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Histological Properties of the Nasal Cavity and Olfactory Bulb of the Japanese Jungle Crow *Corvus macrorhynchos*

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Abstract

The nasal cavity and olfactory bulb (OB) of the Japanese jungle crow (*Corvus macrorhynchos*) were studied using computed tomography (CT) and histochemical staining. The nasal septum divided the nasal cavity in half. The anterior and maxillary conchae were present on both sides of the nasal cavity, but the posterior concha was indistinct. A small OB was present on the ventral surface of the periphery of the cerebrum. The OB–brain ratio—the ratio of the size of the OB to that of the cerebral hemisphere—was 6.13. The olfactory nerve bundles projected independently to the OB, which appeared fused on gross examination. Histochemical analysis confirmed the fusion of all OB layers. Using a neural tracer, we found that the olfactory nerve bundles independently projected to the olfactory nerve layer (ONL) and glomerular layer (GL) of the left and right halves of the fused OB. Only 4 of 21 lectins bound to the ONL and GL. Thus, compared with mammals and other birds, the jungle crow may have a poorly developed olfactory system and an inferior sense of olfaction. However, it has been contended recently that the olfactory abilities of birds cannot be judged from anatomical findings alone. Our results indicate that the olfactory system of the jungle crow is an interesting research model to evaluate the development and functions of vertebrate olfactory systems.

Key words: computed tomography, fused olfactory bulb, jungle crow, juxtaglomerular cells, lectin, nasal cavity

Introduction

It is generally believed that avian species do not have a well-developed sense of olfaction; however, some birds use their olfactory abilities in several situations (Roper 1999). The foraging behavior of kiwis (Wenzel 1968) and petrels (Hutchison and Wenzel 1980; Cunningham et al. 2003), navigation of pigeons (Wallraff 2004), and individual identification of domestic chicks (Burne and Rogers 1996) and petrels (Bonadonna et al. 2003, 2007; Bonadonna and Nevitt 2004) are well-known examples of activities involving olfaction in birds. With the recent advances in research on olfaction in animals, in particular, mammals, the molecular biological and neural circuits in the olfactory systems are being analyzed in many animal species; however, research on avian olfaction remains stagnant (Wenzel 2007). Progress in research on avian olfaction as a general science has been slow because not only the olfactory abilities of birds but also the physiologic significance of olfaction shows a marked species variation. Thus, it is relatively difficult to use birds as a research model of olfaction. However, we think that these marked interspecies differences in the degree of development and physiologic significance of olfaction between avian species may be an interesting and useful research model for the evaluation of the evolution of vertebrate sensory systems.

It has been suggested that the raven (*Corvus corax*), which belongs to the same family as the jungle crow, can distinguish feeds by using olfaction (Harriman and Berger 1986). However, the extent to which olfaction is used by crows in general in their living environments remains completely unknown. The olfactory bulbs (OBs), which are usually a pair of independent structures in vertebrates, are anatomically fused in

crows and sparrows, forming a very unique morphological feature (Crosby and Humphrey 1939). Further, as crows are known to have a very high cognitive ability, they have garnered considerable interest in the field of neuroscience as a brain research model (Izawa and Watanabe 2007). In the downtowns of Japanese cities, foraging in garbage bins by crows and their offensive behavior against humans and domestic animals have become a serious social problem. To solve these problems, it is important to understand how crows depend on olfaction for their activities. For these reasons, crows are considered to be important as a research model not only to clarify the evolution and development of olfaction in birds but also to elucidate the cognitive function of the avian brain. In this study, we analyzed the anatomical and histological characteristics of the nasal cavity and OB of the jungle crow, which is a common crow species in Japan, in order to evaluate the level of olfactory development.

Materials and methods

Sample preparation

Jungle crows (*Corvus macrorhynchos*) were trapped on a farm in Utsunomiya University (Utsunomiya, Tochigi Prefecture, Japan) and at Niza City Hall (Saitama Prefecture, Japan) and then kept in the crow farm in Utsunomiya University. Permission to capture the birds and collect their eggs was previously obtained from Tochigi Prefecture (Urin No. 0010) and Niza City, Saitama Prefecture (No. 01–02), as stipulated by Article 9, Clause 2, of the Law Concerning the Appropriate Conservation and Hunting of Birds.

