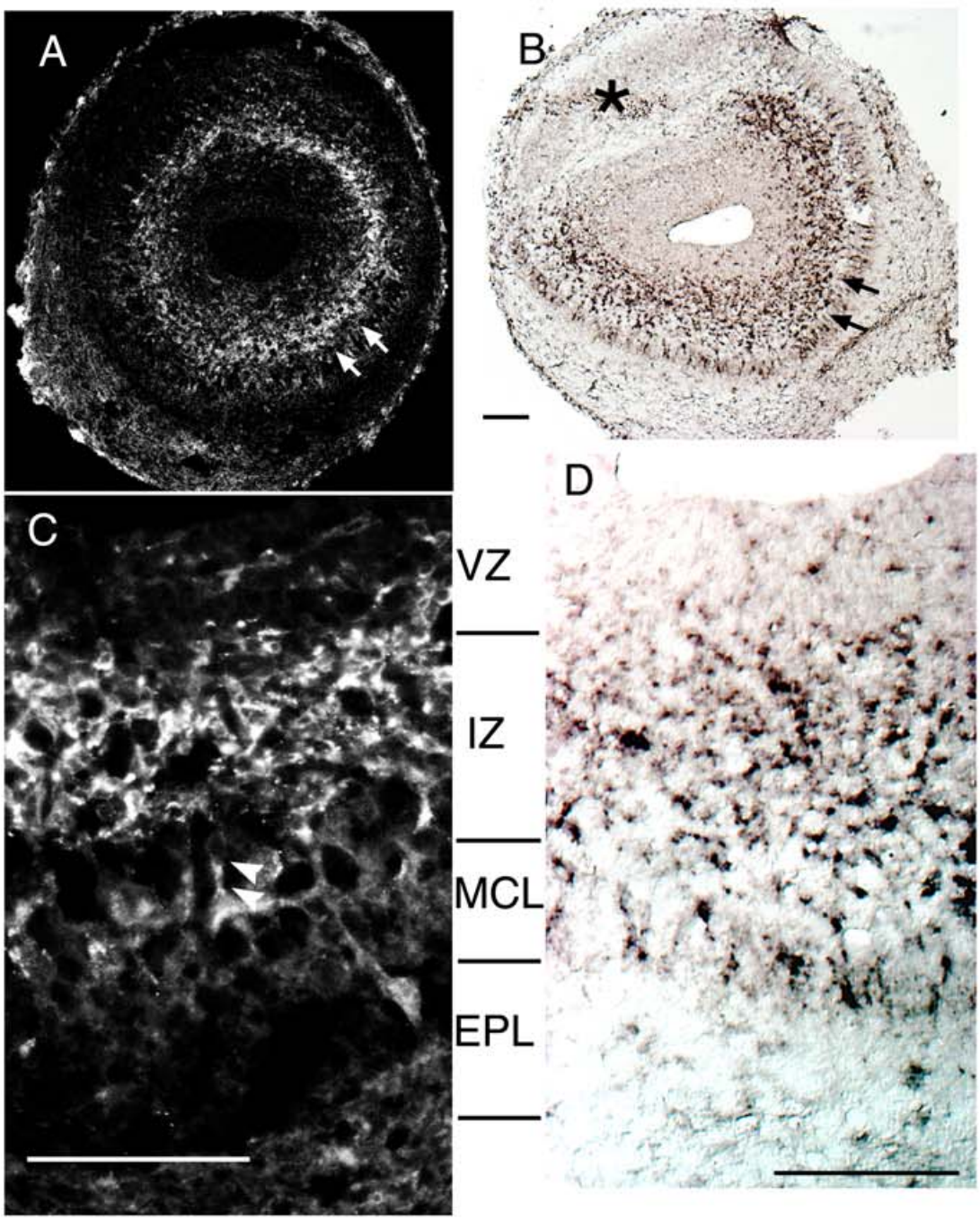

Figure. 3 Distribution of c-kit-positive cells in the olfactory bulb. *A,B,* Coronal sections of the E16.5 OB immunostained with mAb H2C7 (A) and in situ hybridization for c-kit mRNA (B). c-kit-expressing cell in the intermediate zone are indicated by arrows. The asterisk in (B) indicates the accessory olfactory bulb, which has a different cytoarchitecture. *C,D,* Higher magnifications of (A) and (B), respectively. Strong signals are detected in cell bodies in the intermediate zone, which have smaller nuclei than those of mitral cells. Arrowheads indicate that radially-oriented processes of H2C7-positive cells are inserted among mitral cells. Abbreviations; VZ, ventricular zone; IZ, intermediate zone; MCL, mitral cell layer; EPL, external plexiform layer. Scale bar = 100 μm

