The Journal of Veterinary Medical Science

FULL PAPER

Anatomy

Number of olfactory receptor neurons in the Chinese soft-shelled turtle

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J. Vet. Med. Sci.
79(9): 1569–1572, 2017
doi: 10.1292/jvms.17-0326

Received: 13 June 2017
Accepted: 24 July 2017
Published online in J-STAGE: 5 August 2017

ABSTRACT. The olfactory organ of turtle consists of the upper chamber epithelium (UCE) and the lower chamber epithelium (LCE), detecting air-borne odorants and water-borne odorants, respectively. In this study, we investigated the number of olfactory receptor neurons (ORNs) in the UCE and LCE of soft-shelled turtle in order to find their possible differences among terrestrial, semi-aquatic and highly-aquatic turtles. The number of ORNs in the soft-shelled turtle was higher in the LCE than in the UCE, suggesting its close relationship to the environment the turtle lives. In addition, relative abundance of the ORNs in the LCE to the UCE varied in accordance with the size of individuals, although its functional significance remains elusive.

KEY WORDS: electron microscope, olfactory nerve, olfactory organ, *Pelodiscus sinensis*, vomeronasal organ

In many vertebrates, there are two types of olfactory organs: the olfactory epithelium and the vomeronasal organ [9]. In mammals, the olfactory epithelium and the vomeronasal organ are anatomically separated from each other. The olfactory epithelium occupies a dorsal portion of the nasal cavity, whereas the vomeronasal organ is situated at a ventral portion of the nasal cavity. In turtles, the olfactory organ consists of the upper chamber epithelium (UCE) and the lower chamber epithelium (LCE). The UCE and the LCE line the upper and lower chambers of the nasal cavity, respectively, and are generally referred to as the olfactory epithelium and the vomeronasal organ. Although the UCE and the LCE are merely divided by ridges of non-sensory epithelium, they can be distinguished from each other by the presence of associated glands in the UCE and absence of them in the LCE [6, 7]. Thus, the UCE and LCE are supposed to detect air-borne odorants and water-borne odorants, respectively [1, 8].

The olfactory receptor neurons (ORNs) contained in the sensory epithelium of the olfactory organ are bipolar cells, extending a dendrite apically and an axon basally. After leaving the epithelium, the axons of ORNs fasciculate in the lamina propria to form the olfactory nerve and project to the olfactory bulbs situated at the rostral pole of the telencephalon. In the olfactory bulbs, axons of ORNs de-fasciculate and make synapses with secondary neurons. Since axons of ORNs do not branch until they reach the olfactory bulbs [2], the number of axons in the olfactory nerve is equal to the number of ORNs in the olfactory organ. In addition, because the ORNs of turtles send axons separately from the UCE and LCE to the ventral and dorsal parts of the olfactory bulbs, respectively, the number of ORNs in the UCE and LCE can be estimated by the number of axons in the olfactory nerves individually [3].

There are nearly 300 species of turtles. Whereas some are fully terrestrial (the tortoises), most of them are semi-aquatic. Some are highly adapted for aquatic life (sea turtles and soft-shelled turtles), and they land only for egg laying. Although the number of ORNs in the UCE and LCE of terrestrial and semi-aquatic turtles has been reported [3], it has not been investigated in highly-aquatic turtles to date. In the present study, the number of axons in the olfactory nerve of the Chinese soft-shelled turtle *Pelodiscus sinensis* was investigated with the aid of light and electron microscope to clarify the number of ORNs in the UCE and the LCE, and demonstrate the changes in the relative abundance of ORNs in the UCE and the LCE during postnatal development.

MATERIALS AND METHODS

All animals were treated in accordance with the Guide for the Care and Use of Experimental Animals at Iwate University (accepted number: A201431). The Chinese soft-shelled turtles (n=6) were obtained from a turtle farm (Suntopia Shimoda, Shimoda, Japan) and processed for transmission electron microscopy as described before [4]. Briefly, animals were anesthetized

