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# Laryngeal Chemosensory Clusters

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## Abstract

The expression of molecules involved in the transductory cascade of the sense of taste (TRs, α-gustducin, PLCβ2, $IP_3R3$) has been described in lingual taste buds or in solitary chemoreceptor cells located in different organs. At the laryngeal inlet, immunocytochemical staining at the light and electron microscope levels revealed that α-gustducin and PLCβ2 are mainly localized in chemosensory clusters (CCs), which are multicellular organizations differing from taste buds, being generally composed of two or three chemoreceptor cells. Compared with lingual taste buds, CCs are lower in height and smaller in diameter. In laryngeal CCs, immunocytochemistry using the two antibodies identified a similar cell type which appears rather unlike the α-gustducin-immunoreactive (IR) and PLCβ2-IR cells visible in lingual taste buds. The laryngeal IR cells are shorter than the lingual ones, with poorly developed basal processes and their apical process is shorter and thicker. Some cells show a flask-like shape due to the presence of a large body and the absence of basal processes. CCs lack pores and their delimitation from the surrounding epithelium is poorly evident. The demonstration of the existence of CCs strengthens the hypothesis of a phylogenetic link between gustatory and solitary chemosensory cells.

**Key words:** gustducin, neuroendocrine cells, phospholipase, taste, taste receptors



## Introduction

In mammals, taste buds are located in the oral cavity, pharynx and larynx. Laryngeal taste buds have been described in several species, including hamsters (Miller and Smith, 1984), sheep (Bradley *et al.*, 1980), horses (Yamamoto *et al.*, 2001), cats (Shin *et al.*, 1995), monkeys (Khaisman, 1976) and humans (fetus, Lalonde and Eglitis, 1961; adult, Jowett and Shrestha, 1998). These studies described evident species-related differences, probably linked to feeding habits. For example, laryngeal taste buds are numerous in herbivorous mammals, particularly in ruminants (Palmieri *et al.*, 1983) and are rare in insectivorous animals, while an intermediate number seems to be found in omnivorous species (Shrestha *et al.*, 1995).

In rodents, laryngeal taste buds constitute a significant percentage of the total number of taste buds. In the rat, 103 laryngeal taste buds were found versus only 38 pharyngeal buds (Travers and Nicklas, 1990). In the hamster, ~10% of all taste buds are located in the larynx (Miller and Smith, 1984). Other studies provided significantly lower values, but the data may have been influenced by age-related differences: laryngeal taste buds develop postnatally and their total number decreases significantly with age (Yamamoto *et al.*, 2003).

In all the species studied, laryngeal buds are mainly located at the 'entrance' of the larynx, primarily on the laryngeal surface of the epiglottis and aryepiglottal folds (Ide and Munger, 1980; Belecky and Smith, 1990; Travers and Nicklas, 1990; Shin *et al.*, 1995; Yamamoto *et al.*, 1997; Jowett and Shrestha, 1998). In some species, the taste buds are arranged around the opening of the duct of the epiglottic glands (Yamamoto *et al.*, 2001); in other species, accumulations of buds seem to exist at the bases of the epiglottis.

Most studies did not report any structural differences between laryngeal taste buds and those located in the oro-pharyngeal cavity, apart from a small number of α-gustducin-immunoreactive (IR) cells (Boughter *et al.*, 1997). Ultrastructural studies also demonstrated that laryngeal buds are basically similar to oro-pharyngeal taste buds (Sweazey *et al.*, 1994).

Nevertheless, a great functional difference exists between laryngeal and oro-pharyngeal buds. Laryngeal taste buds do not play a role in gustation, but are adapted to detect chemicals that are not saline-like in composition. They are

 

stimulated by the pH and tonicity of a solution and not by its gustatory properties (Bradley, 2000). Therefore, it is generally thought that laryngeal taste buds may work as chemosensory detectors to initiate the reflex reaction to protect the airway from oral substances during swallowing and drinking. Considering that they are described as quite similar structures, the morphological bases of the mismatch between laryngeal and oro-pharyngeal taste bud functions are unknown. A new light on this could be provided by recent developments, which have greatly increased our knowledge of the transductory machinery present in taste cells (Hoon *et al.*, 1999; Adler *et al.*, 2000; Lindemann, 2001; Margolskee, 2002; Montmayeur and Matsunami, 2002; Perez *et al.*, 2003). All the new data have been obtained from oral taste buds, while information about their laryngeal counterparts is lacking. The present study focuses on detection in the larynx of molecules involved in the transductory cascade of taste (i.e. α-gustducin and phospholipase C of the β2 subtype, PLCβ2). The work was undertaken in order to ascertain whether laryngeal and oral buds are the same from an anatomical and immunohistochemical point of view in spite of their functional differences, or whether the chemoreceptor structures of the two areas are differently organized.

