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Morphological and Histochemical Study of the Nasal Cavity and Fused Olfactory Bulb of the Brown-Eared Bulbul, *Hysipetes amaurotis*

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The brown-eared bulbul (*Hysipetes amaurotis*) is commonly found in Japan where it is regarded as a harmful bird that causes damage to agricultural products. Few studies have investigated the sensory apparatus of this bird, and consequently little is known of the sensory modalities it uses. Here we analyzed the anatomical and histological properties of the nasal cavity and olfactory bulb (OB) of the bulbul in order to investigate the functional level of olfaction in this species. Although both anterior and maxillary conchae were observed in the bulbul nasal cavity, there was no structure equivalent to the posterior concha. The OB located on the ventral side of the anterior extremity of the cerebrum and the ratio of olfactory bulb size to that of the cerebral hemisphere were very small. Interestingly, the left and right OBs were completely fused at the midline of the cerebrum. Furthermore, certain types of lectins that bind to the olfactory nerve of vertebrates with a well-developed sense of smell also bound positively to the olfactory nerve and glomerular layers of the bulbul OB. These findings suggest that the brown-eared bulbul has an anatomically and functionally less well developed sense of smell compared to other avian species. Although the molecular and developmental mechanisms underlying the fusion of the OB remain unknown, we suggest that the fused OB may offer a unique model for studying the evolution and development of the central olfactory nervous system in vertebrates.

Key words: bulbul, fused olfactory bulb, nasal cavity, lectin, computed tomography

INTRODUCTION

The brown-eared bulbul (*Hysipetes amaurotis*) (Order Passeriformes, Family Pycnonotidae) is widely distributed in far eastern Russia, northeastern China, South Korea, Japan, Taiwan, and the northern Philippines (Clements, 2007). The bird is particularly common and familiar in Japan, where it acts as a disperser of the seeds of wild plants (Fukui, 1995); however, it is also harmful in Japan, causing damage to various agricultural products, including feed crops, fruits, and vegetables (Matsuoka, 1994) (also see The Wildlife Management Laboratory of the *National Agricultural Research Center*, Japan, http://narc.naro.affrc.go.jp/kouchi/chougai/wildlife/projects_e.htm).

In common with many other passeriform birds, the brown-eared bulbul is diurnal and feeds mainly on the seeds of crops and fruits. It is accordingly considered that this species principally uses vision when searching for food. Recently, in the first study of the sensory functions of the brown-eared bulbul, we reported on the histological features of its retina (Rahman et al., 2008). However, the types of sensory information that the bulbul uses in searching for food and in inter-individual communication remain unclear. A comprehensive understanding of these sensory functions would not only contribute to our knowledge of the ecology and evolution of this avian species, but would also provide important information necessary for developing measures to prevent this bird causing damage to agricultural products.

It is generally believed that avian species do not have well-developed olfactory senses; however, some birds are known to use their olfactory sense to determine the direction of flight (or flight navigation), to search for food, and to identify individuals (Papi, 1991; Roper, 1999; Bonadonna and Nevitt, 2004; Wallraff, 2004; Hagelin, 2006; Bonadonna et al., 2007; Nevitt et al., 2008). It is thought that the olfactory ability of each avian species is correlated with the olfactory bulb-brain ratio (OBBR), which is the ratio between the longest diameter of the OB and the longest diameter of the hemisphere expressed as a percentage (Bang and Cobb, 1968; Bang, 1971). For example, the OBBR of brown kiwis (*Apteryx australis*), which have the most highly developed

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olfactory sense of all birds, is 34.0, whereas in canaries and crows, for which no reports on olfactory functions have been published, the OBBR ranges from 5.0 to 6.0. Steiger et al. (2008) found that the estimated number of avian olfactory receptors is positively correlated with the OBBR. However, they also demonstrated that birds with very small OBBRs, such as canaries, have functional olfactory receptor genes and that the percentage of these is not correlated with the OBBR. These findings indicate that avian olfactory functions cannot be equated solely with the anatomical size of the olfactory systems, and suggest that even birds with a small OBBR have a limited but functional olfactory ability.

In the present study, to investigate the olfactory ability of the brown-eared bubul, and thereby assess the sensory modalities used by this bird, we analyzed the morphology of the nasal cavity and determined the histochemical properties of the OB.

MATERIALS AND METHODS

Sample preparation

The animal protocols used in this study were approved by the Care and Use of Laboratory Animals Committee at Utsunomiya University and by the Nippon Veterinary and Life Science University Postgraduate School Institutional Animal Care and Use Committee.

Brown-eared bulbuls were collected by trapping in the city of Kanuma, Tochigi Prefecture, Japan, under permission (ID. 0004) granted by the Prefectural Governor of Tochigi, Japan. The birds were collected from city areas and physically examined to rule out infections. Two adult males and one adult female with an average weight of 84 g were used in this study. The birds were deeply anesthetized by injection of sodium pentobarbital (30 mg/kg) into the cutanea ulnaris vein and then perfused transcardially with saline followed by a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3; PB). The heads of the birds were separated from their bodies and preserved in fixative solution in specimen bottles overnight at 4°C. The brains were then removed and incubated in 30% sucrose in 0.1 M PB at 4°C until they sank (Yokosuka et al., 2008). Before sectioning the olfactory bulb, we measured the longest diameters of the olfactory bulb and the hemisphere in order to calculate the OBBR. After brain removal, head specimens were preserved in fixative solution in specimen bottles at 4°C until required for examination by computed tomography (CT).

