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REVIEW ARTICLE

Ultrastructural Aspects of Olfactory Signaling

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Abstract

The olfactory area of the nasal cavity is lined with olfactory receptor cell cilia that come in contact with incoming odor molecules. Ultrastructural immunocytochemical studies in rodents have shown that these cilia contain all the proteins necessary to transduce the odorous message into an electrical signal that can be transmitted to the brain. These signaling proteins include putative odor receptors, GTP binding proteins, type III adenylyl cyclase and cyclic nucleotide-gated channels. The rest of the cells, including dendrites and dendritic knobs, showed no discernible labeling with antibodies to these signaling proteins. Furthermore, freeze–fracture and freeze–etch studies have shown that the membrane morphology of olfactory cilia differs substantially from that of non-sensory cilia. Olfactory cilia have many more membrane particles. Transmembrane signaling proteins, such as odor receptors, adenylyl cyclase and cyclic nucleotide-gated channels, conceivably appear as membrane particles. Thus, the long-standing supposition that olfactory cilia are peculiarly adapted to deal with the reception and initial transduction of odorous messages has now been verified in terms of both ultrastructural morphology and cytochemistry. Emerging studies on vomeronasal receptor cell microvilli indicate that the same is true for this organ, even though the actual signaling components differ from those of the main olfactory system. **Chem. Senses 22: 295–311, 1997.**

Introduction

Vertebrate olfactory receptor cells are specialized neurons that have numerous long tapering cilia. The tapering parts of these cilia form the interface between the external odorous environment and the luminal surface of the olfactory epithelium. There is accumulating evidence that these cilia contain the biochemical mechanisms of olfactory signal-transduction (Farbman, 1992; Anholt, 1993; Breer, 1994; Mori and Yoshihara, 1995; Breer *et al.*, 1996; Buck, 1996; Sullivan and Dryer, 1996). Much of this evidence comes from ultrastructural research (Asanuma and Nomura, 1991, 1993; Menco, 1992a, 1994; Spreca and Rambotti, 1994), the topic of this survey.

Olfactory signal transduction begins when odors interact with members of the GTP binding-protein- (or G-protein-) linked odor-receptor superfamily which characteristically traverses the membrane seven times (Buck and Axel, 1991;

Buck, 1996; Sullivan and Dryer, 1996). This stimulus receptor interaction leads to activation of a G-protein, probably G_{olf}, but perhaps G_s as well. Their α subunits, $G_{olf\alpha}$ and $G_{s\alpha}$, most likely activate calcium(Ca^{2+})/calmodulin-sensitive type III adenylyl cyclase (AC), making cyclic AMP (cAMP). The cAMP opens cyclic nucleotide-gated (CNG) ion channels, resulting in an electrical signal (Gold and Nakamura, 1987; Jones and Reed, 1989; Bakalyar and Reed, 1990, 1991; Dhallan *et al.*, 1990; Choi *et al.*, 1992; Breer, 1994; Kleene, 1994; Brunet *et al.*, 1996; Buck, 1996).

Alternative routes, particularly in invertebrates (Fadool and Ache, 1992; Hatt and Ache, 1994; Stengl, 1994), may work through activation of a phospholipase C (PLC)/trisphosoinositide (IP_3) system. G-proteins may be the catalysts for these routes as well (Boyle *et al.*, 1987; Boekhoff *et al.*, 1994). Recent studies (Dhallan *et al.*, 1990; Brunet *et al.*, 1996; Firestein, 1996; Nakamura *et al.*, 1996) have shown that the role played by signaling events involving IP_3 is somewhat obscure in vertebrates. For example, studies using transgenic mice implied that the AC/cAMP/CNG pathway is involved in all odor signaling in the main olfactory system, at least in mammals (Brunet *et al.*, 1996).

Ca^{2+}/calmodulin-activated phosphodiesterase (PDE; Borisy *et al.*, 1992), β-adrenergic receptor kinase-2 and β-arrestin-2 may mediate termination of the signal by regulating AC and guanylyl cyclase (GC) activities (Dawson *et al.*, 1993; Buck, 1996; Kroner *et al.*, 1996b). The latter enzyme, in combination with various phosphatases (Kroner *et al.*, 1996a), could also be involved in the fine-tuning of olfactory responsiveness. Several Ca^{2+}-binding proteins are found in receptor cells of main and vomeronasal olfactory organs (VNO) (e.g. Kishimoto *et al.*, 1993; Iino *et al.*, 1995) and it is likely that Ca^{2+} plays an important role in some of the aforementioned processes (Ronnett and Payne, 1995; Boekhoff *et al.*, 1996). Cytochemical studies using dyes under *in vitro* conditions have provided further evidence of the significance of Ca^{2+}. Transient Ca^{2+} responses were elicited from olfactory receptor cells upon odor stimulation, particularly at the level of the dendritic knobs (Tareilus *et al.*, 1995).

Cryopreparation methods as applied to olfactory chemosensory structures

Cryopreparation methods (Menco, 1986, 1995b; Echlin, 1992; Griffith, 1993; Severs and Shotton, 1995) provide excellent tools for the preservation of the delicate ultrastructural and immunocytochemical features of olfactory cilia. In most of our studies rapidly frozen olfactory tissues were subjected to freeze–fracturing, freeze–etching (Menco, 1983, 1984, 1992b, 1994, 1995b), and freeze–substitution in acetone (unfixed tissues) or methanol (fixed tissues). Freeze–substitution was followed by low-temperature embedding in Lowicryl resins (Van Lookeren Campagne *et al.*, 1991; Menco, 1995b). Tissue sections were immunoreacted with antibodies to proteins presumably important in olfactory signaling with immunogold labels as secondary probes (Hayat, 1989/91; Bendayan, 1995; Menco, 1995b). While the freeze–fracture and freeze–etch studies were particularly useful to study the special membrane morphology of olfactory cilia (Menco, 1983, 1984, 1992b, 1994, 1995a,b), the freeze–substitution studies demonstrated that these cilia contain important components of the olfactory signal-transduction cascade (Menco *et al.*, 1992, 1994, 1997; Menco, 1994, 1995a,b). This review surveys these studies for an overview of the ultrastructure of the vertebrate's olfactory signal-transduction apparatus.

Olfactory cilia; membrane ultrastructure

Modified cilia sprout from basal bodies inside olfactory receptor cell dendritic knobs (Figures 1–4 and 7). The olfactory cilia of mammals are most likely immotile. Their cytoskeletal structures serve to support specialized membranes that are important in olfaction (Menco, 1983, 1992a, b; Lidow and Menco 1984; this review). At the mucous surface, the long and thin distal parts of the cilia are present in a largely parallel arrangement interspersed with the tips of microvilli of olfactory supporting cells (Figures 7 and 11–14). This lattice of cilia and microvilli forms the interface between the luminal surface of the olfactory epithelium and the external environment, the area where interaction with odors takes place. Relative to the nasal surface underneath, the membrane area is enlarged by about 40 times owing to the number and length of the cilia (Menco, 1983), enhancing chances that odor molecules entering the nose will reach the receptor cells. Freeze–fracture and freeze–etch observations have shown that membranes of olfactory sensory cilia have, without exception, higher particle densities than membranes of non-sensory respiratory cilia, irrespective of the species

