Unique blood vasculature and innervation in the cavernous tissue of murine vomeronasal organs

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ABSTRACT
The vomeronasal organ (VNO) is an accessory olfactory device related to reproductive behavior. The soft tissue of the tubular organ is composed of sensory/non-sensory epithelia and a highly developed vasculature, which in the latter the dilation and contraction of blood vessels are thought to contribute to pumping in and out luminal fluid or air, like penile erectile tissue. The present histological observation of the murine VNO revealed a more complicated vasculature than previously evaluated ones with large differences along the rostro-caudal axis. An immunohistochemical study for vasoactive substances displayed extremely dense innervation by cholinergic nerves containing nitric oxide synthase and VIP/PHI in the thick smooth muscle layer surrounding venous sinuses at light and electron microscopic levels. Furthermore, the differential distribution of cholinergic nerves and adrenergic nerves may provide a novel insight into the pumping mechanism of VNO.

INTRODUCTION
The vomeronasal organ (VNO), also called Jacobson’s organ, is the second olfactory system. The VNO, a bilateral tubular structure, is located at the basal region of the nasal cavity and enclosed by the osseous/cartilaginous capsule (Vaccarezza et al., 1981). The vomeronasal duct runs rostro-caudally in the center of the VNO, and while posteriorly closed is anteriorly open to the oral or nasal cavity. The VNO is functionally linked to intraspecies communication including reproductive events, such as sexual attraction and maternal behavior via pheromones (Halpern, 1987; Tirindelli et al., 1998; Keverne, 1999; Martin-Sánchez et al., 2015). It is known that mammary pheromones awaken rabbit neonates to induce the nipple-suckling reflex (Coureaud et al., 2010). Removal of the VNO or other damage reducing the functions and neural outputs causes the impairment of mating behavior in male animals (Winans and Powers, 1977; Meredith et al., 1980; Meredith, 1986). The VNO of adult humans, a small or rudimentary organ, persists even in sexually matured individuals, although the functional significance is still unclear (Bhatnagar and Smith, 2001; Halpern and Martinez-Marcos, 2003).

The vomeronasal duct is covered laterally and medially by a thin non-sensory and a thick sensory epithelium, respectively (Vaccarezza et al., 1981). As a pheromone-detecting organ, many researchers have focused on the structure and function of the vomeronasal sensory epithelium and connection to the accessory olfactory bulb in various animals. On the other hand, a unique and complex vasculature is developed between the non-sensory epithelium and the lateral bony and/or cartilaginous envelope, suggesting its indirect involvement in the sensory function of the VNO (Cantó and Suburo, 1998). Stimulus substances must be forced into the organ by auxiliary devices rather than simple diffusion via the narrow entrance duct. The spongy tissue containing the developed vasculature is a kind of erectile tissue similar to the penis and functions to pump in or out the luminal contents of the vomeronasal duct (Meredith et al., 1980; Eccles, 1982; Villamayor et al., 2018).
Although the accumulated findings on the vascular architecture and innervation support this pumping role, precise, detailed information seems insufficient. The present morphological and histochemical study deals with the architecture of the complicated vascular system and unique innervation by the autonomic nervous system. Previous morphological studies of the VNO have covered various mammalian species; however, information on the mouse VNO is limited. Since the mouse is one of the most useful animal species in investigations associated with a genetic approach, an exact understanding of the mouse VNO would have scientific significance.

