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Anatomy

Structure and zonal expression of olfactory receptors in the olfactory epithelium of the goat, *Capra hircus*

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ABSTRACT. The mammalian olfactory system employs sophisticated mechanisms to detect and recognize an extensive range of smells. In rodents, the olfactory epithelium (OE), situated within the nasal cavity, mainly comprises four defined endoturbinates and several ectoturbinates. Olfactory receptors (ORs) belong to a large family, comprising over 1,000 genes in rodents, which are expressed in olfactory sensory neurons in the OE that detect odor molecules. The rodent OE is divided into four topographically distinct zones, defined by individual OR distribution. However, although the structural complexity and the zonal organization of mammalian OE may contribute to successfully interpreting olfactory information, it remains poorly understood. In this study, we investigated the nasal cavity structure and zonal organization of the OE in goats. Morphological observations revealed that the goat nasal cavity possessed well-developed endoturbinates and ectoturbinates and had a structure similar to that of rodents and sheep, previously reported in other studies. *In situ* hybridization was used to analyze the expression pattern of ORs, NADPH:quinone oxidoreductase 1, and olfactory cell adhesion molecules as markers of zonal organization in the goat OE. Based on the expression patterns of these genes, we concluded that the goat OE was divided into four zones. The well-developed structure of the nasal cavity and distribution of each OR in the OE were similar to those found in rodents, suggesting that these features were highly conserved between mammals and may have fundamental roles in discriminating among numerous odor molecules in the environment.

KEY WORDS: goat, olfaction, olfactory sensory neuron, zonal organization

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Olfaction plays an important role in the survival of individual mammals, most of which use their sense of smell to find food, avoid predators, and seek appropriate partners, *etc*. In the first step of odor reception, odor molecules from the external environment are captured by the olfactory receptors (ORs), which are expressed in olfactory sensory neurons (OSNs) in the olfactory epithelium (OE). The OE is mainly located in the posterior part of the nasal cavity and lies on the septum, endoturbinates, and ectoturbinates in most mammals. As reported previously, the structure of the nasal cavity appears to be quite complex in rodents, dogs, sheep and horses [1–3, 10]. In these species, several winding and branched endoturbinates and ectoturbinates protrude into the lumen of the nasal cavity; in contrast, the primate nasal cavity is a simple structure [6, 8]. Thus, there are large differences in nasal cavity structures among mammalian species.

ORs comprise the largest multigene family in vertebrates, although the number of functional OR genes differs between species. There are 1,130 and 1,207 functional OR genes in the genome of mice and rats, respectively [14]. Other mammalian species possess a relatively similar number of functional OR genes (rabbits, 768; guinea pigs, 796; dogs, 811; cows, 1,186; horses, 1,066). However, primates, including humans, have a relatively small number of functional ORs compared with other mammals (macaques, 309; marmosets, 296; humans, 396) [14].

The expression pattern of each OR in OSNs has two characteristic features. First, each OSN expresses only one type of OR gene, selected from the pool of OR repertoires, known as the "one-neuron-one receptor" theory [5, 11]. Second, the mammalian OE can be classified into topographically distinct zones, defined by the expression pattern of each individual OR [17]. In rodents, OSNs expressing a given OR gene are randomly scattered within specific zones of the OE. The rodent OE has been reported to have approximately four OR expressing zones [19], although recent studies indicate a partial overlap between them [9, 12]. In macaque monkeys, which possess a simpler nasal cavity structure and smaller number of OR repertories compared with the

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majority of mammalian species, the OE has only two broad OR expressing zones [8].

These differences in the zonal organization of the OE between rodents and primates indicate that there may be diverse types of zonal patterning in different species. However, rodents and primates are the only mammals in which the zonal organization of the OE has been reported. It therefore remains unclear whether the four-zonal organization of the OE in rodents is conserved in mammalian species, such as sheep, dogs and horses, that possess a nasal cavity structurally similar to that of rodents. Moreover, because the biological significance of the zonal organization of the OE is still incompletely understood, comparative studies focused on the zonal organization of the OE in several mammalian species may provide important insights.

Although the structure of the sheep nasal cavity is fairly similar to that in rodents [3], both the distribution of the OE in the nasal cavity and the zonal organization of the OE still have not been investigated in ungulates. Among ungulates, the goat olfactory system has been extensively characterized [15, 16, 22, 23]. Moreover, the goat olfactory system is equipped with a unique subset of OSNs that express both vomeronasal type-1 receptor (V1rs) and Gαi2, a V1rs-associated G protein [23]. These OSNs in goats are not observed in the rodent OE, implying that there may be some differences between the rodent and goat olfactory systems.

Therefore, in the present study, we aimed to clarify these differences by examining the structure of the nasal cavity in the Shiba goat (*Capra hircus*). We also performed immunohistochemistry for olfactory marker protein (OMP), a marker of mature OSNs, to investigate the distribution of the OE in the goat nasal cavity. We cloned several genes that encode goat ORs, OMP, dihydronicotinamide-adenine dinucleotide phosphate (NADPH)-quinone oxidoreductase (NQO1) and olfactory cell adhesion molecule (OCAM); these were then used as markers of zone-specific expression for *in situ* hybridization (ISH) analysis, using the whole goat OE.