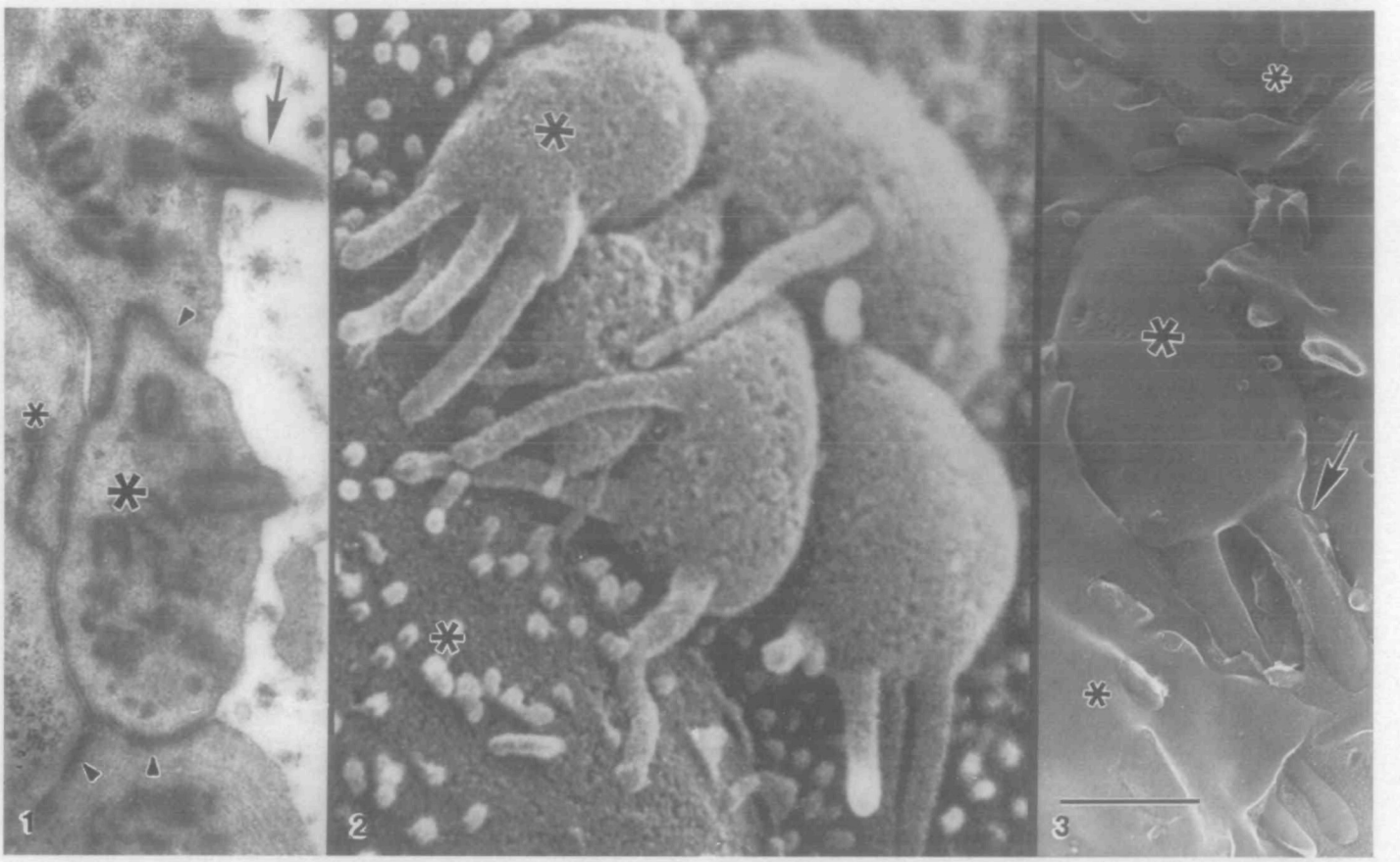


Figure 1 Thin-section transmission electron micrograph of cilium bearing dendritic endings of olfactory receptor cells in an E16 rat embryo (E1 = sperm positive). The arrow points to an olfactory cilium. The large asterisk marks the lumen of a dendritic knob filled with basal bodies. In embryos dendritic knobs are often found in clusters as seen here, while this is not the case in adults. The tight-junctional belt between knobs is marked by arrowheads while between knobs supporting cells this belt is marked by a small asterisk (Menco, 1980b, 1988c). Adapted from Figure 42 in Menco and Farbman (1985a), with permission of Company of Biologists. Scale: see Figure 3.

Figure 2 Scanning electron micrograph of a cluster of short cilium bearing dendritic endings of olfactory receptor cells in an E17 rat fetus (large asterisk). The small asterisk marks surrounding supporting cells with, at this developmental age, short microvilli. Adapted from Figure 35 in Menco and Farbman (1985a), with permission of Company of Biologists. Scale: see Figure 3.

Figure 3 Freeze-fracture platinum/carbon replica transmission electron micrograph of a cilium bearing dendritic ending of an olfactory receptor cell in an E16 rat embryo. The large asterisk marks a dendritic knob with short cilia (arrow; Menco and Farbman, 1985a,b); the small asterisks mark surrounding supporting cells. Adapted from Figure 8 in Menco (1988a), with permission of Springer-Verlag. Scale bar, 1 μm.

examined. Because of this it has been suggested that these particles represent protein entities important in olfaction (Figures 5–7; Kerjaschki and Hörandner, 1976; Menco *et al.*, 1976; Usukura and Yamada, 1978; Menco, 1980a, 1983, 1984; Breipohl *et al.*, 1982).

Freeze–fracture studies with tantalum/tungsten (Ta/W) rotary replication further supported the above supposition on the importance of freeze–fracture particles of olfactory cilia in olfaction. Using this high-resolution replication mode, we found that many particles seem to have pores. That at least some of the pores may be genuine is attested by the fact that different classes of particles, with and without apparent pores, occur within the same membrane leaflets. This is especially clear in Figure 5, where ciliary necklace particles are quite distinct from other particles of olfactory cilia (Menco *et al.*, 1988; Menco, 1995b). Particles with pores could represent ion channels, such as the CNG channels, which are the major current-carrying proteins of olfactory cilia (Gold and Nakamura, 1987; Kurahashi and Kaneko, 1991; Brunet *et al.*, 1996), but also adenylyl cyclase (Krupinski *et al.*, 1989).

