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A morphological study of the vomeronasal organ and the accessory olfactory bulb in the Korean roe deer, *Capreolus pygargus*

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ABSTRACT

The vomeronasal organ (VNO) and accessory olfactory bulb (AOB) of the Korean roe deer (*Capreolus pygargus*) were studied histologically to evaluate their morphological characteristics. Grossly, the VNO, encased by cartilage, has a paired tubular structure with a caudal blind end and a rostral connection through incisive ducts on the hard palate. In the VNO, the vomeronasal sensory epithelium (VSE) consists of galectin-3-positive supporting cells, protein gene product (PGP) 9.5-positive receptor cells, and basal cells. The vomeronasal respiratory epithelium (VRE) consists of a pseudostratified epithelium. The AOB strata included a vomeronasal nerve layer (VNL), a glomerular layer (GL), a mitral/tufted cell layer, and a granular cell layer. All lectins used in this study, including *Bandeiraea simplicifolia* agglutinin isolectin B4 (BSI-B4), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA-I), and *Triticum vulgaris* wheat germ agglutinin (WGA), labeled the VSE with varying intensity. In the AOB, both the VNL and the GL reacted with BSI-B4, SBA, and WGA with varying intensity, but not with UEA-I. This is the first morphological study of the VNO and AOB of the Korean roe deer, which are similar to those of goats.

Introduction

The vomeronasal system (VNS), an olfactory system, consists of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB), the vomeronasal amygdala, and nerves connecting them all (Takami, 2002; Halpern and Martinez-Marcos, 2003; Yokosuka, 2012). The VNS is present in mammals, reptiles, amphibians (Taniguchi and Saito, 2011), but morphological and functional differences clearly exist even between closely related species such as mice and rats (Takigami et al., 2000; Tirindelli et al., 2009). Depending on the species, the VNS is related to a variety of functions, including reproduction through the perception of pheromones (Gelez and Fabre-Nys, 2004; Keller et al., 2009).

In the past several decades, the morphological characteristics of the VNOs of various animals have been studied, including those in mice (Salazar et al., 2001; Salazar and Sanchez Quinteiro, 2003), rats (Salazar and Sanchez Quinteiro, 1998; Lee et al., 2012), golden hamsters (Taniguchi et al., 1992; Taniguchi, 2008) and cats (Salazar and Sanchez-Quinteiro, 2011). Furthermore, comparative morphological studies have been conducted on the VNOs of domestic animals including horses and cattle (Lee et al., 2003; Taniguchi and Mikami, 1985), goats (Takigami et al., 2000), pigs (Park et al., 2012a) and sheep (Salazar et al., 2007). However, little is known about the VNOs of wild ruminants apart from the Scandinavian moose (Vedin et al., 2010).

The behavioral characteristics of wild roe deer have been studied under natural conditions (McLoughlin et al., 2007; Richard et al., 2008). On Jeju Island, Korea, the Korean roe deer *Capreolus pygargus* inhabits fields and meadows, particularly on Halla mountain (Han et al., 2007). Few studies have addressed their morphological characteristics apart from a genetic identification study (Han et al., 2007). Hence, as a first step to understanding the morphological characteristics of this animal, we histologically analyzed two VNS organs, the VNO and AOB of the Korean roe deer.

Abbreviations: AOB, accessory olfactory bulb; BSI-B4, *Bandeiraea simplicifolia* agglutinin isolectin B4; GL, glomerular layer; GrL, granular cell layer; M/TcL, mitral/tufted cell layer; PGP, protein gene product; SBA, soybean agglutinin; UEA-I, *Ulex europaeus* agglutinin I; VNL, vomeronasal nerve layer; VNO, vomeronasal organ; VNS, vomeronasal system; VRE, vomeronasal respiratory epithelium; VSE, vomeronasal sensory epithelium; WGA, *Triticum vulgaris* wheat germ agglutinin.

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Table 1
Description of Korean roe deer used in this study.

Age* (years)	Weight (kg)	Sex
2	20	Male
3	25	Male
3	21	Male
3	21	Male
3	23	Male
3	22	Male

* The estimated age was calculated by the number of horn branches.

Materials and methods

Tissue preparation

VNO and AOB samples from seven (six male, one female) roe deer *C. pygargus* (Han et al., 2007) were obtained from the Jeju Wildlife Rescue Center. Their ages were determined to be two to three years old, respectively (Table 1), based on the number of horn branches (spikes). For light microscopy, the VNO and AOB were removed immediately after death and fixed in 10% buffered formalin for 48 h. All experimental procedures were conducted in accordance with Jeju National University Guidelines for the Care and Use of Laboratory Animals.

