Histochem Cell Biol (2002) 117:493–509
DOI 10.1007/s00418-002-0407-1

ORIGINAL PAPER

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On the chemosensory nature of the vomeronasal epithelium in adult humans

Accepted: 22 March 2002 / Published online: 27 April 2002

Abstract In contrast to many lower vertebrates, the vomeronasal epithelium (VNE) in humans has long been regarded as absent or functionally irrelevant. For example, the neural connection between the VNE and the accessory olfactory bulb has been reported to degenerate during the second half of pregnancy and its presence has not been demonstrated in adults. Further, reports on the organ's occurrence in adult humans have been contradictory. The aims of this study were to collect immunohistochemical data on the neurogenic or epithelial character of the VNE [for example, with antibodies against protein gene product 9.5 (PGP 9.5), olfactory marker protein (OMP), β-tubulin, and cytokeratin], determine its proliferative capacity (for example, proliferating cell nuclear antigen), as well as to examine the differentiation activity of VNE cells and their interactions with extracellular matrix components (for example, hyaluronan receptor CD44, galectins, and caveolin). To this end, we studied the vomeronasal organs (VNOs) of 22 human cadavers, three adult biopsies, one embryo (week 8) and one fetus (week 13) by means of immunohistochemistry. The histology of the VNE appeared extremely heterogeneous. There were sections of stratified, respiratory, and typical "pseudostratified" vomeronasal epithelia consisting of slender bipolar cells. Mostly negative immunohistochemical results for OMP indicated that the human VNE does not function like the mature olfactory epithelium. In addition, the investigations did not support the hypothesis that neural connections between the VNE and central brain structures might be present. On the other hand, the presence of some bipolar cells positive for both PGP 9.5 and soybean lectin (SBA) pointed to a neuron-like activity of a small subset of VNE cells. Proliferation antigens located in the nuclei of basally located cells of the VNE were not regularly expressed. However, positive reactions for CD44 demonstrated a high activity of VNE cells in terms of differentiation and migration. Some bipolar cells showed immunoreactivity for caveolin indicating its possible role in signal transduction and differentiation. In summary, the reaction patterns of most antibodies in the adult human VNE are different from those obtained in the olfactory epithelium and the VNO of the rat. However, the VNE shows a specific pattern of activity unique to the mucosa of the nasal cavity. Considering the histologically well differentiated epithelium and its steady maintenance, the VNE of the adult human appears to be a highly differentiated structure the function of which remains unclear.

Keywords Vomeronasal organ · Human · Immunohistochemistry · Differentiation · Olfactory epithelium · Rat

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Introduction

The mammalian vomeronasal organ (VNO) plays an important role in the reception of environmental chemicals. It is often mentioned in the context of conspecific "pheromone" detection (Beauchamp et al. 1976; Brennan 2001; Meredith 2001). It has been implicated in social or reproductive behavior (for review, see Johnston 2000). In humans it is developed during early gestation, but the vomeronasal nerve connecting the vomeronasal duct (VND) with the accessory olfactory bulb degenerates between week 14 and 28 (Kjaer and Fischer Hansen 1996; Nakashima et al. 1985) leaving the function of the human VNO unclear. Although the VND proper is maintained in many adults there is little data on the histological structure of its epithelium (VNE). Recent electron microscopic evidence indicates that portions of the duct possess a highly differentiated epithelium resembling that of a chemoreceptive organ (Gaafar et al. 1998; Moran et al. 1991).

Detectability of the human VND varies considerably. While most authors report the presence of a VND in many, but not all of the investigated subjects (for review, see Knecht et al. 2001) there is one report which indicates presence of the VND in 100% of the cases (Moran et al. 1991). The orifice of the duct appears as a pit less than 2 mm in diameter which often exhibits a yellow-brownish pigment (Knecht et al. 2001). It is generally described as a blind-ending duct, or a mucosal pouch located in the anterior nasal septum (for review, see Abolmaali et al. 2001), however, there is one report of a VNO with a tubular mucosal structure with anterior and posterior openings (Mangakis 1902). Typically, its length varies between 3 and 22 mm (Anton 1895), however, VNDs with a length of 47 mm have also been described (Abolmaali et al. 2001). Thus, the human VND seems to exhibit considerable variability in size, shape, and detectability/presence. This variability adds to the current argument on the functionality of the adult VND, which is spurred by recent reports of non-functional human vomeronasal receptor genes (Kouros-Mehr et al. 2001). A major issue in this debate is that a neural connection to the olfactory bulb in adult humans has not been detected (see, for example, Bhatnagar and Smith 2001; Trotier et al. 2000). In addition, there are only spurious reports on VNE cells staining for the pan-neuronal marker protein gene product 9.5 (PGP 9.5; Takami et al. 1993) indicating that neuron-like cells are not characteristic of the epithelial lining of the VND. To establish the putative neurochemical nature of the VNE, we used antibodies against olfactory marker protein (OMP; Margolis et al. 1983), β-tubulin (Roskams et al. 1998; Suzuki et al. 2001; Yasuno et al. 2000), and the soybean agglutinin (SBA; Key and Akeson 1993; Key and Giorgi 1986).

The scope of the present study was to investigate possible neuroepithelial properties of VNE cells and the presence of putative afferent nerves. Moreover, it also aimed to further characterize the epithelial structure using additional parameters such as cell adhesion, differentiation, and proliferation. Integrins are important for the mediation of cell-substrate adhesion, as apparent in migrating olfactory neurons. The participation of integrins in neuronal pathfinding, for example, has been described for retinal ganglion cells (Stuermer and Bastmeyer 2000). Furthermore, we studied the distribution of hyaluronan and its receptor CD44 (Porter and Hogg 1998; Sherman et al. 1994) in both the VNE and olfactory epithelium (OE), because rRNA sequences for hyaluronan receptor RHAMM in an immobilized olfactory cell line have been reported to modulate hyaluronan-mediated motility (Zehntner et al. 1998). Since endogenous lectins participate in differentiation processes in the OE (Heilmann et al. 2000; Mahanthappa et al. 1994), immunoreactivities for galectin-1 (Gal-1) and galectin-3 (Gal-3) were also investigated. Finally, because cytokeratins have been reported to occur in horizontal basal cells and non-sensory supporting cells of the OE (Okabe et al. 1997; Satoh and Yoshida 2000), they were also investigated in the VNE.

To put the immunohistochemistry of the adult human VND into a more general context, specimens from the adult human OE and the fetal human VNO as well as both OE and VNE from the newborn rat were investigated with the same antibodies.

Materials and methods

Collection of human specimens

Biopsic material from the vicinity of the VND was obtained from three subjects who underwent nasoplastic surgery after written consent had been obtained prior to surgery (2 males, 1 female; ages 28, 36, and 59 years, respectively). This part of the study was approved by the local ethics committee (application number EK950998). For the entire evaluation of VNDs, nasal septa of 23 cadavers (volunteer donors from the Department of Anatomy; 11 males, 12 females, age range 58–96 years, mean age 80 years) were excised, fixed in buffered formalin, and embedded in paraplast. In addition, the VNOs of an 8-week-old human embryo and a 12-week-old fetus were examined whose cranial sections remained from an earlier study (Witt and Reutter 1998). The septa from the cadavers were perpendicularly cut in the horizontal plane. To avoid possible confusion with nasopalatine ducts or large glandular excretory ducts (Gegenbaur 1886; Jacob et al. 2000; Zuckerkandl 1893), a VND was considered present only when it was lined, at least partially, with VNE (Smith et al. 2001b; Trotier et al. 2000). Biopsies were immediately fixed in buffered formalin and embedded in paraplast.

Animals

Vomeronasal organs were obtained from newborn Wistar rats which were given an overdose of CO_2. The viscerocranium was cut in the coronal plane.

Immunohistochemistry

Deparaffinized sections (thickness 6 µm) were pretreated with microwaves (800 W) or pronase (see Table 1) and exposed to 0.3% aqueous H_2O_2 to block endogenous peroxidases. Sections were then incubated with various primary antibodies as listed in Table 1 (pH 7.2; containing 1% bovine serum albumin) for 1 h at 37°C. After washing in phosphate-buffered saline (PBS), the sections were exposed to secondary biotinylated antibodies for 1 h at room temperature (RT). The reaction products were visualized by an avidin-biotin-peroxidase complex (ABC; Vectastain-Elite; Vector, Burlingame, Calif., USA) followed by incubation with 0.3% diaminobenzidine/H_20_2. Sections were counterstained with hematoxylin. *N*-acetyl-galactosamine residues were detected using biotinylated soybean lectin (SBA; Vector; Debray et al. 1981; Pereira et al. 1974) followed by the ABC technique as described earlier (Witt and Reutter 1988).

Indirect fluorescence

For colocalization studies in rat tissue, a double fluorescence technique was applied. Deparaffinized sections were incubated with anti-OMP (1:400) overnight. After rinsing, an antiserum against PGP 9.5 (1:200) was applied for 1 h at RT, followed by incubation with corresponding secondary FITC- or Texas red-labeled antibodies (1:100; Dianova, Hamburg, Germany). The sections were mounted with PBS-gelatin and analyzed under a confocal laser-scan microscope (Leica, Bensheim, Germany).