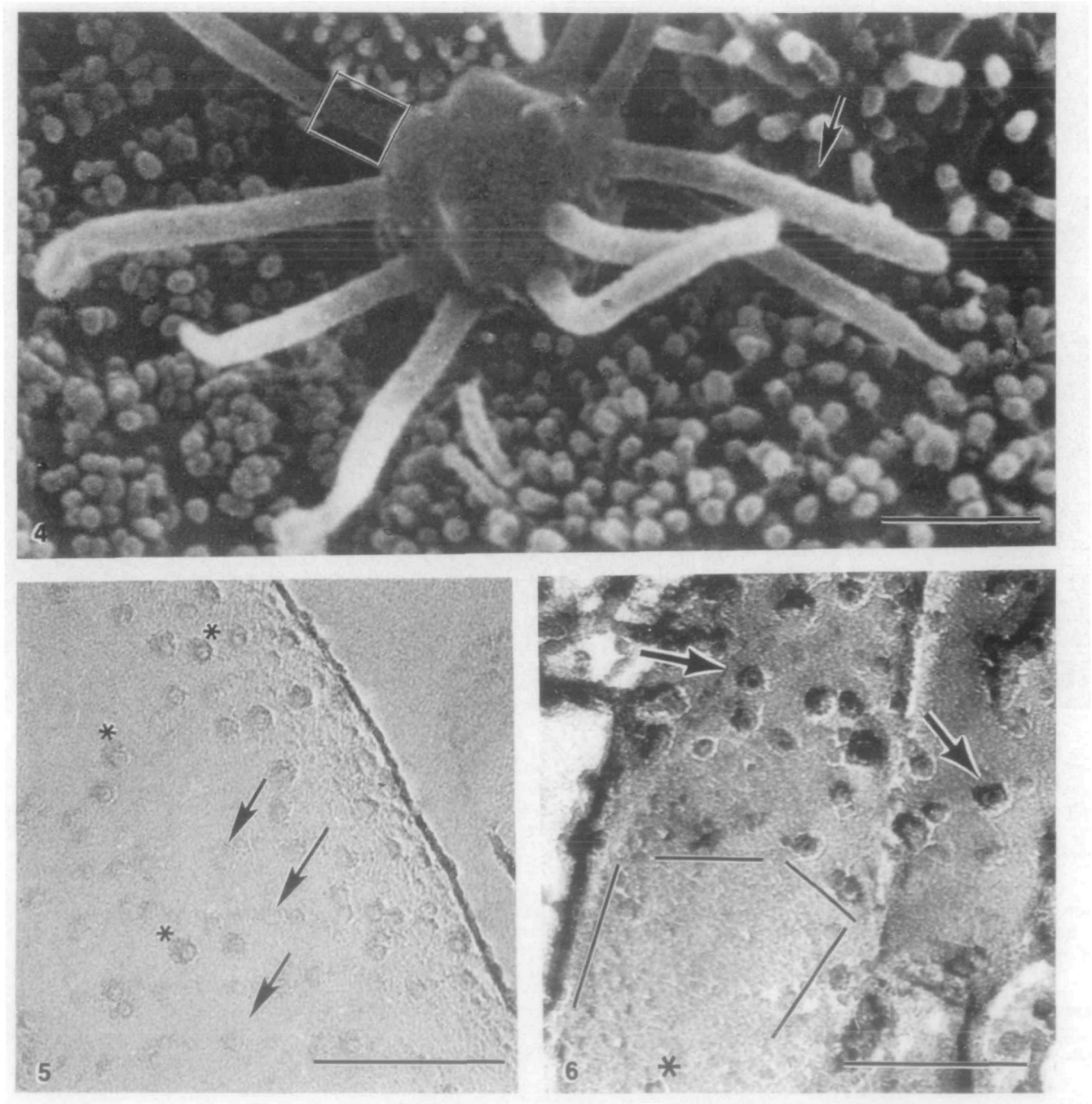


Figure 4 Conventional scanning electron micrograph of a dendritic knob of an olfactory receptor cell neuron in an E20 embryo. The olfactory cilia give the knob the appearance of a sea anemone. These cilia radiate all around the knob, likely enhancing chances of capturing odor molecules that enter the nose from the ambient environment. An area like the framed one is shown in Figure 5. Adapted from Figure 33 in Menco and Farbman (1985a), with permission of Company of Biologists. Scale bar, 1 μm.

Figure 5 High-resolution micrograph of the base of an olfactory cilium in an adult rat (see frame Figure 4). The unfixed rapidly frozen specimen was replicated with tantalum/tungsten at an angle of 20 degrees and at a temperature of −150°C. The replica was examined in a transmission electron microscope. The membranes of the olfactory cilia contain different proteins, reflected here as particles. The aligned shallow ones near the bottom comprise the so-called ciliary necklace (arrows). Others seem to depict pores (asterisks). Adapted from Figure 7 in Menco (1995b; see also Menco *et al.*, 1988), with permission of Wiley-Liss, Inc. Scale bar, 0.1 μm.

Figure 6 Membrane particles of olfactory cilia bind the gold-conjugated lectin wheat germ agglutinin (WGA) (arrows) before replication and metal casting. This means that the glycosyl groups of the proteins reflected as particles contain N-acetyl glucosamine that binds WGA. The lines mark the approximate border between fracture plane and true etched membrane surface. A membrane particle in a fracture plane is marked by an asterisk. The replication material was platinum/carbon evaporated from an angle of 45 degrees. Adapted from Figure 10 in Menco (1992c), with permission of Wiley-Liss, Inc. Scale bar, 0.1 μm.

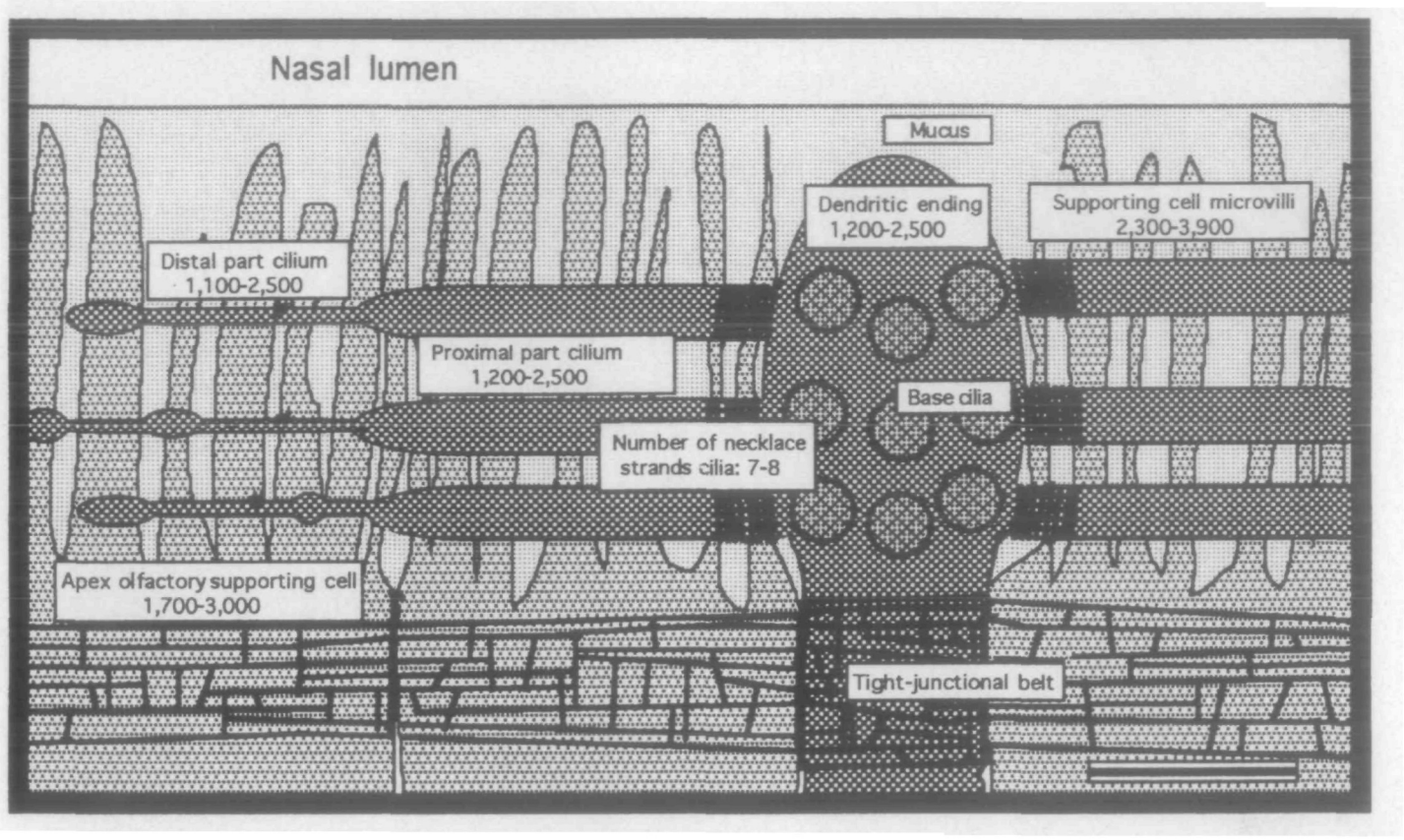
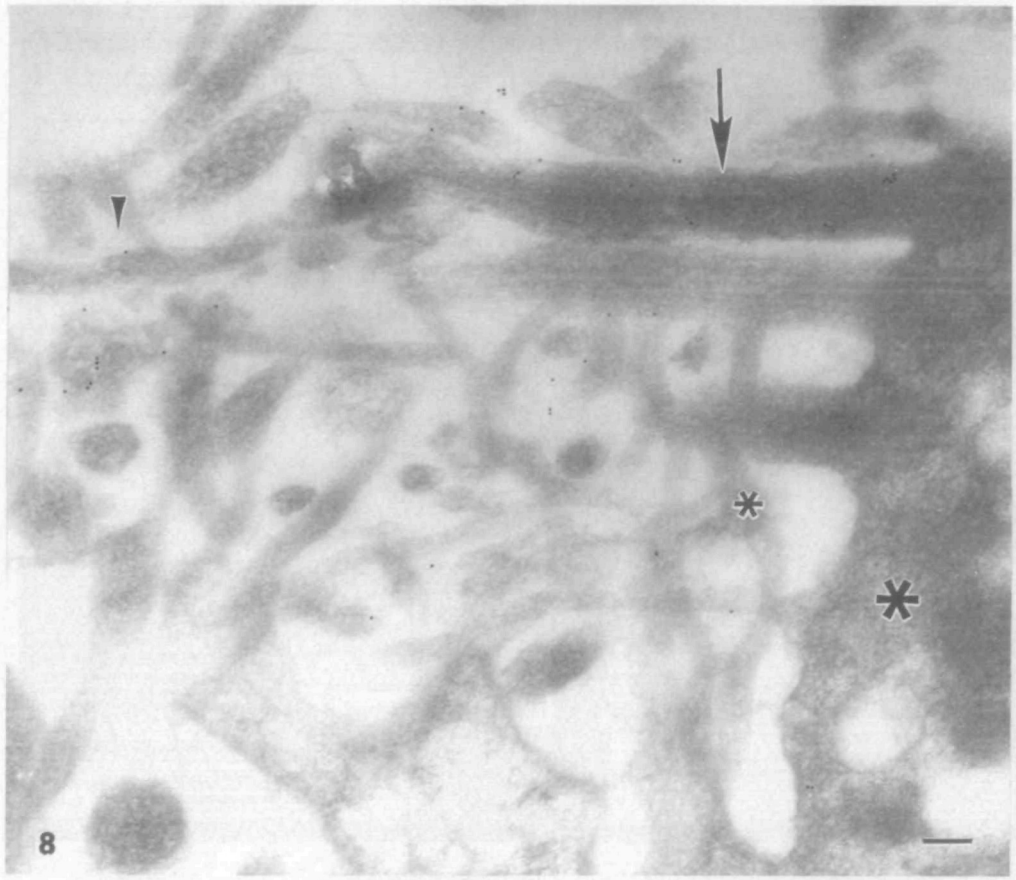