MATERIALS AND METHODS

Animals. Eight-week-old male mice of the ddY strain were supplied by Japan SLC (Hamamatsu, Japan). For immunohistochemistry at the light and electron microscopic levels, deeply anesthetized mice were perfused via the aorta with a physiological saline, followed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The heads were removed, immersed in the same fixative for an additional 48 h, and decalcified with 5% EDTA for 3 weeks at 4°C. Unfixed fresh heads were directly immersed in Bouin’s fluid and decalcified using EDTA as mentioned above. The Bouin-fixed and decalcified heads were embedded in paraffin according to the conventional method, and dewaxed paraffin sections were stained with hematoxylin-eosin (H-E). All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Immunohistochemistry. The formaldehyde-fixed and decalcified tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections, about 14 μm in thickness, were mounted on poly-L-lysine-coated glass slides and subjected to single and double immunofluorescence staining. After pretreatment with 0.3% Triton X-100-containing phosphate buffered saline (PBS, pH 7.2) for 1 h and normal donkey serum for 30 min, the sections were incubated with antibodies against the following molecules: vesicular acetylcholine transporter (VAT, VACHT) (raised in rabbit and guinea pig; VACHT-Rb-Af670 or VACHT-GP-Af1120; Frontier Institute, Ishikari, Japan), tyrosine hydroxylase (TH) (rabbit), neural nitric oxide synthase (NOS) (guinea pig; nNOS-GP-Af740; Frontier Institute), calcitonin gene-related peptide (CGRP) (guinea pig; CGRP-GP-Af280; Frontier Institute), peptide histidine isoleucine (PHI) (rabbit; Y022; Yanaihara Institute, Shizuoka, Japan), CD31 (rat; 550274; BD Pharmingen), and LYVE-1 (rabbit; 11-034; AngioBio, Del Mar, CA). Antigen-antibody reaction was visualized by incubation with Alexa Fluor 488-labeled anti-rabbit or guinea pig or rat IgG (Invitrogen, Carlsbad, CA) or Cy3-labeled anti-rabbit or guinea pig IgG (Jackson Immunoresearch, West Grove, PA). Some of the immunostained sections were counterstained with SyTO13 (SYTOX, Invitrogen) for observation of the nuclei. The stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

For immunostaining of α-smooth muscle actin, dewaxed paraffin sections were preincubated with normal goat serum, incubated with mouse anti-α-smooth muscle actin (A2547; Sigma-Aldrich), and followed with incubation with peroxidase-labeled anti-mouse IgG. The reactions were visualized by incubation in 0.01 M Tris–HCl buffer (pH 7.6) containing 0.01% 3,3’-diaminobenzidine and 0.001% H₂O₂. The specificity of immunoreactions on sections was confirmed according to a conventional procedure, including absorption tests.

Silver-intensified immunogold method for electron microscopy. Frozen sections from the formaldehyde-fixed tissues were mounted on poly-L-lysine-coated glass slides, incubated with the rabbit anti-VAT or rabbit anti-CGRP antibody (1 μg/mL) overnight, and subsequently reacted with goat anti-rabbit IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmificated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

RESULTS

Vascularity

Conventional observation of HE-stained sections showed that a vascularized tissue, often called spongy tissue, occupied the lateral wall of the crescent-shaped vomeronasal duct in each side (arrows in Fig. 1a); near its blind end, the duct rotated at about a 90° angle. At the more rostral segment, one large and
simple blood vessel was located in the center of the lamina propria under a non-sensory epithelium, which is a typical respiratory epithelium. These vessels, venous in type, were associated with small pocket-like invaginations. As the vessels ran caudally, the central vessels branched to display complicated shapes (Fig. 1b) and were associated with irregularly thick smooth muscle layers. These imag-
The vascular tissues in the VMO were essentially double-innervated by cholinergic and adrenergic nervous systems. Especially, cholinergic fibers immuno-reactive for VAT were densely distributed in the perivascular smooth muscle layer (Fig. 2b–d, Fig. 3a). TH-immunoreactive adrenergic fibers were less abundant and gathered in the more peripheral part of the smooth muscle coat (compare Fig. 3a and 3b; Supporting Fig. a). Namely, adrenergic fibers surrounded plexuses of cholinergic fibers, implying differential distributions of cholinergic and adrenergic fibers (Fig. 3a, b), in contrast to the co-distribution of two types of autonomic nerves observed in the penile erectile tissues (Supporting Figs. c, d). VAT-immunoreactive cholinergic nerves largely corresponded to NOS-immunoreactive and vasoactive intestinal polypeptide (VIP)/PHI-immunoreactive nerves (Fig. 3c, Supporting Fig. b), suggesting the co-localization of innervation