Histological examination

Formalin-fixed VNOs were trimmed and decalcified in sodium citrate–formic acid solution with several changes of the solution, until the bony pieces softened as shown in our previous study (Park et al., 2012b). Then, decalcified VNO and olfactory bulbs containing AOB were dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, embedded in paraffin, and sectioned at a thickness of 5 μm. After deparaffinization, the sections were stained with hematoxylin and eosin (H&E). To visualize the AOB nerve fiber tract and strata, Kluver–Barrera staining (0.1% Luxol Fast Blue and 0.1% Cresyl violet) was applied to paraffin sections (Kluver and Barrera, 1953; Geisler et al., 2002). The morphological study of VSE and AOB was accomplished in six male roe deer because only one sample of female VNO was obtained and both the VNO and AOB in the female were histoloigcally similar those of the males.

Antibodies

To confirm the presence of receptor cells and supporting cells in the VSE, immunohistochemistry was performed using a rabbit polyclonal antibody to protein gene product 9.5 (PGP 9.5) or a rat anti-galectin-3 monoclonal antibody, respectively. The rat anti-galectin-3 monoclonal antibody was purified by affinity chromatography from the supernatant of hybridoma cells (clone TIB-166™, M3/38.1.2.8. HL.2; American Type Culture Collection, Manassas, VA, USA) and used at a final concentration of 1–5 μg/mL for immunohistochemistry. This antibody has been used to detect galectin-3 in the tissues of various animals including ungulates, cows (Kim et al., 2008), and pigs (Park et al., 2012b). Rabbit polyclonal antibody to PGP 9.5 (Biotrend, Cologne, Germany) has been used to detect receptor cells in the VNOs of humans and rats (Johnson et al., 1994; Witt et al., 2002).

Immunohistochemistry

Sections (5 μm) of paraffin-embedded tissue were deparaffinized and heated in a microwave (800 W) in citrate buffer (0.01 M, pH 6.0) for 5 min. After cooling the slides, the sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Then non-specific binding was blocked with 10% normal goat serum (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA), washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h, and then allowed to react with the rat anti-galectin-3 antibody (1:1000) for 1 h at room temperature. After washing in PBS, the sections were reacted for 45 min with biotinylated rabbit anti-rat IgG (1:100; Vector Laboratories). Rabbit polyclonal antibody to PGP 9.5 (1:800) was reacted with a biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories). After another wash in PBS, the sections were incubated for 45 min with the avidin–biotin peroxidase complex (ABC Elite Kit; Vector Laboratories) prepared according to the manufacturer's instructions. After washing in PBS, the peroxidase reaction was developed for 3 min using a diaminobenzidine substrate (DAB Kit; Vector Laboratories), prepared according to the manufacturer's instructions. Sequentially, the sections were counterstained with hematoxylin for 30 s, washed in running tap water for 20 min, dehydrated through a graded ethanol series, cleared with xylene, and mounted with Canada balsam (Sigma–Aldrich, St. Louis, MO, USA).

Lectin histochemistry

The following lectins (all of which were purchased from Sigma–Aldrich, St. Louis, MO, USA) were used: *Bandeiraea simplicifolia* agglutinin isolectin B4 (BSI-B4), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA-I), and *Triticum vulgaris* wheat germ agglutinin (WGA). Lectins are commonly used to identify receptor cells and/or their matching area in the AOB (Taniguchi et al., 1993; Salazar and Sanchez Quinteiro, 2003).

Lectin histochemistry was performed as in our previous studies (Park et al., 2012b). In brief, the paraffin-embedded VNO and AOB were sectioned to a thickness of 5 μm using a microtome. The sections were mounted on glass microscope slides, and the paraffin was removed. Then the sections were rehydrated. Endogenous peroxidase activity was blocked through 30 min incubation with 0.3% hydrogen peroxide in methanol. After three washes with PBS, the sections were incubated with either *BSI-B4*-peroxidase (diluted 1:50), SBA-peroxidase (diluted 1:100), UEA-I-peroxidase (1:200), or WGA-peroxidase (1:100) for 3 h at room temperature. Signals were developed using a DAB substrate kit (Vector). The sections were counterstained with hematoxylin before mounting. Negative controls were processed through omission of lectin during the staining procedure to clarify the reactivity of lectin histochemisty.

Results

Gross anatomy

The VNO had a prominent tubular-shaped structure about 80 mm long on the floor of the nasal cavity, adjacent to the vomer (Fig. 1, boxed area). The ducts originated from medially located openings in the rostral hard palate caudal to an incisive papilla (Fig. 1, inset, hollow arrows). From the openings, the VNO was arranged under the nasal septum bilaterally and stretched over two-thirds of the nasal cavity. The vomeronasal nerves (Fig. 1, arrows) were observed in sagittal sections through the nasal septum; they innervated the AOB (Fig. 1, circled area).

Histological features of the VNO

The morphological features of the VNO are shown at low magnification in Fig. 2A. A lamina of cartilage wrapped around the whole structure and connective tissue, glands, vessels, and nerves making up the soft tissue of the VNO were organized around the vomeronasal duct. The vomeronasal glands were most consistently found to communicate with the lumen of the VNO at two locations: