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The Peripheral Olfactory Organ of the Zebrafish, *Danio rerio*: an Ultrastructural Study

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Abstract

The peripheral olfactory organ of the adult zebrafish, *Danio rerio*, was investigated by light as well as scanning and transmission electron microscopy. The olfactory organ consists of several lamellae that insert into a midline raphe, thus forming an oval-shaped rosette. Sensory and nonsensory regions are located separately on each lamella. The olfactory epithelium contains three types of receptor cells: two as described classically for other fishes, bearing either cilia or microvilli, the third being a new sensory cell type—the crypt cell. The crypt cell has no olfactory knob but bears microvilli as well as submerged cilia. Its axon travels together with the axons of the other receptor cells towards the basal lamina. Whereas the classical receptor cells are separated by supporting cells with small protrusions on their apical surfaces, the crypt cell is always surrounded by one or two specialized electron-lucent supporting cells which also bear microvillous-like apices. The nonsensory areas contain the goblet cells, ciliated nonsensory cells and epidermal cells with microridges.

Introduction

The olfactory system mediates responses to a multitude of different stimuli important for the interaction of an organism with its surrounding world as well as with conspecifics. Receptor cells equipped with odorant receptors (Buck and Axel, 1991; Ngai *et al.*, 1993) detect the stimuli and relay the information to the olfactory bulb. All teleosts hitherto examined have shown two morphologically distinct receptor cell types: ciliated receptor cells and microvillous receptor cells. A differential pattern of distribution within the epithelium has been reported for these two morphological distinct receptor cells for char (Thommesen, 1983) and catfish (Erickson and Caprio, 1984), and has been found in goldfish (A. Hansen, unpublished data). Other proposed receptor cell types have been suggested for certain species of fish (Bannister, 1965; Schulte, 1972; Breipohl *et al.*, 1973; Muller and Marc, 1984). Some of these cells remained unaffected by olfactory nerve axotomy (Ichikawa and Ueda, 1977; for review see Zeiske *et al.*, 1992). Recent studies have described the aspects of the developmental morphology (Hansen and Zeiske, 1993) and receptor expression (Barth *et al.*, 1996; Byrd *et al.*, 1996) of the olfactory organ of the zebrafish, *Danio rerio*, which is an important model in fish embryology. Studies on the adult zebrafish, however, are few and deal mainly with questions of the olfactory bulb (Baier and Korsching, 1994; Baier *et al.*, 1994; Byrd and Brunjes, 1995). The present study was undertaken to describe the morphology and ultrastructure of the olfactory epithelium of the adult zebrafish.

Materials and methods

Adult zebrafish (n = 12) were obtained from our own colony. Their ages ranged from 6 months to 2.5 years (total body length 2.8–3.5 cm). The fish were kept in aereated 100 l glass tanks at 26°C on a 14 h light/10 h dark cycle. All animals were killed with an overdose of 3-aminobenzoic acid ethyl ester (MS 222, Sigma Chemical Co.).

Light and transmission electron microscopy (TEM)

The heads of the fish were fixed by immersion in 5% glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) for several hours or overnight. After rinsing in phosphate buffer, the animals were postfixed with 1% osmium tetroxide for 2 h. The fixed specimens were dehydrated in a graded series of ethanol and propylene oxide and embedded in glycid ether 100 (Serva). Semithin sections of 1 μm were stained with toluidine blue and examined by light microscopy. Ultrathin sections (silver to gold) were stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.To estimate the density of the receptor cells, horizontal ultrathin sections of the apical portion of the epithelium of different fish and different lamellae were used (see Figure 9). Sectors of 21 × 28 μm of the tissue (a size that happened to be convenient) were