MATERIALS AND METHODS

Animals

A total of eight adult female Shiba goats (*C. hircus*), each weighing 20–28 kg, were used. All animals were housed under natural conditions at the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO) and provided with a diet of standard pellets, hay and water *ad libitum*. All procedures used for the animal experiments were approved by the Committee for the Care and Use of Experimental Animals at the Institute of Livestock and Grassland Science, NARO.

Tissue preparation

The animals were sacrificed by injection with a lethal dose of sodium pentobarbital under deep anesthesia (50 mg/kg body weight). The heads were then perfused bilaterally through the carotid arteries with 4 *l* of 10 mM phosphate-buffered saline (PBS) containing 3,000 units of heparin/m*l*, followed by a fixative containing 4% paraformaldehyde (PFA) in phosphate buffer. The goat nasal cavities were dissected in order to extract the OEs, which were then immersed overnight at 4°C in the same PFA fixative. The tissues were decalcified by submerging them in 0.45 M ethylenediaminetetraacetic acid (EDTA; pH 8.0) in PBS (pH 7.4) for at least 2 weeks at 4°C. Tissues were then soaked in a 30% sucrose solution until fully immersed and embedded in optimal cutting temperature compound. Fourteen-micron-thick serial sections were obtained using a cryostat and were dried overnight at room temperature. The sections were then maintained at −20°C until use.

To perform western blotting analysis, two goats were sacrificed using the same method as described above. The OE and olfactory bulb (OB) were dissected out and washed with PBS. Tissues were then immediately homogenized in protein homogenate buffer (100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), proteinase inhibitor cocktail; Roche, Mannheim, Germany). After removing the debris by centrifugation, the protein extract was stored at −80°C until use.

Observation of the structure of the nasal cavity

Tissues from two goats were used in an experiment to make observations of the structure. The sections were stained with methyl green-pyronin Y (Muto Pure Chemicals, Tokyo, Japan) for 15 min at room temperature and then washed with water and cover slipped using Entellan new (Merck, Darmstadt, Germany) as the mounting medium. The sections were observed by taking photographs using a microscope (ECLIPSE E800M; Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (AxioCam HRC; Zeiss, Jena, Germany).

OMP immunohistochemistry

To clarify the specificity of the anti-OMP antibody in goat tissue, western blotting was performed. The protein extracts of the goat OE and OB were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the fractionated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, U.S.A.). The membranes were treated with PBS containing 10% BlockAce (Dainippon Pharmaceutical, Osaka, Japan) and 0.1% Tween 20 for 1 hr at 4°C. After washing in 0.1% Triton X-100 in PBS (PBST), anti-OMP antibody (1:3,000) [20] was reacted with the membrane overnight at 4°C. After washing in PBST for 15 min, alkaline phosphatase (AP)-conjugated anti-rabbit IgG (1:5,000; Promega, Madison, WI, U.S.A.) was incubated with the membranes for 1 hr at room temperature. After washing in PBST, the hybridized protein bands were visualized using a Western-blue-stabilized substrate (Promega).

Sections were treated with PBST and 3% H_2O_2 in methanol for 30 min. The sections were then reacted with anti-OMP antibodies (1:1,000 dilution) and 1% normal goat serum for 18 hr at 4°C. After washing in PBST, the sections were reacted with biotinylated goat anti-rabbit IgG (1:800 dilution; Vector Laboratories, Burlingame, CA, U.S.A.) for 1 hr at room temperature

Table 1. Information of genes investigated in this study

Gene	Primer sequences	Direction	Size (bp) (PCR fragment)	GenBank accession No.	% fo Seq. homology (>90%)	Counterpart in mice (expressing zone(s))
OMP	GCCTTCGCAGCTCAGCATGC	F	393	AB274831	100 (goat *OMP*)	*OMP* (zone 1–4)
	ACCTTGCGGATCTTGGCCAGGTC	R				
NQO1	TATGCCATGAACTTCAATCC	F	688	XM_005692193	100 (goat *NQO1*)	*NQO1* (zone 1)
	TTTGATCTGGTTGTCCATTGG	R				
OCAM	GGTGAATCTAAATTCTTCACATGT	F	558	XM_013966137	100 (goat *NCAM2/OCAM*)	*OCAM* (zone2–4)
	GGCGTTAAAGGATTTCTGAGGCAT	R				
OR151	ATCTACTCTGTCACCATGGT	F	780	LC314440	97 (cow *OR151*)	*Olfr160* (zone 1)
	ATTCCTCAAGCTGTAGATCAG	R			90 (cow *OR8A1*)	
OR4X2	ATCCAGAGGTGCAGAAAGTC	F	837	LC314441	96 (cow *OR4X2*)	*Olfr1269* (zone2)
	CATGGCCTTCTTCACTTCAGC	R			95 (cow *OR4S1*)	
OR10A4	ACGTCCTCATAATCCTGGTTAC	F	687	LC314442	97 (cow *OR10A4*)	*Olfr10* (zone3)
	TGCTCTCAGGAGAGTTGCTG	R				
OR5H6	TTGTTCTCACAGGACTAAAGT	F	766	LC314443	97 (cow *OR5H6*)	*MOR183-2* (zone4)
	GATTGGTGTGGATCCAGGACGAT	R				

and avidin-biotin complex solution (15 μl/m*l* PBST; Vectastain Elite ABC kit; Vector Laboratories) for 1 hr. The sections were then immersed in 50 mM Tris-HCl (pH 7.5), followed by reaction with the chromogen solution consisting of 0.04% 3,3′-diaminobenzidine and 0.0026% H_2O_2 in 50 mM Tris-HCl (pH 7.5) for 5 min.