Table 1 Antibodies and lectins used in this study. (*MAB* Monoclonal antibody, *PAB* polyclonal antibody)

Antibody	MAB/PAB	Dilution	Source	Pretreatment
Olfactory marker protein (OMP)	PAB	1:5,000	Dr. F. Margolis, University of Maryland, Baltimore	Microwaves
Protein gene product 9.5 (PGP 9.5)	PAB	1:2,000	Biotrend, Cologne, Germany	Microwaves
β-Tubulin	MAB	1:100	Zymed, San Diego, Calif., USA	–
Hyaluronan receptor CD44 (BBA-10)	MAB	1:500	R&D systems, Minneapolis, Minn., USA	Microwaves
Biotinylated hyaluronan-binding protein (HABP)	–	1:500	Seikagaku (Medak, Wedel, Germany)	–
β1-integrin (CD29)	MAB	Undiluted 1:50	Biogenix Novocastra, (Medak)	Microwaves
Proliferating cell nuclear antigen (PCNA)	MAB	1:50	Dako, Hamburg, Germany	–
Pankeratin antibody MNF	MAB	1:200	Dako	Pronase
Pankeratin antibody I+II	MAB	1:500	Progen, Heidelberg, Germany	Pronase
Galectin-1 (Gal-1)	PAB	1:1,000	Dr. Cooper, UCSF, USA	Pronase
Galectin-3 (Gal-3)	MAB	1:50	Novocastra	Microwaves
S-100	PAB	1:2,000	Dako	Pronase
Biotinylated soybean agglutinin	–	1:400	Vector, Burlingame, Calif., USA	–
Caveolin 1–3	PAB	1:1,000	BD Biosciences, Heidelberg, Germany	Microwaves

Controls

The following controls were carried out: (1) omission of the primary antibody to rule out non-specific binding of the secondary antibody and (2) parallel incubation of tissue (usually rat OE and VNE) previously reported to be immunopositive to the markers tested.

Results

The results of the immunohistochemical studies in vomeronasal and olfactory epithelia are summarized in Table 2.

The human VNE

The human VND appeared as a slightly oblique duct within the nasal septum, the orifice of which was located 1–2 cm distal from the columella and 4–15 mm above the nasal floor (Fig. 1). Despite a careful search at the histological level, VNDs were only detected in 15 of the 23 specimens (8 females, 7 males).

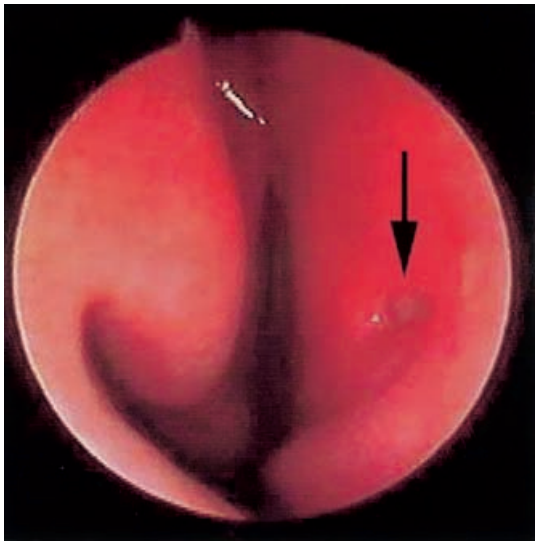


Fig. 1 Endoscopic view of the inferior nasal duct of an 18-year-old female subject. *Arrow* indicates the opening of the vomeronasal duct (VND) in the nasal septum

Immunohistochemistry

The human embryonic VND at 8 weeks after conception exhibited a bilateral and symmetric structure (Fig. 2). The mucosa consisted of a pseudostratified epithelium with slender bipolar cells reactive for PGP 9.5, the nuclei of which appeared in the basal one-third of the epithelium (Fig. 2c). Clusters of ganglion cells in immediate proximity to the VND, as well as nerve fiber profiles, were PGP 9.5 positive. In contrast, these structures did not react with antiserum to OMP (Fig. 2b). There were no structural or immunohistochemical differences between the medial and lateral sides of the duct. In adult samples, however, the VNE exhibited extreme intraindividual heterogeneity. In both cadaver specimens and biopsies, there were sections of (sometimes degenerated) respiratory epithelium including goblet cells followed by restricted areas of pseudostratified epithelium comprised of slender bipolar cells. There were no reproducible structural differences between medial and lateral sides of the VND. Basal cells were present but they were not in a strict linear arrangement in contrast with globose and horizontal basal cells of the OE.

Neuronal markers: OMP, PGP 9.5, β-tubulin, and SBA

In most VND preparations epithelial cells were not reactive with antibodies to OMP, PGP 9.5, and β-tubulin (Fig. 3a, b). Although smart nerve fibers were seen in connective tissue, they failed to interact with the epithelium.

Table 2 Survey about antibodies used in this study, their binding sites in chemosensory epithelia, and their possible functional implications. (VND Vomeronasal duct, OE olfactory epithelium, *n.d.* not determined)

Antibody	Human			Rat		Functional significance for chemoreception	Reference
	VND		OE	VNO	OE		
PGP 9.5	Embryo: receptor cells, nerve fibers, ganglia	Adult: negative, individual positive cells in biopsies	Receptor neurons, nerve fibers	Receptor neurons, nerve fibers	Receptor neurons, nerve fibers	Neuron-like properties	Kjaer and Hansen (1996); Nosrat et al. (2000)
OMP	Embryo: negative	Adult: negative, individual positive cells in biopsies	Mature receptor neurons, nerve fibers	Mature receptor neurons, nerve fibers	Mature receptor neurons, nerve fibers	Marker for mature olfactory neurons (ORN), function unknown	Margolis (1982)
Soybean agglutinin (*Glycine max*)	Bipolar cells		Receptor neurons, supporting cells? some basal cells	Receptor neurons, supporting cells, basal cells	Receptor neurons, supporting cells, basal cells	Marker for (ORN), olfactory bulb (OB) and accessory OB	Key and Akeson (1991); Meyer et al. (1996)
β-Tubulin	Ciliated cells, nerve fibers; some bipolar cells in biopsies		Receptor neurons, nerve fibers	Receptor neurons, nerve fibers	Receptor neurons, nerve fibers	Marker for immature and mature ORN	Roskams et al. (1998)
S-100	Glial cells, unidentified cells		Glial cells	Glial cells	Glial cells	Marker for glial cells that ensheath ORN	Astic et al. (1998)
Gal-1	Suprabasal cells		Basal cells	Basal cells	Glial cells	Involved in axonal pathfinding of ORN in mouse	Mahanthappa et al. (1994)
Gal-3	Supporting cells? Bipolar cells (biopsy)		Mature receptor neurons	Supporting cells?	Supporting cells	Present in mature ORN in humans	Heilmann et al. (2000)
Cytokeratin (MNF 116)	Most epithelial cells, especially basal cells		Supporting cells Basal cells	Supporting cells	Supporting cells	Marker for non-neuronal epithelial cells in the olfactory epithelium and vomeronasal epithelium	Okabe et al. (1997); Trotier et al. (2000)
HABP	Epithelial cell layer (lower one-third)		Receptor neurons (apical)? Basal cells	–	–	Involved in differentiation of taste bud cells during development	Witt and Kasper (1998)
CD44 (BBA-10)	Epithelial cell layer (lower one-third)		Receptor neurons (apical)? Basal cells	n.d.	n.d.		
β1-integrin (CD29)	Supporting cells?		Supporting cells?	n.d.	n.d.	Synaptic plasticity in olfactory bulb Maintenance of structural integrity of the cytoskeleton	Einheber et al. (1996) Littlewood-Evans and Müller (2000)
Caveolin 1–3	Bipolar cells		Bipolar cells (apical)	–	–	Possibly involved in signal transduction in ORN	Schreiber et al. (2000)
PCNA	Basal cells		Basal cells	Basal cells	Basal cells	Indicator for cell proliferation	Ohta and Ichimura (2000)

