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REGULAR ARTICLE

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Extraepithelial cells expressing distinct olfactory receptors are associated with axons of sensory cells with the same receptor type

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Abstract During critical phases of mouse development, axons from olfactory sensory neurons grow out of the nasal neuroepithelium and navigate through the connective mesenchyme tissue towards their targets in the developing telencephalic vesicle. Between embryonic days E11 and E16, populations of cells are located in the mesenchyme which express distinct olfactory receptor genes along with the olfactory marker protein (OMP); thus they express markers characteristic for mature olfactory sensory neurons. These extraepithelial cells are positioned along the axon tracts, and each population expressing a given receptor gene is specifically associated with the axons of those olfactory sensory neurons with the same receptor type. The data suggest that they either might be guide posts for the outgrowing axons or migrate along the axons into the brain.

Keywords Development · Olfactory receptor · Olfactory sensory neuron · Projection · Mouse, transgenic

Abbreviation *ITLZ* · IRES-Tau-LacZ

Introduction

During critical phases of mouse development, axons from olfactory sensory neurons (OSNs) in the nasal neuroepithelium grow out and navigate through the connective mesenchyme tissue towards their synaptic target, the developing telencephalic vesicle. A variety of studies have proposed that guidance of axons to their target is controlled by the interaction of growth cones with extracellular matrix proteins which promote or inhibit the axonal elongation (Treloar et al. 1996; Kafitz and Greer 1997; Tisay and Key 1999), as well as with adhesion molecules and surface components of cells located on the path of the axons (Mahanthappa et al. 1994; Gong and Shipley 1996; Kafitz and Greer 1998). It is, however, still not known how the axons of different neuron populations navigate with high precision to the correct region of the developing bulb to form glomeruli at discrete positions. Previous studies have described that growing axons on their trajectory towards the bulb are in contact with distinct populations of cells (Farbman and Squinto 1985; Schwanzel-Fukuda et al. 1992; Pellier and Astic 1994; Pellier et al. 1994); this has led to the hypothesis that they might play a role as guideposts for outgrowing axons. Some of the cells associated with the fibres were found to express the characteristic olfactory marker protein (OMP; Baker and Farbman 1993; Valverde et al. 1993; Walters et al. 1993; Tarozzo et al. 1994a, 1994b, 1995a, 1995b, 1998). Recently, it has been reported that even cells expressing olfactory receptors (ORs) are located in the mesenchyme tissue between epithelium and bulb (Leibovici et al. 1996; Nef et al. 1996; Saito et al. 1998;) these cells may be particularly attractive candidate guideposts. In the olfactory epithelium itself, cells expressing ORs are actually subpopulations of cells expressing OMP (Wensley et al. 1995); therefore, it was a priority of the present study to evaluate the relationship between OMP- and OR-expressing cells in the nasal mesenchyme.

Axons of olfactory neurons expressing the same OR but being located in different areas of the nasal epithelium have been found to navigate to defined positions in the bulb and converge onto common glomeruli (Ressler et al. 1994; Vassar et al. 1994). The tight linkage between the receptor choice of an individual OSN and the site of its projection within the bulb led to the concept that the receptor itself may be involved in the targeting process. Recent receptor-swapping experiments, in which one particular OR gene has been genetically substituted by the gene encoding another olfactory receptor, and knock-out experiments have provided new evidence that olfactory receptor proteins are involved in the process of guiding olfactory axons to their correct target in

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the olfactory bulb (Mombaerts et al. 1996; Wang et al. 1998; Rodriguez et al. 1999). Based on the notion that ORs may be involved in cell-cell recognition (Singer et al. 1995), it seems conceivable that OR-expressing extraepithelial cells may act as guideposts for exactly those axons which are equipped with the same receptor type. Such a model would imply that axons of different neuron populations are exclusively associated with their subpopulation of extraepithelial cells. To scrutinize this hypothesis, it would be necessary to specifically visualize the respective population of extraepithelial cells and at the same time the chemosensory neurons, including their axons. This can indeed be accomplished by employing transgenic mouse lines in which expression of a defined receptor type is accompanied by the co-expression of a histological marker, thus allowing visualization of the projection of a particular neuron population (Mombaerts et al. 1996; Wang et al. 1998; Strotmann et al. 2000). Using these approaches, we have tried to determine the topological position of extraepithelial cells expressing a distinct receptor type as well as the axons of neurons which express the same receptor type.