Cloning of genes

cDNA was obtained by reverse transcription using total RNA derived from the goat OE as a template. Polymerase chain reaction (PCR) was performed using goat-specific primers for *OMP*, *NQO1* and *OCAM*. Because the complete OR repertoire has not yet been published in goats, we designed specific primers for several ORs based on sequences in cattle (*Bos taurus*), a species phylogenetically close to goats [14]. Since previous studies in mice have reported that *Olfr160*, *Olfr1269*, *Olfr10* and *MOR183-2* are expressed in zones 1, 2, 3 and 4, respectively [8, 9], we searched for homologs of each OR in cattle using the nucleotide search tool in BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The cow ORs corresponding to those in mice were *OR151*, *OR4X2*, *OR10A4* and *OR5H6*, respectively. Then, specific PCR primers were generated, derived from the coding regions of these ORs. Information relating to the PCR primers is shown in Table 1. Each PCR fragment was inserted into the pTA2 Vector (Toyobo, Osaka, Japan), and nucleotide sequences were determined using a DNA sequencing service (Eurofins Genomics, Tokyo, Japan).

In situ hybridization (ISH)

Digoxigenin (DIG)-labeled riboprobes were prepared according to the method described by Wakabayashi *et al*. [22]. The sections were submerged in 4% PFA/PBS solution at room temperature for 5 min. After washing with PBS, the tissues were treated with proteinase K (10 μg/m*l*) in PBS solution at 37°C for 30 min, 4% PFA/PBS for 10 min at room temperature, 0.2 N HCl for 20 min, and hybridization solution (50% formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/m*l* tRNA, 1× Denhardt's Solution, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA [pH 8.0]) for 30 min at room temperature. The sections were then hybridized overnight by probes in hybridization solution at 60°C. After hybridization, the sections were washed with 5× saline sodium citrate (SSC) for 30 min at room temperature, 50% formamide containing 5× SSC at 50°C for 30 min, and TNE buffer (10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA (pH 8.0)) for 5 min. Then, sections derived from four goats were incubated in ribonuclease A (10 μg/m*l*) in TNE buffer at 37°C for 30 min and washed with 2× SSC for 30 min at 50°C, 0.2× SSC for 30 min at 50°C, and TNT buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) for 5 min at room temperature. In order to detect DIG-labeled probes, an AP-conjugated anti-DIG fab fragment antibody (1:500; Roche) was reacted with the sections for 60 min at room temperature. After washing with TNT buffer, the tissue sections were reacted with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) as a chromogen; the sections were then analyzed, and images were taken using a microscope (Nikon Eclipse E800).

RESULTS

General structure of the goat nasal cavity

The midsagittal view of the goat nasal cavity is shown in Fig. 1A. There are four endoturbinates in the goat nasal cavity (indicated as I, II, III and IV in Fig. 1A); the darker area seen on the posterior part of each endoturbinate involved the OE. The structure of the endoturbinates was fairly similar to those in sheep and rodents [1, 2]. Tissue blocks that included the OE were dissected from the goat nasal cavity; the frontal and lateral views of the dissected tissue are shown in Fig. 1B and 1C, respectively.

After staining with methyl green-pyronin Y solution for optimum visualization (Fig. 2), rostral-caudal structural variations

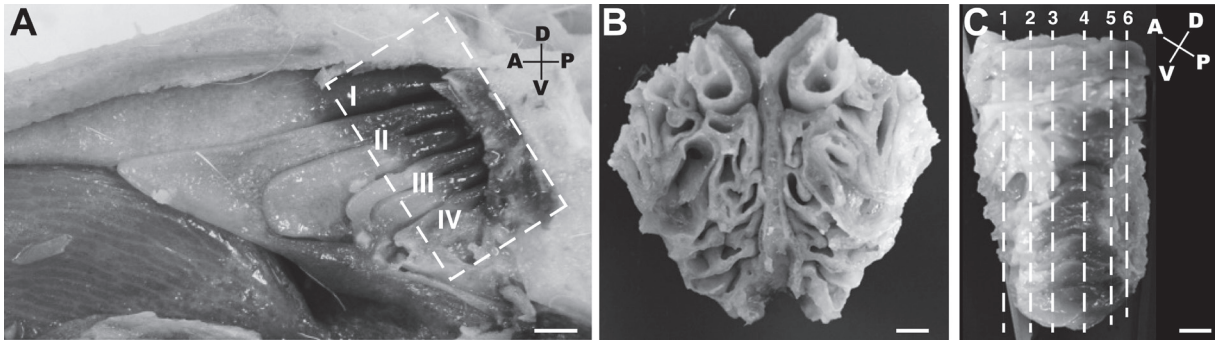

Fig. 1. Structure of the goat nasal cavity. (A) Midsagittal view of the goat nasal cavity showing the four endoturbinates (I–IV). The square (with the dashed border) indicates the location from which the tissue was dissected for use in the experiment. (B) Frontal view of the goat nasal cavity. (C) Lateral view of the goat nasal cavity with the OE; the broken lines correspond to the approximate positions of representative sections taken from the anterior to posterior regions (see Fig. 2). A: anterior, P: posterior, D: dorsal, V: ventral. Scale bars=5 mm.