CT analysis of the nasal cavity

Head samples preserved in fixative solution (Fig. 1A) were sufficiently dehydrated and 3D images of the interior of the nasal cavity were obtained by using CT (InspeXio SMX-90CT Micro Focus X-Ray CT system; Shimadzu, Kyoto, Japan). The area of examination extended from the anterior margin of the nostril to the anterior part of the cerebrum (Fig. 1B). After CT imaging, approximately 2-mm-thick coronal sections of the beak were prepared, and the structures of the interior of the nasal cavity were analyzed (Fig. 2A). Images were taken using a commercial digital camera (Model μ1020; Olympus, Tokyo, Japan).

Histochemistry

Fluorescence-labeled lectins

Using a freezing microtome (Yamoto Koki, Saitama, Japan), serial coronal (one male) and sagittal (one female) sections of the OB (50 μm thick) were prepared from sucrose-treated brain samples for histochemical examination. Lectin histochemistry was performed with 21 fluorescence-labeled lectins (Table 1) (lectin kits FLK -3100 and -4100; Vector Laboratories, Burlingame, USA). The sections were temporarily preserved in 0.1 M PB (pH 7.4), rinsed three times with PB for 5 min, and incubated with each fluorescence-labeled lectin (1:500 in PB). The reactions were performed at room temperature for 1 h in the dark. The sections were then rinsed three times with PB for 5 min and then stained for identification of the OB structures using propidium iodide (PI; Molecular Probes, Eugene, USA) diluted 20,000 times with 0.1 M PB (pH 7.4). The sections were then mounted on poly-L-lysine (PLL)-coated slides (s7441; Matsunami, Kishiwada, Japan) and coverslipped with an anti-fade mounting reagent (Vecta HardSet H-1400; Vector Laboratories). Histological structures of the OB were observed under a fluorescence microscope (Axioskop; Zeiss, Göttingen, Germany) and a confocal laser scanning microscope (LSM 510; Zeiss). Optical sections, usually at consecutive intervals of 1 μm, were imaged through the depth of the labeled areas and saved as image stacks. Using software incorporated in the LSM 510 system, these stacks were collapsed into a single plane, thereby generating a two-dimensional reconstruction of the labeled neuron.

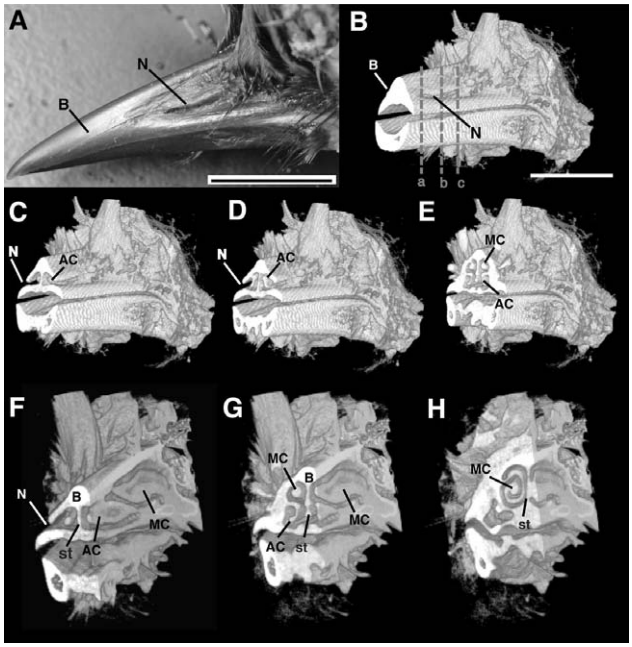

Fig. 1. Nasal cavity of the brown-eared bulbul. **(A)** Left side external view of the beak and nostril of a male bulbul. Scale bar, 10 mm. **(B–E)** Three-dimensional CT images of the interior of the beak and nostril. Dashed lines a–c in (B) indicate the transverse position of panels C–E, respectively. Scale bar, approximately 10 mm (for B–H). (**F–H)** CT images of the inner structures of the left half of the nasal cavity at angles different from B–E. AC, anterior concha; MC, maxillary concha; st, olfactory septum; B, beak; N, nostril.

Mammalian vesicular glutamate transporter 2 (VGLUT2)

Mammalian VGLUT2 immunohistochemistry was performed by using free-floating methods. Serial coronal sections of the OB from one male bird were cut to a thickness of 50 μm with a freezing microtome. These cross-sectional samples were used not only for immunohistochemistry but also for lectin histochemistry and Nissl staining (0.1% cresyl violet solution in distilled water) to study the histological structure of the OB. Sections for mammalian VGLUT2 immunohistochemistry were temporarily preserved in 0.1 M phosphate-buffered saline (PBS, pH 7.4), rinsed three times with PBS for 5 min, and then incubated in 10% Block Ace in 0.3% Triton X-100 in 0.1 M PBS (PBST, pH 7.4) for 30 min to block nonspecific binding sites. Without washing, the sections were incubated with a primary antibody against mammalian VGULT2 (HY-19, C terminus of rat VGLUT2, raised in rabbits; Sigma, Saint Louis, USA) diluted