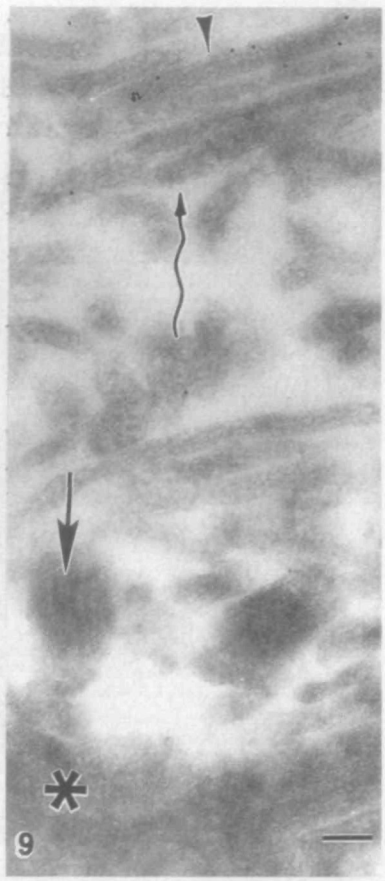
Figure 7 Summary diagram of the cytoarchitecture of the olfactory epithelial surface (modified after Menco, 1995a,b). Olfactory receptor cell dendrites end in dendritic knobs, which give rise to 10–30 olfactory cilia, with thick proximal parts (about 2 μm long) and much longer (about 50 μm) thin distal parts (Seifert, 1970; the # sign indicates that the cilia are much longer than indicated here). The arrays of spiraling membrane particles at the base of the cilia are the ciliary necklaces (Menco, 1988b; see Figure 5). Receptor cells are surrounded by supporting cells which have many microvilli that are oriented perpendicular to the course of the distal parts of the cilia. Dendritic knobs, cilia and microvilli are embedded in a mucous layer, which borders the nasal lumen. The distal parts of the olfactory cilia and the tips of the supporting cell microvilli line the interface between external odorous environment and organism. The diagram includes approximate densities of intramembrane particles per μm^2 membrane in olfactory cilia, supporting cell microvilli, and apices of these cells based on results of freeze–fracture studies (Menco, 1980a, 1983, 1984). Densities of intramembrane particles are approximately the same in the dendritic knobs, and in proximal and distal parts of the olfactory cilia. Compare this figure with Figure 14, which provides summary identifications of some ciliary components. Adapted from Figure 1 in Menco (1995b), with permission of Wiley-Liss, Inc. Scale bar, 1 μm.

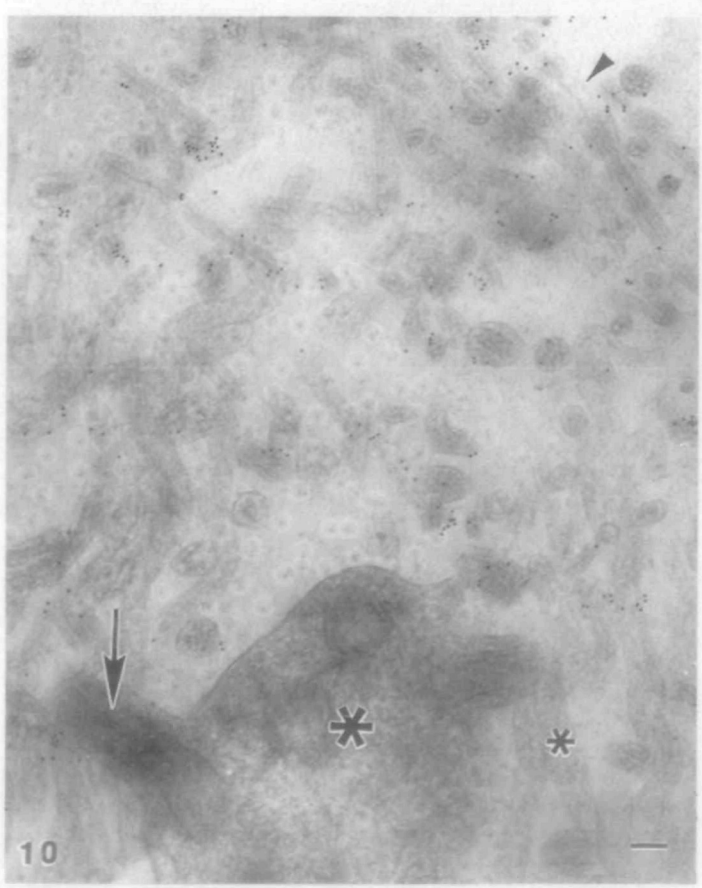
Olfactory cilia; localization of signal-transduction proteins

The label-etch technique was applied to olfactory structures. This technique makes use of cytochemical labeling followed by deep etching, which is readily combined with ultrarapid freezing and allows correlation between membrane particles and labeled surface components on contiguous areas of membrane. It was revealed that gold-conjugated wheat germ agglutinin (WGA) binds to membranes of dendritic knobs and cilia of rat olfactory receptor cells, but not to membranes of supporting cell structures (Figure 6; Menco, 1992c). These studies provide the first direct link between the chemical nature of membrane proteins of olfactory cilia and their topography. The further pursuit of such studies with antibodies to olfactory signaling proteins may make it possible to relate the shape of the particles to their functions.