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DISCUSSION

The most noteworthy findings obtained in the present morphological study of murine VNO are an extensively dense distribution of cholinergic nerves and differential innervation between cholinergic and adrenergic fibers in the developed smooth muscle layers. These figures differ from those observed in the corresponding smooth muscles of penile cavernous tissue. Moreover, the mouse appeared to have the most highly developed VNO among mammals, with a complicated and huge vascular space of venous sinuses.

Characteristics of vasculature

It is generally accepted that the developed vasculature of VNO, often called cavernous tissues or spongy tissues, occupies the loose connective tissue at the lateral side of vomeronasal ducts. The degree of their development varies among mammals, also
Fig. 4  Electron microscopy of VAT- and CGRP-immunoreactive nerve terminals in the perivascular smooth muscle layer. 

**a**: The gold particles showing existence of VAT immunoreactivity gather in nerve terminals among smooth muscles. 

**b**: CGRP-immunoreactive nerves are seen within the epithelium (arrow) and subepithelial regions.
differing according to the location at the rostro-caudal axis. Based on figures presented in the literature, rabbits appear to possess the most developed vascular system, composed of grouped blood vessels with large diameters (Villamayor et al., 2018), being comparable to the mouse VNO examined in the present study. In contrast, animals like the opossum, cat, and wallaby do not have such advanced venous sinuses around non-sensory epithelium (Salazar et al., 1996; Poran, 1998; Schneider et al., 2008). The spongy tissue close to non-sensory epithelium in cows, also dogs and pigs, is characterized by several large veins which are coated with the typical circular muscle layer of the tunica media (Dennis et al., 2003; Salazar et al., 2003, 2008), differing from the cavernous sinuses of rabbit and mouse VNO. Hamsters, which have been physiologically well investigated, possess comparatively developed cavernous tissue (Meredith and O’Connel, 1979). Although rat VNO appeared to have a simple large central vein (Vaccarezza et al., 1981; Garrosa et al. 1992; Cantó Soler and Suburo, 1998; Salazar et al., 1998; Barrios et al., 2014), this may be under-stimulated without considering regional differences.

While the developed blood vessels along the vomeronasal duct are veins or venous sinuses in type, they are associated with irregularly thick layers of smooth muscle, as shown in the present study. The architecture of this smooth muscle considerably differs from the tunica media seen in ordinary veins; thus, this figure has reminded researchers of its relation to penile erectile tissues. One of the differences from the penile cavernous tissues is the lack of helicine arteries. The penile helicine arteries, though small in diameter, possess a thick vascular wall with an irregular lumen and can exchange routes of the blood flow under control by autonomic innervation (Kano et al., 1987; Banya et al., 1989). An observation using corrosion casts of arterial supply in the cat VNO failed to demonstrate such specialized arteries (Salazar et al., 1997). The ordinary arteries as well as functional arteries (helicine arteries in this case) are essentially few in the cavernous tissues of VNO (cat, Salazar et al., 1996; opossum, Poran, 1998; rabbit, Villamayor et al., 2018), as confirmed in the present study. Further studies are needed to prove the existence of blood flow-exchanging devices in the VNO.

**Unique innervation**

Previous immunohistochemical studies concerning the VNO focused on the unique innervation of the vomeronasal cavernous tissue. Nerve fibers containing substance P and particularly CGRP were abundant in and beneath the non-sensory epithelium while they were scarce within the perivascular muscle layer in the mouse and rat VNO (Masuda et al., 1996; Cantó Soler and Suburo, 1998; Uddman et al., 2007). These peptides are representative peptidic transmitters— often co-existing— in primary sensory neurons, possibly derived from the trigeminal ganglion. The rich existence of CGRP/substance P-containing sensory nerves within the epithelium supports an earlier observation that cannulation of the vomeronasal duct showed some signs of irritation in cats despite deep anesthesia (Eccles, 1982).