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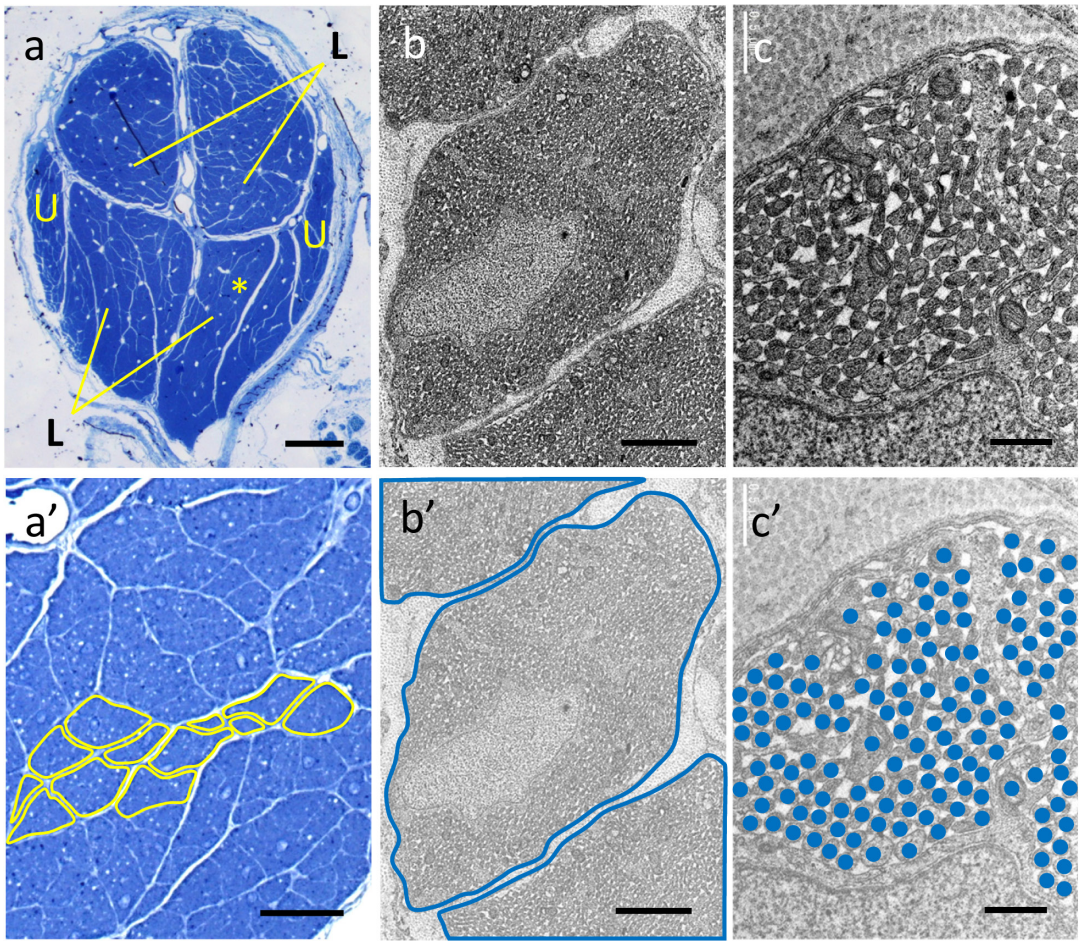

Fig. 1. (a) Toluidine blue-stained semi-thin section of the olfactory nerve. Both left and right olfactory nerves are included in this section. Dorsal region of the olfactory nerve is top. U indicates axons originating from the UCE on the lateral aspect of the olfactory nerves, and L indicates those from the LCE on the medial aspect of the olfactory nerves. (a') Higher magnification view of the area indicated by an asterisk in (a). Some of the cross-sectioned nerve bundles surrounded by the perineurium are traced by yellow lines. (b) Electron micrograph of cross-sectioned nerve bundles containing numerous axons. (b') Traced image of (b) for area measurement. (c) Higher magnification view of (b) showing axons indicated by blue dots in (c'). Scale bars, 200 μm in (a), 20 μm in (a'), 2 μm in (b) and (b'), 0.5 μm in (c) and (c').

by sodium pentobarbital (64.8 mg/kg, i.p.) and euthanized by the cardiac perfusion of Ringer's solution, followed by a fixative solution (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). Olfactory nerves were dissected and kept in the same fixative solution overnight at 4°C. Then, they were postfixed in 1% osmium tetroxide for 1 hr at 4°C, dehydrated in a graded series of ethanol and embedded in epoxy resin Epon 812 (TAAB, Aldermaston, U.K.). Ultrathin sections, stained with TI Blue (Nisshin EM, Tokyo, Japan) and lead citrate, were examined with a transmission electron microscope (JEM-2100, JEOL, Tokyo, Japan).