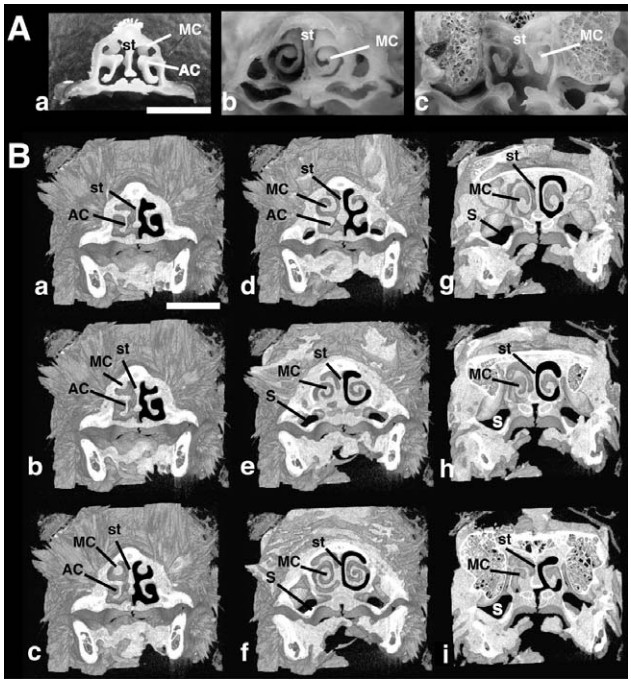

Fig. 2. Internal structure of the MC. **(A)** Transverse internal views of the nasal cavity. **(A-a)** Anterior end of maxillary concha attached to the superior wall of the nasal cavity (end of line MC). Scale bar, 5 mm (for A-a to A-c). **(A-b)** Middle and **(A-c)** posterior parts of the maxillary concha. **(B)** Semi-serial, three-dimensional transverse CT views of the nasal cavity. Maxillary concha from the anterior end **(B-b)** to the posterior end **(B-i)**. Scale bar, approximately 5 mm (for B-a to B-i). The black-filled areas in the left nasal cavity represent suppositional incoming airways. The inside structure of the MC is "scrolled" approximately twice **(B-e, B-f)**. AC, anterior concha; MC, maxillary concha; st, olfactory septum; S, sinus.

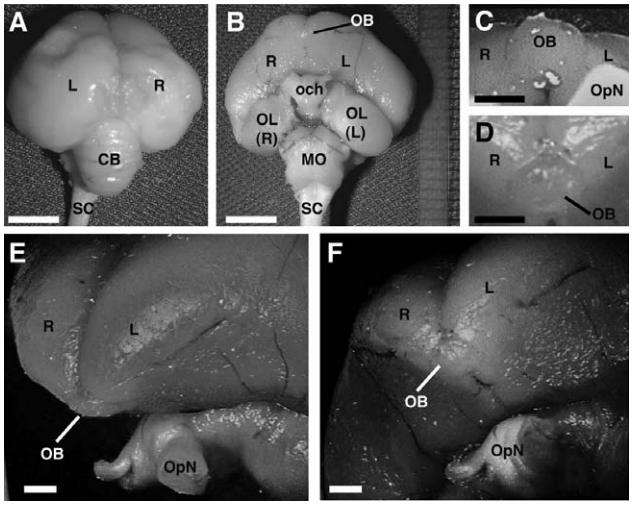

Fig. 3. Bulbul brain and olfactory bulb. **(A)** Dorsal view of the bulbul brain. The cerebrum is swollen toward the outside. Scale bar, 5 mm. (**B)** Ventral view of the brain. A small OB is located on the rostro-ventral aspect of the cerebral hemispheres. Scale bar, 5 mm. **(C–F)** High-magnification photographs of the OB. **(C)** Ventral view. Scale bar, 1 mm. **(D)** Frontal view, Scale bar, 10 mm. **(E)** Left-slanted view. Scale bar, 1 mm. **(F)** Semi-ventral view. Scale bar, 1 mm. The OB is small compared with the cerebrum, and both sides of the OB are totally fused. L, left side; R, right side; OB, olfactory bulb; och, optic chiasm; OpN, optic nerve; OL, optic lobe; CB, cerebellum; MO, medulla oblongata; SC, spinal cord.

1:2000 with 10% Block Ace in 0.1 M PBST for 60 h at 4°C. After washing in PBST, the sections were incubated overnight at 4°C with FITC or Alexa 488-conjugated anti-rabbit antibodies (Molecular Probes) diluted 1:1000 in PBST. The sections were then rinsed, counterstained with PI in PBST (1:20,000) for 5 min before being mounted, coverslipped, and observed under fluorescence and confocal laser scanning microscopes as described above. Immunohistochemical controls included substitution of the first antibody with preimmune rabbit serum (S20-100ML; Chemicon, Temecula, USA) and omission of the primary (VGLUT2) and/or secondary (Alexa 488) antibodies.

RESULTS

Nasal cavity structure

Analysis by CT imaging and gross anatomy revealed that the bulbul nasal cavity was completely separated by the nasal septum into left and right cavities (Figs. 1, 2). The anterior concha (AC) and maxillary concha (or middle concha, MC) were distinctly formed in both nasal cavities (Figs. 1, 2); however, no conchal structure corresponding to the posterior concha (or olfactory concha) (Bang, 1971; Bang and Wenzel, 1985) was observed. Therefore, the bulbul nasal cavity was shown to be segmented by the AC and MC. The MC, which occupies the main part of the nasal cavity, appears as a "pear-like" structure, with its anterior extremity attached to the superior wall of the nasal cavity (Figs. 1E, F; 2Aa, Bc). The inside structures of the MC were "scrolled" approximately two times, but were not branched (Fig. 2Ab, Bd–Bi).

