

Immunohistochemical analysis for G protein in the olfactory organs of soft-shelled turtle, *Pelodiscus sinensis*

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ABSTRACT. In turtles, the epithelia lining the upper and lower chambers of the nasal cavity project axons to the ventral and dorsal parts of the olfactory bulbs, respectively. In a semi-aquatic soft-shelled turtle, *Pelodiscus sinensis*, more than 1,000 odorant receptor genes have been found, but it is not known where they are expressed. In this study, we aimed to clarify the distribution of cells expressing these genes in the olfactory organs of soft-shelled turtles. Immunoreactions for the G α olf, the α subunit of G protein coupled to the odorant receptors, were detected on the surface of epithelia lining both the upper and lower chambers of the nasal cavity. The receptor cells in the epithelium of both chambers possessed cilia on the tip of their dendrites, whereas microvillous, non-ciliated, receptor cells were not found. These data suggest that the odorant receptor genes are expressed by the ciliated receptor cells in the upper and lower chamber epithelia. Precise location of the vomeronasal epithelium is not known at present.

KEY WORDS: cilia, electron microscope, immunohistochemistry, reptile, vomeronasal organ

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Many vertebrates have two distinct olfactory systems, the main olfactory system and the vomeronasal system. The olfactory cells and the vomeronasal receptor cells are olfactory receptor cells distributed in the olfactory epithelium (OE) and the vomeronasal organ (VNO), respectively, and separately project their axons into the main and accessory olfactory bulbs (OB) [17, 30, 31, 33]. The olfactory cells and vomeronasal receptor cells of mammals bear cilia and microvilli on the tip of their dendrites, respectively. These cells express olfactory receptors which can bind chemical substances and sense them as olfactory stimuli. Olfactory receptors, including odorant receptors (OR) and type 1 or type 2 vomeronasal receptors (VRs), belong to the G protein-coupled receptor family: Cells expressing ORs express G α olf, and cells expressing type 1 and type 2 VRs express G α i-2 and G α o, respectively [1, 2, 12]. Many of the studies on G protein expression in the olfactory organs were aimed at mammals [8, 9, 13, 15, 23–25, 29], whereas few of those in reptiles have been reported [10, 14, 18, 34].

The nasal cavities of turtles are divided into the upper and lower chambers and project their axons into the ventral and dorsal parts of the OB, respectively. In aquatic and semi-

aquatic turtles, several lines of evidences suggest that the lower chamber epithelium, usually considered to be the VNO, is an olfactory organ detecting under-water odorants [26]. In addition, it has been reported that the G α olf is expressed in the lower chamber epithelium of some turtles [19, 34]. Previously, we analyzed the expression of G protein α subunits in the olfactory systems of red-eared sliders (*Trachemys scripta elegans*, Emydidae) [19]. In the nasal cavity of red-eared sliders, receptor cells coexpress G α olf and G α o in the upper chamber epithelium. In the lower chamber epithelium, receptor cells in the apical layer coexpress G α olf and G α o, whereas those in the basal layer express G α o. We also found that G α olf and G α o are expressed in the olfactory nerve layer and the glomerular layer of both the ventral and dorsal parts of the OB. Considering that the receptor cells in the upper chamber epithelium of the turtle's nasal cavity are regarded as mammalian olfactory cells and those in the lower chamber epithelium are as mammalian vomeronasal receptor cells, it is interesting that both receptor cells coexpress G α olf and G α o. As mentioned above, G α olf and G α i-2 and/or G α o are involved in the signal transduction in the OE and the VNO of mammals, respectively. In the Reeve's turtle (*Geoclemys reevesii*, Geoemydidae), which belongs to superfamily Testudinoidea along with the red-eared slider, both G α olf and G α o have been suggested to function as signal transduction molecules in the chemoreception of olfactory cells and vomeronasal receptor cells [34].

More than 1,000 OR genes have been found in the genome of soft-shelled turtles (superfamily Trionychia, family Trionychidae, *Pelodiscus sinensis*) [35]. The nasal cavity

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of soft-shelled turtles is divided into the upper and lower chambers as with that of the red-eared slider and Reeve's turtle. The former is considerably smaller than the latter, and axons from there project into the restricted areas in the OB. We speculated that the cells expressing OR are distributed in the lower chamber epithelium as well as in the upper chamber epithelium in the olfactory organs of soft-shelled turtle. To test our hypothesis, expression of G protein α subunit coupling to OR was immunohistochemically analyzed in this study. In addition, we characterized the fine structure of upper and lower chamber epithelia by investigating with a transmission electron microscope (TEM) and a scanning electron microscope (SEM).

MATERIALS AND METHODS

Animals and tissue processing: Six adult soft-shelled turtles were purchased from a local supplier TOBUN (Kamikita, Japan). All procedures were in accordance with the Guideline for Care and Use of Animal Experiments and approved by the Animal Care and Use Committee at Iwate University. In all cases, the animals were anesthetized by the injection of sodium pentobarbital (64.8 mg/kg i.p.). Two animals (1 male and 1 female) were perfused transcardially with Ringer's solution followed by a fixative solution, 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The head was cut off and immersed in the same fixative solution overnight at 4°C. The brain was removed, and the upper jaw was decalcified in 10% ethylenediamine tetra-acetic acid (EDTA) in 0.1 M PB at 4°C for two weeks. All specimens were cryoprotected in sucrose gradient, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and cut at 15–20 μm in thickness with a cryostat. Sections were air-dried and processed for immunohistochemistry. Some of the sections were stained with hematoxylin-eosin for general histological examination.

Immunohistochemistry: Immunohistochemistry was performed with two *Gas/olf* antibodies (Santa Cruz, Dallas, TX, U.S.A., mouse mAb, sc-55545, 1:100 dilution; and Santa Cruz, rabbit pAb, sc-383, 1:1,000 dilution). After washing in 0.1% Triton X-100 in phosphate-buffered saline (PBT) followed by phosphate-buffered saline (PBS), sections of the olfactory organs were treated with 2% normal donkey serum in PBS for 30 min at room temperature (RT) to block non-specific binding. Then, they were incubated with one of the primary antibodies at 4°C overnight. After washing, the sections were incubated for 2 hr at RT with fluorescent-labeled secondary antibodies: Tetramethyl Rhodamine Isothiocyanate (TRITC)-donkey anti-mouse IgG or TRITC-donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A., 1:1,000 dilution each).

Transmission electron microscopy: The olfactory organs of 4 animals (1 male and 3 females) were dissected and fixed in 2.5% glutaraldehyde in 0.1 M PB overnight at 4°C. Specimens were postfixed in 1% osmium tetroxide for 1 hr at 4°C, dehydrated in a graded series of ethanol, substituted with butyl glycidyl ether and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife, stained with uranyl

acetate and lead citrate, and examined with a TEM (JEM-2100, JEOL, Tokyo, Japan).

Scanning electron microscopy: The dehydrated specimens were dried with t-butyl alcohol using a freeze dryer (ES-2030, Hitachi, Tokyo, Japan). The specimens were coated with gold or osmium and examined with a SEM (JSM-7001F, JEOL).

RESULTS

In the soft-shelled turtle, the nasal cavity consisted of two chambers, the upper and lower chambers, communicating with each other (Fig. 1). The upper chamber epithelium was associated with glands (Bowman's glands), whereas the lower chamber epithelium was not.

The OB was divided into two parts, dorsal part and ventral part (Fig. 2A). The former occupied the dorsal and lateral regions of the OB, while the latter occupied the medial ventral regions. The dorsal part of the OB received projections from the lower chamber epithelium and the ventral part of the OB from the upper chamber epithelium. As described in the previous report [28], both dorsal and ventral parts of the OB consisted of seven layers: Olfactory nerve layer, glomerular layer, external plexiform layer, mitral cell layer, internal cell layer, granular cell layer and ependymal layer (Fig. 2B and 2C).

Immunohistochemistry for *G α olf* was performed to clarify the localization of *G α olf* proteins in the upper and lower chamber epithelia (Fig. 3). The immunoreactivity for two *Gas/olf* antibodies (sc-383 and sc-55545) was similar to each other. *G α olf* protein was detected in the somata and axons of olfactory receptor cells in both epithelia (Fig. 3A and 3B). *G α olf* protein was also localized to the cilia on the apical surface of both epithelia (Fig. 3A' and 3B').

In order to clarify the ultrastructural characteristics of the upper and lower chamber epithelia, apical part of them was examined by TEM and SEM (Fig. 4). The cytoplasm of olfactory receptor cells and supporting cells was easily distinguished in both epithelia, since the former (1–2 μm in diameter) had a large number of microtubules, whereas the latter (3–4 μm in diameter) had secretory granules (Fig. 4C and 4F).

In the apical part of the upper chamber epithelium, two types of cells, ciliated olfactory receptor cells and microvillous supporting cells, were observed (Fig. 4B and 4C). The olfactory receptor cells possessed numerous small protrusions as well as several cilia on the tip of their dendrites, whereas the supporting cells possessed a few short microvilli on the free border.