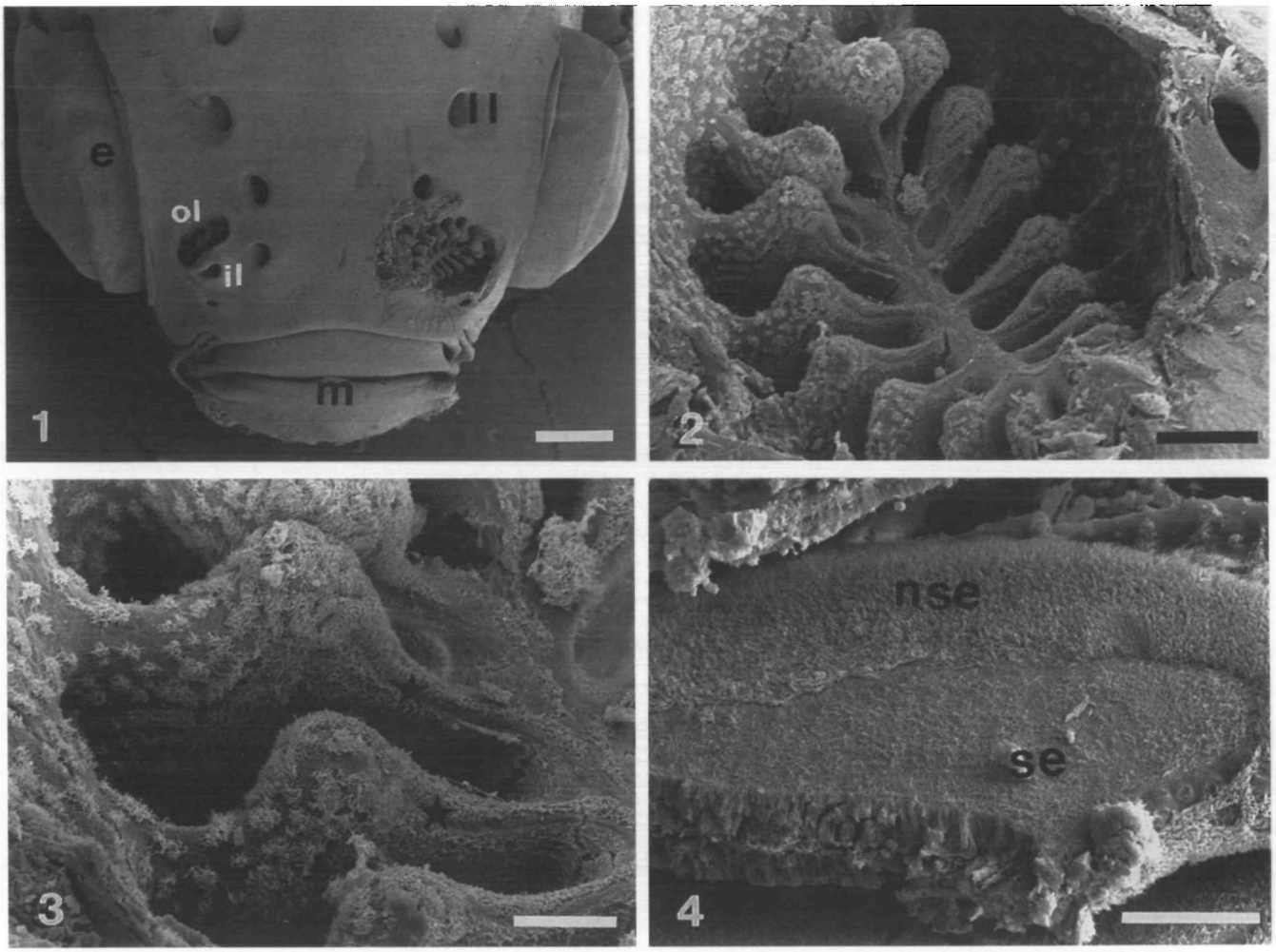


Figure 1 Head of an adult zebrafish. The right side shows the openings of inlet (il) and outlet (ol) of the nasal cavity. On the left side the skin was removed to demonstrate the olfactory organ proper. e, eye; ll, cephalic lateral line system; m, mouth. SEM. Scale bar = 500 μm.

Figure 2 The olfactory rosette is located in the nasal cavity. Lamellae insert in the midline raphe. The smallest lamellae are the youngest. Rostral is to the right, caudal to the left side. SEM. Scale bar =100 μm.

Figure 3 Higher magnification of the lamellae. The sensory epithelium is located on the sides of the lamellae and not visible in this figure. The nonsensory epithelium builds a channel-like system on the upper rim of the lamellae (asterisk). The distal rims and the nasal cavity proper bear tufts of ciliated nonsensory cells. SEM. Scale bar = 5 μm.

Figure 4 On the lamella sensory (se) and nonsensory (nse) areas are strictly separated. The kinocilia of the nonsensory cells are longer than the cilia of the receptor cells. SEM. Scale bar = 50 μm.

photographed at the same magnification to count the dendrites.

Scanning electron microscopy

The heads of the fish were fixed by immersion in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). After rinsing in sodium phosphate buffer, the specimens were dehydrated in a graded series of acetone and isoamylacetate and critical-point-dried in CO_2. Most of the dried olfactory organs were dissected from the head to examine single lamellae and fractions. The specimens were coated with gold and examined with a CamScan DV4 electron microscope. All length values were directly measured from the electron microscope.

Results

General morphology of the olfactory organ

The nostrils of the paired olfactory organ lie on the dorsal side of the head of the zebrafish close to the eyes and the mouth (Figure 1). Water conveying odorants enters the nasal cavity through a funnel-shaped inlet and exits through a posterior outlet. The peripheral organ proper has

the shape of a bilaterally symmetrical rosette (Figure 2). The size of the rosette increases as the fish grows. Thus, the length of an adult organ may vary between 350 and 600 μm, its width between 250 and 350 μm. The center of the rosette is the so-called midline raphe from which the lamellae project outward. The oldest animals examined (2–2.5 years; this period usually corresponds to the life span of a zebrafish) had 19–21 lamellae and smaller animals 12–15 lamellae. The oldest and largest lamellae are arranged radially at the caudal end of the organ, young lamellae being built rostrally at both sides of the midline raphe.

The surface of the midline raphe is covered by nonsensory epithelium. Some of these cells show microridges that are typical for fish epidermis, other cells bear kinocilia. Rows of the ciliated nonsensory cells are arranged in a channel-like system that extends to the upper rims of the lamellae (Figure 3). The nasal cavity is also lined with epidermal cells possessing microridges and ciliated nonsensory cells that are grouped in little tufts (Figure 3). In general, the sensory and the nonsensory epithelium on one lamella are strictly separated (Figure 4), but exceptions are possible. The lamellae of the olfactory rosette contain the sensory epithelium. Each lamella has a rim covered with ciliated nonsensory epithelium (Figure 5) where it inserts into the nasal cavity. The medial part of each side of the lamella bears the olfactory sensory epithelium (Figures 6 and 7). It is arranged continuously and also covers the valleys between two lamellae at the midline raphe. At the interfaces of sensory and nonsensory areas, small groups of receptor cells or even single receptor cells may occur within the nonsensory area. No secondary folds exist on the lamellae of the zebrafish. Goblet cells exist only in the nonsensory areas. Occasionally free nerve endings that have penetrated the basal lamina are found in the olfactory epithelium (Figure 8).