## Material and methods

### Animals

The study was conducted on adult Wistar rats of both sexes (150–200 g; Morini Co., Reggio Emilia, Italy) kept at the departmental animal facility. Animals were anesthetized with ether and handled in accordance with the guidelines for animal experimentation laid down in Italian law.

### Immunocytochemistry

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Larynges were removed and fixed by immersion in the same fixative for 2 h at 4°C. After rinsing in 0.1 M phosphate buffer (PB), specimens were dehydrated through an ascending series of ethanol, transferred to xylene and embedded in paraffin. Serial 7 μm thick sections were cut on a rotating microtome (Leitz 1512; Leitz, Germany), collected on polylysine-coated slides and dried overnight at 37°C. The sections were processed for immunohistochemistry or stained with hematoxylin and eosin. The primary antibodies were raised in rabbits and directed against α-gustducin (1:400; Santa Cruz Biotechnology Inc.) or PLCβ2 (1:2000; Santa Cruz Biotechnology Inc.).

Immunohistochemistry was performed using the avidin–biotin complex (ABC) technique. Sections were deparaffinized and rehydrated through xylene and a descending ethanol series. Endogenous peroxidase was quenched by immersion in 0.3% hydrogen peroxide in methanol for 30 min. After washing in 0.05 M Tris–HCL buffer (pH 7.6), sections were treated with 5% normal swine serum for 20 min. The primary antibody was applied overnight at 4°C. After washes, sections were reacted with biotinylated swine anti-rabbit immunoglobulins (DAKO), diluted 1:400, for 2 h. The immunoreaction was detected using a Vectastain Elite ABC kit (Vector) and visualized by 3,3-diaminobenzidine tetrahydrochloride (DAKO) for 5–10 min. Sections were dehydrated, coverslipped with Entellan and observed in an Olympus BX51 photomicroscope equipped with a KY-F58 CCD camera (JVC). The images were analysed and stored using Image-ProPlus software. Control sections were processed as above, omitting the primary antibodies or after immunoabsorption of the primary antibody with the specific peptide. No immunostaining was observed in control sections.

### Transmission electron microscopy (TEM)

For TEM, the specimens were fixed in 2.5% glutaraldehyde in Sorensen's buffer for 2 h, postfixed in 1% osmium tetroxide in Sorensen buffer for 1 h, dehydrated in graded ethanols, embedded in Epon–Araldite and cut with a Ultracut E ultramicrotome (Reichert-Jung, Wien, Austria). Semithin sections were stained with toluidine blue or PAS. Ultrathin sections were stained with lead citrate and uranyl acetate and observed under an EM10 electron microscope (Zeiss, Oberkochen, Germany).

### Immunoelectron microscopy

Animals were perfused with 4% paraformaldehyde in 0.1 M PB, pH 7.4. Larynges were removed and fixed by immersion in the same fixative for 2 h at 4°C. After rinsing in 0.1 M PB, specimens were put into 30% sucrose in PB overnight and cut to 60 μm thickness on a freezing microtome (Reichert-Jung). Free floating sections were collected in 0.1 M PB, pH 7.4 and processed using the ABC technique. Endogenous peroxidase was quenched by immersion in a solution of 3% hydrogen peroxide in $H_20$ for 30 min. After washing in 0.05 M Tris–HCL buffer, pH 7.6, sections were treated with 10% normal swine serum for 60 min. Subsequently, the primary antibody (polyclonal antibody anti-α-gustducin 1:400 or polyclonal antibody anti-PLCβ2 1:2000), diluted with Tris containing 0.02% Triton X100, was applied overnight. After three washes, sections were then reacted with biotinylated swine anti-rabbit immunoglobulins (DAKO), diluted 1:400, for 2 h. The immunoreaction was detected using a Vectastain Elite ABC kit (Vector) and then visualized by 3,3-diaminobenzidine tetrahydrochloride (DAKO) for 5–10 min. Controls for the specificity of the immunoreactions were performed by omitting the primary antibody.

For ultrastructural examination, after post-fixation in 1% $OsO_4$ in PB for 1 h the tissues were dehydrated in graded concentrations of acetone and embedded in an Epon–Araldite mixture (Electron Microscopic Sciences, Fort Washington, PA). Semithin sections (1 μm thickness) were examined by light microscopy to locate areas containing immunoreactivity. Ultrathin sections were cut at 70 nm

thickness on an Ultracut-E ultramicrotome (Reichert-Jung) and observed unstained on an EM 10 electron microscope (Zeiss).

### Scanning electron microscopy (SEM)

For SEM, the tissues were fixed with glutaraldehyde 2% in 0.1 M PB, postfixed in 1% $OsO_4$ in the same buffer for 1 h, dehydrated in graded ethanols, critical point dried (CPD 030; Balzers, Vaduz, Liechtenstein), fixed to stubs with colloidal silver, sputtered with gold with an MED 010 coater (Balzers) and examined with a DSM 690 scanning electron microscope (Zeiss).