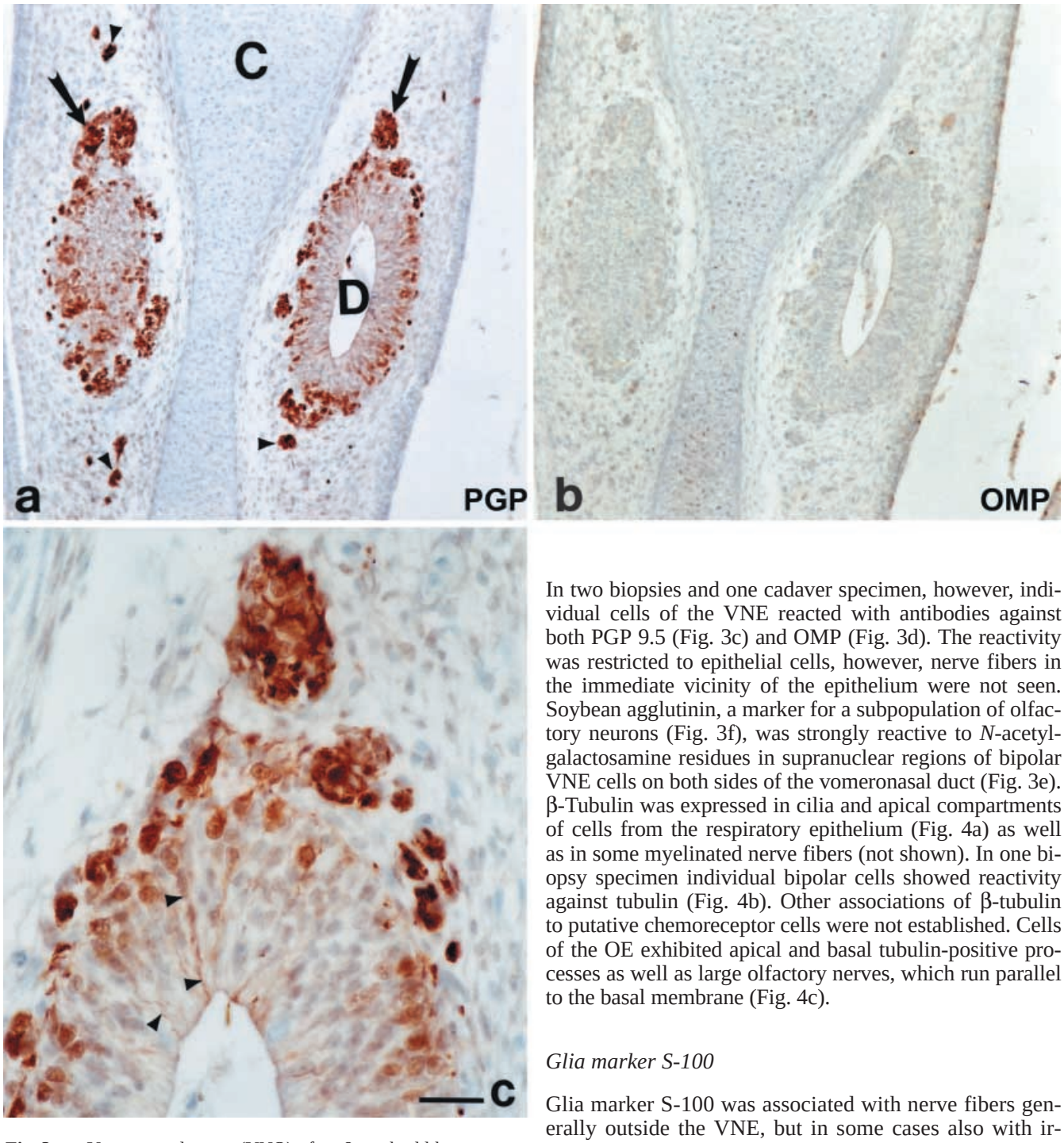


Fig. 2a–c Vomeronasal organ (VNO) of an 8-week-old human embryo. Horizontal section of the nasal septum. **a** Immunoreactivity for protein gene product 9.5 (PGP 9.5) in many cells of the VND (*D*). There is no apparent difference between the staining properties of the medial or lateral side of the epithelium. Some ganglia (*arrows*) and nerve fibers (*arrowheads*) are also reactive. *C* Hyaline nasal cartilage. **b** Adjacent section. Immunoreactivity for olfactory marker protein (OMP) is lacking. **c** Enlargement of **a**. Some cells have tiny apical processes (*arrowheads*); microvilli are not to be seen at this magnification. Counterstain with hematoxylin. *Scale bar* 40 μm in **a**, **b**; 20 μm in **c**

In two biopsies and one cadaver specimen, however, individual cells of the VNE reacted with antibodies against both PGP 9.5 (Fig. 3c) and OMP (Fig. 3d). The reactivity was restricted to epithelial cells, however, nerve fibers in the immediate vicinity of the epithelium were not seen. Soybean agglutinin, a marker for a subpopulation of olfactory neurons (Fig. 3f), was strongly reactive to *N*-acetylgalactosamine residues in supranuclear regions of bipolar VNE cells on both sides of the vomeronasal duct (Fig. 3e). β-Tubulin was expressed in cilia and apical compartments of cells from the respiratory epithelium (Fig. 4a) as well as in some myelinated nerve fibers (not shown). In one biopsy specimen individual bipolar cells showed reactivity against tubulin (Fig. 4b). Other associations of β-tubulin to putative chemoreceptor cells were not established. Cells of the OE exhibited apical and basal tubulin-positive processes as well as large olfactory nerves, which run parallel to the basal membrane (Fig. 4c).

Glia marker S-100

Glia marker S-100 was associated with nerve fibers generally outside the VNE, but in some cases also with irregularly shaped intraepithelial cells (Fig. 4d). In the OE, S-100-reactive structures were observed directly beneath the basal membrane of the epithelium (Fig. 4e).

Galectin-1 and galectin-3

The endogenous lectin Gal-1 occurred inconsistently in individual round cells of the VNE as well as in fibrocytes and endothelial cells (Fig. 5a). In contrast to the OE (Fig. 5d), basal cells were not marked. The staining patterns of Gal-3 were rather heterogeneous. In general, sub-

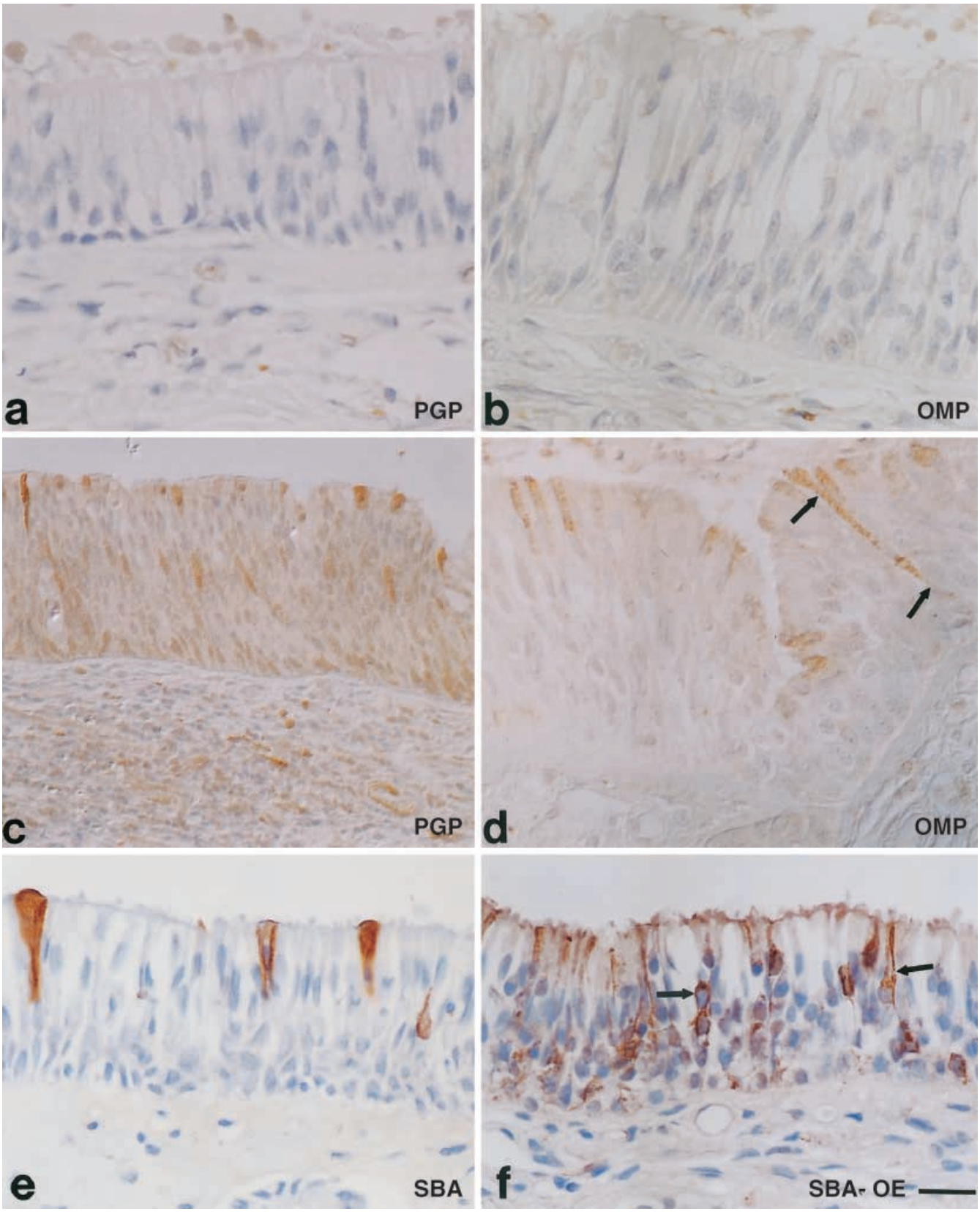


Fig. 3a–f Epithelium of the adult VND. Neither PGP 9.5 (**a**) nor OMP (**b**) is expressed in epithelial cells. The vomeronasal epithelium (VNE) of a biopsy (**c**, **d**), however, shows a small number of cells reactive with antibodies to PGP 9.5 (**c**) and, even less frequently, OMP (**d**). These slender cells extend from the apical to the basal end of the epithelium (*arrows*). **e** In this specimen, the soybean lectin (SBA) binds mostly to cells whose nuclei lie in the upper one-third of the epithelium. **f** In comparison, the olfactory epithelium (OE) exhibits SBA-reactive cells in the layer of olfactory neurons (*arrows*). *Scale bar* 20 µm in **a**, **b**; 40 µm in **c**, **e**, **f**; 8 µm in **d**