Although ultimately topographically less precise than the label-etch technique, immunocytochemical studies employing light microscopy and thin-section transmission electron microscopy have yielded much information about the chemical nature of olfactory cilia. Such studies have demonstrated that olfactory cilia contain putative odor receptors (Figures 8 and 9; Koshimoto *et al.*, 1992, 1994; Krieger *et al.*, 1994; Menco *et al.*, 1997) as well as all important components of the subsequent transduction cascade that involves G_s type G-proteins. These proteins include $G_{s\alpha}$ (Figure 10; Mania-Farnell and Farbman, 1990), $G_{olf\alpha}$ (Figure 11; Jones and Reed, 1989), type III AC (Figure







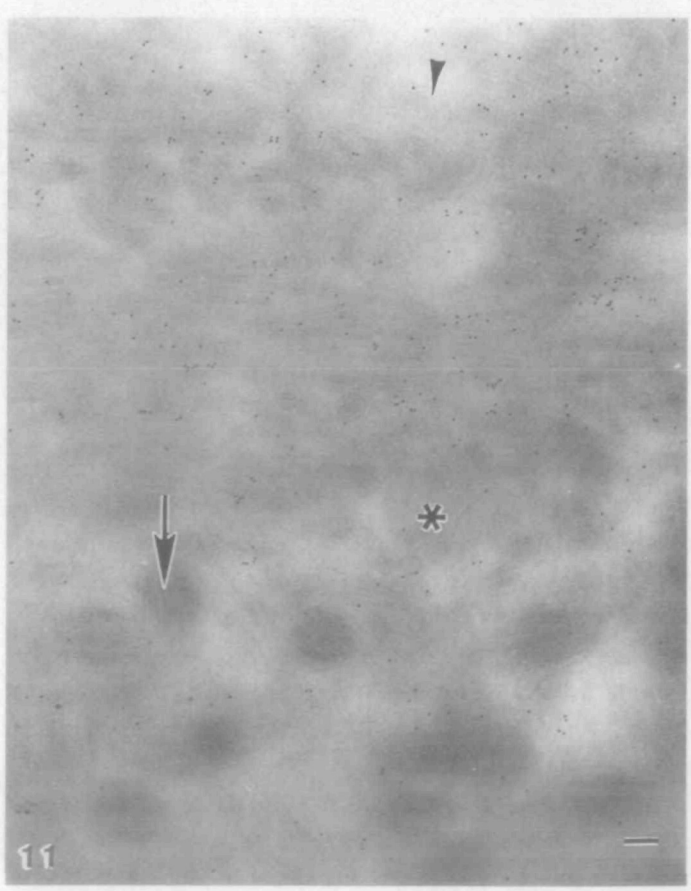


Figure 8 Proximal (arrow) and distal parts (arrowhead) of rat olfactory cilia immunolabeled with antibodies to rat putative odor receptor D3 (dilution: 1:20). Ciliary structures of the same cell labeled in three serial sections. Cilia of very few cells labeled with the antibody. The dendritic knob from which the cilia originate and microvilli of neighboring supporting cell microvilli (small asterisk) did not label. Background was negligible. Paraformaldehyde-fixed cryoprotected tissue was freeze-substituted and immunocytochemistry was applied postembedding on the sections. Goat-anti-rabbit IgG, conjugated to 10nm colloidal gold, was used here as the secondary probe and in the experiments shown in Figures 9–13 (Menco *et al.*, 1997). Scale bar, 0.1 μm.

Figure 9 Some distal olfactory cilium segments of the mouse labeled with antibodies to the M4 (dilution: 1:100) mouse putative odor receptor (arrowhead), while others, nearby, did not (snake-shaped arrow). Proximal cilium segments (arrow) and dendritic knob-structures (asterisk) of a nearby receptor cell also did not label. Experimental conditions are described in Figure 8 (Menco *et al.*, 1997). Scale bar, 0.1 μm.

Figure 10 Distal parts of mouse olfactory cilia (arrowhead) bound antibodies to $G_{s\alpha}$ (dilution: 1:100). Dendritic knobs (large asterisk), proximal cilium parts (arrow), dendritic knob structures (large asterisk) and supporting cell microvilli (small asterisk) did not label. The tissue was rapidly frozen without fixation (Menco *et al.*, 1992, 1994). Scale bar, 0.1 μm.

Figure 11 Distal parts of rat olfactory cilia (arrowhead) bound antibodies to $G_{olf\alpha}$ (dilution: 1:5). Dendritic knobs (large asterisk), proximal cilium parts (arrow) and supporting cell microvilli (small asterisk) did not label. The tissue was rapidly frozen without fixation (Menco *et al.*, 1992, 1994). Scale bar, 0.1μm.

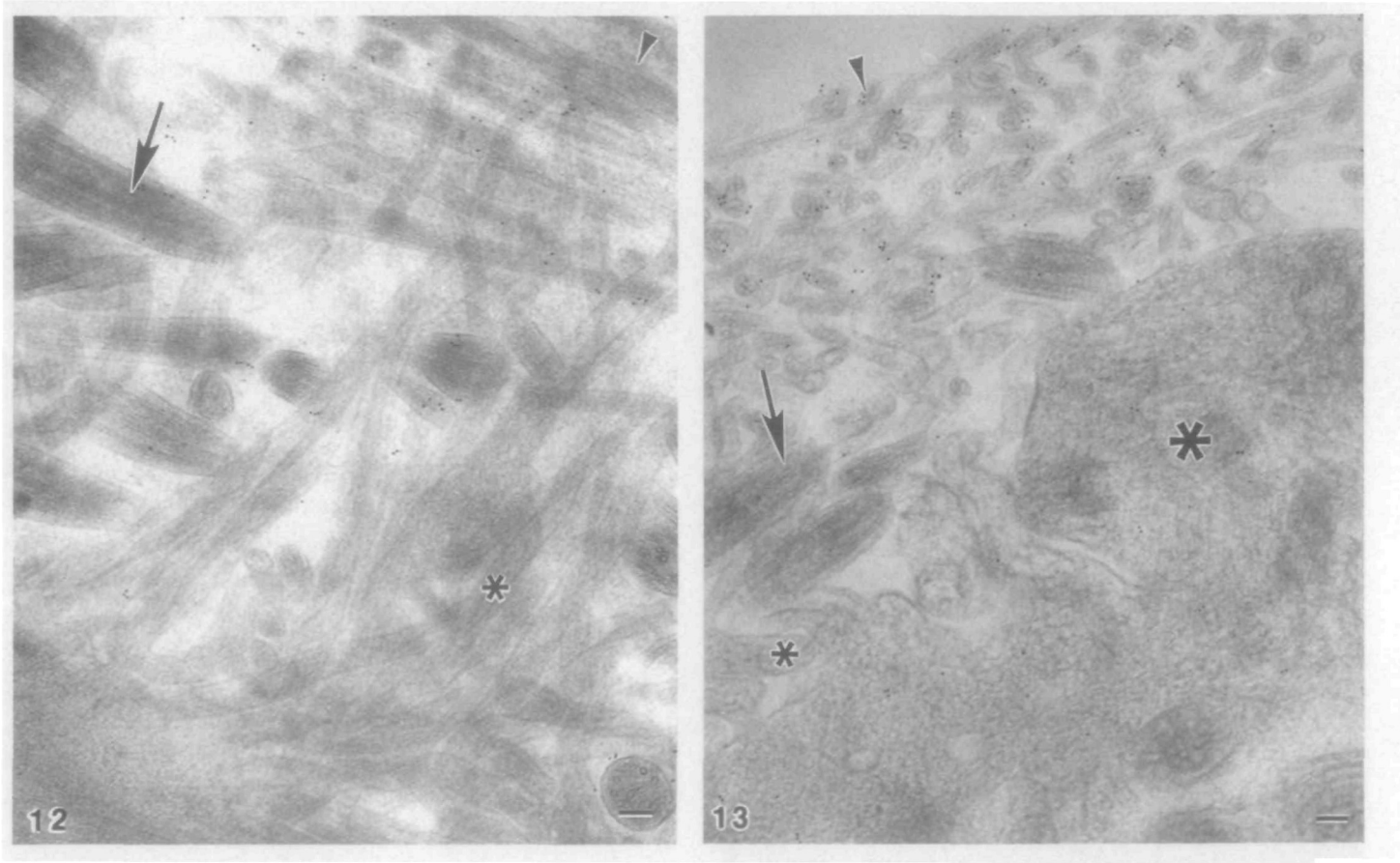


Figure 12 Distal parts of rat olfactory cilia (arrowhead) bound antibodies to type III AC (dilution: 1:8). Dendritic knobs (not shown), proximal cilium parts (arrow) and supporting cell microvilli (asterisk) did not label. The tissue was rapidly frozen without fixation (Menco *et al.*, 1992, 1994). Scale bar, 0.1 μm.

Figure 13 Distal parts of mouse olfactory cilia (arrowhead) bound antibodies to the α-subunits of CNG channels (dilution: 1:25). Dendritic knobs (large asterisk), proximal cilium parts (arrow) and supporting cell microvilli (small asterisk) showed hardly any gold grains. The tissue was rapidly frozen without fixation (see also Matsuzaki *et al.*, 1994; Menco *et al.*, 1995). Scale bar, 0.1 μm.

12; Bakalyar and Reed, 1990) and CNG channels (Figure 13; Matsuzaki *et al.*, 1994). Ultrastructural studies have shown that the majority of these proteins mostly localize in the distal segments of olfactory cilia (Figures 8–14, Table 1; Menco *et al.*, 1992, 1994, 1995; Menco, 1994). However, this is not always the case; antibodies to two putative odor

receptors in both rat and mouse bound equally well to proximal and distal parts of the cilia (Figures 8 and 9; Menco *et al.*, 1997). Also, unlike antibodies to the subsequently activated signaling proteins, which bound to the cilia of virtually all receptor cells, antibodies to the putative odor receptors bound only to cilia of very few receptor cells. When these cilia were labeled, they labeled amply, suggesting that they had many of the same putative receptor molecules. None of the antibodies labeled dendrites or cell somata at ultrastructural levels.

The pattern of ultrastructural labeling with antibodies to the signal-transduction proteins differs from that of antibodies to the still enigmatic (but see Buiakova *et al.*, 1996; Carr and Farbman, 1996) olfactory marker protein (OMP), as OMP antibodies label the cytoplasmic compartments throughout the receptor cells (Menco, 1989, 1994; Johnson *et al.*, 1993; Menco *et al.*, 1992, 1994).

Despite some controversy (Brunet *et al.*, 1996; Firestein, 1996; Smutzer *et al.*, 1997), there is evidence that alternative olfactory signaling routes in vertebrates may work through activation of different G-proteins, those of a PLC/IP_3 cascade (Bruch and Gold, 1990; Breer *et al.*, 1992). Labeling patterns of antibodies to the G protein $G_{q\alpha}$, conceivably involved in the aforementioned cascade (Mailleux *et al.*, 1992; DellaCorte *et al.*, 1996), and antibodies to IP_3 receptors, channel-type proteins possibly involved in the PLC/IP_3 system (Miyamoto *et al.*, 1992; Kalinoski *et al.*, 1993), resemble one another. Antibodies to both proteins bound to receptor cell cilia and supporting cell microvilli (Figure 14, Table 1; Cunningham *et al.*, 1993; Kalinoski *et al.*, 1993, 1994; Menco, 1994; DellaCorte *et al.*, 1996). However, patterns of labeling using antibodies to these proteins were less distinct than those with antibodies to proteins involved in the AC/cAMP cascade.

Several proteins that may be involved in the modulation and/or termination of olfactory signaling (Leinders-Zufall *et al.*, 1996; Ronnett and Payne, 1995), notably GC (Spreca and Rambotti, 1994) and cyclic 3′,5′-nucleotide PDE (Asanuma and Nomura, 1993), were also found in the cilia using ultrastructural techniques. Furthermore, ultrastructural cytochemical studies have demonstrated that the protein Na^+, K^+-ATPase is present in the olfactory receptor cells, including their cilia (Kern *et al.*, 1991; Menco, 1994). It has been suggested that this protein plays an important role in the restoration and maintenance of the cell's resting potential. The only Ca^{2+}-binding protein shown ultrastructurally to localize to olfactory receptor cells, including their cilia, is neurocalcin. This protein may have a role in olfactory signal-transduction (Iino *et al.*, 1995).

Ultrastructural studies have also shown that the mucus surrounding the receptor cell cilia is heterogeneous (Menco and Farbman, 1992; Getchell *et al.*, 1993). In the frog this mucus has at least four distinct domains (Menco and Farbman, 1992, and references therein). Analogous to what is true in insects (Steinbrecht *et al.*, 1995; Steinbrecht, 1996; see below), these mucus domains may reflect regions that contain different odor binding proteins (e.g. Krishna *et al.*, 1995).

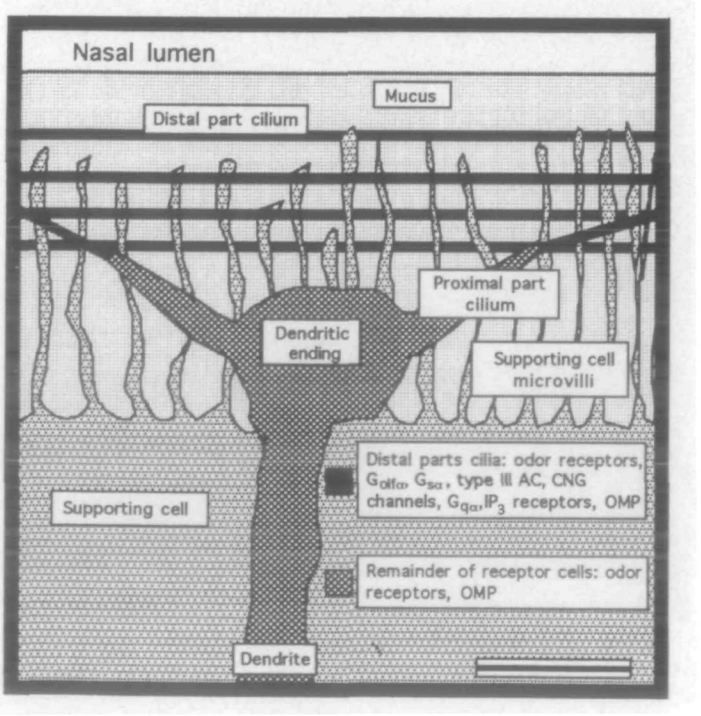


Figure 14 Diagram of the major sites of cytochemical labeling for antibodies to olfactory signal-transduction proteins. In addition to antibodies to the signal-transduction proteins, the cells also labeled with antibodies to the cytoplasmic OMP (Margolis, 1988). These antibodies immunoreacted with cytoplasmic compartments, also of knobs and dendrites (Menco, 1989), whereas antibodies to the signal-transduction proteins mainly labeled cilia (Menco *et al.*, 1992, 1994, 1995, 1997; Cunningham *et al.*, 1993; Matsuzaki *et al.*, 1994; Menco, 1994, 1995a; DellaCorte *et al.*, 1996). Scale bar, 1 μm.

Development of olfactory cilia; membrane ultrastructure

Developmental freeze–fracture studies (Menco, 1988a), combined with detailed scanning and thin-section transmission electron microscopic studies were performed in the rat (Menco and Farbman, 1985a,b, 1987). The results of

these studies imply that the onset of specificity of olfactory receptor cells as measured with electrophysiological means (Gesteland *et al.*, 1982) parallels those stages when the receptor cells become multiciliated, densities of ciliary intramembrane particles increase, and apices of olfactory supporting cells acquire characteristically shaped particles. Also, during development, olfactory cilia always have higher particle densities than respiratory cilia. The developmental data stress that the biochemical entities, represented as membrane particles in olfactory cilia and dendritic knobs, are likely to play major roles in olfactory signal-transduction (Menco, 1988a).

