Characteristics of Olfactory Organs in Sika Deer (Cervus nippon)

Kazuei MATSUBARA¹* , Shugo AKAOGI¹, Shoko NAKAMUTA², Tsunenori TSUJIMOTO³ and Nobuaki NAKAMUTA²

¹) Department of Animal Sciences, Graduate School of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
²) Laboratory of Veterinary Anatomy, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
³) Morioka City Zoological Park, 60-18 Shimoyakida, Shinjo, Morioka, Iwate 020-0803, Japan

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ABSTRACT

In this study, an immunohistochemical analysis was conducted to clarify the distribution of G proteins in the olfactory organ of sika deer (Cervus nippon). A Gαs/off antibody was found to label the free border of the olfactory epithelium and a Gαi-2 antibody labeled the free border of the vomeronasal sensory epithelium; however, a Gαo antibody positive reaction was not observed at any epithelial free border. These results indicate that olfactory receptor cells expressing odorant receptors are distributed in the olfactory epithelium of the deer and those expressing type 1 vomeronasal receptors are distributed in the vomeronasal organ. Therefore, it is suggested that the same olfactory receptor families are expressed in the olfactory organ of sika deer as in many mammals and that sika deer has a similar olfactory system to those many mammals.

Key words: G protein, sika deer, vomeronasal organ

INTRODUCTION

Sika deer (Cervus nippon) is a ruminant belonging to the order Artiodactyla, family Cervidae, genus Cervus. In Japan, the species exists as contiguous or isolated populations from Hokkaido to Honshu, Shikoku, Kyushu and Yakushima [1]. Herbivorous animals, including deer, have well-developed sensory organs, such as visual, auditory, olfactory and taste organs, to enable the rapid detection of predators. One of the means of achieving such detection is the ability to discriminate the excrement of other species. Footprints, odors and excrement are environmental cues that notify herbivores and carnivores of the presence of each other; these cues also play a role for recognition of other intraspecific individuals and territories [2, 3]. Apfelbach et al. [4] reported that herbivores show an aversive reaction to odors originating from carnivores regardless of whether these carnivores are sympatric or allopatric. Pork et al. [5, 6] examined the vomeronasal organ and accessory olfactory bulb of Korea’s roe deer (Capreolus pygargus) histologically and reported the morphological features of the olfactory mucosa. However, the olfactory organs of sika deer have not been fully characterized and to date there are no detailed reports on sika deer olfaction.

In general, olfactory organs contain olfactory cells and vomeronasal sensory cells that respectively bear cilia and microvilli at the tip of the dendrites and express olfactory receptors that bind to chemical substances. Olfactory receptors have been shown to be members of the G protein-coupled receptor family: this family includes odorant receptors that bind Gαolf, type 1 vomeronasal receptors that bind Gαi-2, and type 2 vomeronasal receptors that bind Gαo [7, 8]. Gαolf is expressed in the olfactory epithelium of all mammals, whereas G protein expression in the vomeronasal organ differs among species. In some animals including mouse, rat, rabbit and opossum (Didelphimorphia), cells in the vomeronasal organ expressing Gαi-2 and Gαo are situated in the superficial and deep layers of the sensory epithelium, respectively [9,10].
By contrast, in many mammals, cells in the vomeronasal organ express Gαi-2 but not Gao [10, 11]. However, the range of mammalian species studied to date is limited and it includes mostly livestock and laboratory animals. The olfactory epithelium detects so-called “odorants” and transmits signals to the main olfactory bulb, whereas the vomeronasal organ detects pheromones and transmits signals to the accessory olfactory bulb [12]. Vomeronasal type-1 receptors (V1Rs), which are mainly expressed in the vomeronasal organ, are also partially expressed in the olfactory epithelium of ruminant livestock [13]. Therefore, olfactory sensory function seems to be achieved by a complicated interaction of multiple organs. Olfactory epithelium and a vomeronasal organ have been reported in deer [5, 6, 14], but it is not clear which olfactory receptors are expressed in these organs.