The olfactory epithelium

The thickness of the sensory epithelium varies from ~15 μm in the valleys at the midline raphe to ~20 μm on the lamella. The epithelium consists of three ultrastructurally distinct types of olfactory receptor cells, supporting cells and the small, roundish basal cells. A differential pattern of distribution within the epithelium for these three sensory cell types was not obvious. All cells are closely packed. The apical junctional complexes connecting the epithelial cells are not distinct and a terminal web is only faintly developed. In the lower portion of the epithelium some cells have desmosomes or gap junctions between pairs of supporting cells, between pairs of basal cells, and sometimes between supporting and basal cells. The pseudostratification, typical for olfactory epithelia in other species (Zeiske *et al.*, 1992), is inconspicuous. Receptor cell nuclei and supporting cell nuclei do not form clearly distinguishable layers. Thus, the receptor cell nuclei can lie above or below the supporting cell nuclei. Very often, the long, slender dendrites bear cilia (Figure 6) and the short, thick dendrites bear microvilli. The soma as well as the nuclei of these microvillous receptor cells generally are less basophilic than those of the neurons with the long dendrites bearing cilia. The supporting cells separate the dendrites of the receptor cells. Usually, one supporting cell envelopes several receptor cell dendrites (Figure 9). Based on countings of dendrites in horizontal sections, we estimated ~250 000 dendrites per mm^2 of sensory epithelium. Although the apical dendrites are separated by supporting cells, the perikarya of the receptor cells often lie adjacent to each other. Basally, the olfactory epithelium is lined with a basal lamina (Figure 10) that separates it from the lamina propria. The lamina propria between the two epithelia of the lamella is only a thin layer filled with connective tissue, capillaries and fat cells, and occasionally pigment cells, macrophages and granulocytes. Axons of the receptor cells accumulate within the sensory epithelium and penetrate the basal lamina in bundles. In the lamina propria, these bundles form the fila olfactoria (Figure 11), which in turn aggregate and leave the olfactory organ. Beneath the organ, the fila olfactoria are braided into a cord, the olfactory nerve, that passes through a single opening in the ethmoidal bone and travels to the olfactory bulb, which is not pedunculated as in other cyprinids. The olfactory axons (0.12–0.15 μm in diameter) contain neurofilaments, neurotubules and some mitochondria. The number of neurofilaments and neurotubules varies. The fila olfactoria as well as the olfactory nerve are accompanied by Schwann cells and surrounded by a faint and often hardly visible basal lamina.

Ciliated and microvillous receptor cells

The bipolar receptor cells are primary sensory cells that reach from the lumen of the olfactory cavity to the basal lamina of the epithelium, where their axons enter the lamina propria. The cells are often more electron-dense than the neighboring supporting cells. The receptor cells are rich in polyribosomes, rER and mitochondria. Mitochondria are elongate, particularly within the dendrites. Microtubules are arranged longitudinally. The nuclei are often lobed, and their chromatin shows the typical 'checkerboard' pattern of olfactory receptor nuclei. Ciliated receptor cells have a pronounced olfactory knob, projecting into the lumen of the olfactory cavity. The olfactory knob bears 3–7 cilia. Each cilium has a basal body and a basal foot, but no rootlet. The basal foot contains a small central vesicle. The microtubules of the cilium are arranged in the 9 + 2 pattern. Dynein arms are lacking. The cilium ends in a slender distal lash which is supported by only a few microtubules. The cilia are ~0.25 μm in diameter and ~2–3 μm long as measured in SEM pictures, not accounting for likely shrinkage, especially of the distal lash.

The olfactory knobs of the microvillous receptor cells are less pronounced than those of the ciliated receptor cells and often contain small electron-lucent vesicles. They bear 10 to 30 short microvilli (0.5–0.8 μm, measured in TEM photos)