Olfactory bulb (OB)

The olfactory nerves were observed as a pair (left and right) of independent bundles. The left and right olfactory nerve bundles ran to the ventral side of the anterior extremity of the cerebrum and independently projected to the "mass" of the OB located on the ventral sides of the cerebral hemisphere extremities. The bulbul OB was observed as very small lumps compared with the entire cerebrum (Fig. 3B). The olfactory bulb-brain ratio (OBBR; see Introduction) of the bulbul was 6.1–6.8 (OB diameter 1.3–1.5 mm; hemisphere diameter 21.0–22.0 mm). In terms of gross anatomy, the bulbul OB was observed as a single mass rather than a pair of independent structures (Fig. 3C–F).

Histological structure of the OB

The bulbul OB protruded anteriorly from the bottom of the cerebral hemisphere (Fig. 3). The left and right OBs appeared to be completely fused, not only at the gross anatomical level but also microscopically (Figs. 3, 4). This complete fusion was clearly confirmed in the central portion of the OB in the coronal sections (Fig. 4A). Histologically the bulbul OB could be divided into four indistinct layers: the olfactory nerve layer (ONL), glomerular layer (GL), mitral cell layer (MCL), and granule cell layer (GCL) (Figs. 4B, 6C). In the MCL and GCL, a small number of large cells (diameter ≥10 μm), which were presumed to be mitral cells, were distributed irregularly (Figs. 4B, C; 6C). Owing to this irregular

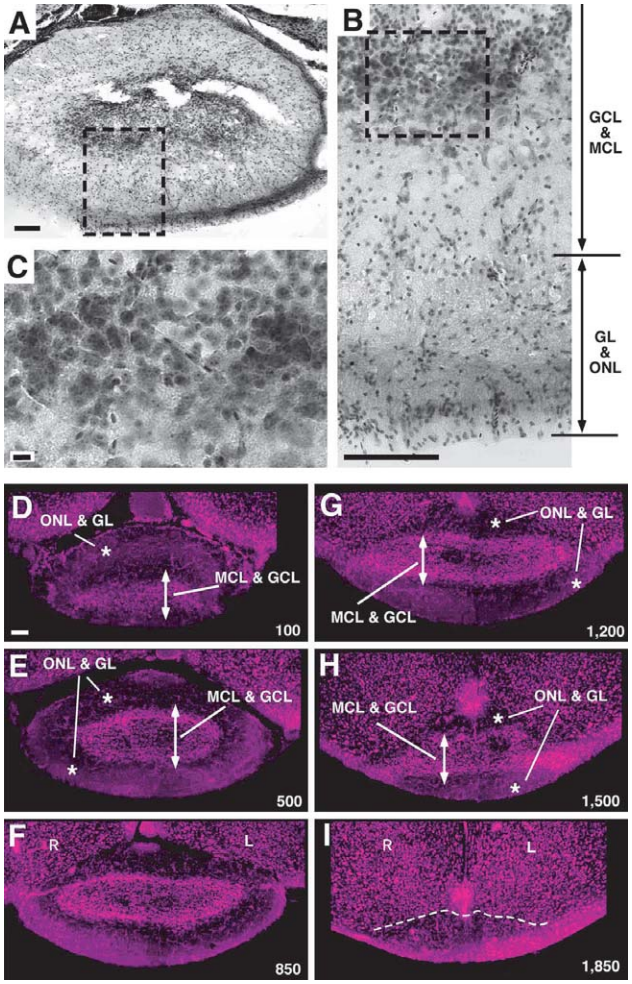

Fig. 4. Histological organization of the fused olfactory bulb. **(A–C)** Nissl-stained (cresyl violet staining) coronal section from the central portion of the "fused" OB. All layers are joined in the midline of the brain and form a "one-loop" structure in the main body of the OB. **(B, C)** High-magnification photographs of the boxed areas in A and B, respectively. **(B)** The border between the olfactory nerve layer (ONL) and the glomerular layer (GL), and the border between the mitral cell layer (MCL) and the granule cell layer (GCL), could not be clearly distinguished by cresyl violet staining. **(C)** Large cells with a diameter of 10 μm or greater, which are presumed to be principal neurons of the OB (mitral cells and tufted cells) are distributed in the MCL and GCL. **(D–H)** Propidium iodide (PI)-stained transverse sections through the rostral **(D)** to caudal **(I)** levels of the OB. In the rostral portion **(D)**, both sides of the glomerular layer have already fused. Scale bar, 100 μm. In the central portion **(E–G)**, all layers of the OB are joined at the midline of the brain and form a "one-loop" structure. This "one-loop" structure continues in the caudal portion of the OB **(H)**. **(I)** In the most caudal portion, the "one-loop" structure almost disappears. The dotted line in (I) indicates the border between the cerebrum and the OB. Distances from the most anterior position are indicated in parentheses at the lower right (μm). ONL, olfactory nerve layer; GL, glomerular layer; MCL, mitral cell layer; GCL, granule cell layer; L, left side cerebrum; R, right side cerebrum.

distribution of the mitral cells, the external plexiform layer (EPL) and the internal plexiform layer (IPL), which are present on the upper and lower sides of the MCL in the mammalian main OB (MOB), could not be clearly identified