In the present study, the expression of the G protein α subunit in the olfactory organs of sika deer was examined immunohistochemically; additionally, the olfactory receptor cells in the olfactory epithelium and the vomeronasal organ of sika deer were characterized.

**MATERIALS AND METHODS**

Five female sika deer (49–56 kg, age unknown) kept at Iwate University for research use were utilized in January. This study has been approved by the Iwate University Animal Experiment Committee (No. A201402) and conducted in accordance with the Iwate University Animal Experimental Rules. Animals were anesthetized using MMB (medetomidine hydrochloride 0.02 mg/kg, midazolam 0.10–0.30 mg/kg, butorphanol tartrate 0.04–0.2 mg/kg), and slaughtered under deep anesthesia. After decapitation, heads were perfused with 10% formalin fixative solution (5 L/animal) bilaterally from the carotid arteries. Subsequently, the olfactory epithelium and the vomeronasal organ were dissected from the head (Fig. 1), and immersed en bloc in a 10% neutral buffered formalin fixative solution. Thereafter, tissues were decalcified in 10% EDTA·2Na solution until bone tissues became softened (approximately for 2 weeks).

In accordance with the conventional method, formalin-fixed tissues were washed with water, dehydrated with an ascending ethanol series, substituted with xylene, and embedded in paraffin. Sections (7 µm thick) were cut with a microtome, adhered to glass slides and dried overnight. The sections were then stained with hematoxylin-eosin (HE) and analyzed using an optical microscope.

Some of the formalin-fixed tissues were washed with water and then cryoprotected with 20% sucrose/0.1 M phosphate buffer (PBS). The tissues were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and 15 µm sections were cut using a cryostat.

Primary antibodies against Gαs/olf (Santa Cruz, USA, sc383), Gαi-2 (Merck, Germany, 05-1403), and Gao (Millipore, MAB 3073) were used here. The Gαs/olf antibody is a rabbit polyclonal antibody against the C-terminal peptide of rat Gαs/olf. The Gαi-2 antibody is a mouse monoclonal antibody against recombinant Gαi-2 protein. The Gao antibody is a mouse monoclonal antibody against bovine brain Gao protein. The Gαs/olf antibody was diluted to 1:1000 with PBS containing 1% bovine serum albumin (BSA-PBS); the Gαi-2 and Gao antibodies were diluted to 1:2000. Biotin-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 711-066-152, West Grove, PA, USA) and donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, 715-066-151) were
used as secondary antibodies at 1:1000 and 1:500 dilutions, respectively.

Frozen sections were washed with PBS containing 0.1% Triton X-100 and immersed in methanol containing hydrogen peroxide for 30 min. After washing, sections were incubated with PBS containing 2% normal donkey serum for 30 min, followed by the primary antibody overnight at 4℃. After washing, they were incubated with the secondary antibody for 1 hr. The sections were then incubated in ABC solution (Vector Laboratories, PK-6100, Burlingame, CA, USA) for 45 min, washed and colorized with 0.05 M Tris-HCl buffer containing diaminobenzidine as a substrate. After nuclear staining with hematoxylin, coverslips were placed over the sections, and analyses were performed using an optical microscope. All procedures were carried out at room temperature unless otherwise stated.

RESULTS

Histological images of HE-stained paraffin sections of the deer olfactory epithelium and the olfactory organ are shown in Figs. 2-4. The olfactory epithelium contained olfactory cells, supporting cells, and basal cells (Fig. 2). From the free border to the basement membrane, the following order of cells was observed: nuclear band of supporting cells, nuclear band of olfactory cells, nuclear band of basal cells and basement membrane. Bowman’s glands, olfactory nerve bundles and blood vessels were distributed in the lamina propria of the olfactory epithelium. No differences in histological appearance were observed between the five individuals examined.