The number of axons originating from the UCE and LCE was analyzed as described by Matsuzaki [3] with slight modifications. Semi-thin sections (1 μm in thickness) were stained by toluidine blue and photographed with a light microscope. Axons originating from the UCE coursed the lateral aspect of the olfactory nerve and projected to the ventral part of the olfactory bulb, whereas those from the LCE coursed the medial aspect of the olfactory nerve and projected to the dorsal part of the olfactory bulb. Thus, at the middle level along the rostrocaudal axis of the olfactory nerve, axons originating from the UCE were situated on the lateral aspect, while those from the LCE were on the medial aspect of the olfactory nerves (Fig. 1a). Each portion surrounded by the perineurium was traced (Fig. 1a'), and whole areas of cross-sectioned nerve bundles in one side of the olfactory nerve were measured using ImageJ software (http://rsb.info.nih.gov/ij/). The number of axons per unit area, or the density of axons, was calculated from the area and number of axons in the electron micrographs of cross-sectioned olfactory nerves (Fig. 1b, 1b', 1c and 1c'). Finally, the density of axons was multiplied by the area of cross-sectioned olfactory nerves to obtain the total number of axons or the total number of ORNs.

RESULTS

The area of cross-sectioned nerve bundles varied among individuals depending on the body size. It was larger in the large-sized animals than in the small-sized animals. The area of nerve bundles from the UCE (6.82×10^3–73.8×10^3 μm^2) was smaller

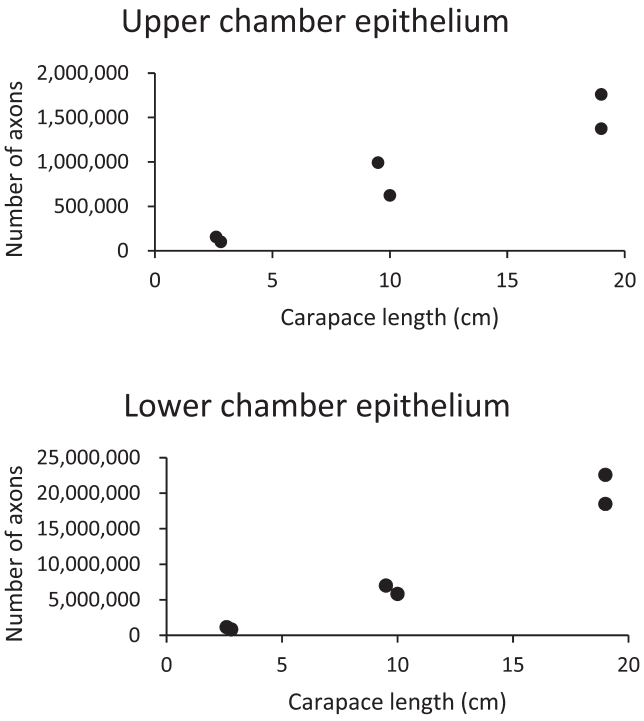

Fig. 2. Number of axons in one side of the olfactory nerve.

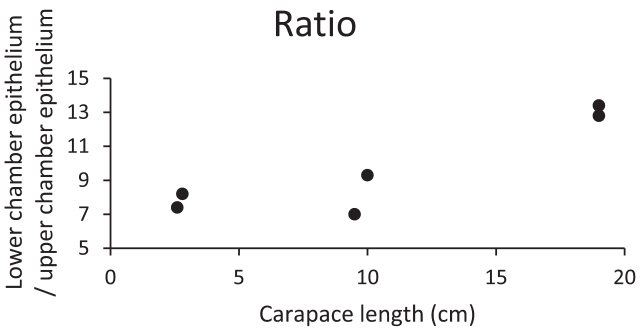

Fig. 3. Relative abundance of axons in one side of the olfactory nerve.