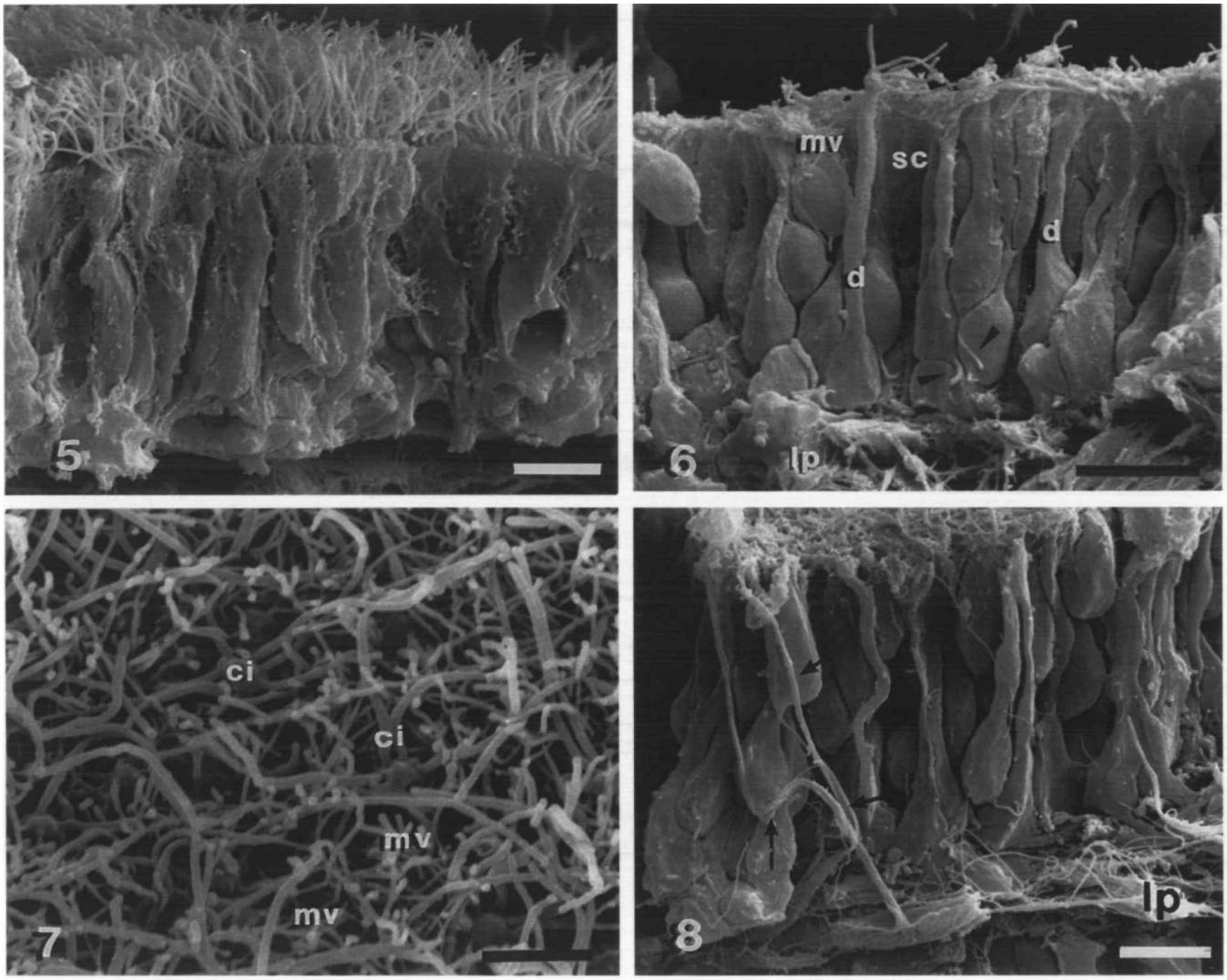


Figure 5 Fraction of the nonsensory epithelium showing ciliated nonsensory cells. The cells are cylindrical and each cell bears numerous kinocilia. SEM. Scale bar = 5 μm.

Figure 6 Fraction of the sensory epithelium. The receptor cells are closely packed. Some have very long dendrites (d) with cilia on their olfactory knobs. Other cells are roundish with a short dendrite. These cells often bear microvilli (mv). The supporting cells (sc) are more or less cylindrical. Arrowheads, axons; lp, lamina propria. SEM. Scale bar = 5 μm.

Figure 7 The surface of the sensory epithelium shows olfactory knobs with receptor cilia or microvilli that protrude from less pronounced knobs. ci, olfactory cilia of ciliated receptor cells; mv, microvilli of microvillous receptor cells. SEM. Scale bar = 2 μm.

Figure 8 Fraction of the sensory epithelium showing free nerve endings (arrows) that leave the lamina propria (lp) and reach to the apical portion of the sensory epithelium. SEM. Scale bar = 5 μm.

which sometimes branch. The upper part of the dendrite just beneath the olfactory knob often contains several centrioles.

Crypt cells

In addition to the two classical receptor cell types decribed above, a cell type is present in the sensory area of the olfactory epithelium of the zebrafish which we call crypt cell. The cell bodies of these cells are egg-shaped and appear in the upper quarter of the epithelium of all zebrafishes examined. Although crypt cells occur regularly in all lamellae, the absolute number of this cell type is low. The most striking characteristic of this cell is the fact that it bears cilia as well as microvilli. The cilia (up to seven per cell) are submerged in the upper portion of the cell body (Figure 13), and the microvilli are located around the cilia on the apical rim of the cell (Figure 14). These cilia show the