In the vomeronasal organ, medially situated sensory epithelium and laterally situated non-sensory epithelium faced each other across the lumen (Fig. 3). The sensory epithelium contained vomeronasal sensory cells, supporting cells and basal cells (Fig. 4C). From the free border toward the basement membrane (Fig. 4C), the following order of cells was observed: mucus layer, nuclear band of supporting cells, nuclear band of vomeronasal sensory cells, and nuclear band of basal cells (Fig. 4C). The vomeronasal nerve bundles and blood vessels were intermingled in the lamina propria of the sensory epithelium (Fig. 4A). By contrast, the non-sensory epithelium contained ciliated cells and basal cells (Fig. 4D). The order of cells from the free border to the basement membrane was mucus layer, nuclear bands of ciliated cells, basal cells, and basement membrane (Fig. 4D). The luminal openings of the ducts of Jacobson’s glands (Fig. 3A) were scattered near the junction of sensory and non-sensory epithelia. Jacobson’s glands and large
Fig. 3  HE-stained image of the sika deer vomeronasal organ. A, ventral part; B, dorsal part. SE, sensory epithelium; NSE, non-sensory epithelium; BV, blood vessels; N, vomeronasal nerve bundles; JG, Jacobson’s glands. Asterisks, lumen of the vomeronasal organ; arrowheads, luminal opening of Jacobson’s glands.

Fig. 4  HE-stained images of the sensory and non-sensory epithelia in the sika deer vomeronasal organ. A, sensory epithelium; B, non-sensory epithelium; C, enlarged view of the vomeronasal sensory epithelium. 1, free border; 2, nuclear zone of supporting cells; 3, nuclear zone of sensory cells; 4, nuclear zone of basal cells; 5, lamina propria. D, enlarged view of the non-sensory epithelium. 1, free border; 2, nuclear zone of ciliated cells; 3, nuclear zone of basal cells; 4, lamina propria. VC, vomeronasal sensory cells; SC, supporting cells; CC, ciliated columnar cells; BC, basal cells; JG, Jacobson’s glands; N, vomeronasal nerve bundles; BV, blood vessels. Dashed line, basement membrane; asterisk, lumen of the vomeronasal organ.
blood vessels were present in the submucosal tissue underlying the non-sensory epithelium (Fig. 4B). A large number of blood vessels, of greater size than those under the sensory epithelium, were distributed below the non-sensory epithelium (Fig. 3).

Frozen sections of the olfactory epithelium (Fig. 5) and the vomeronasal organ of sika deer (Fig. 6) were analyzed after immunohistochemical staining for Gαolf/s, Gαi-2, and Gαo.

No differences in immunohistochemical staining were observed between the five individuals examined. In the olfactory epithelium, the anti-Gαolf/s antibody intensely labeled the mucus layer (Fig. 5A, D) and the olfactory nerve bundles (Fig. 5A). However, the anti-Gai-2 antibody showed no positive reaction in the olfactory epithelium (Fig. 5B, E). The anti-Gαo antibody intensely labeled the cell body of sensory cells (Fig. 5C, F) and the axons, but showed no staining in the free border.

In the vomeronasal sensory epithelium, the anti-Gai-2 antibody intensely labeled the free border (Fig. 6B, E) and the axons, but showed no staining in the free border. In the vomeronasal sensory epithelium, the anti-Gai-2 antibody intensely labeled the free border (Fig. 6B, E), and faintly labeled the cell body of vomeronasal sensory cells (Fig. 6B) and the axons (Fig. 6B). The anti-Gao antibody intensely labeled the cell body of vomeronasal sensory cells (Fig. 6B) and the axons (Fig. 6B). The anti-Gao antibody intensely labeled the free border (Fig. 6B, E).
labeled the axons (Fig. 6C, F), but did not stain the free border. The anti-Gαolf/s antibody did not label the vomeronasal sensory epithelium (Fig. 6A, D).

**DISCUSSION**

Macroscopic and microscopic analyses showed that the olfactory organs of sika deer had similar anatomical features to roe deer as well to those of cattle. Flehmen, a sexual behavior displayed by deer during the breeding season, is also seen in ruminant animals such as cattle and goats. The flehmen behavior has the effect of uptake of pheromones into the vomeronasal organ through opening the vomeronasal duct and exposure to air [15]. Furthermore, in cattle, the blood vessels around the lumen of the vomeronasal organ pulse rhythmically to increase and decrease their size. This pulsation induces a negative pressure in the vomeronasal organ, which has the effect of pulling air into the vomeronasal organ like a pump [16, 17]. In the present study, large-sized and small-sized blood
vessels were found in the vomeronasal organ, suggesting that the blood vessels of the sika deer vomeronasal organ can function as a pump, similarly to cattle.