Materials and methods

Mice

The analyses were performed on four transgenic mouse lines: mOR37B-ITLZ(B-lacZ) and mOR37C-ITLZ(C-lacZ) mice (Strotmann et al. 2000), P2-ITLZ(P2-lacZ) mice (Mombaerts et al. 1996) and OMP-GFP mice. In the receptor transgenic lines (B-lacZ, C-lacZ and P2-ITLZ), the reporter enzyme β-galactosidase is expressed from a bi-cistronic message derived from the genuine genomic locus of the associated olfactory receptor. The OMP-GFP line was kindly provided by Chen Zheng, Paul Feinstein and Peter Mombaerts. In this line the OMP promoter drives expression of the green fluorescent protein (GFP).

Tissue preparation

Homozygous B-lacZ, C-lacZ or OMP-GFP mice, respectively, were mated for 2 h and subsequently examined for a vaginal plug; for double-labeling experiments, animals double homozygous for *B-lacZ* and *OMP-GFP* (*B-lacZ* × *OMP-GFP*) were mated; 24 h later was dated E1.0. The head of each embryo was fixed for 60 min in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature and then incubated in 30% sucrose at 4°C for 48 h. The tissue was embedded in "tissue freezing medium" (Reichert and Jung) and frozen on dry ice. Coronal and sagittal sections (30 µm thick) were cut on a Reichert and Jung Frigocut 3000 at –17°C and adhered to Superfrost microslides (Fisher).

X-gal staining