Figure. 4

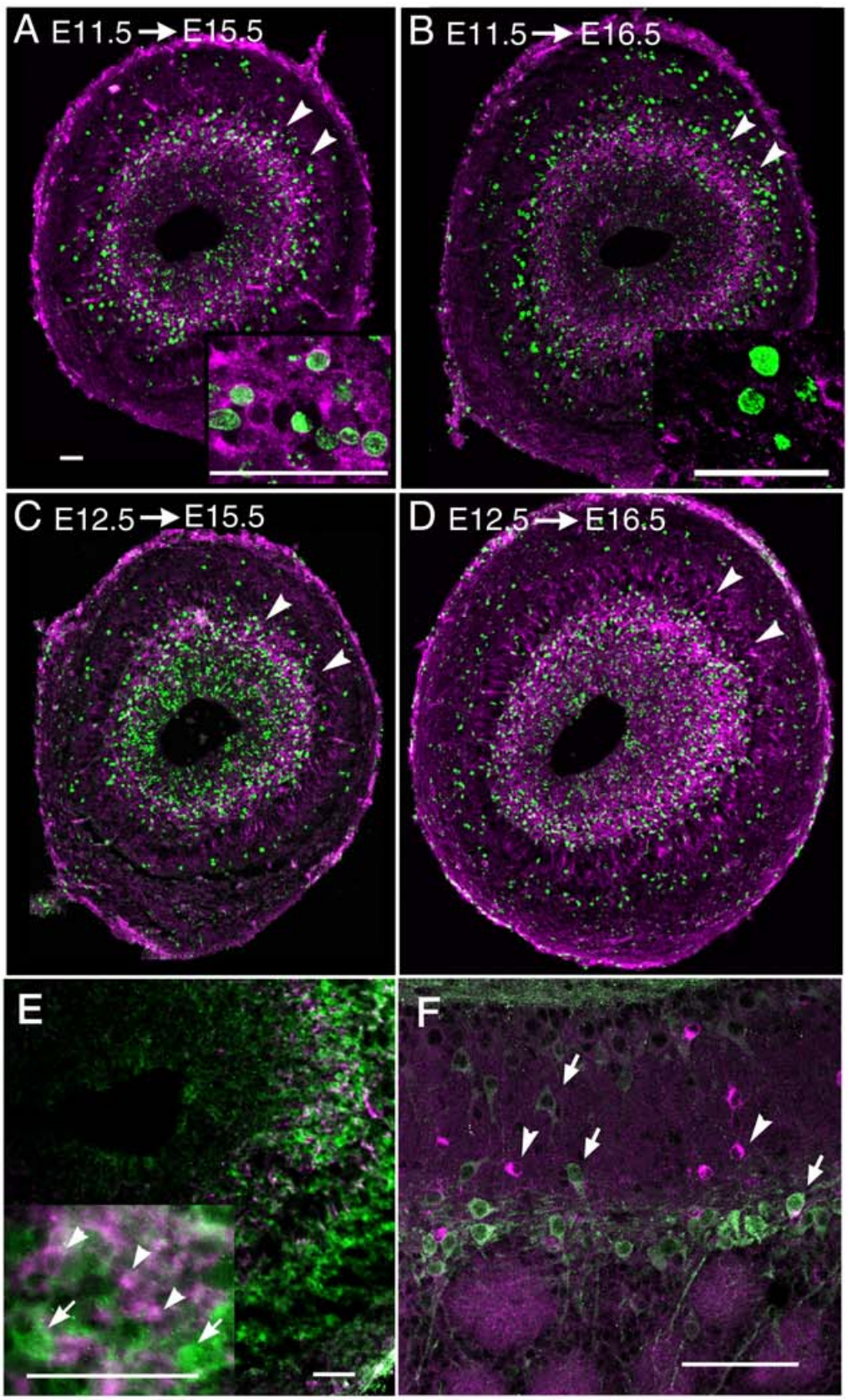

Figure. 4 Development and migration of OB neurons. *A-D,* Double immunostaining of the OBs with mAb H2C7 (magenta) and anti-BrdU antibody (green). BrdU was injected at E11.5 (A,B), or E12.5 (C,D) and detected at E15.5 (A,C) and E16.5 (B,D). The inset in (A) shows an enlarged view of BrdU/c-kit double labeled cells and the insert in (B) shows BrdU-positive and c-kit-negative cells. Arrowheads indicate the position of the mitral cell layer. *E,* Double-immunostaining of the E16.5 OB with mAb H2C7 (magenta) and anti-GAD67 antibody (green). H2C7-positive cells are distinguished from GAD-positive cells. The inset in (E) shows an enlarged view of the cell in the intermediate zone. *F,* Double-immunostaining of the P7 OB with mAb H2C7 (magenta) and anti-cholecystokinin-8 antibody (green). H2C7-positive cells (arrowheads) are small and mostly located in the EPL, whereas cholecystokinin-8-positive cells (arrows) are larger and positioned more superficially. Scale bar = 20 µm (A-D); 50 µm (E, F)

Figure. 5

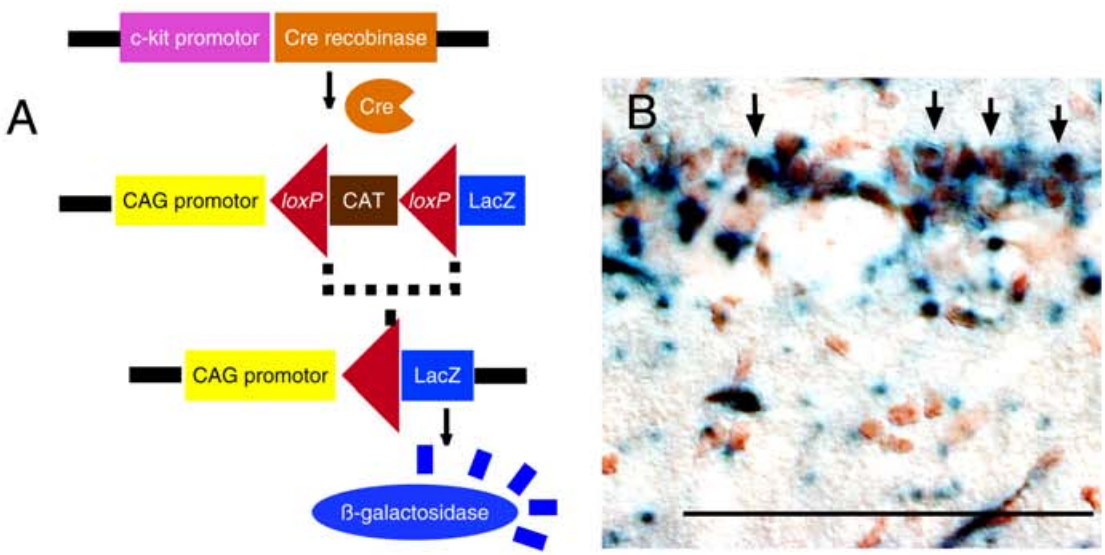
A
c-kit promotor
Cre recobinase
Cre
CAG promotor
loxP
CAT
loxP
LacZ
CAG promotor
LacZ
ß-galactosidase
B

Figure. 5 Identification of the c-kit-expressing cells. *A,* A schema of the c-kit (151) cre/CAG-CAT-Z double- transgenic mouse system. *B,* LacZ staining of the OB from a P7 c-kit (151) cre/CAG-CAT-Z double-transgenic mouse counterstained with hematoxylin. Most mitral cells express lacZ (arrows). Scale bar = 50 μm

Figure. 6

A

Stage of BrdU injection	Stage of BrdU detection: E14.5	E15.5	E16.5	E17.5	P0	P10
E11.5	IZ	IZ	MCL	MCL	MCL	MCL
E12.5	VZ	VZ	IZ	MCL	MCL EPL	MCL EPL
E13.5			IZ	IZ	IZ MCL	MCL EPL GCL
E14.5				IZ	IZ EPL	EPL GCL