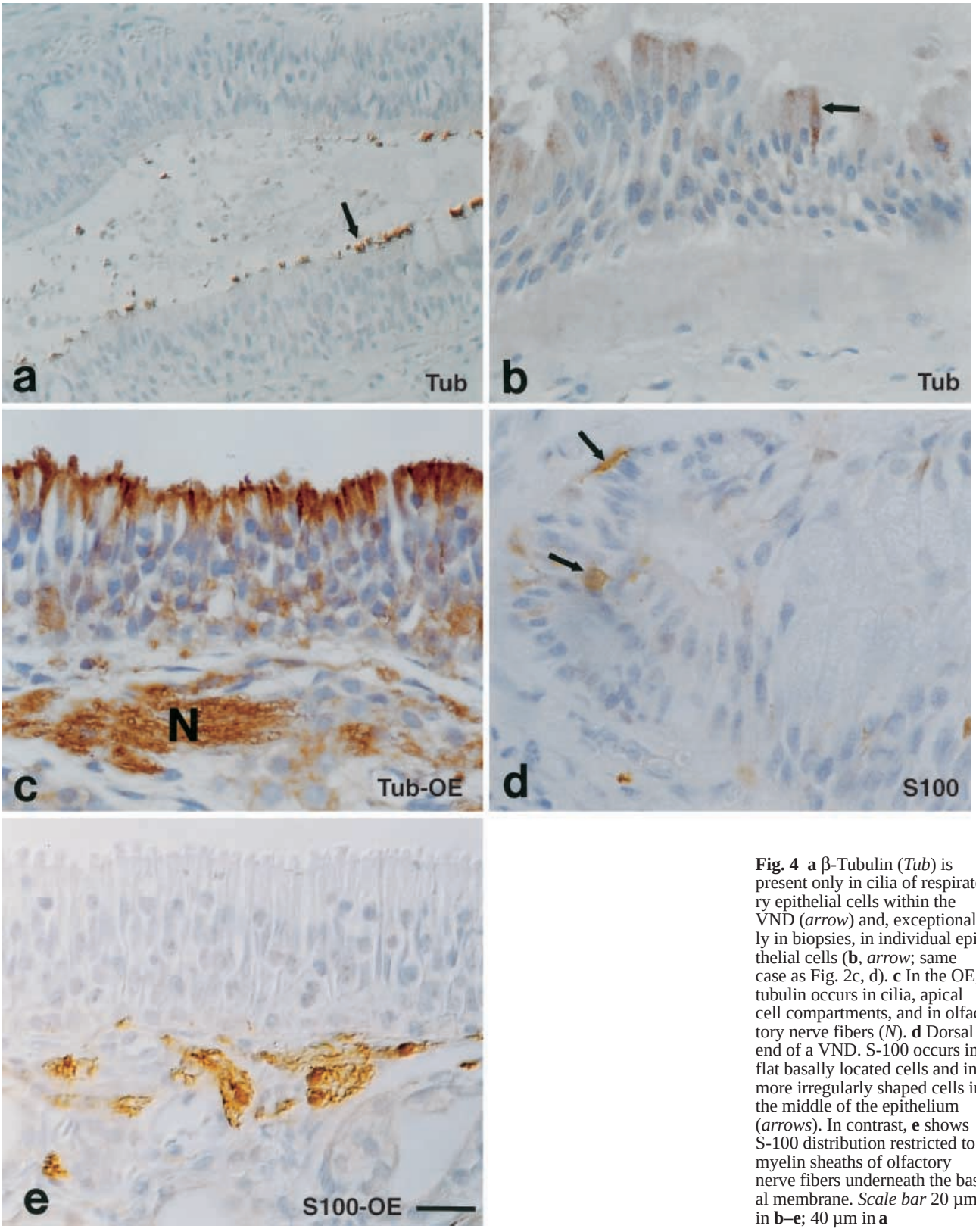


Fig. 4 **a** β-Tubulin (*Tub*) is present only in cilia of respiratory epithelial cells within the VND (*arrow*) and, exceptionally in biopsies, in individual epithelial cells (**b**, *arrow*; same case as Fig. 2c, d). **c** In the OE, tubulin occurs in cilia, apical cell compartments, and in olfactory nerve fibers (*N*). **d** Dorsal end of a VND. S-100 occurs in flat basally located cells and in more irregularly shaped cells in the middle of the epithelium (*arrows*). In contrast, **e** shows S-100 distribution restricted to myelin sheaths of olfactory nerve fibers underneath the basal membrane. *Scale bar* 20 µm in **b–e**; 40 µm in **a**

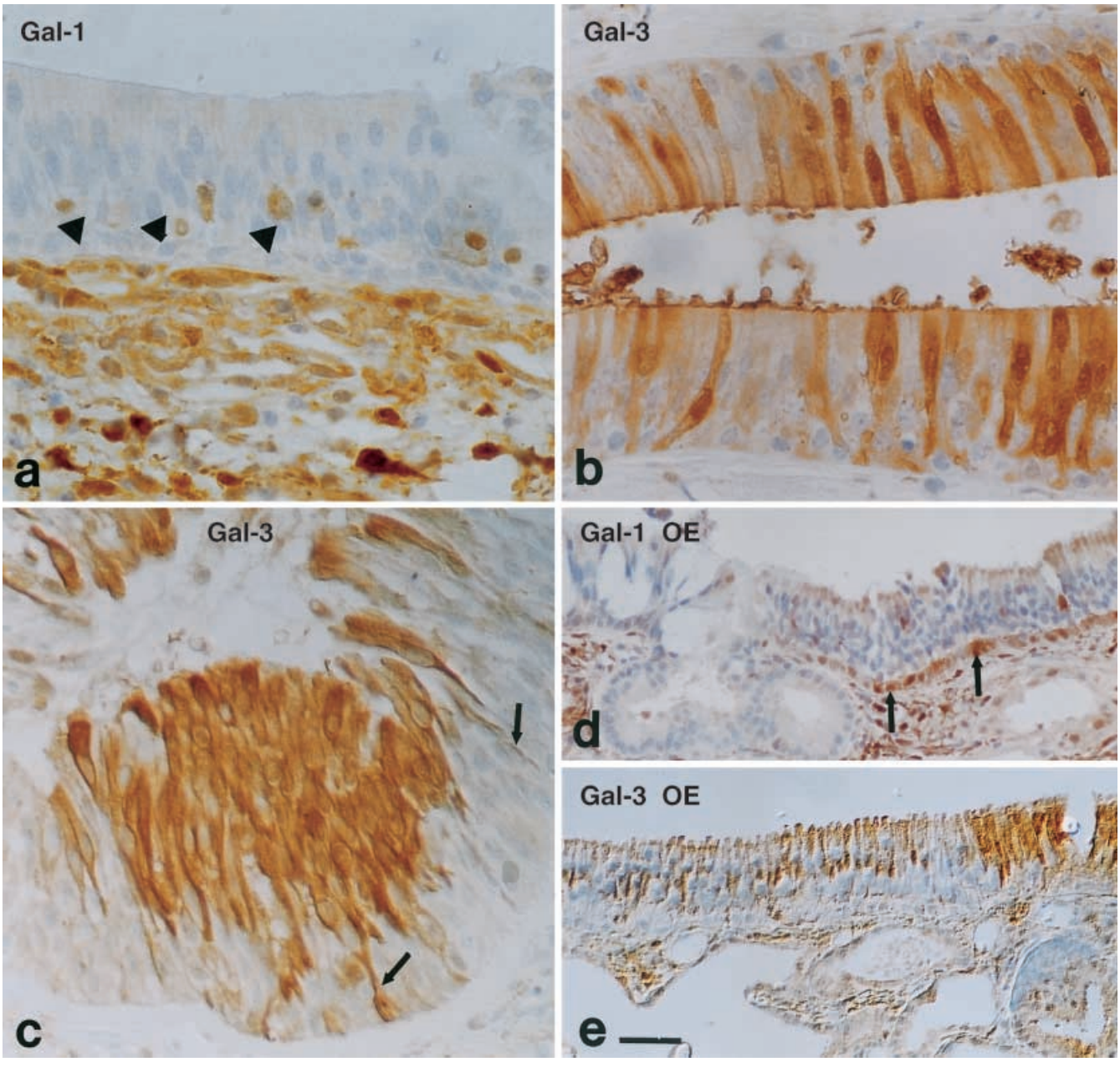


Fig. 5 **a** Galectin-1 (Gal-1) is strongly expressed in cells of the lower one-third of the VNE. In contrast to the pattern in the OE (**d**), basal cells (*arrowheads*) are not labeled. Fibrocytes, vascular endothelial cells in the subepithelial connective tissue react strongly. **b** Galectin-3 (Gal-3) is expressed in a subpopulation of slender bipolar cells, the basal processes of which reach to the basal membrane. Basal cells do not stain. **c** Biopsy specimen, dorsal end of the VND. Most epithelial cells are reactive with Gal-3. Thin processes reach the basal membrane (*arrows*). Extra-epithelial structures do not stain. **d** The OE shows reactivity for Gal-1 in undifferentiated basal cells and connective tissue cells. Only basal cells of the OE are reactive (*arrows*), whereas those of the neighboring respiratory epithelium (*left part*) do not stain. **e** Galectin-3 is present in a subpopulation of receptor neurons of the OE, and in olfactory nerve fibers. The respiratory epithelium (*right side*) reacts intensely. Nomarski optics. *Scale bar* 20 μm in **a–d, f**; 40 μm in **e**

populations of fusiform cells of the VND were positive whereas morphologically similar cells as well as basal cells lacked immunoreactivity (Fig. 5b). There were, however, biopsy specimens with a very dense staining pattern of slender Gal-3-positive cells (Fig. 5c). In the OE, Gal-3 was detected in mature olfactory neurons which also showed immunoreactivity for OMP (Fig. 5e).

Cytokeratins

The pan-cytokeratin MNF showed reactivity in most VNE cells (Fig. 6a). The OE, in contrast, exhibited strongly reacting basal cells and superficial supporting cells, whereas the layer of olfactory receptor neurons (ORNs) remained cytokeratin negative (Fig. 6b).