## Results

### General morphology

The most rostral portion of the larynx is lined with a pluristratified squamous epithelium. Caudally to this squamous portion, the laryngeal inlet is lined by the so-called transitional (intermediate) epithelium, which shows gradations ranging from stratified squamous through stratified cuboidal to ciliated stratified low-columnar types (Nakano and Muto, 1987; Nakano *et al.*, 1989). According to the classic descriptions of Smith (1977) and Lewis and Prentice (1980), this relatively unusual cuboidal epithelium is present within a band-like transitional area in the ventrolateral aspect of the larynx at the level of arytenoid projections and in the ventral pouch.

### Light microscopy and immunocytochemistry

In a further step of the study, we tested whether two gustatory markers were expressed in buds having a morphology similar to those visible in the tongue. Light microscopy and immunocytochemistry for detection of α-gustducin (Figure 1A,B) and PLCβ2 (Figure 1C,D) demonstrated the expression of these proteins in structures at the laryngeal inlet (generally located at the internal wall of the epiglottis and aryepiglottic folds). In size and shape, the IR structures appeared quite unlike the lingual taste buds (Figure 1E,F). Small buds were regularly found, but the most common morphological feature was the 'chemosensory cluster' (CC), composed of two or three IR cells. Precise quantification of the described structures was difficult because the pattern changed with the level of the section. Rostrally (at the laryngeal inlet) the buds were more numerous, whereas caudally, clusters were the commonest structures. Isolated IR cells (solitary chemosensory cells, SCCs) were also found, but were rare in this portion of the larynx, being more numerous distally (Sbarbati *et al.*, 2004). Typical buds composed of more than five or six IR cells were also rare and restricted to a thin rostral area. With respect to lingual taste buds, laryngeal buds showed lesser height and diameter, as was evident from a comparison with control specimens obtained from the vallate papilla (Figure 1E,F). In the taste buds sampled in the latter, for each of the antibodies used more than five IR cells were usually visible.

In laryngeal CCs, delimitation from the surrounding epithelium was poorly evident and pores were lacking. In laryngeal CCs, the two antibodies stained a similar cell type that appeared rather unlike the α-gustducin-IR and PLCβ2-IR cells visible in lingual taste buds. The laryngeal IR cells (Figure 1A–D) were shorter than the lingual ones and had poorly developed basal processes; in particular, the apical process was shorter and thicker. Some cells showed a flask-like shape due to the presence of a large body and to the apparent absence of basal processes.

### Transmission electron microscopy

Ultrastructural examination of the laryngeal inlet confirmed the presence of elements with a morphology ranging from small buds (Figure 2A) to CCs (Figure 3A,C,D ) and solitary chemosensory cells (SCCs, Figure 3E).

The laryngeal buds were located at the laryngeal inlet, embedded in a pluristratified epithelium (Figure 2A). The distinction between the bud and the non-receptor epithelium was evident. The cells in the laryngeal buds appeared to be less elongated than those visible in lingual taste buds (Figure 2B). In laryngeal buds, the extremity of the cells often protruded from the free surface (Figure 2C,E) and the dense material in a pore which is visible in lingual taste buds (panel D) was lacking. In general, the apical portion of the laryngeal bud was less complex than the corresponding portion of lingual taste buds (Figure 2D,E).

The cells forming the laryngeal CCs showed the basic features of receptor elements (i.e. apical ends contacting the lumen and innervation). Generally, their microvilli resembled those of type II lingual taste cells (i.e. short, brush-like microvilli). However, a comparative evaluation by ultrastructural methods confirmed the presence of several differences between lingual taste buds and laryngeal CCs. The cells located in a CC were not clearly distinguished from the surrounding epithelium and they were usually shorter than those found in lingual buds (Figures 2B and 3B). In laryngeal CCs, an apical pore was short or absent (Figure 3A). The dense material usually present in the taste pore of lingual buds (Figure 3B) was regularly absent in laryngeal CCs. In the latter, the absence of a true pore and of material covering the cells allowed direct visualization of microvilli by SEM (Figure 3F); this was generally not the case with lingual taste buds.

Ultrastructural immunocytochemistry for α-gustducin was used to identify the submicroscopic characteristics of chemoreceptor elements (Figure 4). The results confirmed the presence of IR cells disposed in clusters of two or three elements, not organized in buds. These elements showed nucleo-cytoplasmic characteristics quite different from those of the surrounding epithelial cells. In particular, the latter showed numerous bundles of filaments that were virtually absent in IR cells. In transversal sections, the IR