In addition to the ciliated olfactory receptor cells and microvillous supporting cells, cells bearing both cilia and rod-like protrusion were observed in the apical part of the lower chamber epithelium. The rod-like protrusions appeared to be fused cilia, because of the presence of basal bodies at their base and bundles of microtubules within them (Fig. 4E and 4F). Cilia on the olfactory receptor cells in the lower chamber epithelium (1–2 μm in length, Fig. 4E) were slightly shorter than those in the upper chamber epithelium

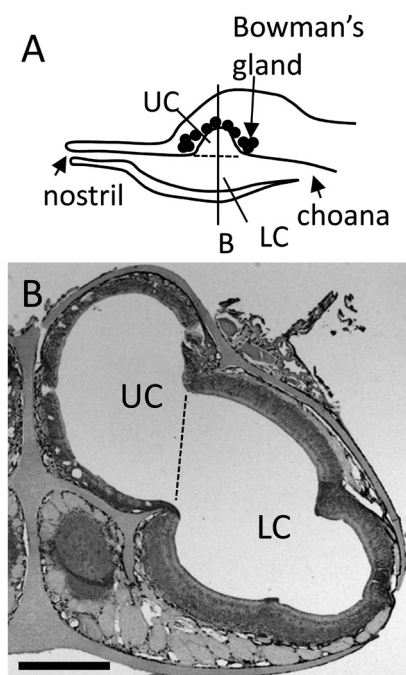


Fig. 1. The olfactory organs of a soft-shelled turtle. (A) A schematic drawing of the sagittal view. (B) Coronal section at line B. Dorsal is top, and lateral is right. UC, upper chamber; LC, lower chamber. Scale bar: 1 mm.

(2–3 μm in length, Fig. 4B). The supporting cells of lower chamber epithelium (Fig. 4F) had fewer secretory granules than those in the upper chamber epithelium (Fig. 4C).

DISCUSSION

In general, semi-aquatic turtles sense smells both on lands and under water [3]. Since semi-aquatic turtles make buccal oscillation (sniffing behavior) under water, the upper chamber of the nasal cavity is occupied by air and the lower chamber is filled with water while they are in water. Thus, in the nasal cavity of semi-aquatic turtles, the upper chamber epithelium and the lower chamber epithelium are considered to be the olfactory organs detecting air-borne odorants and water-borne odorants, respectively [3, 26]. The fact that the upper chamber epithelium is associated with glands but the lower chamber epithelium is not, as shown in this study, supports the idea that the former is an olfactory organ that functions in the air and the latter in the water. Since soft-shelled turtles demonstrate most behaviors, such as the feeding and reproduction, under water, the olfactory organs detecting water-borne odorants are supposed to play more important roles in soft-shelled turtles than in other semi-aquatic turtles. In fact, the lower chamber was much larger than the upper chamber in the nasal cavity of soft-shelled turtles. Assuming that the lower chamber epithelium is an olfactory organ detecting odorants under water, it is very reasonable that the lower chamber occupies a larger part of the nasal cavity in the soft-shelled turtles, which are highly adapted to the

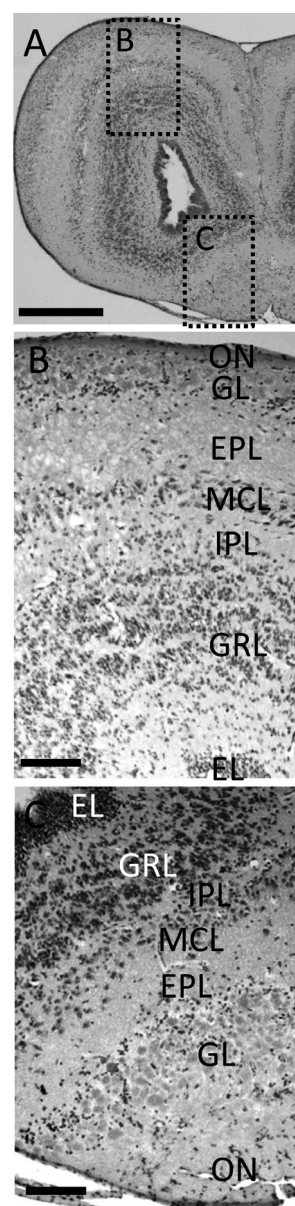


Fig. 2. The olfactory bulbs of a soft-shelled turtle. (A) The olfactory bulb (OB) is divided into two distinct parts: dorsal (d-OB) and ventral (v-OB). Dorsal is top, and medial is right. Higher magnification views of d-OB and v-OB are shown in B and C. (B and C) These OBs consist of seven layers: Olfactory nerve layer (ON), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granular cell layer (GRL) and ependymal layer (EL). Scale bars: 500 μm in A, 100 μm in B and C.

aquatic life.