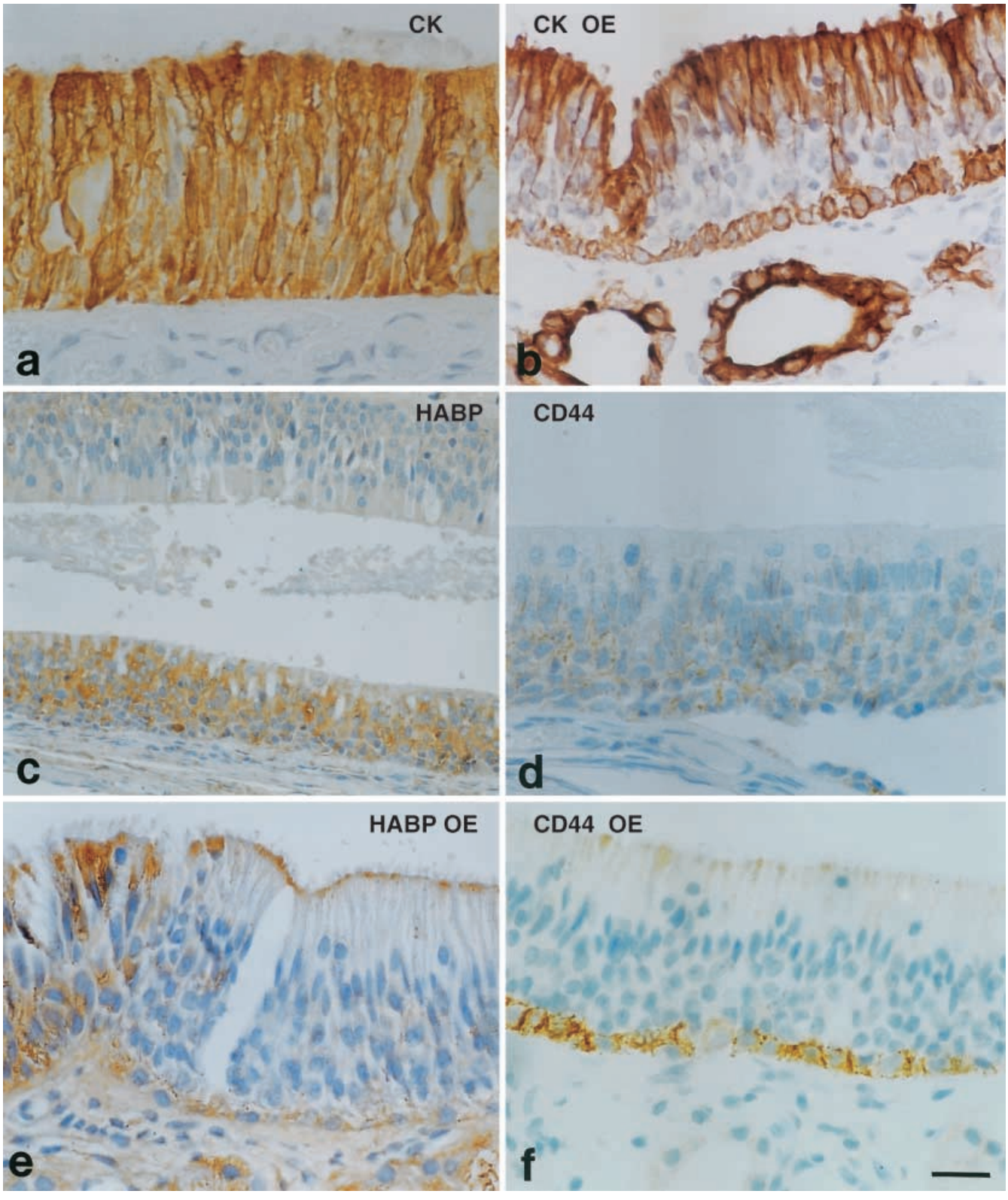
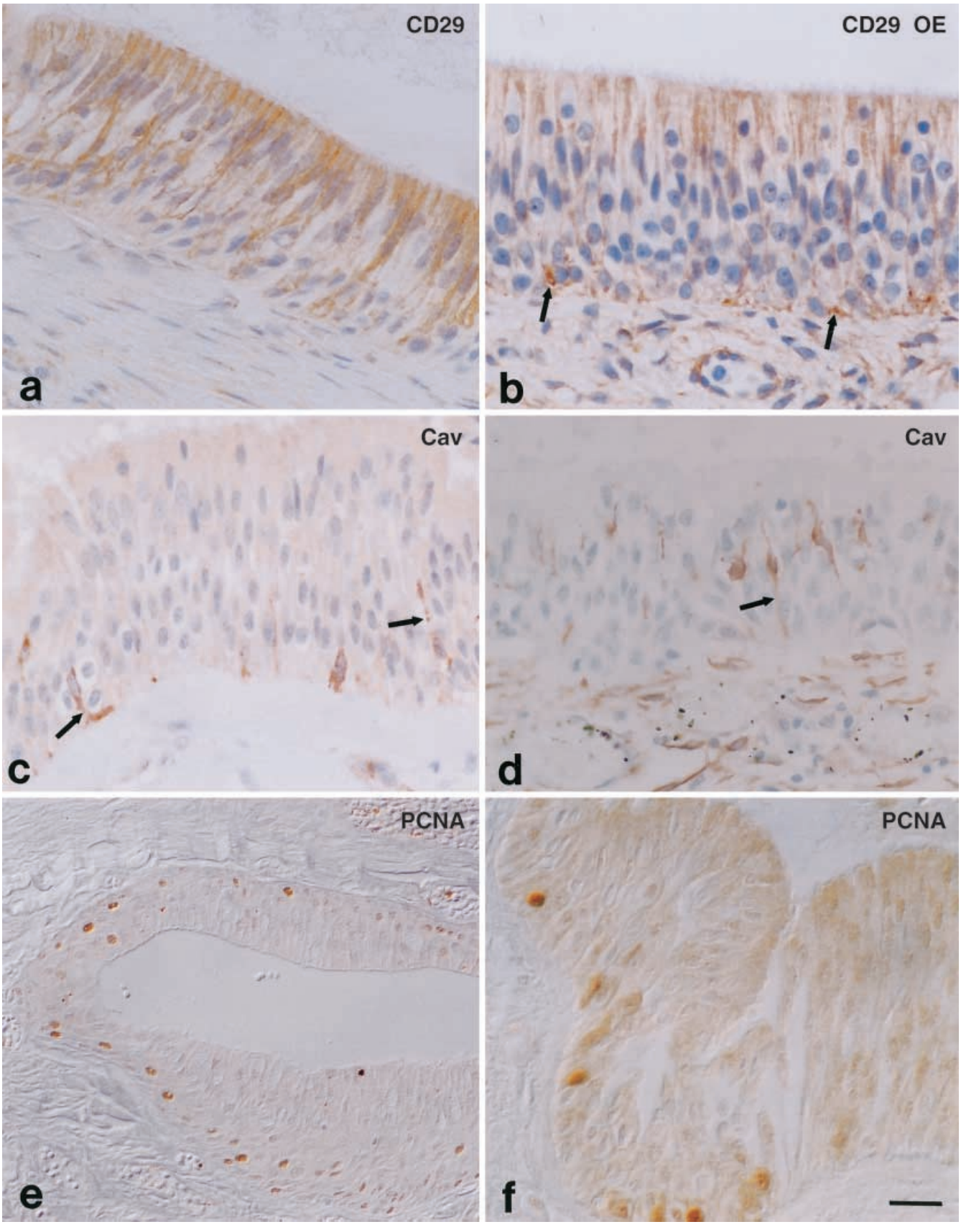


Fig. 6 **a** Cytokeratin (CK; MNF 116)-positive cells are present throughout the entire VNE. In this section, only two to three cells do not stain, whereas in the OE (**b**) most of the olfactory receptor neurons are MNF-negative. **c** Hyaluronan-binding protein (HABP) shows hyaluronan mainly in cells and cell membranes of the medial (lower) side of the VND. **d** The respective receptor for hyaluronan, CD44, is detectable on cell membranes on correspondent sites (adjacent section). **e** The OE possesses binding sites for HABP on basal cells and on apical dendrites. The respiratory epithelium (*left side*) has more binding sites for hyaluronan than OE. **f** Accordingly, the antibody against CD44, binds to basal cells and apical dendrites. *Scale bar* 20 µm in **a**, **b**, **d–f**; 40 µm in **c**