We used 14 jungle crows (11 males and 3 females; weight, 580–800 g) that were judged to be aged 1 year or older on the basis of the degree of black pigmentation of the oral cavity (Kitagawa 1980; Tamada and Fukamatsu 1992). Each crow was deeply anesthetized by the injection of sodium pentobarbital (40 mg/kg; Somnopenyl, Kyoritsu Seiyaku) and then perfused transcardially with saline followed by a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer [PB], pH 7.3). After the birds were fixed by perfusion of the fixative solution, we decapitated 3 male birds and examined the heads by using computed tomography (CT). We also examined the heads of 3 other male birds and differentiated the olfactory nerve bundles from the connective and bone tissues in the head by tracing the connections of the olfactory nerve bundles of both sides with the nasal cavity and the brain. These nerve bundles were subsequently used for a nerve tracer study by 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) injection. The heads of these 6 males were preserved in specimen bottles containing the fixative solution at 4 °C until immediately before use in each experiment. The brains of the remaining birds (5 males and 3 females) were removed, postfixed overnight in the same fixative solution at 4 °C, and incubated in 30% sucrose in 0.1 M PB at 4 °C until they sank (Yokosuka et al. 2008).

CT analysis of the nasal cavity

The heads preserved in the fixative solution (Figure 1A) were sufficiently dehydrated, and 3-dimensional images of the internal structure of the nasal cavity were analyzed using a CT system (Microfocus X-Ray CT system, inspeXio SMX-90CT; Shimadzu). We scanned the area from the anterior portion of the nostril to the anterior part of the cerebrum (Figure 1A,C). After CT imaging, 5-mm-thick coronal specimens of the beak were prepared, and the structures in the nasal cavity were also analyzed by gross anatomical examination (Figure 1D,E). The specimens were photographed using a commercial digital camera (Model μ1020, Olympus).

DiI injection

DiI crystals (D-282, Molecular Probes) were injected into either the left or right olfactory nerve bundle through a microneedle (diameter, 0.15 mm). After DiI injection, the heads were returned to the fixative solution (4% paraformaldehyde in 0.1 M PB) and preserved in the dark in an incubator whose temperature was adjusted to 37 °C. After 14 days, the OB along with the cerebrum was removed from the heads, and serial 50-μm-thick coronal sections were prepared using a microslicer (DSK-1000, Dosaka). The region from the end of the olfactory nerve bundle in the OB to the caudal end of the OB was sectioned. The sections were nuclear stained using 4′,6-diamidino-2-phenylindole (DAPI; Sigma) diluted 10 000 times with 0.1 M PB (pH 7.4). They were then mounted on poly-L-lysine–coated slides (s7441, Matsunami) and coverslipped with an antifade reagent (H-1400, Vectashield HardSet). The stained cells were observed under a fluorescence microscope (Axioskop, Zeiss) 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. By collapsing this stack into a single plane using the summation options in the confocal software, we generated a 2-dimensional reconstruction of the stained histochemical section.

Lectin histochemical analysis

Prior to histochemical examination, we treated the brain samples with sucrose and prepared 50-μm-thick serial coronal and sagittal sections of the OB by using a freezing microtome (Yamato). These cross-sectional samples were histochemically examined. They were then subjected to Nissl staining using 1% cresyl violet solution (in distilled water) and examined again in order to study the histological structure of the OB. Histochemical analysis for lectin was performed using 21 fluorescence-labeled lectins (FLK-2100, FLK-3100, and FLK-4100; Vector). Sections were prepared using a freezing microtome and temporarily preserved in 0.1 M PB (pH 7.4), rinsed 3 times (5 min each) with PB, and allowed to react with a 500-fold dilution of each lectin in PB. The reactions were