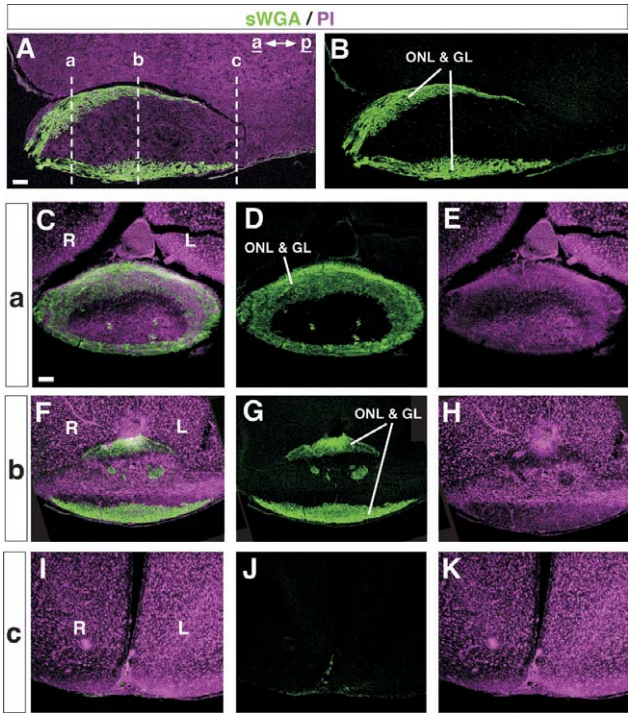

Fig. 5. Lectin histochemistry. **(A, B)** Sagittal section of the olfactory bulb. The lectin sWGA (light green) binds to the olfactory nerve layer (ONL) and the glomerular layer (GL). OB cells are stained purple with propidium iodide (PI). Dashed lines a–c in (A) indicate the transverse position of panels C–K: a, C–E; b, F–H; c, I–K. **(C–K)** Coronal sections from the rostral (a, C–E), central (b, F–H), and caudal (c, I–K) portions of the fused OB. Lectin histochemistry confirmed the complete fusion between the left and right olfactory bulbs in all layers (see C and D). a, anterior; p, posterior; L, left side cerebrum; R, right side cerebrum. Scale bars, 100 μm.

in the bulbul OB.

In coronal sections of the most anterior portion of the OB, the bilateral olfactory lobe joined at the midline (Fig. 4D) and formed a "one-loop" structure (Fig. 4E–G). This complete fusion of all layers was observed in sections anterior to the posterior margin of the OB. In the most posterior portion, the lamination of the olfactory bulb had almost disappeared and the OB tissues were completely fused (Fig. 4I).

Lectin histochemistry and glomerulus of the OB

The comparative biochemical properties of the olfactory system of several vertebrate species have been studied by lectin histochemistry (see Introduction of Saito et al., 2003). Moreover, lectin binding patterns in the ONL and GL of the OB of vertebrates have shown species specificity (see Discussion). In this study, we examined lectin binding in the bulbul OB using 21 types of fluorescence-labeled lectin. Four of these 21 lectins, *Lycopersicon esculentum* lectin (LEL), *Phaseolus vulgaris* agglutinin-L (PHA-L), *Solanum tuberosum* lectin (STL), and succinylated wheat germ agglutinin (sWGA), exhibited intense binding with the ONL and GL. Moreover, another two lectins, peanut agglutinin (PNA) and wheat germ agglutinin (WGA), exhibited positive binding with the same layers (Figs. 5, 6; Table 1).

Using these lectins, the complete fusion between the left

Table 1. Binding patterns of 21 types of fluorescence-labeled lectin in the olfactory nerve and glomerular layer of the bulbul OB. The extent of lectin binding varied from intense to negative. –, negative or faint staining; +, positive staining; ++, intense staining; ONL, olfactory nerve layer; GL, glomerular layer.

Lectins		Binding to olfactory nerve and glomerular layer	
	Abbreviation	ONL	GL
Bandeiraea simplicifolia lectin-I	BSL-I	–	–
Bandeiraea simplicifolia lectin	BSL-II	–	–
Concanavalin A	ConA	–	–
Dolichos biflorus agglutinin	DBA	–	–
Datura stramonium lectin	DSL	–	–
Erythrina cristagalli lectin	ECL	–	–
Jacalin	JAC	–	–
Lycopersicon esculentum lectin	LEL	++	++
Lens culinaris agglutinin	LCA	–	–
Phaseolus vulgaris agglutinin-E	PHA-E	–	–
Phaseolus vulgaris agglutinin-L	PHA-L	++	++
Peanut agglutinin	PNA	+	+
Pisum sativum agglutinin	PSA	–	–
Ricinus communis agglutinin-I	RCA-I (RCA120)	–	–
Soybean agglutinin	SBA	–	–
Sophora japonica agglutinin	SJA	–	–
Solanum tuberosum lectin	STL	++	++
Succinylated wheat germ agglutinin	sWGA	++	++
Ulex europaeus agglutinin-I	UEA-I	–	–
Vicia villosa agglutinin	VVA	–	–
Wheat germ agglutinin	WGA	+	+

and right OBs in all layers was also confirmed (Fig. 5C–E). Individual glomeruli in the glomerular layer could not be distinguished using lectin histochemistry because the glomeruli of the bulbul OB are incompletely surrounded by small numbers of juxtaglomerular (JG) cells (Figs. 4B, 6A–C). The diameter of each glomerulus was, however, predicted to be approximately 20–30 μm. Using lectin histochemistry, it was also difficult to distinguish the EPL and IPL in the bulbul OB; these structures are present on the upper and lower sides of the MCL in the mammalian MOB.

Mammalian VGLUT 2-like immunoreactivity in the glomerulus

Vesicular glutamate transporter 2 (VGLUT 2) accumulates the neurotransmitter glutamate into synaptic vesicles at presynaptic terminals of the glutamatergic axon terminal, and its antibody serves as a good marker for olfactory nerve axon terminals in mammals (Nakamura et al., 2005; Gabellec et al., 2007). We performed mammalian VGLUT2-like immunohistochemistry to confirm the presence of glomerular structures in the OB and to confirm that the bulbul olfactory nerve is also glutamatergic. As shown in Fig. 6D–I, the inner side of the GL exhibited strong mammalian VGLUT2-like immunoreactivity. Moreover, this mammalian VGLUT2-like immunoreactivity suggested that the diameter of individual glomeruli is approximately 20–30 μm (Fig. 6G vs. H), which is the same as the estimate obtained from PHA-L binding (Fig. 6A–C).

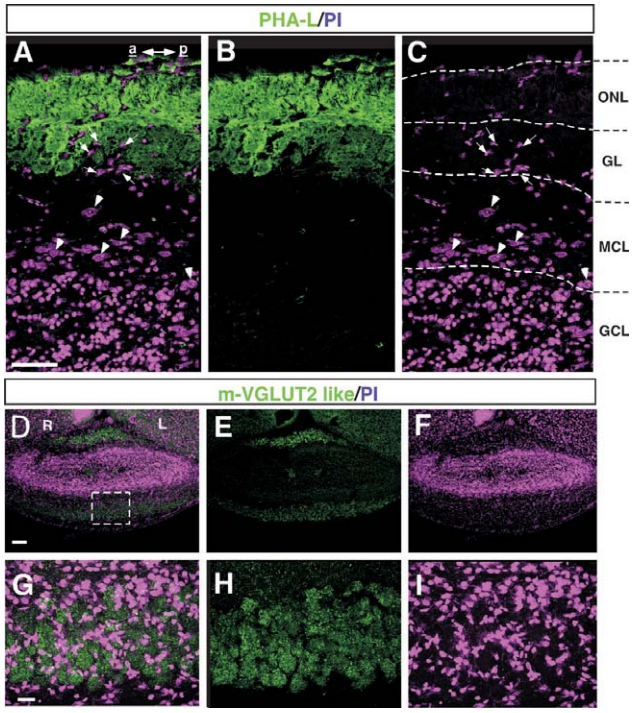

Fig. 6. Layered structure of the bulbul olfactory bulb. **(A–C)** The lectin PHA-L (light green) exhibits strong binding to the ONL and GL in sagittal sections of the OB. OB cells are stained (purple) with propidium iodide (PI). The glomerulus is surrounded by a small number of juxtaglomerular cells (small arrows). Large, unevenly distributed cells (diameter ≥10 μm) in the MCL appear to be mitral cells (arrowheads). Layers corresponding to the external and internal plexiform layers observed on the upper and lower sides of the MCL in the mammalian main OB could not be identified in the bulbul OB. ONL, olfactory nerve layer; GL, glomerular layer; MCL, mitral cell layer; GCL, granule cell layer; a, anterior; p, posterior. Scale bar, 50 μm. **(D–I)** Mammalian VGLUT2-like immunoreactivity (mammalian VGLUT2-like, light green) is evident in the GL and confirms the complete fusion between the left and right OBs. Scale bar, 100 μm. **(G–I)** High-magnification photographs for panels D–F of the boxed area indicated in panel D. Scale bar, 20 μm. Labeling by means of mammalian VGLUT2-like immunoreactivity in the GL allowed us to estimate the diameter of individual glomeruli to be 20–30 μm. L, left-side cerebrum; R, right-side cerebrum.

DISCUSSION

In this paper, we describe for the first time the olfactory system of the brown-eared bulbul and identify several notable features. First, a “scrolled” maxillary concha (MC) occupies the main part of the nasal cavity, and there is no posterior concha. Second, the OB is very small relative to the entire cerebral hemisphere (OBBR 6.1–6.8), and the left and right OBs are completely fused at both the gross anatomical and histological levels. Third, of the 21 lectins analyzed, LEL, PHA-L, PNA, STL, sWGA, and WGA bound to the ONL and GL of the OB. Finally, the OB glomeruli exhibited mammalian VGLUT2-like immunoreactivity.

Absence of the posterior concha

The structure of the avian nasal cavity varies widely among species, but is usually separated by three conchae,

namely, the anterior concha (AC), maxillary (or middle) concha (MC), and posterior concha or olfactory concha (PC), as observed in the domestic chicken (red jungle fowl, *Gallus gallus*) (Bang, 1971; Bang and Wenzel, 1985). The MC occupies a large part of the nasal cavity of all birds. However, species differences are observed in the internal structure of the MC. The structure of the MC is more complicated in avian species with a well-developed olfactory ability such as the brown kiwi (*A. australis*). However, in birds with a common level of olfaction, such as the domestic chicken, the MC is simply a "scrolled" structure (Bang and Wenzel, 1985). Therefore, the anatomical structure of the MC of the brown-eared bulbul suggests that the olfactory ability of this species is at the same or at a lower level than that of avian species with a commonly developed olfactory function, such as the domestic chicken.