CD29
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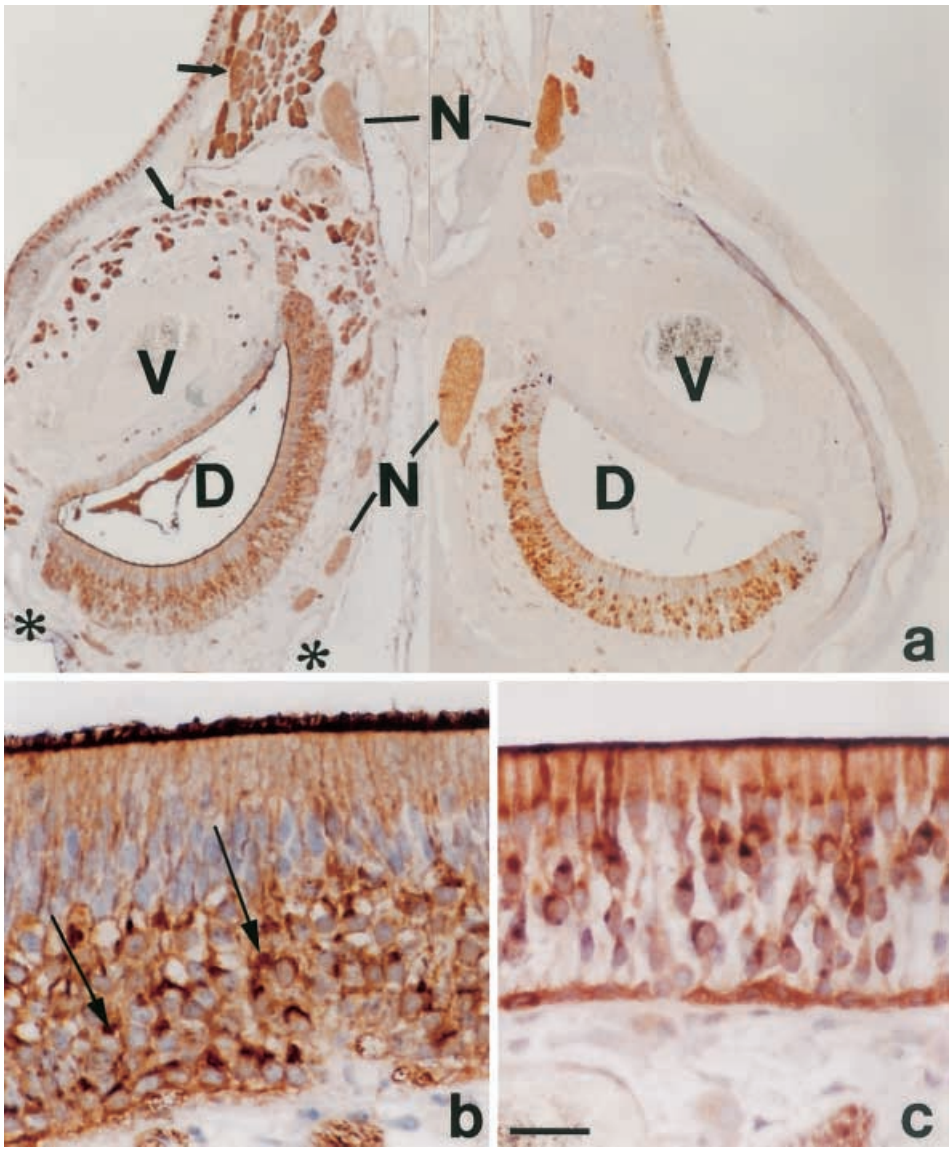

Fig. 8 **a** Frontal section through the ventral part of the nasal septum of a newborn rat. The figure shows a bilateral symmetrically situated VNO consisting of the concave VNE which sits on a fine bone clip (*asterisks*). Dorsal to the non-chemosensory convex epithelium lies the large vomeronasal vein (*V*) surrounded by a dense contractile apparatus (not stained). The *left half* of this image shows the reaction with soybean lectin, SBA, which binds to VNE cells (magnification in **b**), profiles of the vomeronasal nerve (*N*), and numerous glands (*arrows*) ending at the dorsomedial edge of the VND (*D*). The *right half* shows a reaction with OMP, which is confined to cells of the VNE and the vomeronasal nerve (*N*). **b** Soybean agglutinin binds to supranuclear caps of most cells of the VNE, but most intensely to vomeronasal receptor neurons (*arrows*). **c** For comparison, SBA is bound by olfactory neurons as well, but basal cells and supporting cells are also marked. *Scale bar* 160 µm in **a**; 20 µm in **b**, **c**

Hyaluronan-binding protein (HABP) and hyaluronan receptor CD44

A biotinylated HABP was used to localize hyaluronan in cells of the VNE (Fig. 6c). Hyaluronan-binding protein was expressed mainly by cells located in the lower one-third of the epithelium following the staining pattern of the CD44 antibody BBA-10 (Fig. 6d). In contrast, in the OE, reactivity of HABP and CD44 was confined to basal cells and the apical surface of olfactory neurons (Fig. 6e, f).

◀ **Fig. 7** **a** The antibody against CD29 (β1-integrin) binds to cell membranes of nearly all cells of the VNE. **b** A similar reaction pattern can be seen in the OE. Here, the reaction is more dense at the base of the cellular processes (*arrows*). **c** Caveolin (Cav) reactivity is restricted to individual slender cells of the VNE, the basal processes of which touch the basal membrane (*arrows*; same case as Fig. 2d). **d** The VNE shows caveolin-positive cells, the apical and basal processes of which run through the entire width of the epithelium (*arrow*). In the subepithelial connective tissue, endothelial cells and fibroblasts are also stained. **e** Proliferating cell nuclear antigen (*PCNA*) antibody binds to proliferating basal cells of the VNE, which are not equally distributed, but rather focal. Nomarski optics. **f** The VNE of a biopsy (same case as Figs. 2d, 6c) with a similar appearance of proliferating basal cells. *Scale bar* 20 µm in **a**–**d**, **f**; 40 µm in **e**

β1-Integrin (CD29)

CD29 was observed on membranes of slender bipolar cells of the VNE (Fig. 7a) and the OE (Fig. 7b). Similar to hyaluronan receptors, the reactivity of CD29 exhibited considerable variation within the different sections of the VNE. There was no preference to either the medial or lateral side of the VND.

Caveolin

The caveolin isotypes 1–3 were expressed in individual cells of the VNE ranging from a subpopulation of basally

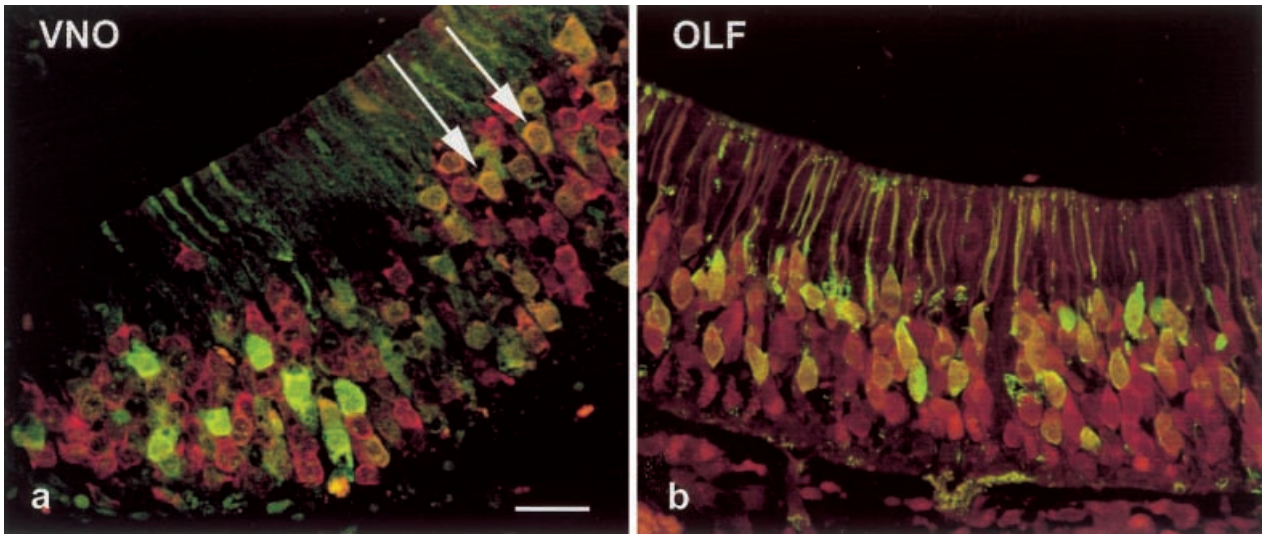

Fig. 9a, b Double immunofluorescence of the rat VNE and OE. This reaction procedure was made on the same section. **a** The majority of VNO chemoreceptor neurons is PGP 9.5 positive (*red fluorescence*); the smaller number of cells is reactive to OMP (*green*). There is an especially dense cluster of OMP-positive cells toward the medial edge of the VND (*left side*). Cells colocalizing both antigens are dominant in the middle (*right side*) of the epithelium (*orange*; *arrows*). **b** In the OE, mature OMP-positive olfactory receptor neurons are mostly located in the middle one-third of the epithelium, whereas the vast majority of chemoreceptor neurons is reactive only for PGP 9.5. Cells reactive for both antibodies express *yellow color*. *Scale bar* 20 µm