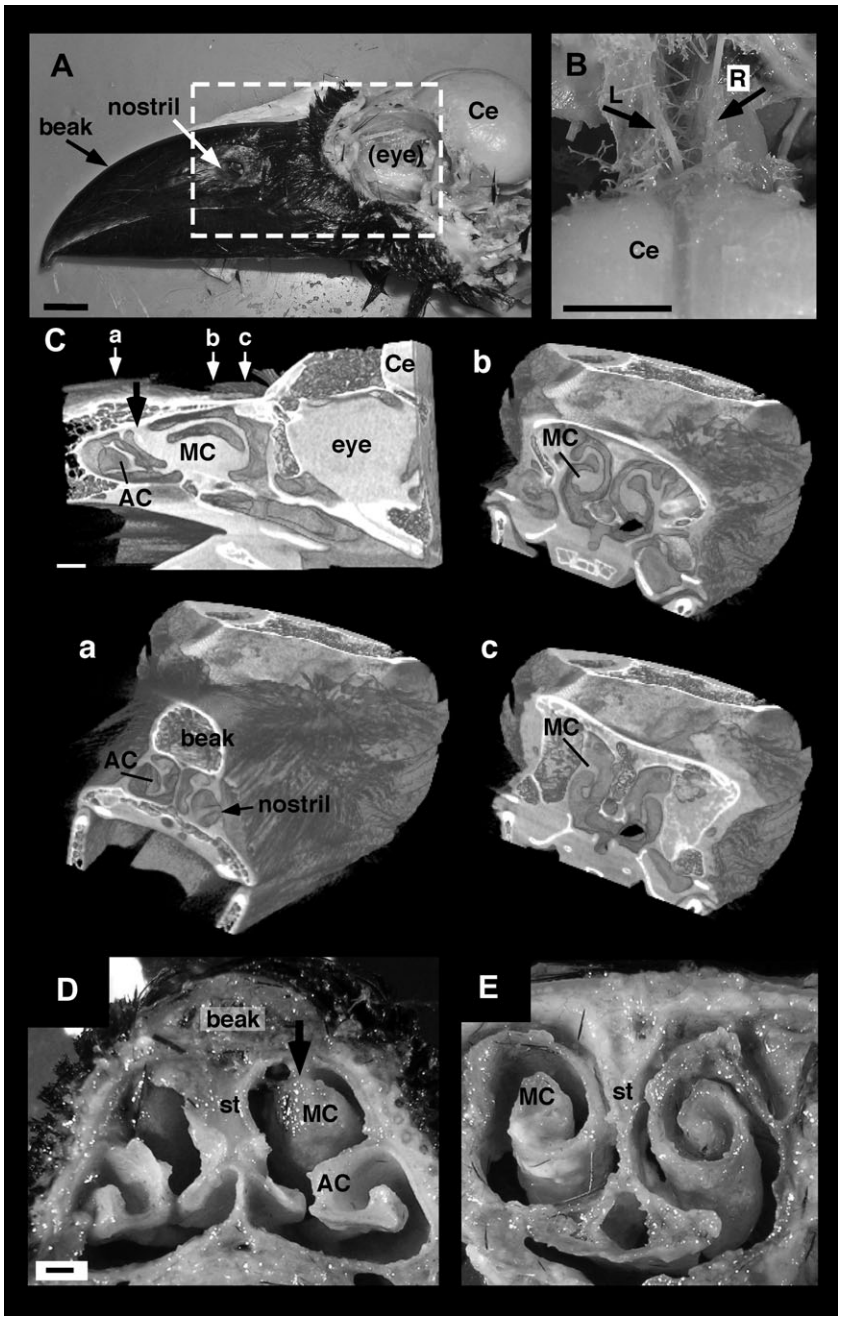

Figure 1 Nasal cavity of the jungle crow. **(A)** Head of the jungle crow (the cranial bone and eyeball have been removed). Bar = 10 mm. **(B)** The left and right olfactory nerve bundles independently project to the brain. L, left olfactory bundle; R, right olfactory bundle. Bar = 10 mm. **(C)** Sagittal and coronal 3-dimensional structural CT images of the nasal cavity. The small white arrows (a–c) in the sagittal image indicate the positions through which the planes of the transverse CT images pass. Bar = 10 mm. **(D** and **E)** Transverse sections of the nasal cavity through the rostral end (D) and middle portion (E) of the MC. The bold black arrows in (C and D) indicate the rostral end of the MC. Bar in (D) = 1 mm. Ce, cerebrum; st, olfactory septum.

performed at room temperature for 1 h in the dark. The sections were again rinsed 3 times (5 min each) with PB, and nuclear staining for the identification of brain structures was facilitated by allowing the sections to react for 5 min with propidium iodide (PI; Molecular Probes) diluted 20 000 times with PB. After the sections were reacted with PI, they were rinsed 3 times (5 min each) with PB, embedded using the same method as for the sections obtained after DiI injection, and analyzed using a confocal LSM, LSM 510.

Quail and mouse OB samples

Quail and mouse OB tissue sections were prepared for structural comparisons with the crow OB sections. We used 7-week-old male Japanese quails (n = 2; Nisseiken) and

40-day-old male C57BL/6J mice (n = 2; Nippon Crea). As in the case of the crows, the mice and quails were deeply anesthetized with sodium pentobarbital and fixed by perfusion of a fixative solution (4% paraformaldehyde in 0.1 M PB). The animals were then treated with sucrose, and 50-μm-thick sagittal sections of their OBs were prepared using a freezing microtome, mounted on gelatin-coated glass slides, and subjected to Nissl staining with cresyl violet by using the same procedure as that used for the crow OB sections.

The animal protocols used in this study were approved by the Institutional Animal Care and Use Committees of Utsunomiya University (in the case of the crows) and the Nippon Veterinary and Life Science University Postgraduate School (in the case of the Japanese quails and mice).