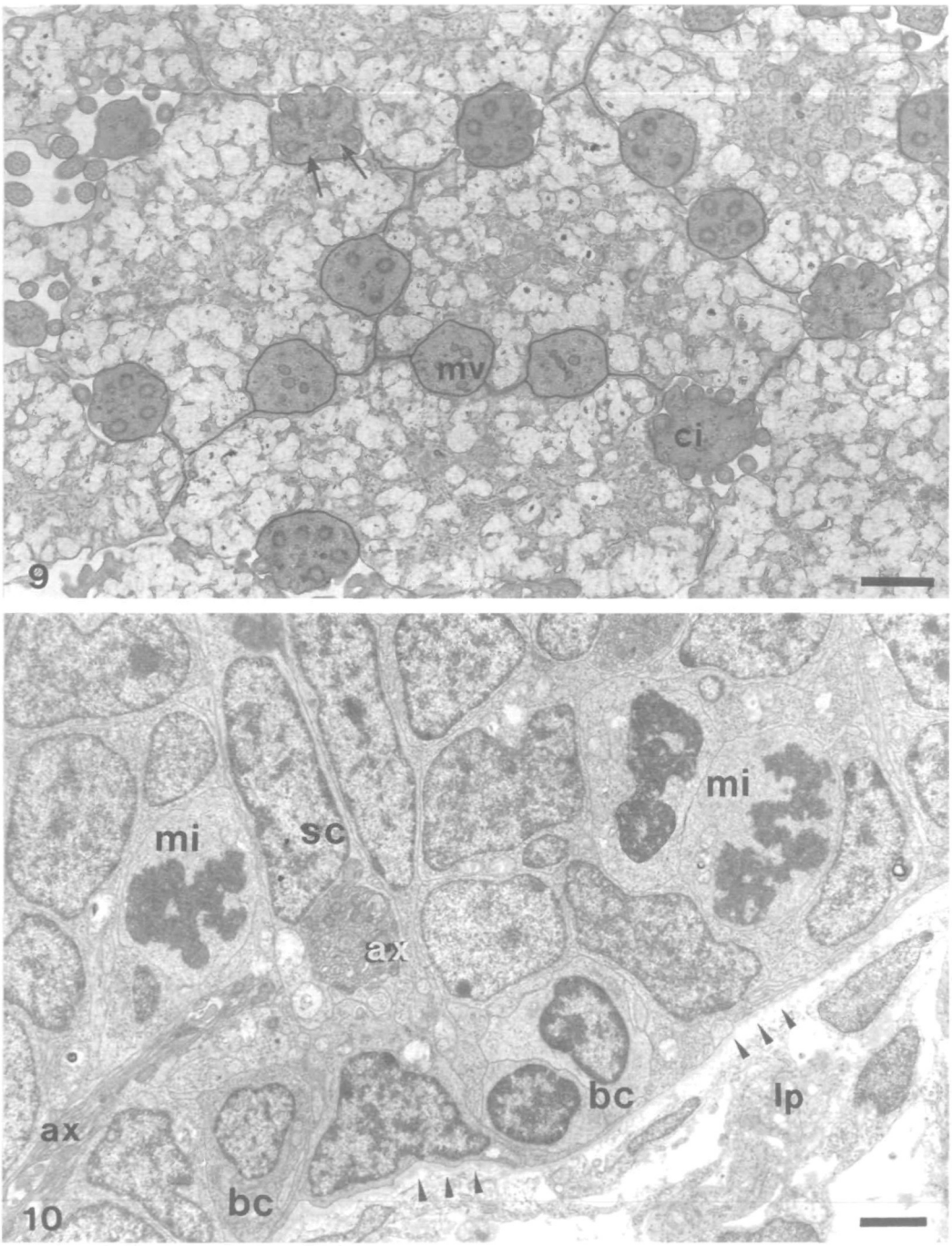
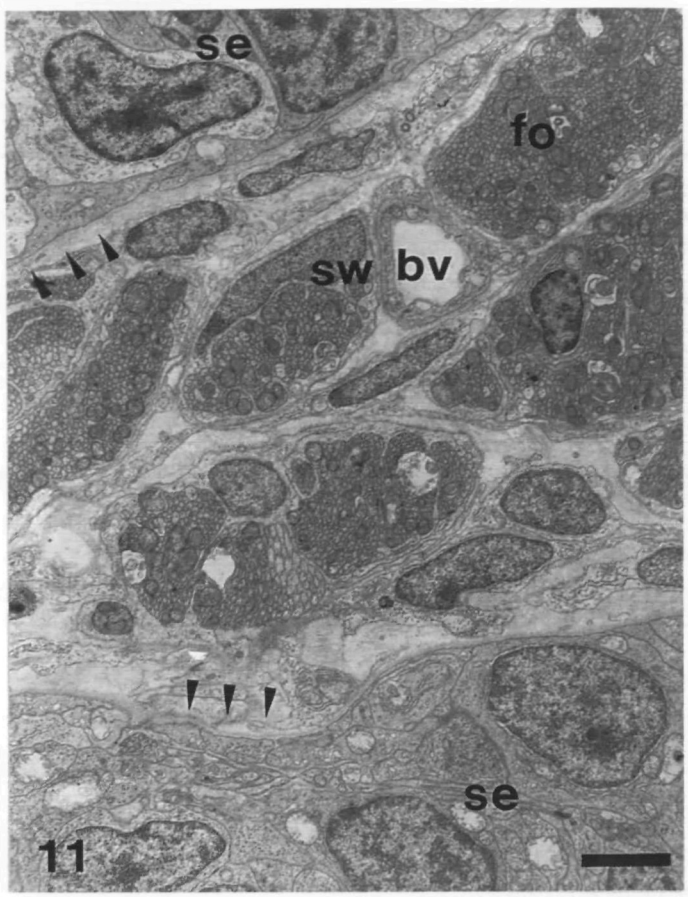


Figure 9 Horizontal section of the sensory area of the olfactory epithelium. The knobs of microvillous (mv) and ciliated (ci) receptor cells are surrounded by supporting cells. The apical ends of the supporting cells are filled with electron-lucent vesicles. The knobs of ciliated receptor cells show the basal bodies of the cilia. The basal bodies have one basal foot pointing to the center of the knob (arrow). In microvillous receptor cells, mitochondria reach into the knobs. TEM. Scale bar = 1 μm.

Figure 10 Basal cells (bc) close to the basal lamina (arrowheads). Mitotic figures (mi) are visible in the basal cell layer. ax, axons; lp, lamina propria; sc, supporting cell. TEM. Scale bar = 2 μm.

typical 9 + 2 patterns of microtubules (Figure 15). The nucleus of a crypt cell fills about one-third of the cell. Its upper part is often flattened. The cytoplasm of this cell is electron-dense: mitochondria are large and free ribosomes abundant. Serial sections reveal that this cell type has an axon (Figure 16) that aggregates with the axons of other receptor cells near the basal lamina. The crypt cells are surrounded by one or two specialized supporting cells which



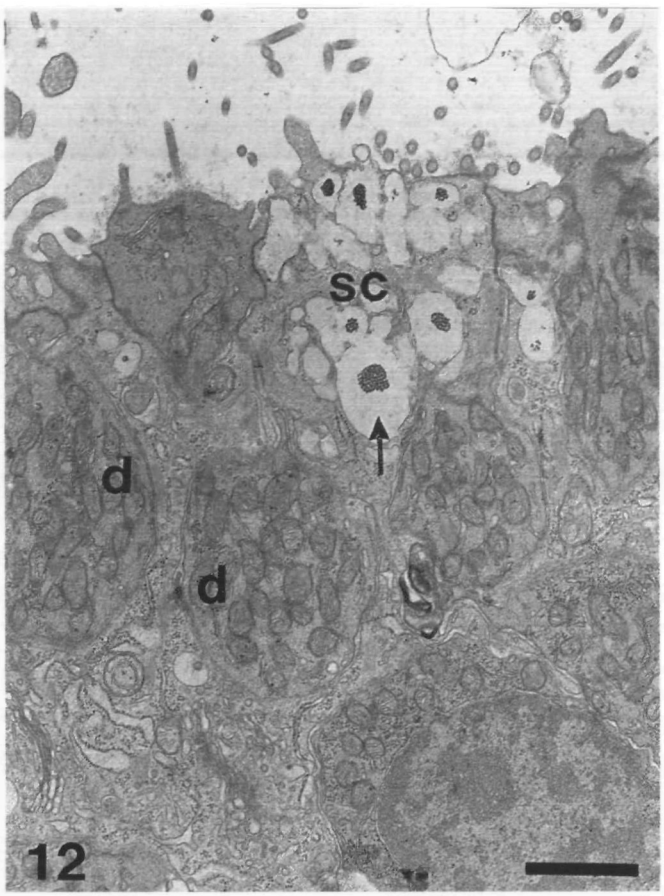


Figure 11 The lamina propria between the two sensory epithelia (se) of a lamella are filled with fila olfactoria (fo) and Schwann cells (sw), blood vessels (bv) and fibrocytes. Arrowheads, basal lamina. TEM. Scale bar = 2 μm.