In birds generally, the olfactory epithelium, which contains the olfactory receptor cells, is formed on the nasal cavity surface of the PC. This is why the PC is also called the "olfactory concha" in birds. Indeed, it has been reported that the PC tends to be well developed in birds (e.g., kiwi) with a well-developed sense of olfaction (Bang, 1971; Bang and Wenzel, 1985). The bulbul has a "scrolled" MC, similar to that of the domestic chicken, occupying the main part of the nasal cavity, but no PC was observed (Figs. 1, 2). However, the bulbul does have olfactory nerve bundles, and markers of the olfactory nerve (lectins and VGLUT2-like immunoreaction) showed clearly positive reactions (Figs. 5, 6). Thus, olfactory receptor cells (olfactory epithelium) must be present in some areas of the nasal cavity. Bang and Wenzel (1985) reported that the olfactory epithelium formed an arch in the ceiling of the nasal cavity in the white-bellied swiftlet (*Collacalia esculenta*), another bird that has no PC. Indeed, in general, the olfactory epithelium is formed in the upper half or ceiling of the nasal cavity in terrestrial animals, including many types of mammal (Clerio et al., 2003; Ding and Dahal, 2003). Therefore, in the bulbul, the olfactory epithelium, from which the bilateral olfactory nerves originate, was expected to be located in the ceiling of the nasal cavity. Unfortunately, however, our histological examination of the bulbul nasal cavity ceiling failed to identify an olfactory epithelium. Further investigations, involving tract tracing of the olfactory nerve and electron microscopic and molecular biological studies, are currently underway to address this issue.

Small, fused olfactory bulb

It is generally accepted that avian olfactory ability is well reflected by the OBBR (see Introduction). The OBBR of the brown-eared bulbul (6.1–6.8) determined in this study strongly suggests that, compared with other birds, bulbuls have a poorly developed sense of smell.

Of particular interest, was our observation that the bilateral OBs of the brown-eared bulbul were fused along the midline of the cerebral hemisphere, both anatomically and histologically. This is the most significant discovery in this study. Actually, a "fused" OB was reported in some passeriform birds, including the canary, house sparrow, common crow, and finch (Crosby and Humphrey, 1939; Bang, 1971). Although research on olfaction and neural aspects of the olfactory system has progressed rapidly since the discovery of olfactory receptor molecules by Buck and Axel (1991), little attention has been paid to this unique "fused" OB. Our discovery establishes a shared characteristic of the Passeriformes, namely, that those species with low OBBR values (below 7.0) (Bang 1971) have a "fused" OB.

Within the OB, the scattered distribution of mitral cells in the MCL prevented the ready distinction of the EPL and IPL, which are present on the upper and lower sides of the MCL in the mammalian MOB. In addition, the small number of JG cells surrounding the glomeruli made identification of the individual glomerular structures difficult using routine neurostaining methods, such as the Nissl and PI staining performed in this study. JG cells are well developed in the more evolutionarily advanced mammals, but are poorly developed in reptiles and fish, which, nevertheless, have a well-developed sense of smell (Andres, 1970). Although a sparse distribution of JG cells does not imply a low level of olfactory function, the JG cells have been shown to play an important role in the control of olfactory receptor information in mammals (Wachowiak and Shipley, 2006) as well as in the Drosophilidae (Root et al., 2008). In some birds (e.g., the wood duck) that have a well-developed sense of smell, large OBs are observed (OBBR 25–30) (Bang, 1971), and many JG cells are distributed in the GL of the OB (Rebiere et al., 1983). Thus, individual glomerular units are easily identified in the duck OB using routine neurostaining methods. Therefore, the anatomical and histological differentiation levels of the bulbul OB (low OBBR, fused olfactory bulb, small number of JG cells, and uncertain presence of the EPL and IPL) tend to indicate that the bulbul has a poorly developed sense of smell, even by comparison with other birds.

Nothing, however, is known of the developmental period of bilateral OB fusion, nor of the underlying molecular mechanism. It is also unknown whether the bulbul OB develops in the fused state from the beginning. In *Rana pipiens*, a ranid frog, the right and left OBs were demonstrated histologically to be completely independent; however, at the level of gross anatomy, the ventral aspect of the olfactory bulb was revealed to be "partly fused" (Scalia, 1976). Ranid frogs generally have a well-developed sense of smell, and their OB structure is also well developed at both the gross anatomical and microscopic levels. Therefore, an anatomically "fused" OB does not necessarily reflect a degeneration of olfactory function. At present, it is difficult to assess to what extent this "fused" OB reflects olfactory ability in the bulbul and/or other Passeriformes.

In the bulbul, the left and right olfactory nerve bundles independently project to the "mass" of the OB. Although in the present study we did not examine the projection pattern of the bilateral olfactory nerve bundles to the "fused" OB, we expect that these bundles project independently to the left and right halves of the ONL/GL of the OB. In *Xenopus*, when a unilateral olfactory nerve innervates one side of the rostral telencephalon, a unilateral olfactory bulb develops on that side and no olfactory bulb forms on the contralateral side (Byrd and Burd, 1993). In mammals, the axons of olfactory receptor cells expressing the same type of olfactory receptors project to particular glomeruli on the same side of the OB, and the relative positions of these glomeruli do not show any inter-individual differences (Mombaerts et al., 1996). Evaluation of the relationship between the molecular mechanism underlying the maintenance of the independent

projections of the left and right olfactory nerve bundles to the left and right halves, respectively, of the fused OB, and factors determining the laminar structure of the OB, may provide important information that will help to clarify the mechanisms leading to the development and regression of the olfactory system in vertebrates. Thus, the "fused" OB may become a unique research model, not only for evaluating the relationship between olfactory ability and the anatomical properties of the OB, but also for clarification of the biological significance of the left and right OBs existing as a pair in vertebrates and the molecular mechanisms preserving the independence of the left and right olfactory nerve circuits.