In this study, immunopositive stainings for *G α olf* were observed in the sensory epithelium lining both the upper and lower chambers of the nasal cavity. In addition, similar immunoreactivity for two *Gas/olf* antibodies (sc-383 and sc-55545) strongly suggests that they specifically recog-

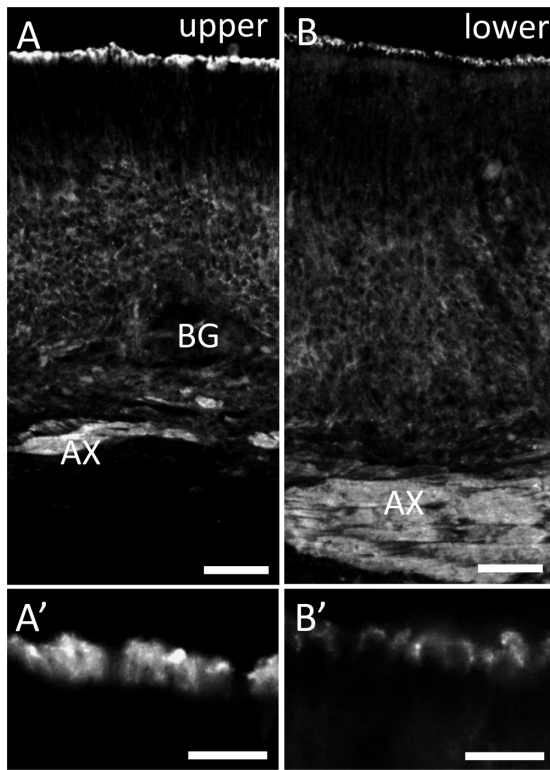


Fig. 3. Immunohistochemistry for $G\alpha_{olf}$ in the upper (A) and lower (B) chamber epithelia. Higher magnification views of the epithelial surface are shown in A' and B'. (A, B) The olfactory receptor cells and axon bundles (AX) are immunopositive for $G\alpha_{olf}$ in both epithelia. (A', B') The cilia on the free border are immunopositive for $G\alpha_{olf}$ in both epithelia. BG, Bowman's glands. Scale bars: 25 μm in A and B, 10 μm in A' and B'.

nized the $G\alpha_{olf}$ protein of soft-shelled turtle. The fact that soft-shelled turtles have more than 1,000 functional OR genes—exceptional in non-mammals [35]—suggests wide distribution of OR-expressing olfactory receptor cells in the nasal cavity. In support of this idea, distribution of cells expressing $G\alpha_{olf}$, the α subunit of G protein coupled with ORs, in both upper and lower chamber epithelia was demonstrated in this study. The vertebrate ORs can be classified into fish-type ORs and mammalian-type ORs, namely, class I ORs involved in the olfaction under water and class II ORs in the air [5, 6, 27]. Unlike other tetrapods, a higher ratio of class I ORs has been found in the genome of soft-shelled turtles [35]. In the olfactory organ of the amphibian *Xenopus laevis*, the OE detecting air-borne odorants expresses class II ORs, and the middle chamber epithelium detecting water-borne odorants expresses class I ORs [4, 16]. In soft-shelled turtles, class I ORs and class II ORs may be expressed in the olfactory receptor cells in the lower and upper chamber epithelia, respectively. Further investigation is required to test this speculation.

In this study, we demonstrated the distribution of ciliated receptor cells in the lower chamber epithelium of the nasal cavity and the expression of $G\alpha_{olf}$ at the cilia on the tip of