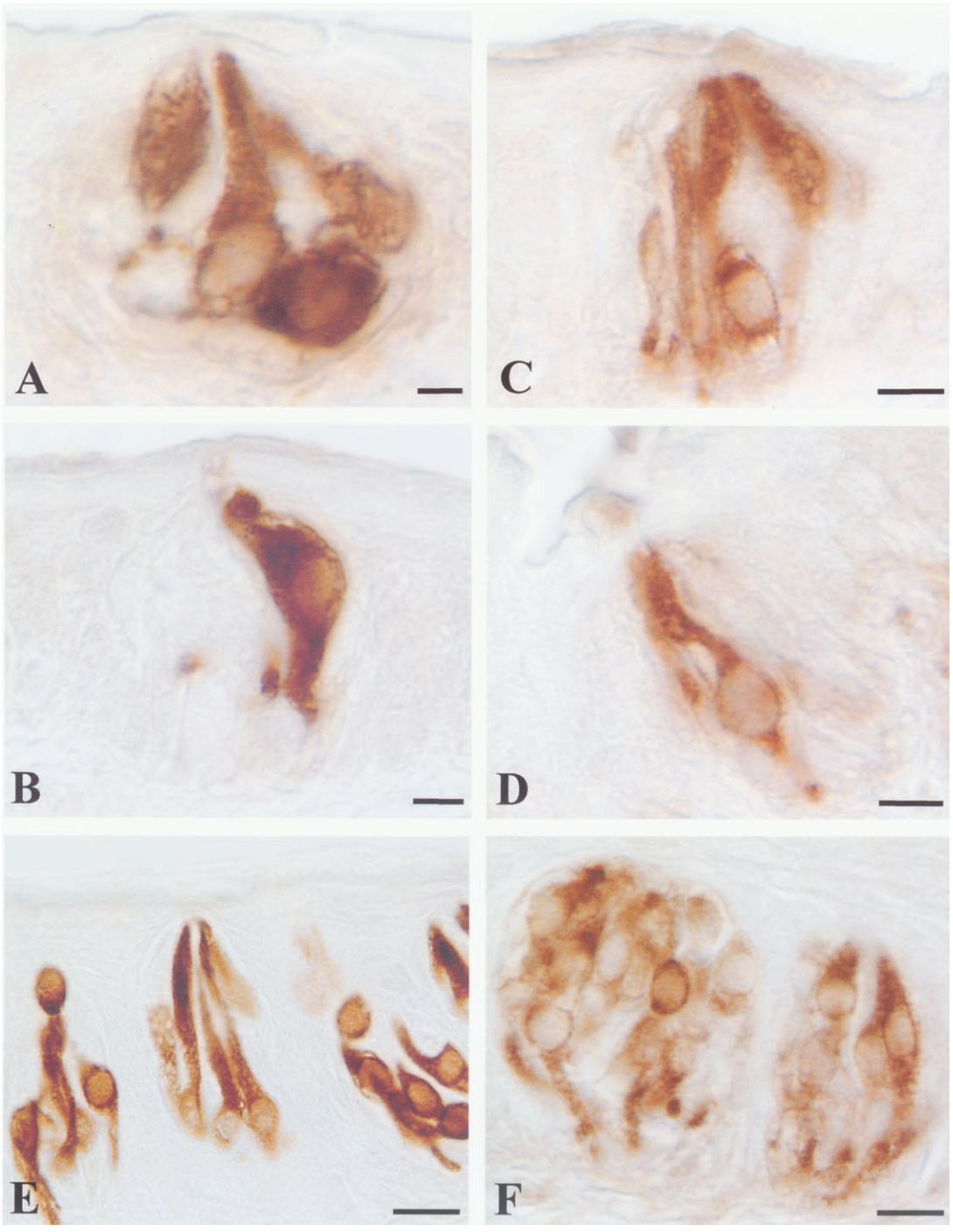


**Figure 1** Immunocytochemistry for α-gustducin or PLCβ2. **(A)** Immuncytochemistry for α-gustducin of a bud at the laryngeal inlet. **(B)** Immunocytochemistry for α-gustducin of a CC at the laryngeal inlet. **(C)** Immunocytochemistry for PLCβ2 of a bud at the laryngeal inlet. **(D)** Immunocytochemistry for PLCβ2 of a CC at the laryngeal inlet. **(E)** Immunocytochemistry for α-gustducin of buds on the vallate papilla of the tongue. **(F)** Immunocytochemistry for PLCβ2 of buds on the vallate papilla of the tongue. Scale bars = 2.5 μm (A); 5 μm (B–D, F); 20 μm (E).

cells appeared as round elements with a variable amount of perinuclear cytoplasm. Associated elements were usually not visible but, in some cases, the surrounding keratinocytes wrapped the IR cells with thin cytoplasmic processes (Figure 4).