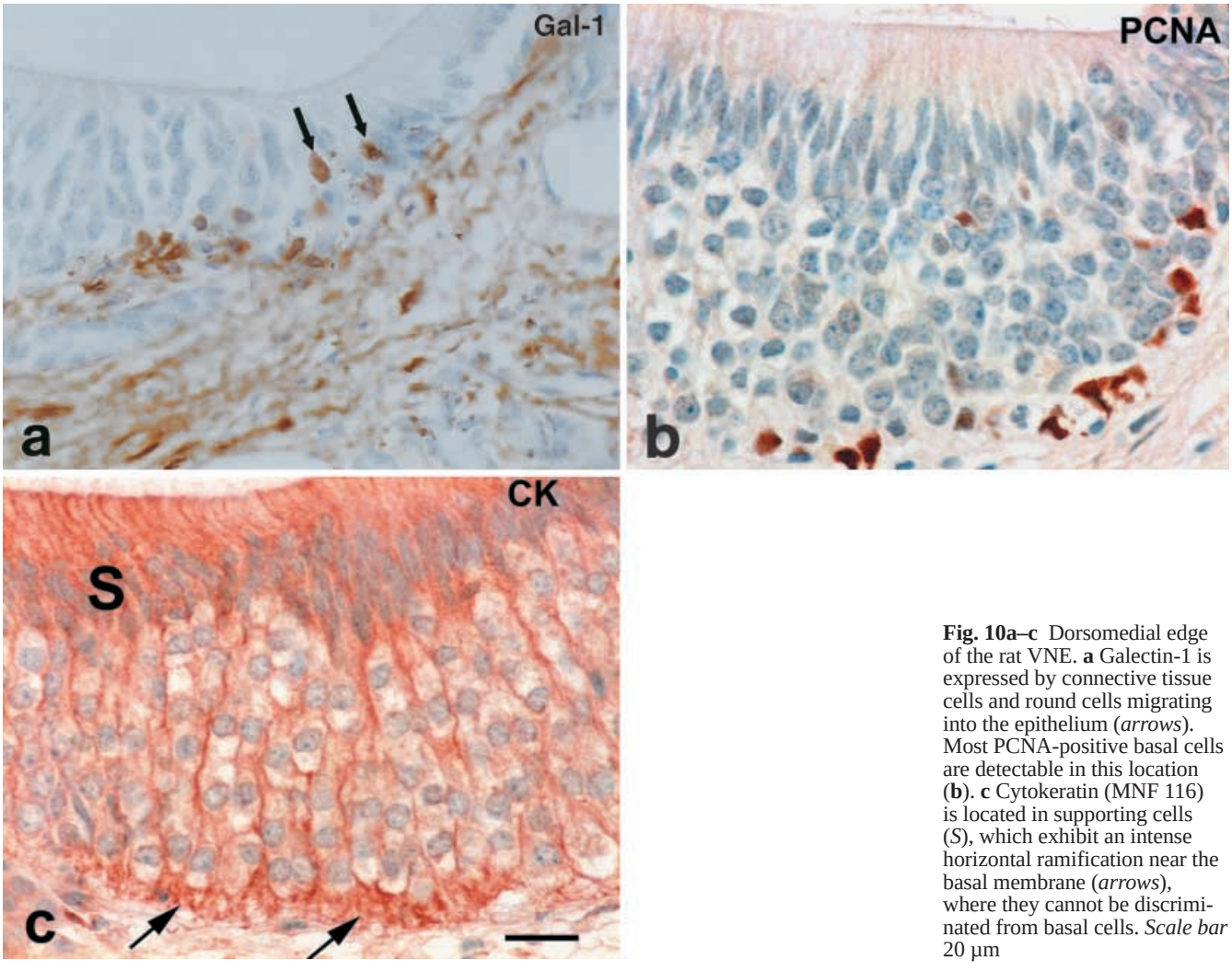

Fig. 10a–c Dorsomedial edge of the rat VNE. **a** Galectin-1 is expressed by connective tissue cells and round cells migrating into the epithelium (*arrows*). Most PCNA-positive basal cells are detectable in this location (**b**). **c** Cytokeratin (MNF 116) is located in supporting cells (*S*), which exhibit an intense horizontal ramification near the basal membrane (*arrows*), where they cannot be discriminated from basal cells. *Scale bar* 20 µm

located cells (Fig. 7c) to elongated slender bipolar cells (Fig. 7d).

Proliferating cell nuclear antigen (PCNA)

The proliferation marker PCNA was detected in a few basal cells of the VNE (Fig. 7e, f). The OE exhibited a consistent basal cell layer reactive for PCNA.

The vomeronasal system of the rat

The VNO of the rat was used as an example for a fully developed chemosensory organ that consists of a VND with two different epithelial linings, associated glands, a vomeronasal nerve, a large vein with a well-developed smooth muscle apparatus, and a shielding clip of vomeronasal bone (Fig. 8a).

The pseudostratified chemosensory epithelium lies medial to the nasal septum and possesses layers of basal cells, chemosensory neurons, and, most apically, supporting cells. The chemoreceptor cells were positive for antibodies against PGP 9.5, OMP, β-tubulin, and the lectin SBA (Fig. 8). Mature chemoreceptor cells (marked by OMP) were diffusely distributed within the layer of chemoreceptor neurons, in contrast to the situation in the OE, where olfactory neurons appeared more apically (Fig. 9). Many cells colocalized with OMP, but there was a small subpopulation of cells which did not react with antiserum to PGP 9.5. In contrast, some cells were reactive for PGP 9.5 alone. Soybean agglutinin bound to carbohydrate residues in perinuclear regions of chemoreceptor cells, but, less intensely, also to basal cells and supporting cells (Fig. 8b). A similar distribution pattern was seen in the OE (Fig. 8c).

Chemoreceptor neurons of both VNE and OE were not reactive for antibodies against cytokeratin. Galectin-1 bound to a subpopulation of basally located cells in the caudomedial edge of the organ (Fig. 10a), in proximity to the main site, where most proliferating basal cells reactive for PCNA were seen (Fig. 10b). Cytokeratin antibodies bound to supporting cells (Fig. 10c).

Discussion

Using the bat as a model, Bhatnagar and Meisami (1998) proposed the term “vomeronasal organ complex” that includes: (a) seromucous glands entering the VND, (b) paravomeronasal ganglion cells, (c) an internal vein located immediately beyond the basement membrane of the VNE suggesting a dual pumping mechanism, (d) a surrounding osseous clip, and (e) the accessory olfactory bulb. This complex is significantly different in humans. Here, the VND is believed to be a remnant of a chemosensory organ which resembles the olfactory mucosa with some minor differences in that cilia are absent and replaced by microvilli (Eisthen 1992; Menco et al. 2001). Both the present data and the literature indicate that, using endoscopic and standard histological methods, the VND cannot be detected in all individuals: Johnson et al. (1985) reported 39% of patients and 70% in postmortem specimens, Trotier et al. (2000) up to 26% of a group of 2,000 subjects, Gaafar et al. (1998) 76% of 200 subjects, and Knecht et al. (2001) 65% of 184 subjects. In addition, a functional connectivity between brain structures and the VNE has not yet been demonstrated and the accessory olfactory bulb is absent in adults (Meisami and Bhatnagar 1998). Finally, a uniform epithelium with proposed anterior-posterior differences is lacking (Moran et al. 1991). According to Moran et al. (1995) the medial side of the VND is outlined with pseudostratified epithelium, whereas the lateral side has respiratory epithelium. Such consistent differences were not described by a number of other groups (see, for example, Bhatnagar and Smith 2001; Smith et al. 2001a) nor did we observe such differences in our study. On the contrary, the epithelium of the VND is extraordinarily heterogeneous in that it shows areas with ciliated (respiratory) epithelial cells, spots of stratified epithelium, and pseudostratified epithelium. This pseudostratified epithelium appears locally, sometimes on the medial side and sometimes on the lateral side of the duct. However, even if two morphologically similar epithelial sections were opposite to each other, there is the possibility that they will differ, as seen in the differential staining of CD44 (Fig. 6c) or β-integrin.

Human development

The vomeronasal groove appears at Carnegie stage 16, approximately 37 days after conception (Bossy 1980; Müller and O’Rahilly 1989). The VNO, including the vomeronasal nerve and associated ganglion cells, is first recognizable around week 8 after ovulation (Kreutzer and Jafek 1980). Results of the present study suggest an even earlier appearance because a well-developed VNO was present already after 8 weeks. During embryonic development, neuron-specific markers bind to the VNE indicating chemosensory activity of the VNO (Fig. 2a, c). Although the existence of two separate nerve fiber bundles in the nasal submucosa, innervating the VNE and the OE, have been demonstrated by Kjaer and Hansen (1996), the lacking OMP reactivity in an 8-week-old human embryo in our study suggests an incomplete functional maturity. The vomeronasal nerve in young fetuses carries gonadotrophic (LHRH-positive) cells (Schwanzel-Fukuda and Pfaff 1994), which migrate towards the hypothalamus. The gonadotrophic activity around the VNO disappears after the 12th gestational week (Kjaer and Hansen 1996).

The vomeronasal epithelium in adults

In adult humans, our results demonstrated that a very small number of VNE cells maintain neuron-like proper-

ties. The pan-neuronal marker PGP 9.5 has been used as a reliable marker for gustatory and olfactory chemosensory cells and their central projections (see, for example, Kjaer and Fischer Hansen 1996; Nakajima et al. 1998; Nosrat et al. 2000; Witt and Reutter 1998). However, the occurrence of PGP 9.5 in individual VNE cells is rather exceptional (Takami et al. 1993; Yamamoto 2001). In accordance with these authors, PGP 9.5-reactive cells were found only in a few cells of four specimens, which questions the chemosensory activity of the VNE. The same is true for other neurochemical markers used in this study, especially OMP, which, to our knowledge, has not yet been detected in adult human VNE. Also, in line with other recent studies (Trotier et al. 2000), we neither found signs for the presence of nerve fibers in association with the VNE nor OMP-positive cells which are normally present in mature olfactory neurons and also in a major subset of VNE cells of many animals (Halpern et al. 1998; Shapiro et al. 1997; Tarozzo et al. 1998; Figs. 8, 9). In some cases, the faint reactivity of S-100-positive cells may indicate a (residual?) glia-like function of these structures. However, immunocompetent dendritic cells may also contribute to S-100 reactivity, such as Langerhans cells (Cocchia et al. 1981).