Development of olfactory epithelia; localization of signal-transduction proteins

Light microscopy and electron microscopy were used to immunolocalize α-subunits of G-proteins, especially of the stimulatory $G_{olf\alpha}$ and $G_{s\alpha}$, $G_{s\beta}$, and also type III AC and CNG channels, in developing rat olfactory epithelia (Figure 15; Menco and Farbman, 1985a,b; Mania-Farnell and Farbman, 1990; Menco *et al.*, 1994; the appearance of CNG channels was only studied in E19 and E22 fetuses; unpublished observations). Some olfactory cilia were immunoreactive with antibodies to $G_{s\alpha}$ and type III AC as early as E15 (E1 = sperm positive; E23 = P1 = day of birth), but immunolabeling with antibodies to $G_{olf\alpha}$ was not observed until E16. From then on numbers of receptor cells with immunopositive cilia increased for all three probes. Immunoreactivity for antibodies to the signaling proteins tends to parallel cilium development at the site of the dendritic knobs, though immunoreactivity to $G_{olf\alpha}$ lags somewhat behind. Newly formed cilia label along their lengths; mature cilia label predominantly along their long

Table 1 Binding of signaling protein and OMP antibodies to cilia and microvilli of rodent olfactory and nasal respiratory epithelia

Antibody probes[a,b]	Olfactory			Respiratory	
	Cilia receptor cell	Microvilli supporting cell	Microvilli microvillous cell[c]	Cilia ciliated cell	Microvilli ciliated cell
Anti-odor receptor + GAR IgG-gold[d]	+ fixed[e]	–	–	–	–
Anti-$G_{olf\alpha}$ + Pr G- or GAR IgG-gold[f]	+ distal	–	–	–	–
Anti-$G_{s\alpha}$ + Pr G- or GAR IgG-gold[g]	+ distal	–	–	–	–
Anti-type III AC + Pr G- or GAR IgG-gold[h]	+ distal	–	–	–	–
Anti-CNG channel GAR IgG-gold[i]	+ *distal*	–	–	–	–
Anti-$G_{o\alpha}$ + GAR IgG-gold[g,j]	–	– apex: +	–	–	–
Anti-$G_{q\alpha}$+ GAR IgG-gold[g,k]	± fixed	+ fixed	–	–	–
Anti-IP_3 receptor + GAR IgG-gold[l]	+ fixed	+ fixed	–	+ fixed	+ fixed
Anti-OMP (goat or rabbit) + Pr G- or GAR IgG-gold[m]	+	–	–	–	–

[a]Positive labeling = +; no discernible labeling = –; labeling hardly noticeable = ±; not determined = ? Respiratory structures served as tissue control.

[b]Protein G-gold (Pr G) or secondary goat-anti-rabbit IgG-gold (GAR IgG) was used for localization of binding sites of primary antibodies (Aurion, Electron Microscopy Sciences, Fort Washington, PA).

[c]Microvillous cells that occur rather sparsely in the olfactory epithelium and that differ from supporting cells (Carr *et al.*, 1991; Menco, 1992c).

[d]Antisera provided by Dr R. R. Reed (Howard Hughes Foundation, Johns Hopkins University, Baltimore, MD; Menco *et al.*, 1997).

[e]Fixed means chemically fixed and cryoprotected before freeze-substitution as opposed to freeze-substituted without fixation.

[f]Antiserum provided by Dr R. R. Reed (Jones and Reed, 1989; Menco *et al.*, 1992, 1994).

[g]Antisera to $G_{s\alpha}$, $G_{q\alpha}$ and $G_{o\alpha}$ supplied by NEN Research Products (Boston, MA; ## NE1-805, NEI-809 and NEI-804; Menco *et al.*, 1994).

[h]Antiserum provided by Dr R. R. Reed (Bakalyar and Reed, 1990; Asanuma and Nomura, 1991; Menco *et al.*, 1992, 1994).

[i]Antiserum provided by Dr G. V. Ronnett (Johns Hopkins University, Baltimore, MD; Dhallan and Reed, 1990; Matsuzaki *et al.*, 1994).

[j]See also Anholt *et al.* (1987) and Shinohara *et al.* (1992).

[k]See also Mailleux *et al.* (1992) and DellaCorte *et al.* (1996).

[l]Antiserum provided by Dr D. L. Kalinoski (Monell Chemical Senses Institute, Philadelphia, PA; Cunningham *et al.*, 1993; Kalinoski *et al.*, 1993, 1994; Menco *et al.*, 1993).

[m]Antiserum provided by Dr F. L. Margolis (University of Delaware, Baltimore, DE; Margolis, 1988; Menco, 1989; Johnson *et al.*, 1993).

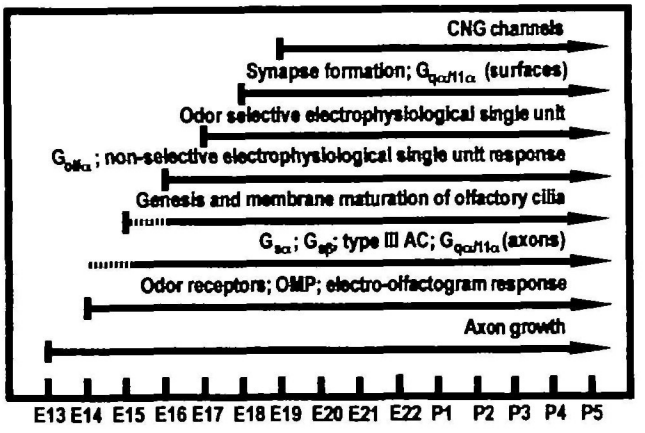


Figure 15 Time lines of expression of morphological, chemical and physiological features of the rat possibly important in the onset of olfactory functioning (modified after Menco, 1995a). Only earliest appearances reported are included (literature sources: Gesteland *et al.*, 1982; Menco and Farbman, 1985a,b; Menco, 1988a, 1995a; Mania-Farnell and Farbman, 1990; Farbman, 1992; Baker and Farbman, 1993; Margalit and Lancet, 1993; Dulac and Axel, as cited in Vassar *et al.*, 1994; Koshimoto *et al.*, 1994; Menco *et al.*, 1994; Strotmann *et al.*, 1995b; Sullivan *et al.*, 1995). Dashed: the onset or termination of the feature might have occurred somewhat earlier; arrows: the feature was retained into adulthood; short side bars: day that a feature appeared or was no longer seen.

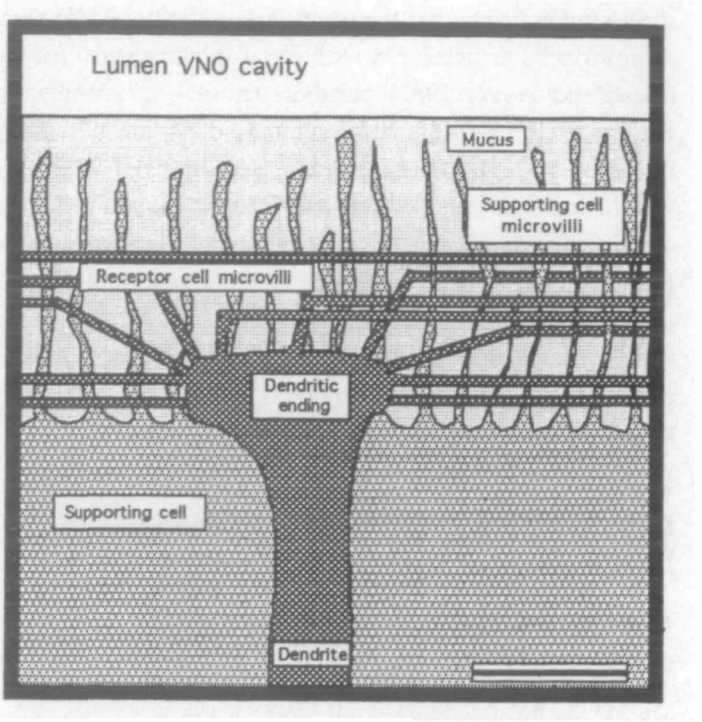


Figure 16 Diagram of the major structural components of the mammalian VNO olfactory epithelium. Microvilli rather than cilia are the odor receptive organelles. Whereas the olfactory cilia line the interface between mucus and nasal cavity (Figures 7 and 14), the VNO sensory microvilli are positioned much closer to the epithelial surface, while the tips of the supporting cell microvilli reach the VNO cavity. Scale bar, 1 μm