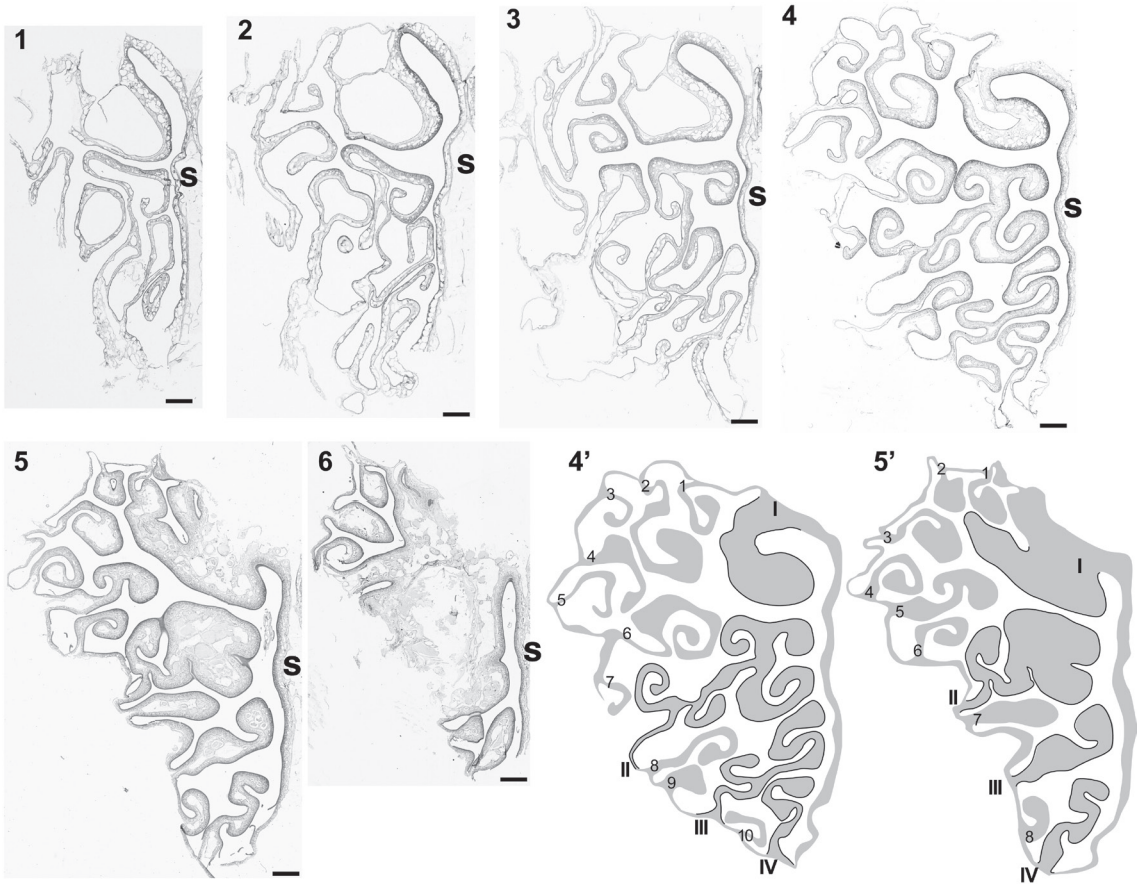

Fig. 2. Representative sections of the goat nasal cavity from its anterior (1) to posterior (6) extent, stained with methyl green-pyronin Y. Each section corresponds to the approximate position highlighted in Fig. 1C (broken lines). (4') and (5'), are traces of section nos. 4 and 5, respectively. The endoturbinates and ectoturbinates are indicated by Roman (I–IV) and Arabic (1–10) numerals, respectively. Thick lines indicate surface areas of endoturbinates. S=septum. Scale bars=2 mm.

were depicted in those sections spanning the anterior to posterior extent of the whole nasal cavity. The most anterior section had a relatively simple structure (data not shown). As the section position advanced toward the posterior, the structure was gradually enlarged in both the ventral and lateral directions. The goat nasal cavity had a complex structure, consisting of branched turbinates characterized by numerous scrolls and folds (Fig. 2-4 and 2-5). Four well-developed endoturbinates were observed (Fig. 2-4' and 2-5'; Roman numerals, solid lines) in the goat nasal cavity. Multiple ectoturbinates were also observed, although the exact number depended on the position of each tissue section (ten ectoturbinates in Fig. 2-4 and 2-4', eight in Fig. 2-5 and 2-5'; Arabic

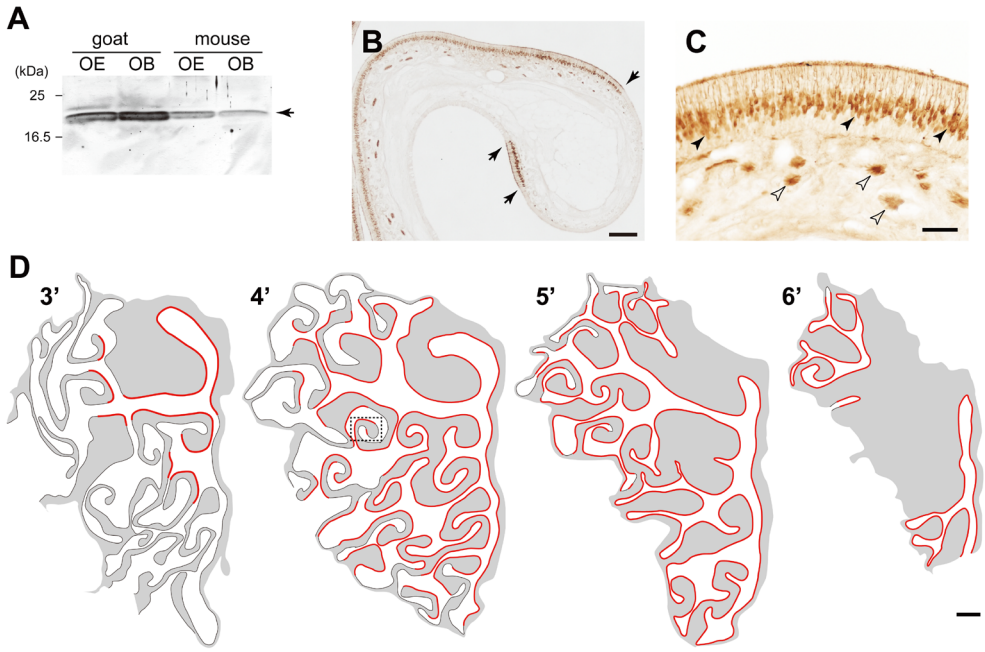

Fig. 3. Distribution of the olfactory epithelium (OE) in the goat nasal cavity. (A) Western blotting of the goat OMP. Extracts from the goat OE and OB and the mouse OE and OB were used. A single band was detected in each lane. The molecular weight of the bands was approximately 19 kDa (arrow). The molecular weights of marker proteins are indicated on the left side. (B) and (C) Sections of the goat nasal cavity stained with anti-OMP antibodies. The borders between the OE and nonsensory epithelium are indicated by arrows in B. Somata (arrowheads) and axon bundles (open arrowheads) of OSNs were stained with anti-OMP antibodies. (D) Illustrations of the special pattern of the OMP-positive area in the goat nasal cavity (red lines). 3', 4', 5' and 6' show OMP immunohistochemistry using adjacent sections of 3, 4, 5 and 6 in Fig. 2, respectively. Most regions of the nasal cavity were covered with OMP-positive OSNs in 4', 5' and 6'. The square in 4' indicates the position of photograph in B. Scale bars=200 μm in B, 50 μm in C, 2 mm in D.

numerals). The whole structure of the nasal cavity derived from two separate goats was investigated. These individuals possessed the same structure of the nasal cavity.

Distribution of the goat OE

To investigate distribution of the OE in the goat nasal cavity, we performed immunohistochemistry using anti-OMP antibodies. First, to clarify the specificity of this antibody in goat tissues, western blotting was performed. The positive signal appeared as a single band in each protein derived from the OE and OB (Fig. 3A, arrowhead), and size of the band in each lane was approximately 19 kDa, similar to that in mice. Thus, anti-OMP antibody used in this study were expected to react specifically with goat OMP. In goats, mature OSNs located in the middle part of the OE express OMP, whereas immature OSNs found basally in the OE do not express OMP [23]. Immunoreactivity was found in cell bodies in the middle area of the OE and axon bundles of OSNs (Fig. 3B and 3C) but not in the basal area of the OE. The most anterior section of the goat nasal cavity had a relatively simple structure, with no OE (data not shown). However, the OE did appear in a small portion of the dorsal area in Fig. 3D-3' (red lines). As the section position advanced toward the posterior, the OE was gradually enlarged in both the ventral and lateral directions (Fig. 3D). In the sections shown in Fig. 3D-4' and 3D-5', almost the entire surface of both the septum and turbinates was covered with the OE.