Figure 12 Apical portion of the sensory area of the olfactory epithelium. Electron-lucent vesicles of a supporting cell (sc) contain dark bead-like inclusions (arrow). d, dendrite. TEM. Scale bar = 1 μm.

are particularly electron-lucent and bear microvilli-like apices (Figure 14).

Supporting cells

Supporting cells are more or less cylindrical (Figure 6). Their oval nuclei are more electron-lucent than those of the receptor cells. Bundles of longitudinally oriented intermediate filaments are characteristic. Mitochondria are round. The apical surface of the supporting cell has small irregular protrusions that reach into the lumen. Two types of vesicles can be distinguished in the distal portion of the cell: vesicles with a homogeneous fine-grained matrix (Figure 9) or electron-lucent vesicles with small, dark, bead-like inclusions (Figure 12). Neighboring supporting cells often show the same type of vesicles.

Basal cells

The small, polyhedral basal cells lie between the basal parts of the supporting cells and the axons of the receptor cells adjacent to the basal lamina. Their large nuclei are oval or round. The number of basal cells is not high, but more basal cells seem to occur in the valleys close to the midline raphe and at the margins of the sensory area than in the medial portion of the sensory epithelium. Sometimes mitotic figures are recognizable (Figure 10).

Ciliated nonsensory cells

Ciliated nonsensory cells surround the olfactory sensory area on the lamella (Figure 5) and build clusters in the olfactory cavity (Figure 3). They are cylindrical and extend from the basal lamina to the epithelial surface. Their electron density is similar to that of the supporting cells. Their electron-lucent nuclei are of various shapes and sometimes lobed. The flat cell apex is broad and gives rise to plenty of kinocilia (up to 60 kinocilia have been counted in one cell). Each kinocilium has a rootlet of 3–4 μm that is inserted diagonally in the basal body of the kinocilium. The 9 + 2 pattern of the axoneme is more clearly visible than in

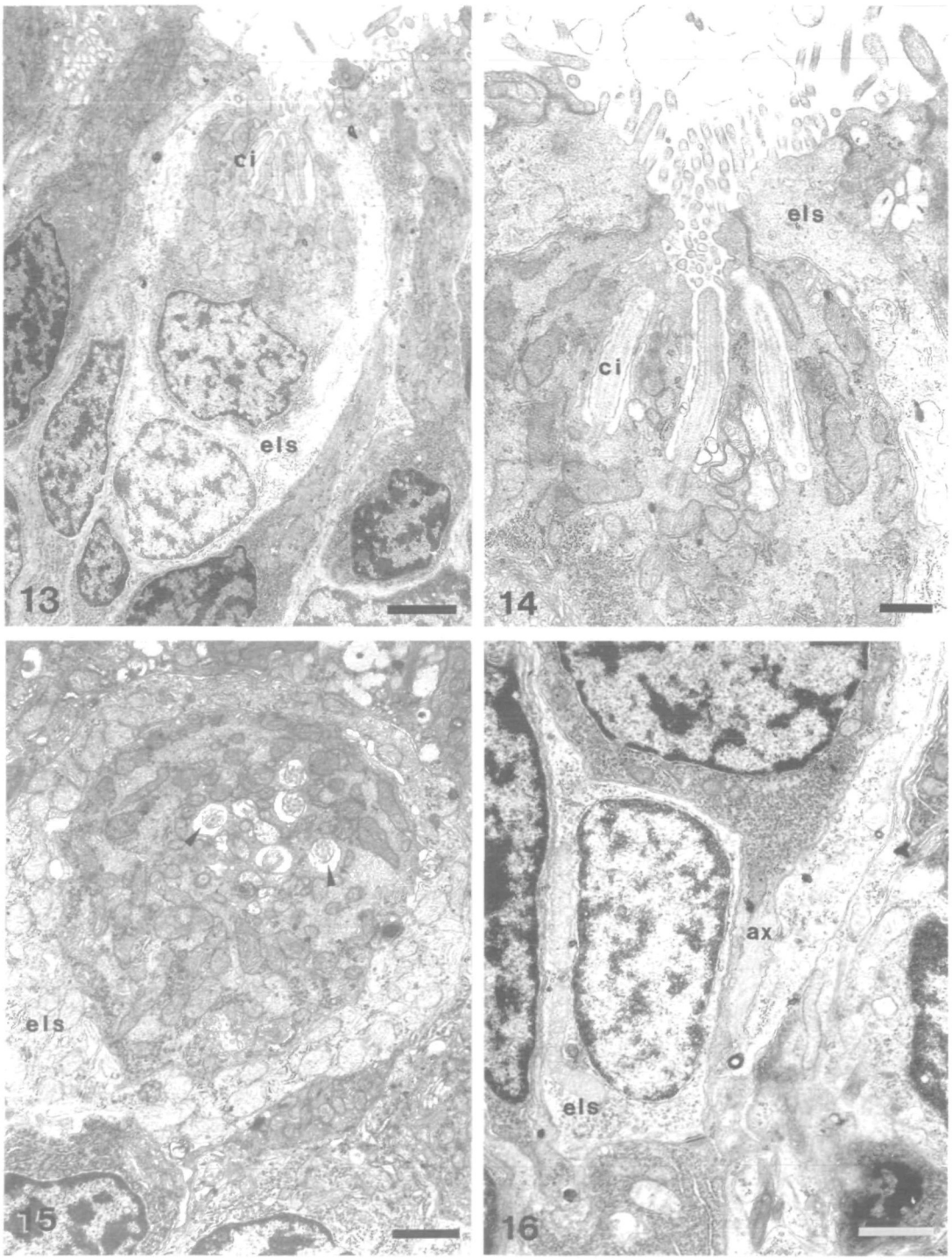


Figure 13 Crypt cell with in-sunk cilia (ci). The nucleus lies in the lower portion of the cell. An electron-lucent supporting cell (els) surrounds the crypt cell. TEM. Scale bar = 2 μm.