The sections were air-dried and stained with 5-bromo-4-chloro-3-indolyl-β-*d*-galactopyranoside (X-gal) as follows. They were washed with buffer A (100 mM phosphate buffer, pH 7.4, 2 mM $MgCl_2$ and 5 mM EGTA) once for 5 min and once for 25 min at room temperature, followed by two incubations of 5 min at room temperature in buffer B (100 mM phosphate buffer, pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet P-40). The blue precipitate was generated by exposure at 37°C to buffer C (buffer B with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal).

Immunohistochemistry

Double fluorescence immunohistochemistry on sections through the olfactory system of *B-lacZ* × *OMP-GFP* animals was performed using a mouse monoclonal anti-β-galactosidase antibody (Clontech; 1:500) at 4°C overnight, followed by a secondary goat-anti-mouse antibody conjugated to Alexa568 (Molecular Probes) 1:500 for 2 h at room temperature. The GFP signal was enhanced using a polyclonal rabbit anti-GFP antibody (Molecular Probes; 1:500) at 4°C overnight, followed by a goat-anti-rabbit Alexa488 (Molecular Probes; 1:500) secondary antibody incubated for 2 h at room temperature. Sections were mounted in Vectashield (Vector Laboratories).

Microscopy and photography

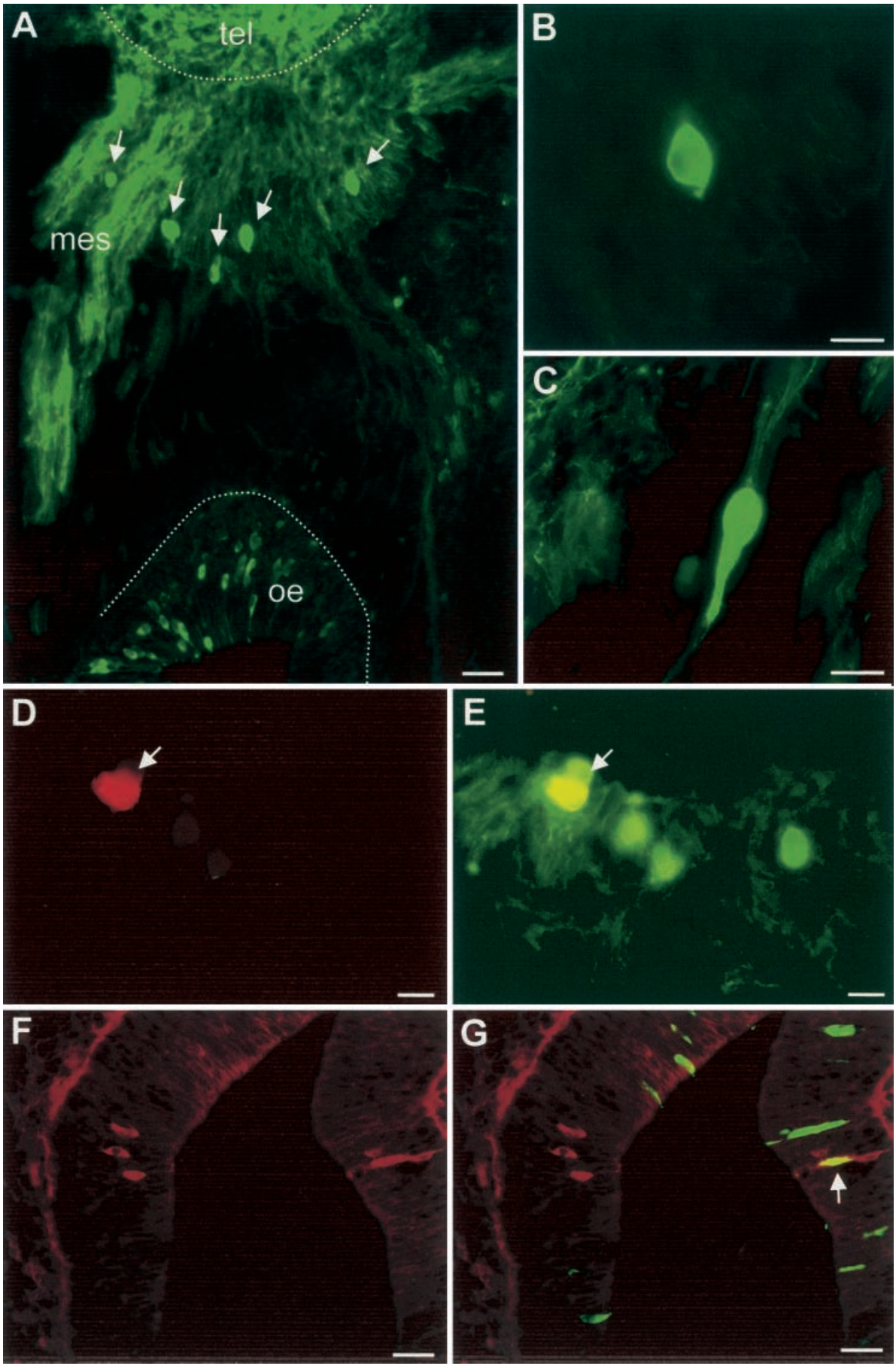
Sections were photographed using a Zeiss Axiophot. Fluorescence was examined with a CCD camera (SensiCam; PCO Computer Optics) and the Zeiss Axiovision imaging system, with the appropriate filter sets for Alexa488 and Alexa568. Photomicrographs were transferred to Corel Photopaint (Corel Corporation) for further editing.