Expression patterns of NQO1 and OCAM in the goat OE

In order to investigate the expression patterns of *NQO1* and *OCAM* in the goat OE, we performed ISH using adjacent sections for *OMP*, *NQO1* and *OCAM* (Fig. 4). First, to identify the location of the goat OE in sections, we performed ISH for *OMP* (Fig. 4A). Localization of *OMP*-expressing OSNs corresponded to that of OMP-positive OSNs (Fig. 3B and 3C). *NQO1* was expressed in mature OSNs (Fig. 4B), whereas *OCAM* was expressed in parts of immature and mature OSNs (Fig. 4C, indicated by arrowheads). These expression patterns for *NQO1* and *OCAM* in goats were similar to those described in rodents [7], and nonoverlapping expression of *NQO1* and *OCAM* was observed (Fig. 4E and 4F). The *OMP*-expressing region was completely covered by both the *NQO1*- and *OCAM*-expressing areas (Fig. 4D–F). *NQO1* was mainly expressed in the dorsomedial area (Fig. 4G and 4H; red line), whereas *OCAM* was mainly localized in the ventrolateral region of the OE (Fig. 4G and 4H; blue line). Notably, no individual differences in the expression patterns of *OMP*, *NQO1* and *OCAM* were detected in the OE.

Expression patterns of ORs in the goat OE

To examine the zonal expression of ORs in the goat OE, we performed ISH for several ORs. Based on previous studies [8, 9], we

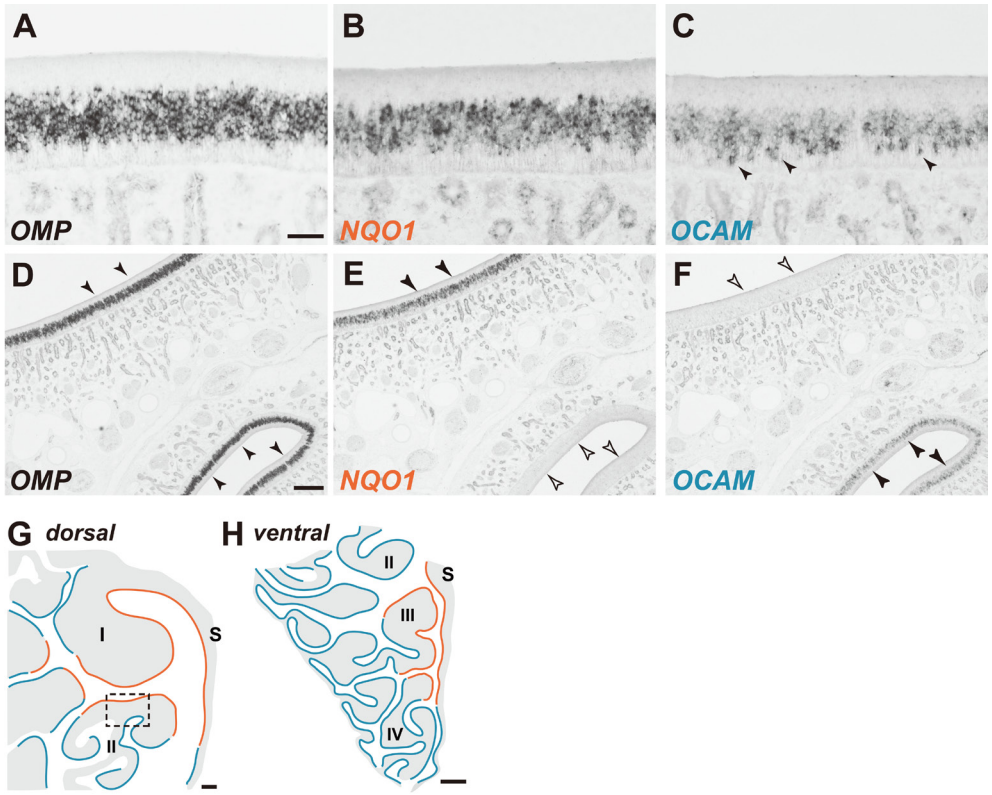

Fig. 4. Expression patterns of *OMP*, *NQO1* and *OCAM* in the goat olfactory epithelium (OE). (A) *OMP* mRNA was expressed in mature olfactory sensory neurons (OSNs). (B) The distribution of *NQO1*-expressing OSNs was similar to that of OMP-expressing OSNs. Expression of *NQO1* could not be observed in the OCAM-expressing area. (C) *OCAM* was expressed by both mature and some of immature OSNs (arrowheads). A region of *OCAM*-positive OSNs was located slightly above the basal cell layer (arrowheads). Expression of *OCAM* could not be observed in the NQO1-expressing area. (D) *OMP*-expressing OSNs were distributed across the whole OE. (E) *NQO1*-positive (arrowheads) and -negative (open arrowheads) regions are shown in the OE. (F) The position of *OCAM*-positive (arrowheads) and -negative (open arrowheads) regions. (G, H) Illustrations of the spatial distribution patterns of *NQO1*- and *OCAM*-expressing OSNs. *NQO1* and *OCAM* were expressed by OSNs in a nonoverlapping manner. Red and blue lines represent the positive regions for *NQO1* and *OCAM*, respectively. Squares indicate the position of photographs in D–F. The endoturbinates are indicated by Roman numerals (I–IV). S: septum. Scale bars in A–C=50 μm; D–F=200 μm; G and H=2 mm.