B

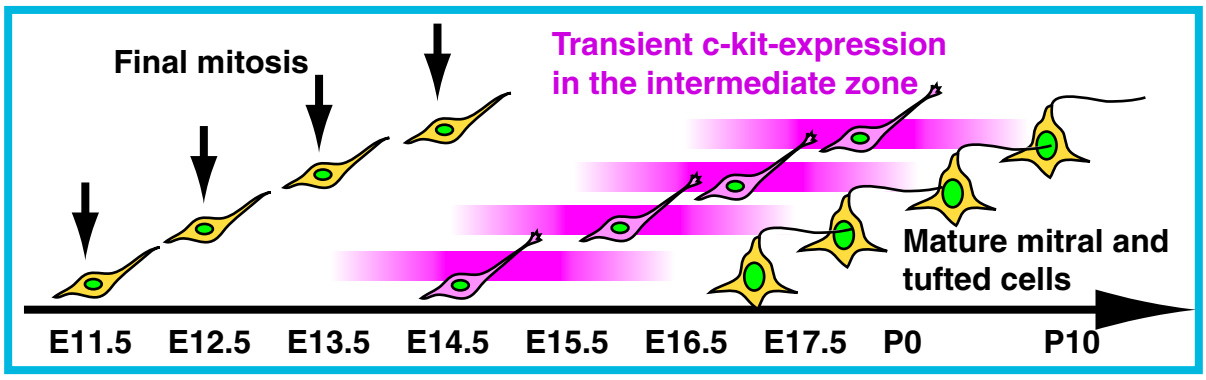

Figure. 6 *A,* Location of BrdU-labeled cells and c-kit-positive cells. The major layers in which BrdU-positive cells are located are indicated by the abbreviations. When the BrdU-labeled cells are also positive for c-kit, the boxes are magenta. The open boxes mean ”not examined”. Abbreviations; VZ, ventricular zone; IZ, intermediate zone; MCL, mitral cell layer; EPL, external plexiform layer; GCL, granule cell layer. *B,* Schematic diagram of transient c-kit expression in the mitral and tufted cells. After final mitosis, immature mitral and tufted cells express c-kit in the intermediate zone and migrate to the mitral cell layer and the external plexiform layer to differentiate into mature mitral and tufted cells.