distal parts; dendritic knobs and ciliary necklaces showed little or no labeling. At E22 most multiciliated cells were immunopositive for $G_{s\alpha}$, $G_{olf\alpha}$ and type III AC. The data suggest that G_s is the predominant G protein in cilia of immature olfactory receptor cells, whereas G_{olf} is probably the predominant G protein in cilia of mature cells (Menco *et al.*, 1994; see also Margalit and Lancet, 1993). Antibodies to G_o, $G_{i1\ \&\ i2}$, and especially those to $G_{q\alpha}/G_{11\alpha}$, immunoreacted with various epithelial structures. The latter antibodies labeled receptor cell cilia and axons, primarily of the VNO nerve, and supporting cell microvilli (DellaCorte *et al.*, 1996). However, the patterns of labeling were not as clear as those with antibodies to $G_{s\alpha}$, $G_{olf\alpha}$, type III AC and CNG channels (Figures 10–13; Menco *et al.*, 1994, 1995).

Epithelial topography and signal transduction

In situ hybridization studies from various laboratories have shown that the rodent's olfactory epithelium has four distinct regions in which most putative odor receptors are located (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Strotmann *et al.*, 1994a,b; Buck, 1996; reviewed in Sullivan and Dryer, 1996). These studies also demonstrate that putative odor-receptor zones form around the time (Strotmann *et al.*, 1995; Sullivan *et al.*, 1995; Dulac and Axel, as cited in Vassar *et al.*, 1994; Breer *et al.*, 1996), or even before (Nef *et al.*, 1996) the complex turbinate structures form, the olfactory epithelial surface becomes recognizably olfactory, and differentiation sets in (Menco and Farbman, 1985a,b); that is, several days before synapse formation (Farbman, 1992; Sullivan *et al.*, 1995; Sullivan and Dryer, 1996). To determine whether morphological features accompany this biochemical patterning, olfactory epithelial surfaces of rat nasal endoturbinates and septa were examined with scanning electron microscopy. The results showed that a distinct topographic pattern emerges during development of the rat olfactory epithelium. The pattern involves both receptor and surrounding cells, and its topography roughly matches the zones seen by *in situ* hybridization (B.Ph.M. Menco and J.E. Jackson, unpublished data; Vassar *et al.*, 1993).

VNO and invertebrate olfactory sensilla; membrane ultrastructure

Whereas cilia are used as signal-capturing organelles in the

main olfactory organ of vertebrates, it is, rather, specialized microvilli that subserve this function in the VNO (Figure 16; Adams, 1992; Eishten, 1992; Farbman 1992; Liman, 1996). These microvilli are found much closer to the epithelial surface and deeper inside the mucus layer than olfactory cilia (Vaccarezza *et al.* 1981; Yoshida *et al.*, 1995; Ichikawa, 1996; B.Ph.M. Menco, unpublished observations; compare Figure 16 with Figures 7 and 14), a distinction to be considered when thinking about the specific sensory functioning of either system.

Freeze–fracture studies of rodent sensory microvilli of the VNO (Breipohl *et al.*, 1982) and also of insect olfactory cilia, especially cilium regions underneath the cuticular pores of the sensilla (Steinbrecht, 1980; Menco and Van der Wolk, 1982; Menco, 1992b), have shown that particle densities are within the range of those of vertebrate olfactory cilia. From these data, we can infer a presence of similarly high densities of signaling proteins in the odor capturing structures of VNO and insect olfactory chemosensory systems.

VNO and invertebrate olfactory sensilla; immunocytochemistry

Light microscopic studies using *in situ* hybridization and immunocytochemistry have demonstrated that the microvilli of the VNO use different heptahelical putative odor receptors (Dulac and Axel, 1995) and signaling proteins than the cilia of the main olfactory organ (Halpern *et al.*, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996). The data suggested that VNO sensory microvilli utilize $G_{o\alpha}$, $G_{i\alpha 2}$, and type II AC for signaling (Halpern *et al.*, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996), rather than the $G_{olf\alpha}$, $G_{s\alpha}$ and type III AC used by the cilia of the main olfactory organ. Preliminary ultrastructural cytochemical studies have demonstrated that differences between the main olfactory organ and the VNO extend to the supporting cells of each of these olfactory systems underscoring the different chemosensory functions of the main olfactory organ and the VNO (Yoshida *et al.*, 1995; Menco, 1997; see also Krishna *et al.*, 1995). Whereas the main olfactory organ serves primarily as a general odor-receptor organ, the VNO probably serves more for kin and conspecies recognition (Stoddart, 1990; Farbman, 1992).

Despite the above differences, apical structures of main and VNO olfactory receptor cells also share cytochemical properties of entities conceivably involved at some level in signal transduction. Notably, antibodies to the cytoplasmic proteins OMP and neurocalcin bind to cytoplasmic areas of main and VNO olfactory receptor cells, including their dendritic knobs. This has been discerned using both light microscopy and ultrastructural techniques (Johnson *et al.*, 1993). However, there are some quantitative differences; OMP antigenicity is much more intense in the main system than in the VNO olfactory system (Shnayder *et al.*, 1993; unpublished observations).

Olfactory systems in insects have differences analogous to those between the main olfactory organ and the VNO in vertebrates. Ultrastructural studies of insect olfactory systems have provided proof that sensilla used for capture of pheromones differ, both morphologically and cytochemically, from sensilla used for capture of general odorants (Steinbrecht *et al.*, 1995). There are also subtle differences regarding the localization of pheromone binding protein among various moth species (Steinbrecht, 1996).

Conclusion

The distal parts of olfactory cilia play a special role in olfactory signal-transduction. The morphological specialization in proximal short and thick, and distal long and thin parts of vertebrate olfactory cilia corresponds to a functional one; the latter structures—those regions that contact odors first—label especially well with most of the antibodies to signal-transduction proteins (the exception being antibodies to putative odor receptors which label both regions equally well). The dendritic knobs showed virtually no immunolabeling with antibodies to any of the signaling proteins under consideration. Specialization of olfactory cilia takes place during development, when the signal-transduction proteins are first expressed in both proximal and distal parts of the cilia; in mature cells these proteins are mainly present in the distal parts of the cilia. The exact identity of the chemical entities represented by most membrane particles of receptor cell dendritic knobs and cilia visualized with freeze-fracturing and -etching is as yet unresolved. But it is likely that many of these particles reflect the transmembrane signaling proteins considered here, such as odor receptors (Menco *et al.*, 1997), AC (Menco, 1992c; Menco *et al.*, 1992), CNG channels (Matsuzaki *et al.*, 1994; Menco *et al.*, 1995) and possibly

also IP_3 receptors (Cunningham *et al.*, 1993; Kalinoski *et al.*, 1994). The accumulated evidence from the freeze–fracture, freeze–etch, and ultrastructural (immuno)-cytochemical studies together with other, especially electrophysiological (Gold and Nakamura, 1987; Kurahashi and Kaneko, 1991; Kleene *et al.*, 1994; Trotier and Døving, 1996), provides compelling evidence that olfactory cilia possess all properties necessary to transform odor–receptor interaction into an electrical signal. Thus, olfactory cilia are highly specialized organelles that resemble in many respects the modified cilia that form vertebrate retinal photoreceptor outer segments (Menco, 1992b, 1994; Menco *et al.*, 1992).

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