selected four ORs (*OR151*, *OR4X2*, *OR10A4* and *OR5H6*) as representative of each of the four zones found in the rodent OE. Small subsets of each OR-expressing OSN were found to be scattered on the goat OE (Fig. 5A–D; arrowheads). *OR151*-expressing OSNs were mainly located in the dorsomedial area (Fig. 5A; blue dots). *OR151*-expressing OSNs were mainly expressed on the dorsal area of the septum, medial region of endoturbinate I, and dorsomedial regions of endoturbinates II, III and IV. The dorsal parts of several ectoturbinates were also found to possess *OR151*-expressing OSNs. This overall expression pattern was reasonably similar to that of *NQO1* (Fig. 5E, red lines). The *OR4X2*-expressing OSNs were mainly adjacent to the *OR151*-expressing region (Fig. 5B; green dots), but were located on the more ventral part. We observed *OR4X2*-expressing regions on all four endoturbinates and on the medial area of most of the ectoturbinates. *OR10A4* was expressed in the lateral area of the endoturbinates and ectoturbinates (Fig. 5C; red dots). Some *OR10A4*-expressing regions overlapped with the *OR4X2*-expressing area (Fig. 5B and 5C). *OR5H6* was mainly expressed on the most ventral region of the septum and on the most lateral area of endoturbinates I–IV and ectoturbinates (Fig. 5D). It should be noted that the *OR5H6*-expressing region was never adjoined to the *OR151*-expressing area. Areas expressing *OR4X2*, *OR10A4* and/or *OR4X2*/*OR10A4* were observed between the *OR151*- and *OR5H6*-expressing areas in the goat OE. Almost all regions occupied by the *OR4X2*-, *OR10A4*- and *OR5H6*-expressing areas overlapped with the *OCAM*-expressing region (Fig. 5). We also investigate whether this zonal expression of each OR was maintained along with the anterior-posterior axis (Supplemental Fig. 1). Although the ventral part did not include an *OR151*/*NQO1*-expressing area in the anterior region (Supplemental Fig. 1A), a zonal distribution of each OR was maintained along with the anterior-posterior axis as was observed previously in rodents and primates [8, 9]. No individual differences were detected in the OR expression patterns in the OE.

DISCUSSION

In the present study, we investigated the structure of the goat nasal cavity and found it to be complex, comprising several branched turbinates. We found that the goat nasal cavity possessed four well-defined endoturbinates. Several mammalian species

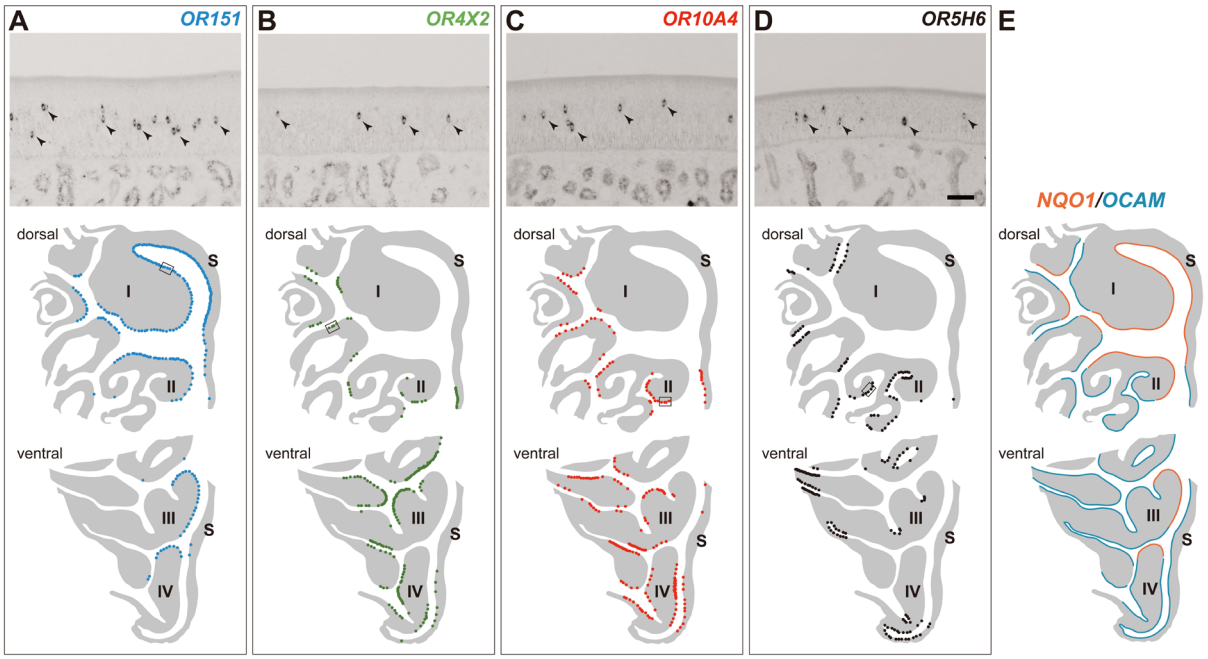

Fig. 5. Distribution of *OR151*-, *OR4X2*-, *OR10A4*- and *OR5H6*- expressing neurons in the goat olfactory epithelium (OE). (A–D, upper panels) The goat *OR151*, *OR4X2*, *OR10A4* and *OR5H6* genes, representing zones 1, 2, 3 and 4 of the OE, respectively, are expressed in a small subset of olfactory sensory neurons (OSNs; arrowheads). (A–D, lower panels) Illustrations showing the distributions of OR-expressing OSNs in representative sections. Position of *OR151*- (A, blue dots), *OR4X2*- (B, green dots), *OR10A4*- (C, red dots), and *OR5H6*-expressing OSNs (D, black dots) are shown. In some areas, the *OR4X2*- and *OR10A4*-expressing regions overlapped in one area of the OE. Squares in illustrations indicate the position of photographs in A–D. (E) Illustration of the expression patterns of *NQO1* (red lines) and *OCAM* (blue lines) in the adjacent sections. The endoturbinates are indicated by Roman numerals (I–IV). S: septum. Scale bar in D=50 μm.