Figure. 7

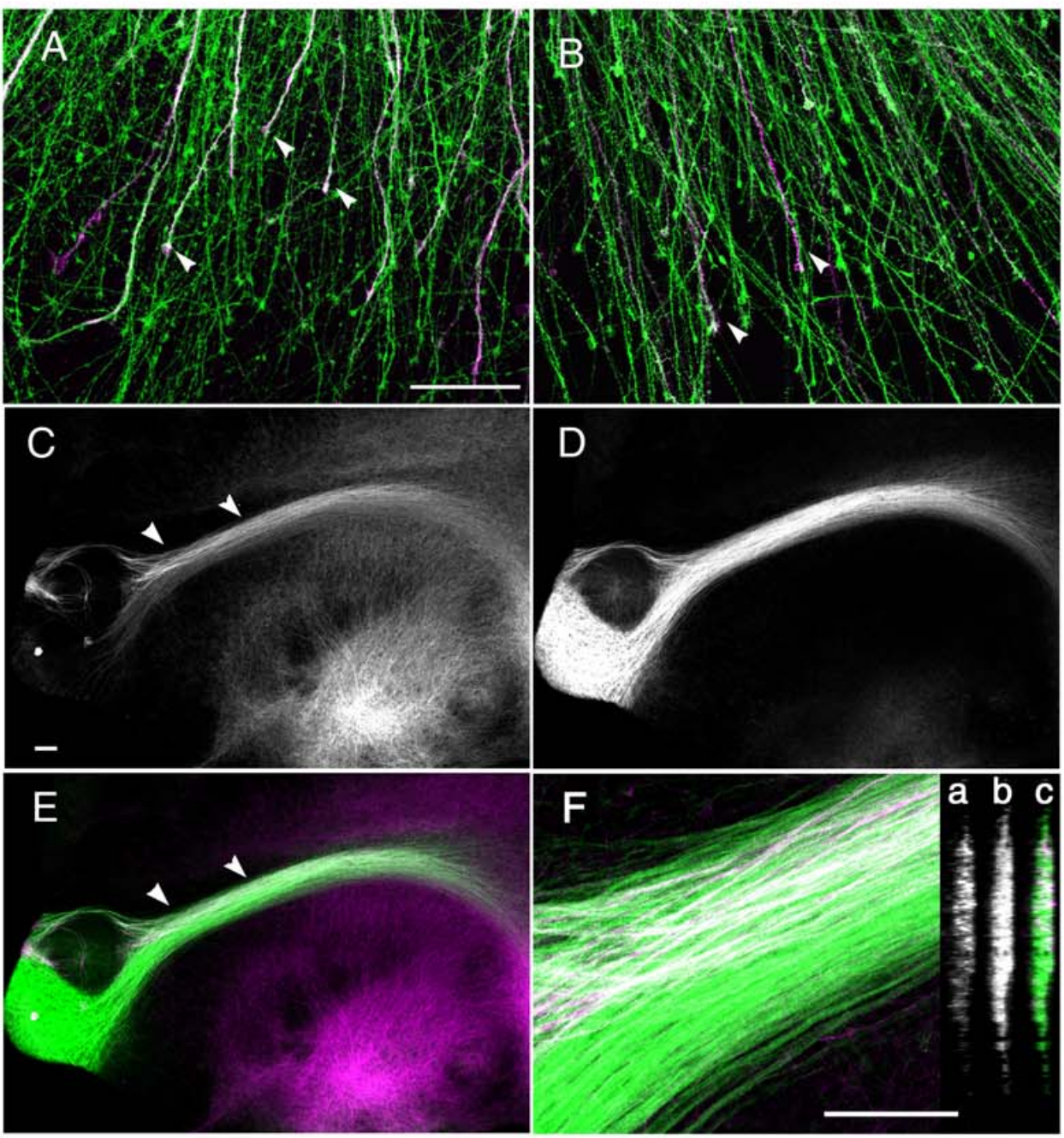

Figure. 7 Development of the mitral/tufted cells projection in culture. *A,B,* c-Kit-positive (arrowheads, magenta) and –negative (green) neurites elongating from the E14.5 OB fragments cultured for 3 days (A) and 7 days (B). *C-E,* Whole-mount preparation of E12.5 telencephalons cultured on collagenn-coated membrane filters for 3days (C, D and E) were stained with mAb H2C7 (C and magenta in E) and anti-neuropilin-1 antibody (D and green in E). Neuropilin-positive mitral and tufted cell axons form LOT-like fiber bundle. c-kit-positive axons get together within the LOT (arrowheads). *F,* Higher magnification of LOT axons in (F). The inset in (F) is the vertical section of LOT axons with a confocal microscope. (a), (b) and (c) indicate mAb H2C7, anti-EGFP antibody and merged image, respectively. The surface of the explant is to the right. C-kit positive axons (magenta) are positioned superficial of the LOT. Scale bars = 100 µm