The expression of *N*-acetyl-galactosamine (Gal*N*Ac) residues in the VNE, detected by a lectin from SBA (*Glycine max*), is of special interest. Some studies have revealed that SBA and another Gal*N*Ac-reactive lectin, *Dolichos biflorus* agglutinin, selectively label a portion of neurons in amphibian and mammalian primary olfactory systems (Key and Akeson 1991, 1993; Menco 1992; Meyer et al. 1996). On the other hand, not all SBA-positive VNE cells identify these cells as typical chemosensory ones, but Gal*N*Ac residues found in this particular conformation may be either remnants or prerequisites for the fraction of chemosensory cells that have lost their neuronal characteristics during ontogeny.

Cytokeratins

Cytokeratins (CK) have been regarded as complementary markers for the discrimination of non-sensory cells from sensory cells in the OE (Okabe et al. 1997; Satoh and Yoshida 2000) and VNE (Trotier et al. 2000). The use of complex cytokeratin antibodies which react with horizontal basal cells in the OE (for example, CK 5, 14) have contributed to the opinion that the globose basal cell layer rather than the horizontal cell layer is the immediate precursor of ORNs (Yamagishi et al. 1989). Horizontal basal cells are believed to maintain the structural integrity of the OE (Holbrook et al. 1995). Nevertheless, there remains some doubt which cell population may act as the stem cells proper for ORNs (for reviews: Mackay-Sim and Chuahb 2000; Schwob 2002). While horizontal basal cells of the OE are usually strongly cytokeratin positive (Holbrook et al. 1995; Satoh and Yoshida 2000; this study), our results in the VNE often show neither a consistent basal cell layer comparable to that of the OE, nor respective cytokeratin-free "gaps" within the VNE which may point to putatively differentiated chemosensory cells. Similar observations have been made by Trotier et al. (2000). In most specimens, there was a consistent outline of cytokeratin-containing epithelial cells, but evidence for an absence of chemosensory potency from this observation is, of course, only indirect.

Apart from the lack of consistent neuroepithelial properties of VNE cells rendering them "non-functional", immunohistochemical detection of proteins reflecting particular cell functions allows us to give a somewhat different picture. In this study, we have looked specifically at galectins, integrins, caveolins, and HABPs. The staining patterns were often selective to certain subpopulations of cells, which, admittedly, do not necessarily represent typical VNE functions (see Table 2).

Galectins

The effects of both endogenous lectins (carbohydrate-binding proteins), Gal-1 and Gal-3, range from apoptosis to cell maturation. They appear to act in an antagonistic manner that is dependent on the physiological activity of the tissue. For example, Gal-1 appears to play a role in differentiation and maturation. It is expressed in developing brain tissue, but not in the mature brain (Joubert et al. 1989). In rat OE, Gal-1 has been shown to be associated with glia-like structures, apparently of importance to the guiding capacities of developing ORNs (Mahanthappa et al. 1994; St John and Key 1999). Knockout mice for the Gal-1 gene have severe deficits in olfactory pathfinding (Puche et al. 1996). Our previous studies on the expression of Gal-1, however, lead to a different function of this endogenous lectin in humans since it appeared selectively in basal cells of the OE (Heilmann et al. 2000). In the VNO of the rat, Gal-1 occurs in areas known for a higher proliferation activity than normal (Weiler et al. 1999), namely in the medial and lateral endings of the chemosensory epithelium, as demonstrated by the PCNA distribution pattern (Fig. 10). The reaction pattern of Gal-1 in the human VNE, however, varies from irregular staining of suprabasal cells to that of cells in the upper one-third of the epithelium. Some reactions were even negative (data not shown). Typical basal cells, i.e., those lying immediately above the basal membrane, were inconsistently reactive. Provided Gal-1 is associated with maturation processes of basal-like cells (as seen in the human OE and the rat VNO), we may suggest an atypical location of those cells and postulate rather unique maturation dynamics in the human VNE.

Gal-3, which is present in a subpopulation of mature ORNs as shown previously by colocalization with OMP (Heilmann et al. 2000), exhibited an ambiguous staining pattern in the human VNE. It was expressed in slender, bipolar cells, which did not express neuronal markers. However, while in some cases it appeared to be more

prevalent at the lateral wall of the VND, sometimes it was also found equally on both sides. Consistent with the situation in the OE, no basal cells contained Gal-3. On the other hand, in biopsies Gal-3 bound to many slender bipolar VNE cells (Fig. 5c) indicating its role in the differentiation of these cells (see, for example, Le Marer 2000).

β-Integrin (CD29) and caveolin

Integrins are heterodimeric cell adhesion molecules that constitute a molecular link between the extracellular matrix (ECM) and the cytoskeleton. They assist in the regulation of cell migration and differentiation (Howe et al. 1998). We have introduced integrins in this study because olfactory sensory cells develop and regenerate in cooperation with specialized ECM proteins. In neuronal tissues, integrins are involved in the regulation of axonal and dendritic growth of some neurons in the developing central nervous system and may participate in the formation, maintenance, or plasticity of synapses in the olfactory bulb (Einheber et al. 1996) and other sensory structures. In auditory hair cells, integrins maintain the structural integrity of the cytoskeleton during stimulation (Littlewood-Evans and Müller 2000). The pathfinding of retinal ganglion cells can be inhibited after downregulation of β1-integrins (Stuermer and Bastmeyer 2000). In insects, integrins have been associated with olfactory memory (Connolly and Tully 1998). β1-Integrin was expressed to a higher degree on membranes of bipolar chemoreceptor-like cells of the VNE and ORN than on relatively "simple" stratified or degenerated respiratory epithelia found on the wall of the nasal septum or partially within the VND. We can therefore deduce an increased activity of VNE cells to establish relationships with ECM proteins, compared to the situation in non-vomeronasal epithelia.

Recently, more data have been presented on additional proteins involved in cell differentiation and many other cell-matrix relationships. One example for physically associated "partner" proteins for integrins are caveolins (Porter and Hogg 1998). Caveolins are 22-kDa phosphoproteins that form specialized plasma membrane invaginations (caveolae) and are involved in transcytosis as well as ECM-mediated promotion of cell proliferation, signal transduction, or terminal differentiation (for review, see Porter and Hogg 1998). In the human OE, the polyclonal antibody against all three isoforms of caveolin showed a regular binding pattern on apical cell compartments including the microvillous/ciliary domains. This is consistent with a study from Schreiber et al. (2000), who found high densities of caveolin in the membranes of ORN, which formed complexes with olfactory G proteins. This led to the idea that caveolins could be specifically involved in olfactory signaling mechanisms. Although a definite chemosensory-related function for caveolin has not yet been established, antibody binding to a subset of individual bipolar cells in the human VNE (Fig. 7c, d) may suggest a particular activity of caveolins in signal transduction pathways.

Transmembrane glycoprotein, CD44

The transmembrane glycoprotein, CD44, is one of the two specific hyaluronan receptors that have been characterized and cloned so far (for review, see Rudzki and Jothy 1997; Sherman et al. 1994). The multitude of functions is structurally represented by glycosylation and several isoforms, one of which, the epithelial variant V6, occurs almost exclusively in epithelia (Günthert et al. 1991). CD44 has been used in earlier studies on the gustatory system (Witt and Kasper 1998) which showed reactivity of marginal cells in developing taste buds. In the retina, CD44 is known to be expressed by apical microvilli of Müller cells. When photoreceptor cells were damaged, Müller cells responded with an increased expression of CD44 (Kuhrt et al. 1997). Such observations on supporting function for sensory cells are in certain agreement with the CD44 binding of basal cells in the OE, assuming these are horizontal basal cells which have supporting cell functions (see cytokeratin section). The binding pattern of CD44 is similar to that of HABP (Fig. 6c, d). In the VNE, however, the more diffuse expression of CD44 in the basal one-third of the epithelium indicates that the maturation dynamics of VNE cells are different from those in the OE.

Several authors have reported that there is no correlation between age and VNO length or volumes (Abolmaali et al. 2001; Bhatnagar and Smith 2001; Smith et al. 2001a). Since the spatial extension of the duct gives no direct information on the biological activity, especially with regard to chemoreception, future studies should focus more on quantitative immunohistochemical approaches. Interestingly, we found in biopsies of younger individuals (mean age 41 years) more cells positive for neuronal markers and differentiation antigens than in cadaver specimens (mean age 80 years). Although a careful quantitative evaluation is still missing, these data indicate that cellular relicts of an earlier chemoreceptor function might decline with advancing age.

Taken together, bipolar cells of the human VNE are structurally similar to olfactory receptor cells. However, in their vast majority they do not express neuronal characteristics such as OMP or PGP 9.5 reactivity which casts doubts on the functionality of the human VNE. Specifically, the present results raise the question whether pheromone-like substances or odorants thought to be mediated by the VNE (Grosser et al. 2000; Monti-Bloch et al. 1998; Savic et al. 2001) may actually be perceived by the olfactory or trigeminal intranasal system. On the other hand, the VNE is equipped with a highly specialized and, compared to neighboring nasal epithelia, unique arrangements of cell adhesion molecules possibly indicating specific chemosensory functions. Thus, the VNE appears as a highly differentiated structure the function of which remains unclear.

Acknowledgements Parts of this work were presented at the 24th meeting of the Association of Chemoreception Sciences held in Sarasota, Florida, 24–28 April 2002. The authors are indebted to Dr. D. Cooper, University of California, San Francisco, for the antibody against galectin-1, and to Dr. F. Margolis, University of Maryland, Baltimore, for the antibody against olfactory marker protein. Ms J. Ito and Dr. K. Touhara, University of Tokyo, assisted with critical reading of the manuscript and helpful discussions.

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