## Discussion

### The laryngeal CC

In the present study, we found that the mucosa covering the laryngeal inlet expressed two molecules involved in taste

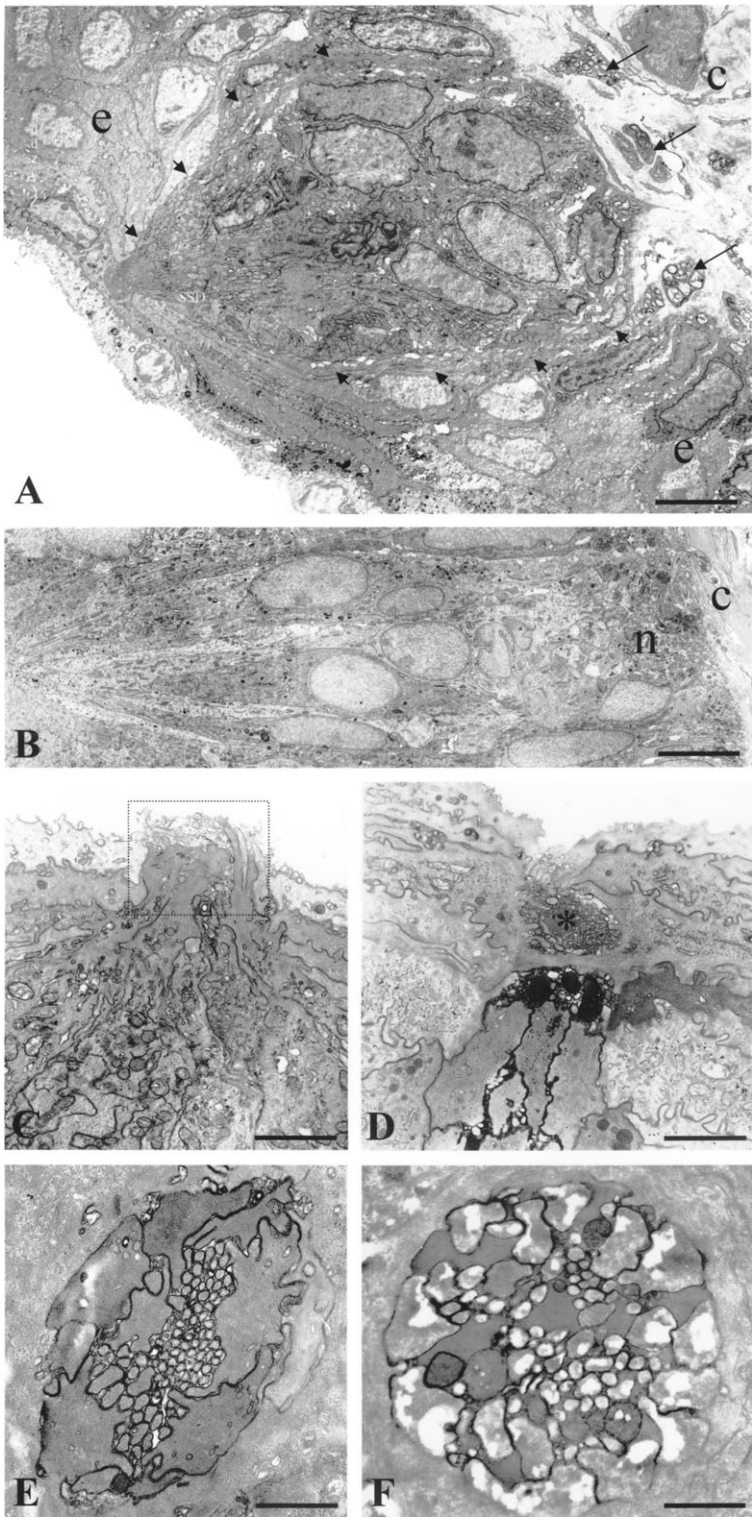


**Figure 2** TEM. In panel **(A)** a small bud at the laryngeal inlet is visible. The bud is embedded in pluristratified epithelium (e). The distinction between the bud and the non-receptor epithelium is evident (short arrows). Nerve fibers (arrows) are visible in the connective tissue (c). The cells appear to be less elongated than those visible in lingual taste buds **(B)**, vallate papilla). In the latter, a well-developed nerve plexus is visible (n) in the basal portion of the gustatory epithelium, near the connective tissue (c). **(C–F)** Apical pores of laryngeal (C, E) or lingual buds (D, F). The images shown in (C) and (D) are vertical sections (i.e. parallel to the major axis of the cells). The images shown in (E) and (F) are horizontal sections. In laryngeal buds, the extremities of the cells protrude from the free surface (squared area in panel C) and the dense material in a pore visible in lingual taste buds (asterisk in panel D) is lacking. Comparison of panels (E) and (F) shows that the apical portion of the laryngeal bud is less complex than the corresponding portion of lingual taste buds. Scale bars = 4.3 μm (A, B); 1.1 μm (C); 0.3 μm (D, F); 0.5 μm (E).