The present study is the first to confirm the ultrastructural localization of CGRP-immunoreactive nerve terminals containing large dense granules in non-sensory epithelium of VNO. Thus, the predominant localization of CGRP/substance P-containing nerves around the non-sensory epithelium suggests their afferent perceptive function, though these peptides exert some vasoconstrictive action.

Many previous studies documented the rich existence of vasoactive substances, such as VIP, NO, noradrenalin (TH-positive), and neuropeptide Y (NPY), in the cavernous tissues of VNO. Nerve fibers containing NOS and/or VIP are numerous around blood vessels of VNO; a moderate number of them were immunoreactive for TH and NPY (Kulkarni et al. 1994; Masuda et al., 1996; Cantó Soler and Suburo, 1998; Zancanaro et al., 1999; Uddman et al., 2007). The present study largely confirmed these findings and emphasized the predominance of acetylcholine-containing nerve fibers. Usually, autonomic vasomotor nerves are controlled by both vasoconstriction and relaxation on balance. This is true in the VNO (Meredith and O’Connel, 1979). Equal numbers of the two types of nerves are distributed with the same distributional density in the penile erectile tissues (Kano et al., 1987; Supporting Fig. c, d). In the mouse VNO, however, the population of cholinergic nerves containing NOS and VIP/PHI was much higher than adrenergic ones. This dense cholinergic distribution may correspond to the heavily histochemical labeling of NADPH diaphorase and NOS in the lateral wall of venous sinuses in the middle and distal segments of rat VNO (Cantó Soler and Suburo, 1998). Interestingly, the same authors noted an asymmetric muscle layer around the vomeronasal duct, namely the lateral thicker wall and medial thinner wall; the former displayed a very large amount of nerves with NOS-immunoreactivity (possibly cholinergic), while the latter showed a predominant NPY-immunoreactivity (possibly adrenergic...
Involvement of VNO erectile tissues in olfactory function

Some vasoactive mechanisms are needed in this sequestrated and blindly-ended organ to inhale the chemical stimuli in the vomeronasal duct or flush them out. Earlier studies had focused on the changes in spongy tissue occupying the large area on the lateral side of VNO for this achievement (Hamlin, 1929; Meredith and O’Connel, 1979; Eccles, 1982). Concerning the vasculature, Hamlin (1929) first postulated a pumping mechanism which allows the inflow and outflow of mucus in the lumen. Then Meredith and colleagues (Meredith, 1977; Meredith and O’Connel, 1979) physiologically confirmed his hypothesis in hamsters that the pumping mechanism is controlled by sympathetic and parasympathetic nerves. Especially, the experimental results suggested that the suction phase of the VNO is under control of the adrenergic sympathetic system (Meredith and O’Connel, 1979; Meredith, 1994); when the volume of blood in the cavernous tissue is reduced, the lumen of vomeronasal ducts expands to draw luminal contents deep inside. In another physiological study using cats, administration of adrenaline caused outflow of mucus from the VNO. Administration of acetylcholine caused outflow of intraluminal fluids in the vomeronasal duct to flush out the lumen after the perception of a pheromone, though the glandular secretion is not attributable to the suction of external air. Furthermore, we cannot rule out the involvement of the tongue or rhythmic movements of the upper lips, analogous to the flehmen behavior in other mammals. During flehmen, the intake of breath conveys pheromone-containing air into the nasopalatine ducts, and further to the VNO (Estes, 1972; Meredith, 1994).

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Supporting Figures  

**a**: Double staining of VAT and TH in the VNO.  
**b**: Immunostaining of PHI in the VNO.  
**c, d**: Immunostaining of VAT (c) and TH (d) in the penile cavernous tissue. Nuclei are labeled with SYTOX Green in Fig. b–d.  
S: venous sinuses  
Bars = 50 μm