Results

Cells outside the olfactory epithelium express OR and OMP

In a first approach, we have analyzed whether cells which are located outside the olfactory epithelium but express ORs also express OMP. For this purpose two transgenic mouse lines were crossed; one expressing GFP under control of the OMP promoter (OMP-GFP) and one expressing β-galactosidase under control of the mOR37B promoter (B-lacZ). Figure 1A shows a cross section through the posterior region of the developing nasal cavity from a double homozygous E14 embryo. In the ventral region, a small segment of the olfactory epithelium with OMP-GFP-positive OSNs is visible; the GFP-fluorescent axons of these cells are detectable passing through the mesenchyme that separates the nasal cavity and the telencephalon. Close to the telencephalon, large numbers of axons come closer together and enter the telencephalon. In that region, a few strongly fluorescent, thus OMP-expressing cells, are visible. Interesting-

Fig. 1 **A** Coronal section through the head of an OMP-GFP (olfactory marker protein-green fluorescent protein) mouse at E14. OMP-positive extraepithelial cells (*arrows*) are located in the mesenchyme (*mes*) between the olfactory epithelium (*oe*) and the telencephalon (*tel*); positive cells are associated with axons from olfactory sensory neurons (OSNs) growing towards the bulb. **B** Round-shaped extraepithelial cell expressing OMP. **C** OMP-positive extraepithelial cell with a cytoplasmic process; the cell is associated with OMP-positive axons. **D** B-lacZ-positive extraepithelial cell (*arrow*) from a *B-lacZ/OMP-GFP* double homozygous animal; β-galactosidase was visualized by immunohistochemistry using an Alexa568-labelled antibody. **E** Visualization of OMP-GFP-positive extraepithelial cells and B-lacZ-expressing cells. The B-lacZ-expressing extraepithelial cell (*arrow*) is also positive for OMP. **F** B-lacZ-expressing OSNs in the nasal neuroepithelium. **G** Double staining for B-lacZ and OMP-GFP reveals that only one of the B-lacZ neurons (*arrow*) is also positive for OMP-GFP. *Bars* 10 µm in **B–E**; 20 µm in **A, F, G** ▶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ly, fluorescence intensity of these cells is even higher than that of the OMP-GFP-expressing neurons in the olfactory epithelium. Typically, the OMP-GFP-expressing cells outside the epithelium were round-shaped (Fig. 1B); some cells extended in short cytoplasmic processes (Fig. 1C). In all cases, the cells were located in immediate vicinity to OMP-GFP-positive fibres, axons which originate from OSNs located in the epithelium (Fig. 1A, C). In the same individual, a small number of B-lacZ-positive cells were detectable in the mesenchyme between the epithelium and bulb (Fig. 1D). Double staining revealed that most B-lacZ cells were also OMP-GFP-positive (Fig. 1E). In their immediate vicinity, there are OMP-GFP-expressing cells that are apparently not expressing this particular receptor type (Fig. 1E). Surprisingly, a small fraction of cells expressing B-lacZ were not OMP-GFP-positive (data not shown). This raised the question of whether these cells represent a different population or whether – at this early phase of development – their OMP expression level may be too low to be detectable. To approach this topic, OSNs in the epithelium, which in adult animals co-express these two genes, were analyzed. Figure 1F shows a representative section through the olfactory epithelium at stage E14. A few B-lacZ cells are visible, and double staining for OMP (Fig. 1G) revealed that only one of the cells on this section contained enough GFP fluorescence to result in a yellow color in the overlay. In other B-lacZ cells, the level of OMP expression is too low. The situation in the epithelium thus mimics that outside the epithelium and suggests that at this early stage at least in some cells receptor expression precedes OMP expression.

OR-expressing cells outside the olfactory epithelium are restricted to distinct developmental stages

At developmental stage E11.25, the first B-lacZ-expressing cells appeared outside the olfactory epithelium and at the same time in the epithelium. Only very few B-lacZ-expressing cells were found in each compartment at that age (Fig. 2); at subsequent developmental stages, the numbers increased significantly. The number of cells outside the epithelium reached a maximum around E12.25 and thereafter declined to an intermediate level at E14. Between E16 and birth, cells expressing B-lacZ were rarely found outside the epithelium and were completely absent after birth. In contrast, in the epithelium the number of B-lacZ-expressing neurons increased almost exponentially between E12 and E16.

Extraepithelial cells are associated with axons of neurons expressing the same receptor

Since in the transgenic mice neurons expressing *mOR37* genes have labelled axons, the question could be raised of whether these axons are associated with extraepithelial cells which also express mOR37 receptors.

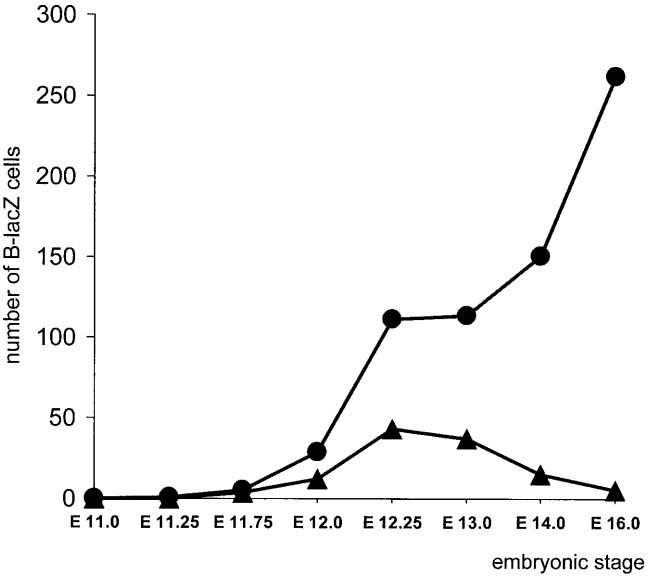

Fig. 2 Quantification of OSNs expressing B-lacZ in the olfactory epithelium (*filled circles*) and in the mesenchyme between the epithelium and the telencephalon (*filled triangles*) at distinct developmental stages of the mouse; the *numbers* include cells from both nasal cavities

Figure 3A, B shows consecutive sagittal sections through the epithelium and bulb from a B-lacZ mouse at stage E12. Cells expressing B-lacZ are detectable in the olfactory epithelium. In Fig. 3A, the axon of one particular neuron is visible extending from the epithelium into the underlying mesenchyme; it first makes a left turn and then changes direction again towards the telencephalon. At a certain position, it disappears into another focal plane; close to that site, on the consecutive section, an X-gal-stained cell is located (Fig. 3B). From this point onwards, the axon grows straight towards the telencephalon until it reaches another X-gal-positive cell. At that position it turns again and can be followed further, parallel to the border of the telencephalon (see Fig. 3A).

Figure 3C, D shows typical results obtained with another member of the mOR37 receptor subfamily, mOR37C, at stage E13. C-lacZ neurons are found at distinct positions in the epithelium and their axons are visible extending into the mesenchyme. In Fig. 3C, again the axon of an individual neuron is visible growing towards the bulb (arrowhead). In the mesenchyme it is apparently joined by more C-lacZ axons and from that point onwards they take the same direction until they reach an X-gal-stained cell close to the telencephalon. High magnification of the extraepithelial cell (Fig. 3D) shows that it is not located at the very tip of the axon bundle. Figure 3E shows another section from the series; a few C-lacZ cells in the epithelium are visible, their axons in the mesenchyme growing parallel to the border of the telencephalon; two X-gal-stained cells (arrows) are detectable on their route. High magnification of the cells (Fig. 3F) shows that, in these cases, OR-expressing cells outside the epithelium appeared to be closely associated with axons from neurons

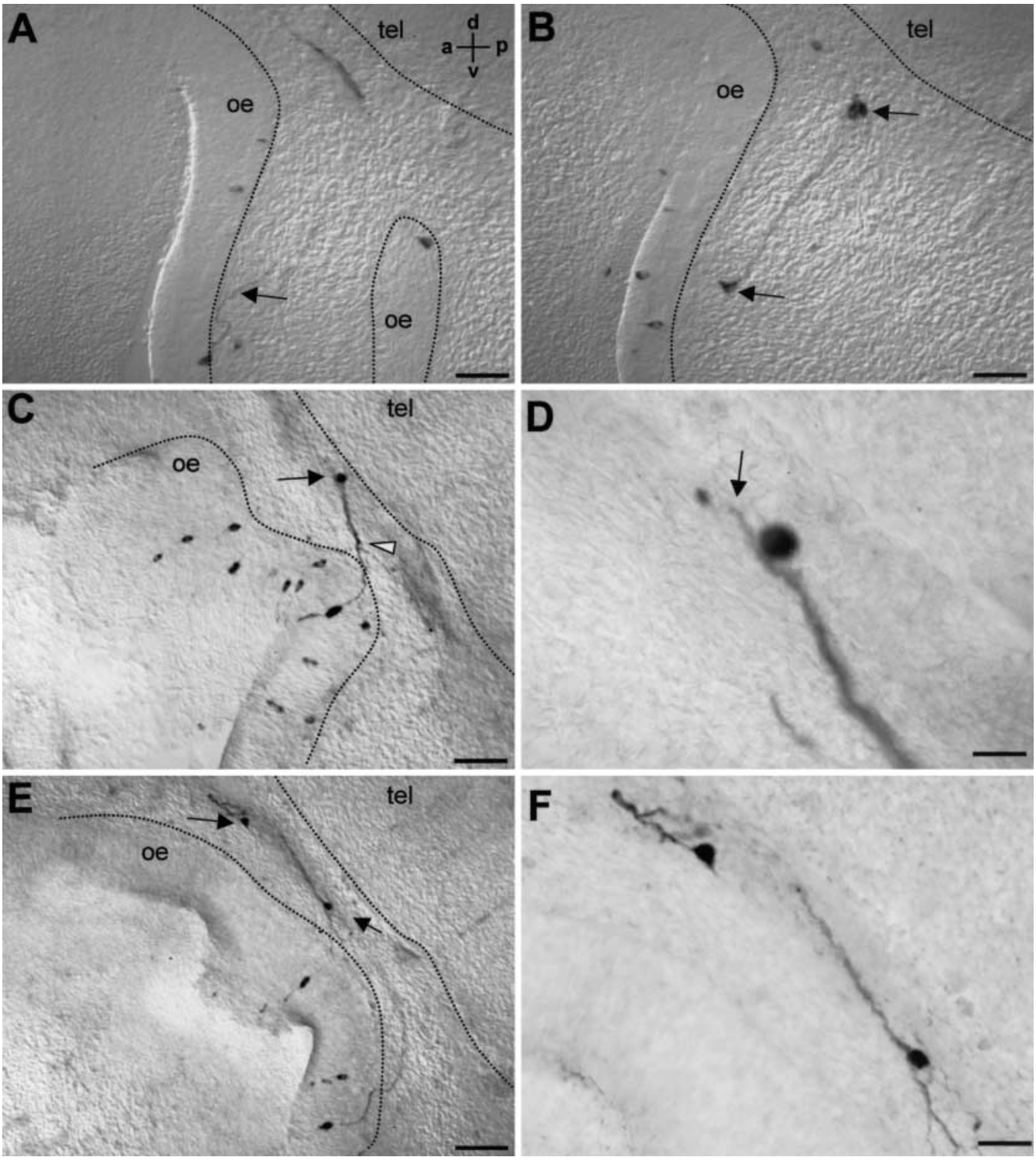

Fig. 3 **A** 5-Bromo-4-chloro-3-indolyl-β-*d*-galactopyranoside (X-gal)-stained sagittal section through the head of a B-lacZ mouse at E12; B-lacZ-positive OSNs are visible in the olfactory epithelium (*oe*). The axon of one particular cell is visible growing into the mesenchyme (*arrow*). The *dotted line* indicates the border of the telencephalic vesicle (*tel*). **B** Adjacent section to **A**; two extraepithelial cells expressing B-lacZ (*arrows*) are located within the mesenchyme between the epithelium (*oe*) and the telencephalon (*tel*). **C** X-gal-stained sagittal section through the head of a C-lacZ mouse at E13; C-lacZ-positive OSNs are visible in the olfactory epithelium (*oe*). Stained axons are visible in the mesenchyme; an extraepithelial C-lacZ-expressing cell is located close to the stained axon bundle (*arrow*). **D** High magnification of the C-lacZ-positive extraepithelial cell shown in **C**; the cell is closely associated with the C-lacZ axons; it is not located at the very tip (*arrow*) of the axon bundle. **E** X-gal-stained sagittal section through the head of a C-lacZ mouse at E13; C-lacZ-positive OSNs are visible in the olfactory epithelium (*oe*). C-lacZ-expressing extraepithelial cells are located on the trajectory of stained axons. **F** Higher magnification of extraepithelial C-lacZ cells from **D**; both cells are located in immediate vicinity to axons from neurons expressing C-lacZ (*a* anterior, *d* dorsal, *p* posterior, *v* ventral). *Bars* 50 µm in **A–C, E**; 10 µm in **D**; 20 µm in **F**