Immunohistochemical analysis of G proteins in the olfactory organ of sika deer showed that cilia in the free border of the olfactory epithelium had a positive reaction only to the anti-Gαolf/s antibody. Generally, Gaolf is a G protein that is coupled to odorant receptors [7]. In addition, the olfactory cells are the only cells in the olfactory epithelium equipped with cilia. Thus, in the olfactory epithelium of sika deer, the only G protein coupled with olfactory receptors is likely to be Gaolf. The cell body also showed a positive reaction to the anti-Gαo antibody. However, in the nervous system, Gαo is involved not only in the olfactory reception but also in axonal elongation, synaptic junctions, and intercellular communication [8]. Therefore, it is inferred that the positive reactions observed in the present study are not coupled with olfactory receptors. On the other hand, the free border of sensory cells in the vomeronasal sensory epithelium stained positively with the anti-Gαi-2 antibody, but did not stain with the anti-Gαolf/s and anti-Gαo antibodies. Since Gαi-2 is a G protein coupled with V1R [9], the only G protein coupled with olfactory receptors in sika deer vomeronasal system is likely to be Gαi-2.

In the present study, the results indicated that the olfactory epithelium of sika deer solely expressed OR-Gαolf. In many animals, the chief olfactory receptor expressed in the olfactory epithelium is OR-Gαolf [8]. Thus, it is inferred that the olfactory receptors expressed in the olfactory epithelium of the deer is similar to that of other animals. Shi and Zhang [9] suggest that V2R may be deleted in the ancestral species by genome level analysis in artiodactyls including deer. In this study, the vomeronasal organ of Sika deer only expressed V1R-Gαi-2, and expression of V2R was not observed. The vomeronasal organ of reptiles and amphibians, such as the Japanese striped snake and the African clawed frog, expresses only V2R-Gαo [18, 19]. The vomeronasal organ of some mammals including rodents expresses both V1R-Gαi-2 and V2R-Gαo [9, 10, 11], whereas the vomeronasal organ of most mammals expresses only V1R-Gαi-2 [20]. Therefore, it is speculated that expression of V1R-Gαi-2 in the vomeronasal organ occurred during evolution from amphibians to mammals.

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原著論文 解剖学

ニホンジカ（Cervus nippon）の嗅覚器の特徴

松原和衛1)*, 赤荻周悟1), 中牟田祥子2), 辻本恒徳3), 中牟田信明2)

1) 岩手大学大学院農学研究科動物科学専攻 〒 020-8550 岩手県盛岡市上田3-18-8
2) 岩手大学農学部共同獣医学科 〒 020-8550 岩手県盛岡市上田3-18-8
3) 盛岡市動物公園 〒 020-0803 岩手県盛岡市新庄下八木田60-18

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要約

本研究では, ニホンジカの嗅覚器のGタンパク質の存在を明らかにするため, 免疫組織化学的な検討を行った。その結果, 嗅上皮の自由縁にはGαs/olf抗体陽性反応, 銀鼻感覚上皮の自由縁にはGαi-2抗体陽性反応が観察され, いずれの上皮自由縁にもGαo抗体陽性反応は観察されなかった。したがって, シカの嗅上皮には匂い受容体, 銀鼻器には1型銀鼻受容体を発現した嗅覚受容細胞が分布していることを示唆する。これにより, ニホンジカの嗅覚器には多くの哺乳類と同様の受容体が発現しており, 多くの哺乳類と類似した嗅覚構造を持つことが示唆された。

キーワード: Gタンパク質, 銀鼻器, ニホンジカ

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* 責任著者: 松原和衛 (E-mail: kazuei@iwate-u.ac.jp)