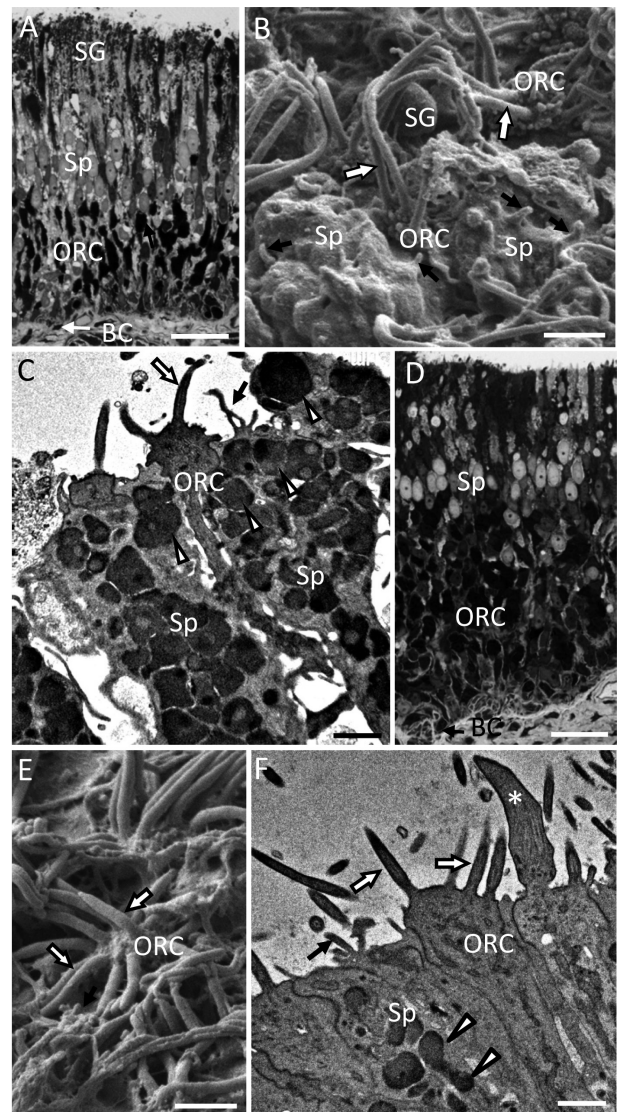


Fig. 4. Ultrastructure of the upper (A-C) and lower (D-F) chamber epithelia. (A, D) Toluidine blue-stained semithin sections showing nuclei of the supporting cells (Sp), olfactory receptor cells (ORC) and basal cells (BC). SG, secretory granules. (B, E) Scanning electron micrographs of the apical surface of epithelium, showing the ORC bearing cilia (white arrows) and the Sp bearing microvilli (black arrows). Apical end of the ORC also bears small protrusions. (C, F) Transmission electron micrographs of the apical region of the epithelium. Sp and ORC bear microvilli (black arrows) and cilia (white arrows) on their free borders, respectively. Arrowheads indicate secretory granules. In the lower chamber epithelium, some cells bear both cilia (white arrows) and a rod-like protrusion (asterisk) on the apical surface. Scale bars: 25 μm in A and D, 1 μm in B, C, E and F.

their dendrites. On the other hand, in other semi-aquatic turtles, such as red-eared slider and Reeve's turtles, microvillous receptor cells are distributed in the lower chamber epithelium of the nasal cavity and express $G\alpha_{olf}$ at the microvilli on the tip of their dendrites [19, 34]. Differences in

the ultrastructure of receptor cells expressing Gaolf among these turtles can be explained as follows: A common ancestor of these turtles possessed lower chamber epithelium containing ciliated receptor cells and microvillous receptor cells, both expressing Gaolf, but one of them has been lost during evolution. Although the reason why one of the receptor cell types has been lost is uncertain, ciliated receptor cells in some turtles and microvillous receptor cells in soft-shelled turtles may have been lost due to their different living environments.

Most vertebrates in higher order than amphibians possess two distinct olfactory organs, the OE and the VNO. In most cases, ciliated receptor cells are clustered in the OE and microvillous receptor cells in the VNO [30, 31]. As in other tetrapods, it has been thought that vomeronasal receptor cells of turtles are generally microvillous based on the electron microscopic studies of gopher tortoises, box turtles [7], Reeve's turtles [11, 32, 34] and red-eared sliders [19]. However, there are approximately 300 extant turtle species, and gross anatomy of the olfactory organs varies among species according to their habitats (aquatic, terrestrial and semi-aquatic) [20–22]. Since only a limited number of species have been investigated on their ultrastructure of olfactory organs, the VNO of some turtle species might contain ciliated receptor cells, not the microvillous ones.

Since the expression of G proteins coupled with VRs (Gao and Gai-2) in the nasal cavity of soft-shelled turtle has not been examined to date, the presence and/or the distribution of receptor cells expressing VRs remain unknown. The VRs and ORs might be coexpressed in a single receptor cell, or the receptor cells expressing VRs might be clustered somewhere in the nasal cavity that was not examined in this study. Further investigation is needed to reveal the distribution of receptor cells expressing VRs in the olfactory organs of soft-shelled turtles.

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