Figure 14 Higher magnification of the upper portion of a crypt cell. Large mitochondria are located around the in-sunk cilia (ci). The crypt cell as well as the electron-lucent supporting cell (els) have microvilli-like apices. TEM. Scale bar = 0.5 μm.

Figure 15 Horizontal section of a crypt cell showing the cilia (arrows) in their membranous bags. The crypt cell is surrounded by electron-lucent supporting cells (els). TEM. Scale bar = 1 μm.

Figure 16 Higher magnification of the lower portion of a crypt cell. Abundant free ribosomes are visible below the nucleus. Electron-lucent supporting cells (els) surround the crypt cell and its axon (ax). TEM. Scale bar = 1 μm.

receptor cilia, and the two microtubules in the center of the axoneme of one cell or even of several adjacent cells are oriented in the same direction (Figure 17). The kinocilia are 7–8 μm long and 0.25 μm in diameter.

Goblet cells and rodlet cells

Goblet cells are restricted to the nonsensory areas. They are surrounded by ciliated nonsensory cells or epidermal cells bearing microridges. Goblet cells are oval in shape. Their nuclei lie basally. About two-thirds of the goblet cell is filled with large granules. Mature goblet cells secrete these granules into the lumen of the olfactory cavity (Figure 18). The epithelia of several of the zebrafish examined contained rodlet cells. These cells have a thick cuticula-like wall and are filled with typical rodlets as well as electron-lucent vesicles (Figure 18).

Discussion

The gross morphology of the olfactory organ of *D. rerio* corresponds to the pattern described for all cyprinids described to date (Holl, 1965; Yamamoto and Ueda, 1978). However, in addition to the ciliated and the microvillous receptor cells usually found in fish olfactory epithelium, a third sensory cell type—the crypt cell—has been found in the olfactory epithelium of the zebrafish. This putative olfactory receptor cell bears microvilli as well as sunken cilia. Similar cells have been reported before only by Andres (1975) and Zeiske *et al.* (1976). The drawing of the olfactory epithelium of fish in Andres' review shows a sensory cell with short cilia that reach into a crypt built by surrounding cells. The species where this cell type was found are not mentioned. Zeiske described these cells as regenerating cells in the epithelia of the swordtail *Xiphophorus*. In the zebrafish, the crypt cells do not resemble de- or regenerating cells, and the consistency of their complex morphology does not indicate an artefact. Crypt cells have now also been found in catfishes, swordtails and needlefishes (Hansen *et al.*, 1997). Submerged cilia occur in various tissues, e.g. rudimentary cilia (Sorokin, 1962) or primary cilia (Tucker and Pardee, 1982). But these cilia are called 'primary' or 'rudimentary' due to their 9 + 0 pattern of microtubules, i.e. they lack the central microtubule doublet. Hence, this type of cilia differs from the submerged cilia of crypt cells, which—except for the cyprinodonts—usually show 9 + 2 doublets. The existence of an axon reaching the olfactory bulb (Hansen *et al.*, 1997) supports our hypothesis that the crypt cell is a receptor neuron.

The sensory epithelium of the zebrafish is thin (15–20 μm) compared with other fish whose olfactory epithelia measure from 35 μm (piranha; Schulte and Riehl, 1978) to 110 μm (pike; Holl, 1965). However, a thin epithelium does not necessarily imply a modest olfactory capacity since the expanse of the sensory epithelium as well as the density of receptor cells are important. Compared with the receptor cell densities reported for various fish species (Yamamoto, 1982, Zeiske *et al.*, 1976) ranging from 48 000 to 500 000 receptor cells per mm^2, the estimated density of 250 000 neurons per mm^2 in the zebrafish lies in the intermediate range. To estimate the total number of receptor cells based on these countings would be too speculative since the sizes of the lamellae and thus their sensory areas are highly variable within one organ and even more between individual fishes. Time-consuming measurements of an adequate number of complete olfactory organs would be necessary to obtain statistically reliable results.

The distribution of sensory and nonsensory areas in the olfactory epithelium is variable in fish (Yamamoto, 1982). Even within such closely related groups as the cyprinids, considerable differences occur. In contrast to the situation in zebrafish where continuous sensory areas even cover the valleys between the lamellae, in the cyprinids *Tinca*, *Phoxinus* and *Cyprinus*, islands of small clusters of olfactory receptor cells can be found in a large expanse of nonsensory epithelium (Holl, 1965). In other cyprinids, *Ctenopharyngodon* and *Carassius*, small islets of nonsensory epithelium may lie in a large area of sensory epithelium (Yamamoto and Ueda, 1978; Zippel *et al.*, 1997). In zebrafish, this parsimonious distribution of sensory and nonsensory areas could be due to the small size of the rosette. In order to have more room for receptor neurons, the nonsensory cells may be restricted to the rims of the lamellae.

Cyprinids belong to the group of isosmates that have no accessory ventilation sacs (Døving *et al.*, 1977). Kinocilia have to propel water and/or mucus over the lamellae. The ciliated nonsensory cells build a channel system that seems to be convenient for this transport. Other than the receptor cilia, kinocilia have dynein arms, indicating their motility (Zeiske *et al.*, 1992). The central microtubules of the kinocilia that are oriented in the same direction for large areas in zebrafish imply that the kinocilia beat metachronously in the same direction, thus providing for directionally preferred movements.