Figure. 8

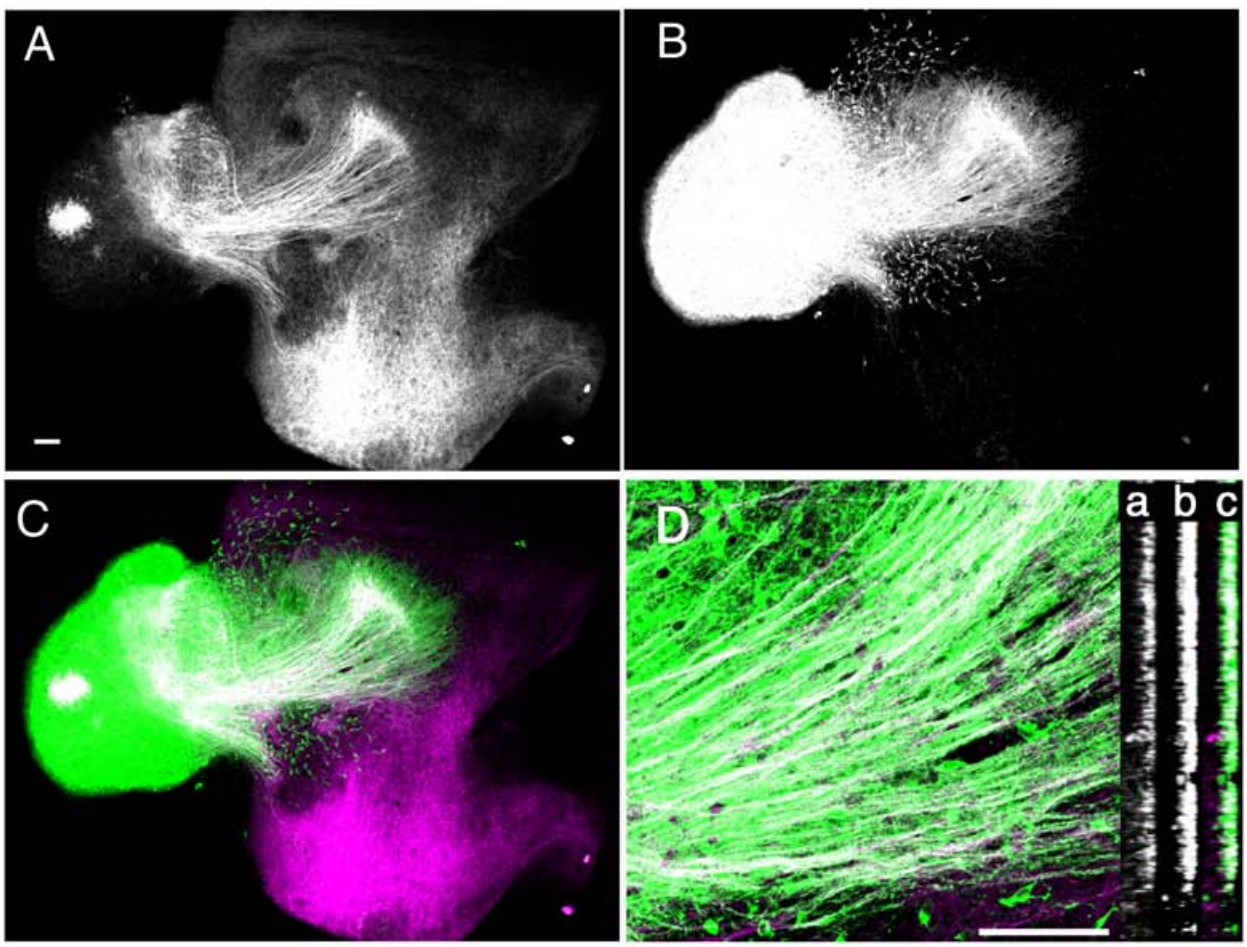

Figure. 8 Elongation of c-kit-positive and –negative axons in co-culture. *A-D*, Co-culture of the OB from the E14.5 green mouse embryo and LOT position of the E14.5 *W*/*W* mutant embryo immunostained with mAb H2C7 (A and magenta in C) and anti-EGFP antibody (B and green in C). The E14.5 telencephalon explant was prepared from *W*/*W* mutant embryo, because the explant lacks endogenous expression of c-kit protein that bothers detection of the newly elongated c-kit-positive axons. *D,* Higher magnification of LOT axons in (D). The inset in (D) is the vertical section of LOT axons with a confocal microscope. (a), (b) and (c) indicate mAb H2C7, anti-EGFP antibody and merged image, respectively. The surface of the explant is to the left. c-Kit-positive axons (magenta) are distributed even in the deep part of the LOT.Scale bars = 100 µm