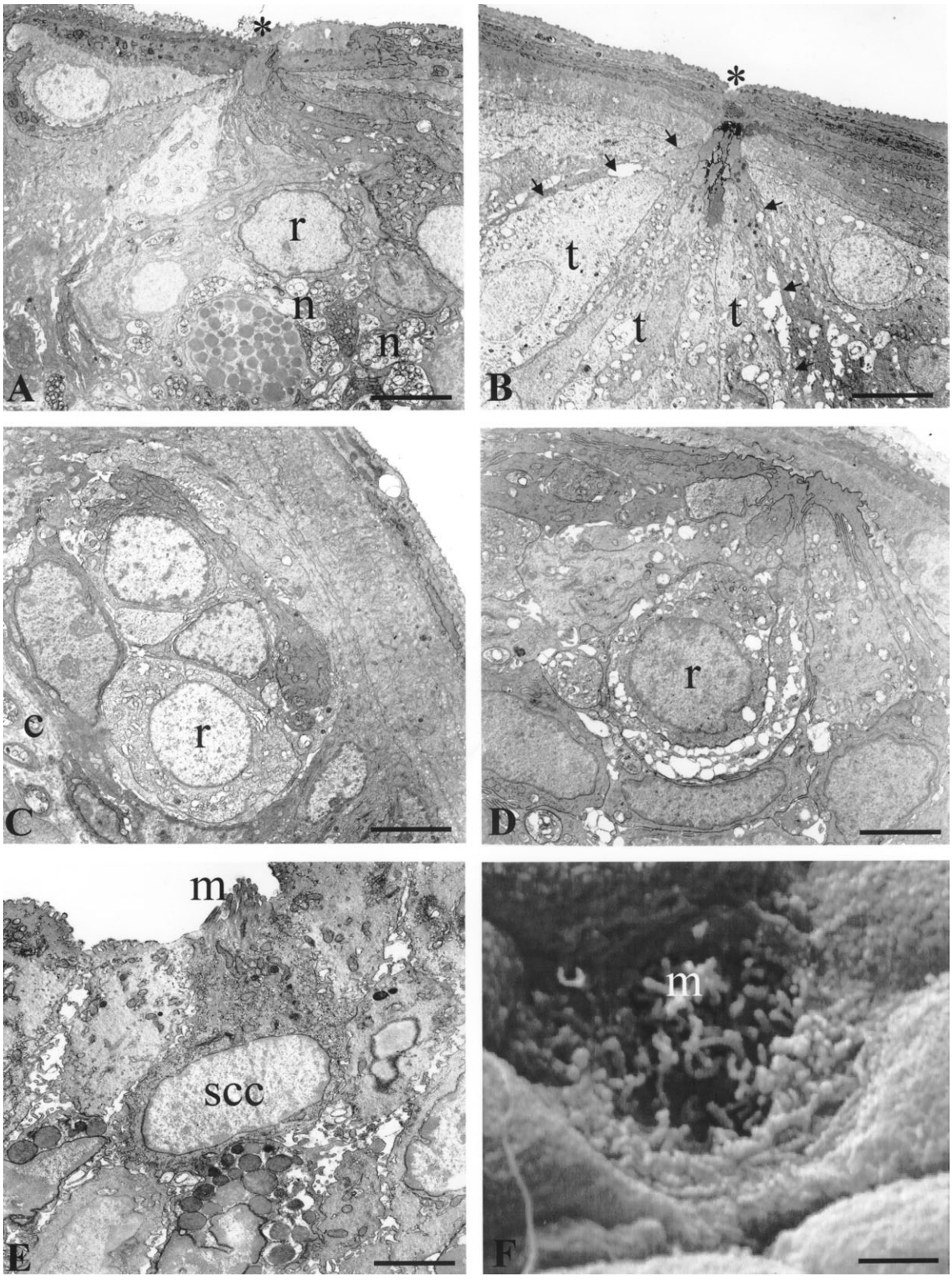


**Figure 3** TEM. Panels **(A–D)** illustrate the difference between lingual taste buds (B) and laryngeal CCs (A, C, D). In panel (B) the asterisk marks the pore. In panel (A) one can see that a true pore is lacking (asterisk). Several nerve fibers are visible (n). The lingual taste bud is composed of numerous taste cells (t) and its border is clearly visible (short arrows). Only a few elements with ultrastructural features of chemoreceptor cells (r) compose the CCs and the delimitation from the non-receptor epithelium is not clear. **(E)** A laryngeal SCC (scc) ending with microvilli (m) at the free surface. **(F)** Detail of the mucosal surface at the laryngeal inlet as visible by SEM. The round area with microvilli (m) probably corresponds to the apical end of a small bud or cluster. In panel (C), a small portion of connective tissue (c) with nerve fibers is visible. Scale bars = 3.6 μm (A, C); 2.2 μm (B); 2.5 μm (D); 1.4 μm (E,F).