Thommesen (1983) described a higher density for microvillous cells in the central areas of the secondary folds in the char. Erickson and Caprio (1984) reported that in catfish microvillous receptor cells occur more abundantly in the dorso-medial portion of the lamellae. The same has been observed in goldfish (A. Hansen, unpublished data). In the zebrafish olfactory epithelium, an obvious distribution pattern of the receptor cell types could not be found with the methods applied. All sensory cell types seem to be distributed randomly over the lamellae.

In the zebrafish—as in *Phoxinus* (Bannister, 1965)—goblet cells are confined to the nonsensory areas of the olfactory epithelium, although in other cyprinids, e.g. goldfish, they also occur in the sensory areas (Breipohl *et al.*, 1973). Rodlet cells, which often appear in the olfactory epithelium of freshwater and marine fish, are not related to the olfactory system. They also appear in other organs

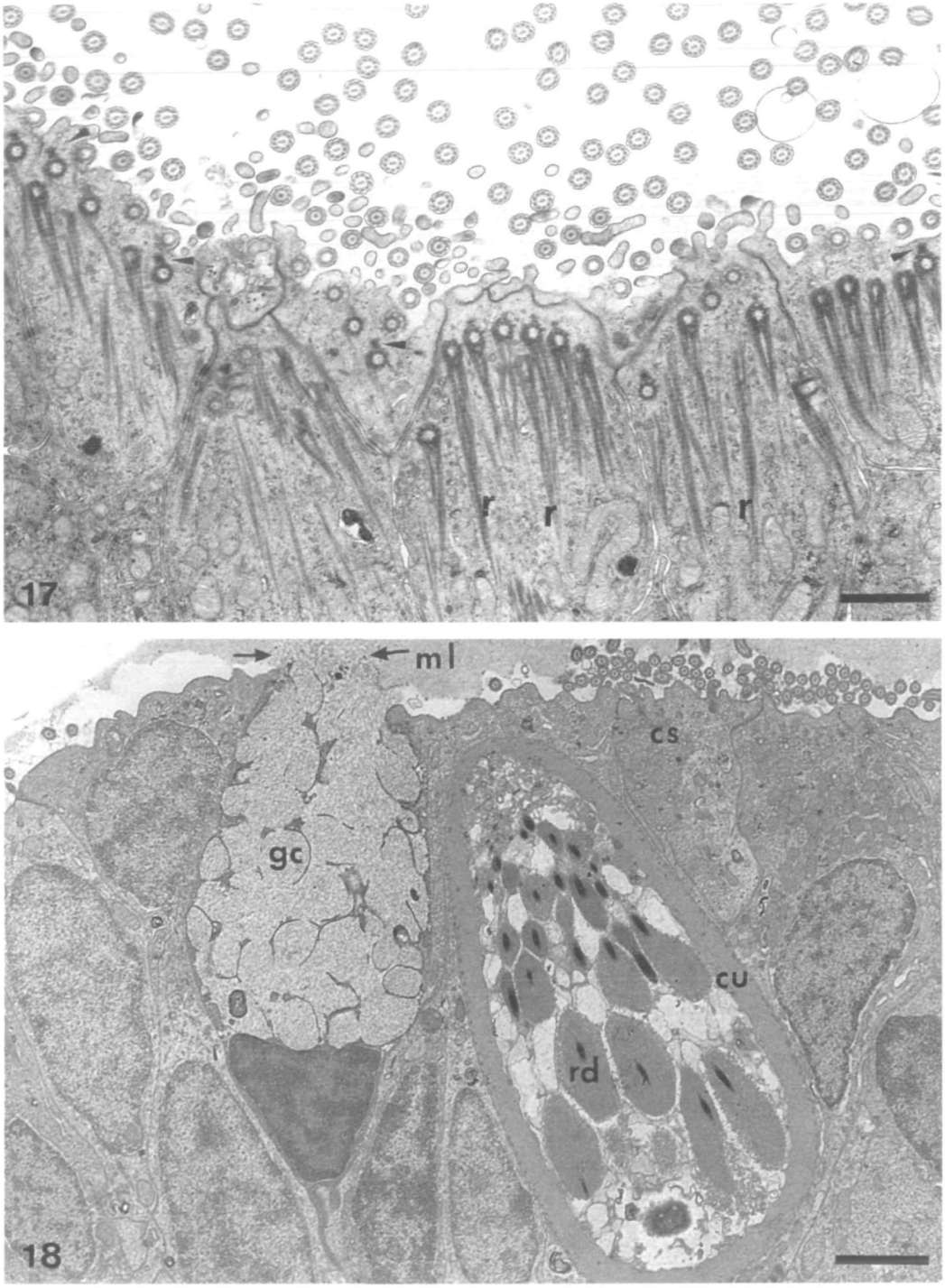


Figure 17 Ciliated nonsensory cells with striated rootlets (r). Note that the central doublets of all axonemes are oriented in the same direction. Arrowhead, basal foot. TEM. Scale bar = 1 μm.

Figure 18 Nonsensory epithelium of the olfactory organ with a goblet cell (gc) and a rodlet cell. The supranuclear region of the goblet cell is filled with mucous granules which are about to be discharged into the lumen of the nasal cavity (arrows). The surface of the epithelium is covered by a mucus layer (ml). The rodlet cell is identified by the thick cuticula (cu) and its typical rodlets (rd) and abundant vesicles. TEM. Scale bar = 2 μm.

such as gut, kidney, liver, gills and gonads. Whether these cells are parasites (Thélohan, 1892) or have an unknown function (for review see Morrison and Odense, 1978) is still unclear.

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