Figure. 9

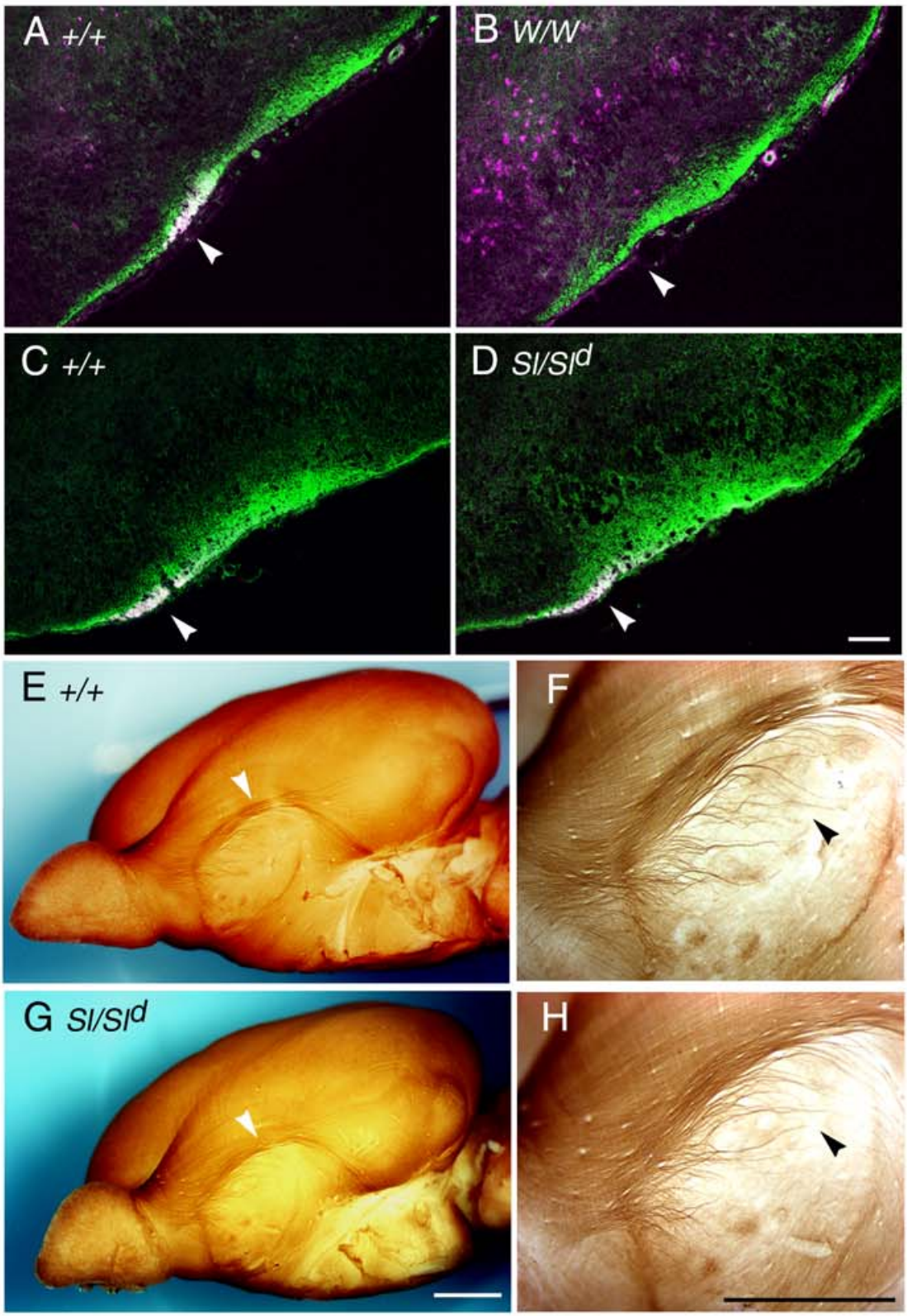

Figure. 9 Projection of the LOT axons in *W*/*W* and *Sl*/*Sl*^{d} mutant mice

A-D, Coronal sections of P2 wild-type (A,C), *W*/*W* (B) and *Sl*/*Sl*^{d} (D) mouse telencephalons double–immunostained with mAb H2C7 (magenta) and anti-neuropilin-1 antibody (green). Arrowheads indicate c-kit-positive axons in the LOT. Note the staining of axons is missing in (B). *E-H,* The P2 wild-type (E,F) and *Sl*/*Sl*^{d} (G,H) mutant telencephalons whole-mount immunostained with mAb H2C7. Arrowheads indicate H2C7-positive axons in ventral part of the LOT. *F,H,* Higher magnification of (E) and (G) respectively. H2C7-positive axon directly projects into the OT (arrowheads). Scale bars = 100 µm (D); 1.0 mm (H)