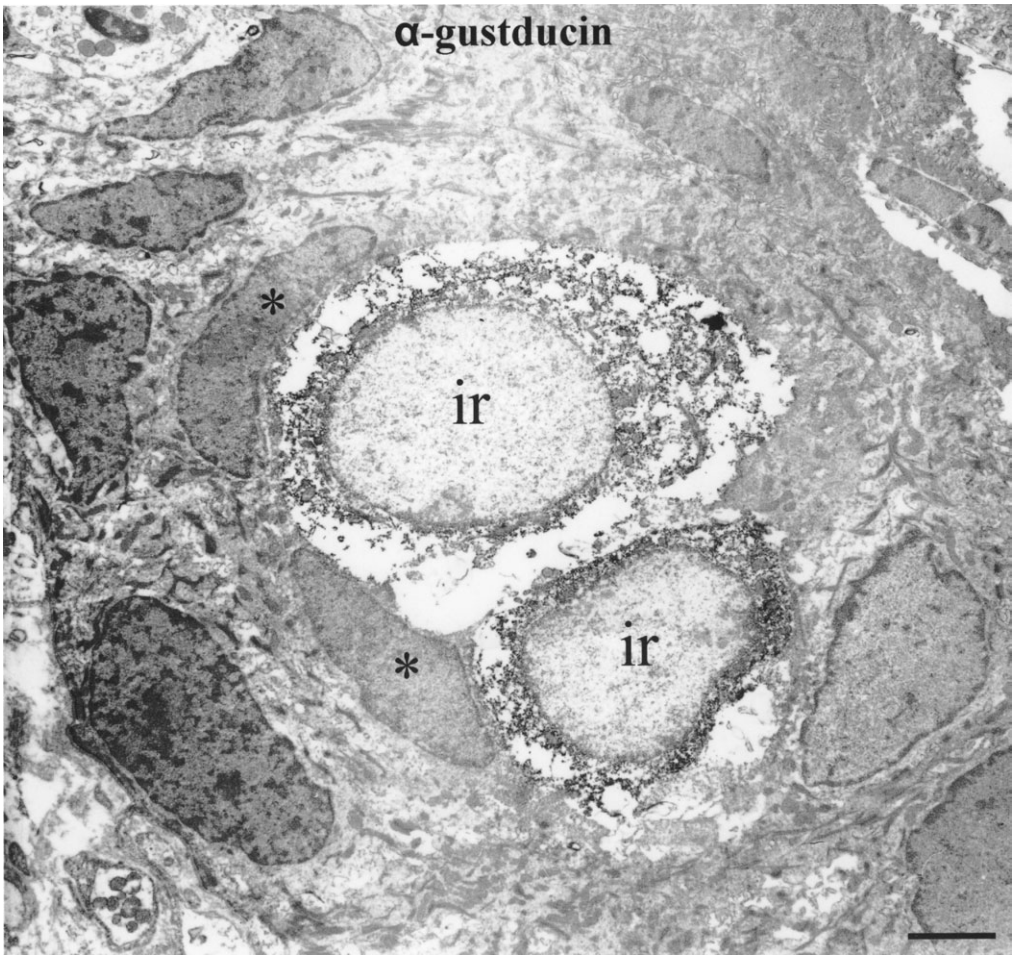


**Figure 4** Ultrastructural immunocytochemistry for α-gustducin. Two IR cells that form a CC are visible (ir). The asterisks indicate keratinocytes surrounding the IR cells.

transduction which are considered to be markers of taste cells (Rossler *et al.*, 1998; Perez *et al.*, 2002). These findings demonstrate an evident link between laryngeal chemoreceptor cells and the taste cells located in the tongue. However, we also demonstrated that differences exist between laryngeal chemoreceptors and the 'classic' taste buds located in the oro-pharyngeal cavity. Laryngeal chemoreceptors constitute a polymorphic population. Buds similar in shape to oral taste buds are few and are generally found in a thin rostral portion of the larynx, while in most cases laryngeal chemoreceptors are organized in structures smaller than oral taste buds, i.e. laryngeal CCs. The small size of these structures may be linked to their location in a thin epithelium, but structural differences are also evident. In particular, the basal processes of laryngeal α-gustducin-IR cells are poorly developed. This feature is not specific to the larynx, since similar α-gustducin-IR cells have been observed in lingual taste buds and immunocytochemical studies have described the wide-ranging polymorphism of these cells (Boughter *et al.*, 1997; Cho *et al.*, 1998; Pumplin *et al.*, 1999; Yang *et al.*, 2000; Clapp *et al.*, 2001; Yee *et al.*, 2001).

1Ultrastructural examination confirmed the presence of structural differences between taste buds and laryngeal CCs. Most CCs display an organization quite different to that of taste buds and only two or three α-gustducin or PLCβ2-IR cells can be found in each CC. The biggest laryngeal CCs resemble the immature taste buds that can be found in the tongue during development (Sbarbati *et al.*, 1998, 1999) or post-neurectomy regeneration (Kusakabe *et al.*, 2002).

The new data seem to be in accordance with the old observation by Khaisman (1976) of the incongruity of the term 'taste bud' in relation to the epiglottis and with observations of the human epiglottis which found 'taste' buds in only three out of five cases, in which they were restricted to the stratified squamous epithelium, they were smaller than typical lingual taste buds and it was rare to find a pore (Jowett and Shrestha, 1998).