than that from the LCE (55.7×10^3–995×10^3 μm^2) in every specimen examined. There were no marked differences between the density of axons originating from the UCE (13.5–23.8 /μm^2) and that originating from the LCE (12.9–22.7 /μm^2) in each individual. In addition, there were no marked differences in the density of axons between individuals with different body size. Total number of axons in one side of the olfactory nerve, calculated from the area of cross-sectioned nerve bundles and the density of axons, was 0.15–1.8×10^6 for that originating from the UCE and 0.82–23×10^6 for those originating from the LCE (Fig. 2). In general, the olfactory nerves in larger animals contained larger number of axons both from the UCE and LCE. The ratio of axon number from the UCE to that from the LCE ranged from 1:7 to 1:13 (Fig. 3). Generally, the ratio was higher in large-sized animals than in small-sized animals. The results are summarized in Table 1.

DISCUSSION

In the Chinese soft-shelled turtle, the dorsal part of the olfactory bulb is far larger than the ventral one [5]. In addition, relative size of the dorsal part of the olfactory bulb to the ventral part is different between small-sized animals and large-sized animals. Since the dorsal and ventral parts of turtle olfactory bulbs are histologically similar to each other, the difference in the size of dorsal and ventral parts of olfactory bulbs reflects the difference in the number of axons they receive, i.e., the number of ORNs in the olfactory organ. A present study demonstrated that the number of ORNs in the LCE was higher than that of the UCE at any developmental stages examined. In the olfactory organ of a terrestrial turtle, four-toed tortoise *Agrionemys horsfieldii*, the UCE contains higher number of ORNs than the LCE [3]. In semi-aquatic turtles, Reeve's turtle *Meuremys reevesii* and the Japanese pond turtle *M. japonica*, the number of ORNs contained in the UCE is almost equal to that of the LCE [3]. Consequently, presence of higher number of ORNs in the LCE than in the UCE in the olfactory organ of soft-shelled turtle indicates that the olfactory organ of highly-aquatic turtles which spend much of the time in water, tends to contain higher number of ORNs in the LCE than in the UCE, suggesting that the relative abundance of ORNs in the UCE to that of the LCE reflects the differences in the environments the turtles live.

In addition to the increase in the number of ORNs in both UCE and LCE during the postnatal development, relative abundance of the ORNs in the LCE to those in the UCE changed in accordance with the size of individuals; the ratio was 1:7–1:9 in small-sized and medium-sized animals, whereas 1:13 in large-sized animals. Present results indicate that differences in the relative size of the UCE and LCE between different-sized animals are derived from the marked increase in the number of ORNs in the LCE as compared to that in the UCE, but not from the arrest of increase in the number of ORNs in the UCE nor the decrease in the number of ORNs in the UCE. Since functional significance of the differences in the relative abundance of ORNs in the UCE and LCE between different-sized animals is unknown, studies addressing the developmental changes in the olfactory receptor genes expressed in the UCE and LCE of soft-shelled turtle and studies on the terrestrial turtles which inhabit an environment different from that of the soft-shelled turtle must be undertaken.

Table 1. Summary of area, density and number of axons in one side of the olfactory nerve

Sex	Carapace length (cm)	Body weight (g)	Nerve bundles from the upper chamber epithelium			Nerve bundles from the lower chamber epithelium			Ratio (UCE:LCE)
			Area ($\times 10^3$ μm^2)	Density ($/\mu m^2$)	Axon number ($\times 10^6$)	Area ($\times 10^3$ μm^2)	Density ($/\mu m^2$)	Axon number ($\times 10^6$)	
n. d.	2.6	4.3	6.82	22.6	0.15	55.7	20.5	1.1	1:7
n. d.	2.8	5.1	7.40	13.5	0.10	63.4	12.9	0.82	1:8
n. d.	9.5	156.5	43.7	22.7	0.99	336	20.8	7.0	1:7
n. d.	10.0	163.3	35.7	17.5	0.62	347	16.8	5.8	1:9
male	19.0	1,053	73.8	23.8	1.8	995	22.7	23	1:13
female	19.0	1,087	70.9	19.4	1.4	955	19.3	18	1:13

n. d.: The sex of animals was not determined, because their gonads were still immature.

ACKNOWLEDGMENT. The authors thank Dr. Toshina Ishiguro-Oonuma in the Laboratory of Veterinary Physiology, Iwate University, for his helpful discussions.

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