Lectin staining pattern

There have been many studies of the biochemical properties of the main olfactory system (olfactory epithelium, olfactory nerve, and main OB) and the vomeronasal system (vomeronasal epithelium, vomeronsal nerve, and accessory OB) using lectin histochemistry. Examples include the newt (Franceschini and Ciani, 1993; Saito et al., 2003), *Bufo* toads (Saito et al., 2006), *Xenopus* frogs (Key and Giorgi, 1986; Hofmann and Meyer, 1991, Saito and Taniguchi, 2000), lizard (Franceschini et al., 2000), opossum (Shapiro et al., 1995), rat (Ichikawa et al., 1992; Takami et al., 1994), common marmoset (Nakajima et al., 1998), and goat (Mogi et al., 2007). These studies suggest that different lectin binding patterns reflect functional differences between the olfactory and vomeronasal systems. In the bulbul olfactory bulb, six lectins, namely, LEL, PHA-L, PNA, STL, sWGA, and WGA, exhibited positive binding to the ONL and GL. Of these, LEL and STL also bound to the olfactory and vomeronasal nerves of the newt, *Bufo*, *Xenopus*, and rat (Ichikawa et al., 1992; Saito et al., 2000; Saito et al., 2003; Saito et al., 2006). sWGA strongly bound to the olfactory and vomeronasal nerves of *Bufo* and the vomeronasal nerve of *Xenopus,* but showed weak or negative binding in the newt and rat. PHA-L bound strongly to the *Xenopus* vomeronasal nerve, bound weakly to the newt olfactory and vomeronasal nerves, and exhibited faint or negative binding in *Bufo* and the rat. Finally, PNA and WGA bound strongly to the olfactory and vomeronasal nerves of the newt, but exhibited only faint or negative binding in *Bufo*, *Xenopus*, and the rat. When we consider the lectin binding patterns of the bulbul and the results in other vertebrates overall, LEL and STL are lectin markers that commonly bind to the olfactory nerve in vertebrates. On the other hand, the binding pattern of other lectins will reflect variation among animal species. As lectins are sugar-binding proteins or glycoproteins of non-immune origin, lectin binding studies suggest that varying expression of surface glycoconjugate molecules may reflect different properties of the olfactory nerve in different animal species. Thus, our results suggest that in bulbuls certain properties of the surface glycoconjugate molecules of the olfactory nerve (identified by LEL and STL) are shared in common with other vertebrates, whereas other properties (identified by PHA-L, PNA, sWGA, and WGA) are specific to the bulbul. Although we are currently unable to assess the relationship between olfactory acuity and the binding of lectins to the olfactory nerve, our results may indicate that the bulbul's olfactory nerve has molecular properties that differ from those of other vertebrates.

VGLUT 2-like immunoreactivity

Glutamate is the principal excitatory neurotransmitter of the central nervous system. Within the mammalian OB, glutamate is released by the axon terminals of olfactory neurons and by the mitral and tufted cells, which establish dendrodendritic synapses with interneurons (including JG cells and granule cells) (Berkowicz et al., 1994; Ennis et al., 1996; Broman et al., 2004; Shepherd et al., 2004). Vesicular glutamate transporter 2 (VGLUT2) is a selective marker of glutamatergic synapses. A common feature of glutamate-releasing neural terminals is their ability to accumulate glutamate in synaptic vesicles prior to exocytotic release. Thus, the VGLUT2 antibody is a useful marker for the mammalian OB glomerulus, which includes olfactory nerve axon terminals and dendrites of the mitral and tufted cells (Nakamura et al., 2005; Gabellec et al., 2007). Unfortunately, in birds, it has yet to be proven that the olfactory nerve is glutamatergic. Recently, Islam and Atoji (2008) reported VGLUT2 mRNA distributed in large neurons of the MCL and glutamate receptor 1 (GluR 1) mRNA in the GL of the olfactory bulb of the pigeon. This report strongly suggests that glutamate-releasing neural terminal and glutamate receptors are present in the glomerulus of the pigeon olfactory bulb. Although we were unable to obtain an avian-specific VGLUT2 antibody, we did, nevertheless, discover that glomeruli of the bulbul OB display mammalian VGLUT2-like immunoreactivity. Taking these observations into account, we think it conceivable that the olfactory nerve of the brown-eared bulbul is indeed glutamatergic. Moreover, our results also suggest the possibility that the immnohistochemistry of VGLUT2 will serve as a selective marker for the glomerulus of the avian OB.

Conclusions

The brown-eared bulbul has no posterior concha in its nasal cavity, and the mass of the OB is very small (OBBR: 6.1–6.8). Moreover, in common with the sparrow and crow (Crosby and Humphrey, 1939), the bulbul has a fused OB. These anatomical and histological properties of the olfactory systems strongly suggest that the bulbul has poorly developed olfaction. However, we observed that some lectins that bind to the OB of animals with a well-developed sense of smell also bind to the glomerulus of the bulbul OB. Moreover, the glomerulus displayed mammalian VGLUT2-like immunoreactivity. These observations indicate that, although the brown-eared bulbul does not have a well-developed sense of smell, it does have a certain level of olfactory acuity. According to Steiger et al. (2008), avian olfactory abilities cannot be evaluated solely on the basis of conventional indices such as the size of the OB and anatomical characteristics of the olfactory system. Therefore, the relationship between the anatomy of the bulbul olfactory system and its actual olfactory functions must be carefully analyzed. On the basis of our results, we suggest that the bulbul olfactory system may become a unique research model, not only for evaluating the relationship between olfaction and linked morphological characteristics, but also for clarification of the biological significance of the paired vertebrate left and right OBs, and the molecular mechanisms preserving the inde-

pendence of the afferent circuit of the left and right olfactory systems.

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