### CCs and SCCs

Laryngeal CCs appear to be a transitional structure between the rostrally located buds and the SCCs more distally located in specific areas of the larynx (Sbarbati *et al.*, 2004). SCCs are elements which were described long ago in fishes (Whitear and Kotrschal, 1988; Whitear, 1992) and which recently were also found in mammals (for a review, see Sbarbati and Osculati, 2003). They were first seen in the developing gustatory epithelium (Sbarbati *et al.*, 1998) and then their presence was confirmed in the developing palate. SCCs are also located in the nasal cavity (Zancanaro *et al.*, 1999) and Finger *et al.* (2003) demonstrated in both rats and mice that in this location, SCCs form synaptic contacts with trigeminal afferent nerve fibers and express T2R 'bitter-taste' receptors and α-gustducin. Functional studies indicate that bitter substances applied to the nasal epithelium activate the trigeminal nerve and evoke changes in respiratory rate. By extending to the surface of the nasal epithelium, these chemosensory cells serve to expand the repertoire of compounds that can activate trigeminal protective reflexes (Finger *et al.*, 2003). On the basis of these findings, Finger *et al.* (2003) hypothesized that trigeminal chemoreceptor cells are likely to be remnants of the phylogenetically ancient population of SCCs found in the epithelium of all anamniote aquatic vertebrates.

The absence of a clear delimitation between laryngeal CCs and the surrounding structures makes them resemble groups of SCCs more than true taste buds. From a structural point of view, they can be considered analogous to clusters of SCCs present on the dorsal fin of the rockling fish (Kotrschal *et al.*, 1993). The presence of such clusters strengthens the analogy between the chemoreceptorial system of the larynx and that found in fish skin and oral cavity, which has already been noted (Sbarbati *et al.*, 2004). In both cases, SCCs or clusters of chemosensory cells are located in an area rich in intraepithelial axons and not particularly exposed to drying.

### Laryngeal chemoreceptors and neuroepithelial cells

Our work demonstrates that the laryngeal chemoreceptorial system is composed of three organizations of cells which can be identified by specific molecular markers, i.e. SCCs, CCs and buds. These structures show a clear topographical pattern with buds, CCs and SCCs arranged in the supraglottic portion of the larynx in a rostro-caudal sequence. A similar variable organization has been described for the neuroendocrine elements of the airways (Sorokin *et al.*, 1997), which can be present in the form of isolated elements, clusters or more complex neuroepithelial bodies (NEBs). For these cells, an age-related progression was reported, with isolated elements proving more numerous in the fetus and NEBs more numerous in the adult. Further, a rostro-caudal progression was also observed, with a prevalence of isolated cells proximally and of NEBs distally. For gustatory cells also, a progression during development from isolated cells to buds was documented in the tongue (Sbarbati *et al.*, 1999). For the larynx, we have no developmental data to demonstrate a progression from CCs to buds.

### Functional considerations

Our data show cells with expression of molecules linked to taste detection in laryngeal CCs. Previous work demonstrated that SCCs also contain G-protein and other molecules of the taste cascade. Such structures could be involved in protective action against exogenous air-borne molecules or material coming from the pharynx. Therefore, the new data seem to indicate that at the laryngeal inlet also, cells expressing molecules of the 'taste machinery' carry out that sentinel action which has been demonstrated in other zones of the respiratory apparatus (Finger *et al.*, 2003).

Physiological studies demonstrated the non-gustatory nature of the response elicited by stimulation of the proximal portion of the larynx (Bradley, 2000), but anatomical studies simply described the laryngeal chemoreceptors as taste buds. The present work contributes to clarifying this mismatch by demonstrating that the laryngeal chemosensory system expresses molecules involved in the taste cascade, but displays three levels of organization: buds, CCs and SCCs. In the nasal cavities, it was recently reported that chemoreceptors with molecular markers of taste cells can operate as detectors of irritating stimuli (Finger *et al.*, 2003). It is possible that the laryngeal chemosensory system has a similar role.

### General conclusions

This work represents a step in the definition of a diffuse chemosensory system composed of taste cell-related elements. In recent years, the identification of cells with gustatory characteristics located outside the oro-pharyngeal cavity has been permitted by the discovery of the molecular machinery of taste transduction. This first allowed the chemical coding of the gustatory cells in taste buds and subsequently allowed the detection of cells with a similar chemical code in other organs. To date, cells with similar characteristics have been identified in endodermic derivatives (i.e. the digestive and respiratory apparatuses), but they always appear in the form of isolated elements—solitary chemosensory cells or brush cells (Hofer *et al.*, 1996; Hofer and Drenckhahn, 1998). In this study, taste cell-related elements were detected in a multicellular form of organization, the CC. The existence of CCs strengthens the hypothesis of a phylogenetic link between the gustatory and the SCC system.

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