also have a similarly complex structure [1–3, 10]. For example, mice, dogs and sheep possess four endoturbinates in their nasal cavity [1–3]. In addition, ectoturbinates are located within the lateral wall of the nasal cavity in the goat, similar to other mammalian species [1–4]. This complex structure of the nasal cavity in some mammals is absent in primates [4], which instead have unbranched endoturbinates. These results therefore indicate that the structure of the goat nasal cavity is similar to that of rodents and the majority of mammalian species, but differs from that of primates. This structural complexity of the nasal cavity may be linked to the degree to which the sense of smell is developed in different species groups.

We performed ISH for *NQO1* and *OCAM* as markers of the zonal organization of the goat OE in the present study and observed a non-overlapping expression pattern for *NQO1* and *OCAM*, with preferential distribution in the dorsomedial and ventrolateral areas, respectively. In rodents, region in which *NQO1* was expressed has also been found to be completely separated from that for *OCAM* [7]. Similarly, *NQO1* and *OCAM* are mainly expressed in the dorsomedial and ventrolateral areas, respectively [7]. Our results, together with those from previous reports in rodents, indicated that the distributions of these genes within the OE may be conserved between mammalian species. Although the precise functions of NQO1 and OCAM in the OSNs are unknown, NQO1 and OCAM have also been detected in the axons of rodent OSNs. NQO1- and OCAM-expressing OSNs extend their axons to the dorsomedial and ventral regions of the OB, respectively [7]. Further analysis is needed in order to clarify the projection patterns of NQO1- and OCAM-positive axons in the goat OB.

In the present study, we performed ISH for several ORs in order to determine the zonal organization of the goat OE. ORs comprise a multigene family, containing over 1,000 genes in some mammals, including cows [13, 14]. Each OR probe in the goat OE detected only a small subset of OSNs, indicating that a single OSN may express only a single OR gene selected from the large group of OR gene repertoires [18].

The distribution of each OR-expressing OSN had a clear topographical preference along the dorsal-ventral and medial-lateral axes; the goat OE appeared to be organized into four distinct zones. These results indicate that the zonal organization of the goat OE is similar to that of rodents, but not primates. In rodents, the expression of ORs has been shown to partly overlap near the boundaries between the conventional olfactory zones [9, 21]. The OE of macaque monkeys has only two broad zones, and several ORs that are mainly expressed in the dorsal zone extend over the boundary defined by the presence or absence of *OCAM* into the ventral zone [8]. In the present study, zones 1 and 4, located in the most dorsomedial and ventrolateral areas, respectively, were found to be clearly distinct from other zones. This was in contrast to the indistinct border between zones 2 and 3. Moreover, these latter two zones partly overlapped with each other in the goat OE. Taken together, these results suggest that the borders between OE zones in mammals are more indefinite than initially expected [9, 12, 21]. We could not rule out the possibility that several of

the ORs detected by our probes may have caused this overlap, suggesting that further analysis will be needed.

Primates have a small number of functional ORs (approximately 300 genes) in their OR repertoire, comprising only two broad zones in their OE, and the structure of their nasal cavity is fairly simple. In contrast, rodents possess a large number of functional OR genes (over 1,000 genes) [13, 14] and have a complex four-zone OE structure. Although the complete OR repertoire has not yet been analyzed in goats, the number of functional ORs it contains may be similar to that of cattle (1,186 genes), a species phylogenetically close to goats [14]. In the present study, the nasal cavity of goat was observed to be complex and OE was organized into four distinct zones defined by the expression pattern of each OR. It is speculated that the degree of structural complexity of the mammalian nasal cavity, as well as its OE organization, is concomitant with the number of functional ORs in the repertoire of each species. It is fascinating to investigate the elephant olfactory system because this species possesses the largest repertoire of ORs in mammals (1,948 functional ORs) [14]. More comparative studies using several mammalian species will be needed to fully understand the biological significance of the zonal organization of the OE during the course of mammalian evolution.

In conclusion, we investigated the structure of the nasal cavity and zonal organization of the OE in Shiba goats. This species was found to possess a well-developed nasal cavity structure and had an OE that was divided into four zones defined by the expression patterns of individual ORs. This is the first report investigating the distribution of the OE in the nasal cavity and showing the presence of four distinct zones defined by OR expression in ungulates. This well-developed structure of the nasal cavity and four-zonal organization in the goat OE were fairly similar to those observed in rodents. This implies that these features are highly conserved between mammals and may provide fundamental roles in discriminating between the numerous odor molecules in the environment.

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