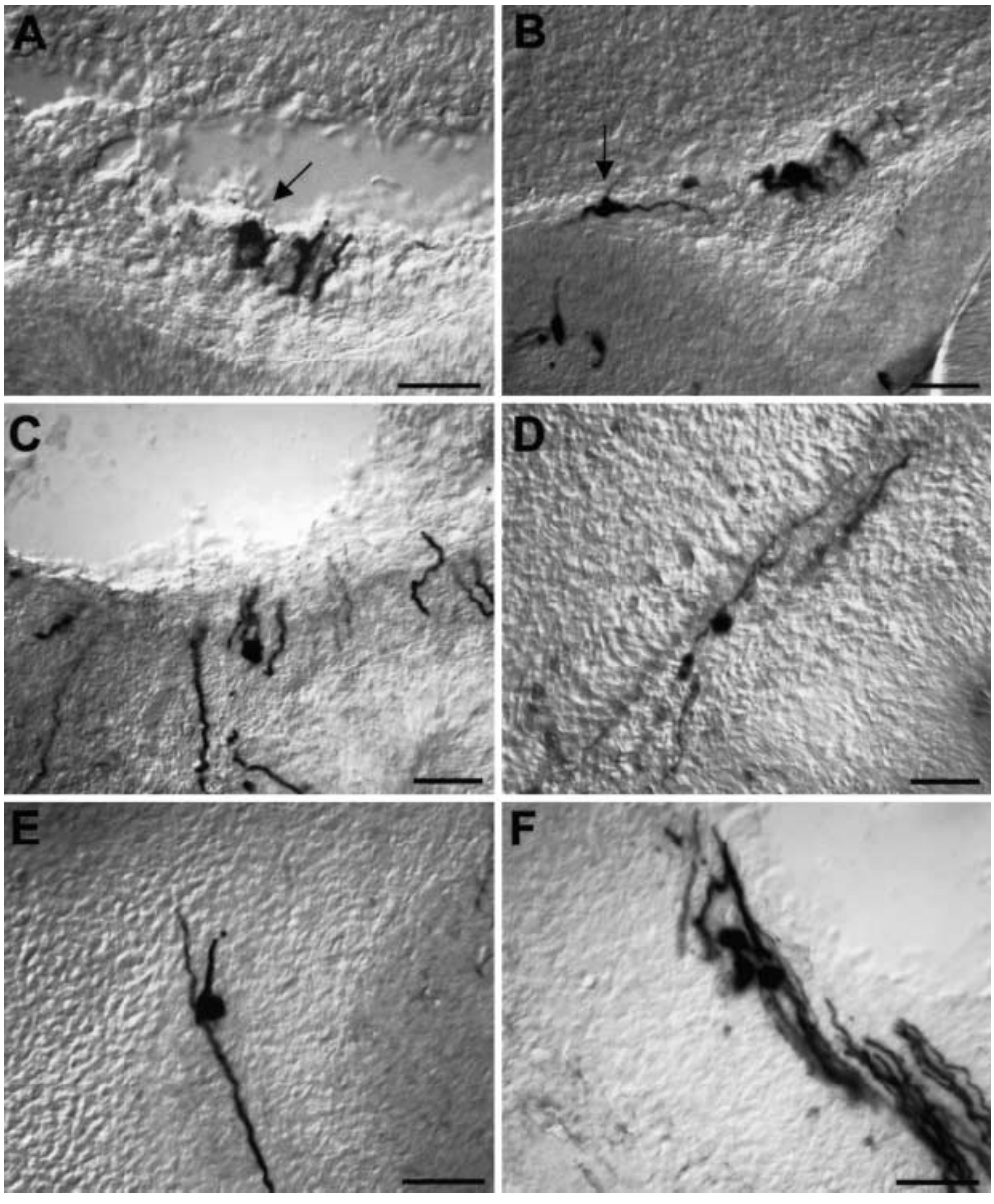

Fig. 4 **A** X-gal-stained coronal section through the head of a B-lacZ mouse at stage E12.25. A few B-lacZ axons and a closely associated extraepithelial B-lacZ cell (*arrow*) are visible. **B** X-gal-stained section through the head of a B-lacZ mouse at stage E12.25. Extraepithelial B-lacZ-expressing cells are closely apposed to B-lacZ-positive axons. Individual cells have an elongated morphology (*arrow*). **C** Coronal section through the head of a C-lacZ mouse at stage E13-stained with X-gal. An individual extraepithelial C-lacZ cell is associated with a stained fibre. **D** Sagittal section through the head of a C-lacZ mouse at stage E13 stained with X-gal; a C-lacZ-positive cell is located on the trajectory of C-lacZ axons. **E** Coronal section through the head of a B-lacZ mouse at stage E14 stained with X-gal. An extraepithelial B-lacZ cell is associated with the corresponding B-lacZ axons. **F** A group of B-lacZ extraepithelial cells is associated with the respective axons of B-lacZ OSNs. *Bars* 40 µm

expressing the same receptor. This notion was next analyzed in more detail for a large number of cells. Figure 4 shows representative results obtained for two mOR37 subtypes at different developmental stages. Fig. 4A, B shows B-lacZ cells at E12.25; at this plane of section, only short segments of axons are visible at distinct positions; X-gal-positive extraepithelial cells are detectable exclusively at these sites. At E14 a very similar picture emerged (Fig. 4E); each X-gal-positive cell (n=90, analyzed in four different animals) was located in the immediate vicinity of axons from the same subtype. At some positions close to the telencephalon, where several stained axons came close together, also groups of extraepithelial cells were found (Fig. 4F). In Fig. 4C, D, representative pictures from C-lacZ cells (n=75, analyzed in three different animals) located outside the epithelium are shown; all C-lacZ cells were so closely apposed to axons of the same subtype that an immediate contact appears likely.

The close association of mOR37-lacZ-expressing cells outside the olfactory epithelium with the axons of corresponding neurons raised the question of whether this is a general phenomenon valid also for other OR types. Therefore cells were analyzed which expressed the P2 receptor (Mombaerts et al. 1996), which is unrelated to the mOR37 subfamily. Figure 5A, B shows cross sections through the head of a P2-ITLZ(P2-lacZ) mouse at stage E13 and E14, respectively; X-gal-stained P2-lacZ cells are visible in the epithelium and their fibres project towards the developing bulb. Close examination of the fibre tracts revealed blue cells within the mesenchyme; cells which express the P2 receptor. In all cases, these cells (n=79; analyzed in three animals) were exclusively associated with axons of the P2 neurons; no extraepithelial P2 cell was found in a region without P2 fibres.

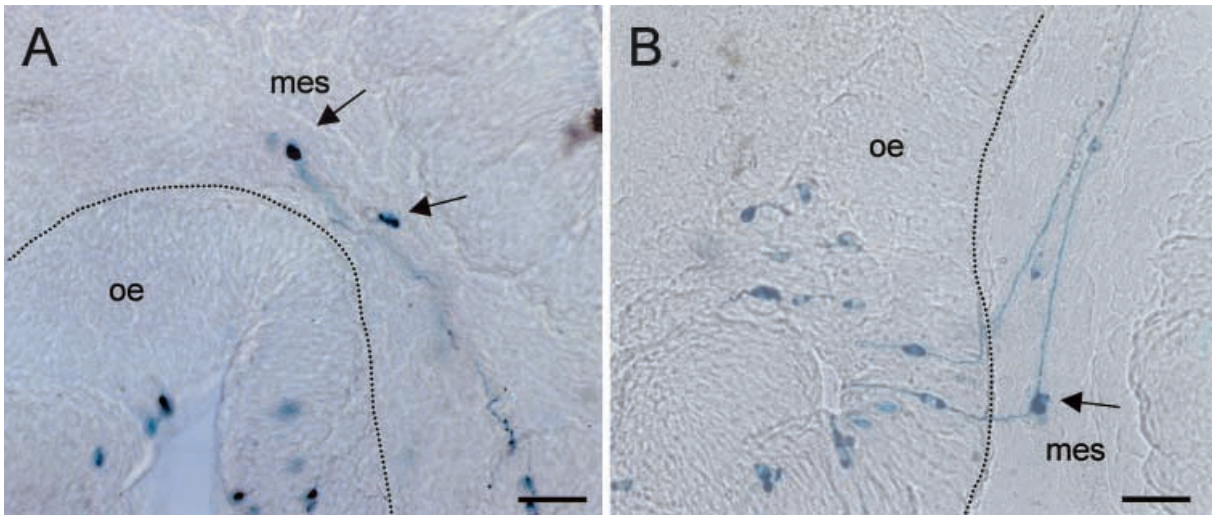

Fig. 5 **A** X-gal-stained coronal section through the head of a P2-lacZ mouse at E13. P2-lacZ-expressing OSNs are located in the olfactory epithelium (*oe*). Their axons pass through the mesenchyme (*mes*); P2-lacZ-expressing extraepithelial cells (*arrows*) are associated with the corresponding axons. The outline of the epithelium is indicated by the *dotted line*. **B** X-gal-stained coronal section through the head of a P2-lacZ mouse at E14. P2-lacZ-expressing extraepithelial cells are associated with P2-lacZ axons in the mesenchyme (*mes*). One extraepithelial cell (*arrow*) is located where a P2-lacZ axon changes its direction of growth. The outline of the epithelium is indicated by the *dotted line*. *Bars* 50 μm

Discussion

In the present study we characterized cells which express distinct ORs, but are located outside the olfactory epithelium. Here, we provide the first evidence that these cells are subpopulations of the OMP-positive neurons that have been described previously (Baker and Farbman 1993; Valverde et al. 1993; Walters et al. 1993; Tarozzo et al. 1994a, 1994b). Based on the expression of two typical olfactory markers, they appear to be closely related to OSNs. The data also indicate that during development ORs occur prior to OMP in epithelial sensory neurons as well as in the extraepithelial cells. Although OR-positive neurons are restricted to the OMP-positive layer of the neuroepithelium in adult animals (Ressler et al. 1993; Vassar et al. 1993), which may suggest an onset of OMP-expression before that of OR-expression, a recent study has demonstrated that also postnatally OR expression in individual OSNs precedes that of OMP (Iwema and Schwob 2000).

What might be the functional implications of OR expression in cells outside the olfactory epithelium? Due to their location on the trajectory of olfactory axons, it is tempting to speculate that they might serve as guideposts for growing olfactory axons. This notion is supported by the finding that in several instances they are located at positions where outgrowing axons change direction (Figs. 3, 5). This suggests that the cells might be transient targets for outgrowing axons of related neurons. Also the fact that these cells are only present during the critical phase, when the first axons have to establish the route to the bulb, can be viewed in favour of this concept. Furthermore, the existence of different groups of cells which are equipped with distinct receptor types makes it conceivable that axons are directed by these posts onto precise positions in the developing bulb. How could a role as guideposts be mediated by the cells? Their close association with axons of neurons which express the same OR gene suggests a functional implication of the receptor protein itself. Recent receptor knockout experiments have indeed shown that elimination of the receptor-coding region results in severe mistargeting of the axons (Wang et al. 1998).

In a different scenario, however, OR-expressing extraepithelial cells might not *guide* growing axons to their correct position, but rather may *use* the axons as tracks for their migration. Migration of cells from the olfactory placode into the brain has frequently been described (Mendoza et al. 1982; Monti-Graziadei 1992; for reviews, see Tarozzo et al. 1995a; Schwanzel-Fukuda 1999). Although a true migration is difficult to prove experimentally, there seems to be some evidence for gonadotropin-releasing hormone (GnRH)-expressing cells (Wray et al. 1989; Schwanzel-Fukuda et al. 1989; Murakami et al. 1992; Schwanzel-Fukuda et al. 1994). The observation that some extraepithelial cells extend cytoplasmic processes (Fig. 1C), which appears to be typical for migrating cells (Gregory et al. 1988; Sheetz et al. 1999; Yee et al. 1999), favors this idea. The close association of extraepithelial cells with their "sister" axons may indicate that they indeed use the fibres as a selective pathway.

Could the receptor protein be involved in the cell-cell interactions? It has been proposed that extracellular domains of olfactory receptor proteins might interact with complementary molecular elements (Singer et al. 1995). However, neither such interaction partners nor the principle of molecular interaction are known; also any evidence that receptor proteins are actually present in the membrane of olfactory axons – an essential prerequisite

for this concept – is still lacking. In the special situations as described in this study, where a tight association between extraepithelial cells and axons of neurons which share the same receptor type exists, it is conceivable that a homophilic interaction of identical receptor proteins on the surface of each cell may occur.

The fate of OR-expressing extraepithelial cells is elusive. Since they are no longer detectable after a certain developmental stage, they may either die by apoptosis or just cease to express OMP and ORs. It has been suggested that this type of cell upon arrival in the bulb may differentiate, e.g. into periglomerular cells and become integrated into the neuronal network of the bulb (Valverde et al. 1992). The migration along a particular population of axons would specifically target them to the position in the developing bulb, where a glomerulus of the related neuron population is forming.

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