Results

Structure of the nasal cavity of the crow

We performed CT imaging and gross anatomical examination to observe the basic structure of the nasal cavity of the crow. The crow nasal cavity was completely divided by the nasal septum into the left and right cavities (Figure 1C–E). Distinctive anterior concha (AC) and maxillary concha (or middle concha: MC) were identified in both nasal cavities (Figure 1C–E). In contrast, the structure corresponding to the posterior concha (or olfactory concha) (Bang 1971; Bang and Wenzel 1985), which is usually observed in the nasal cavity of birds with well-developed olfaction, was quite indistinct. Therefore, in principle, the crow nasal cavity was found to be segmented by the AC and MC. The MC, which occupies the central part of the nasal cavity, had a ''pear-like'' appearance, and its anterior end was attached to the superior wall of the nasal cavity (Figure 1C,D). In the coronal sections, the left and right MCs appeared as open symmetric helices that were rotated downward and to the right and downward and to the left, respectively, when viewed from the front (Figure 1C-b,E).

Olfactory nerve and fused OB

The olfactory nerves of the crow were observed as a pair of independent bundles (Figure 1B). The left and right olfactory nerve bundles ran along the ventral surface of the anterior part of the cerebrum and independently projected to a mass that was identified as the OB and located on the ventral aspect of the anterior regions of the cerebral hemispheres (Figure 2B,C). The crow OB was grossly observed to be a single mass rather than a pair of independent structures (Figure 2C,D). It was very small compared with the entire cerebrum (Figure 2B). The OB–brain ratio (OBBR) is the ratio between the longest diameter of the OB and the longest diameter of the cerebral hemisphere, as measured in terms of percentage (Bang 1971). The OBBR of the jungle crow was 6.13 (mean diameter of the mass identified as the OB after perfusion fixation, 8.5 mm; mean diameter of the hemisphere after perfusion fixation, 138.6 mm; n = 6).

Histological structure of the crow OB

The crow OB protruded anteriorly from the inferior aspect of the cerebral hemisphere (Figures 2B,C,E,F and 3A). The left and right OBs appeared completely fused not only on gross anatomical examination but also on microscopic examination (Figures 4 and 5). In the coronal sections, 2 distinct olfactory lobes were seen in the anterior-most portion of the OB (Figure 4B), but in the rest of the OB, the right and left olfactory nerve layers (ONLs) joined in the midline (Figure 4C). Every nerve layer in the OB was joined in the midline and formed a ''one-loop'' structure (Figures 4E,F and 8E,F). This complete fusion of all layers was observed in the sections anterior to the posterior margin of the OB (Figure 5; from 450 to 1900 μm). Moreover, this one-loop structure divided the caudal portion of the OB into 2 lobes (Figures 4H and 5). The laminar structure disappeared in the posterior-most portion of the OB, and with widening of the distance between the left and right cerebral hemispheres, gross fusion of the left and right OBs was not seen (Figure 5; from 2150 to 2300 μm).

On microscopic examination, the crow OB was found to be clearly divided into 4 layers: ONL, glomerular layer (GL), mitral cell layer (MCL), and granule cell layer (GCL) (Figure 3C). The area of the OB occupied by the ONL and GL progressively decreased toward the posterior region of the OB (Figure 3A). In the MCL, large cells (diameter ≥ 10 μm), which were presumed to be the principal neurons of the OB (mitral cells and/or tufted cells), were found to be irregularly distributed (Figure 3C,F). For this irregular distribution of the principal cells, layers corresponding to the external plexiform layer (EPL) and internal plexiform layer (IPL) observed above and below the MCL in the mouse and quail OBs could not be clearly identified in the crow OB (Figure 3C vs. D,E).

Lectin histochemical analysis and glomerular structure

The biochemical properties of the olfactory systems of several vertebrate species have often been compared using lectin histochemical analysis (Saito et al. 2003). In vertebrates, the lectin-binding patterns of the olfactory (and/or vomeronasal) receptor cells and their axons exhibit species specificity (see Discussion). Therefore, to compare the biochemical properties of the olfactory nerves of the crow with those of the olfactory nerves of other vertebrates, we examined the binding patterns of 21 types of fluorescence-labeled lectins in the crow OB. Of the 21 lectins that reacted with the crow OB, *Lycopersicon esculentum* lectin (LEL), *Solanum tuberosum* lectin (STL), and succinylated wheat germ agglutinin (sWGA) intensely reacted with the ONL and GL of the OB. *Erythrina cristagalli* lectin (ECL) also showed a clearly positive reaction with the ONL and GL (Figure 6, Table 1).

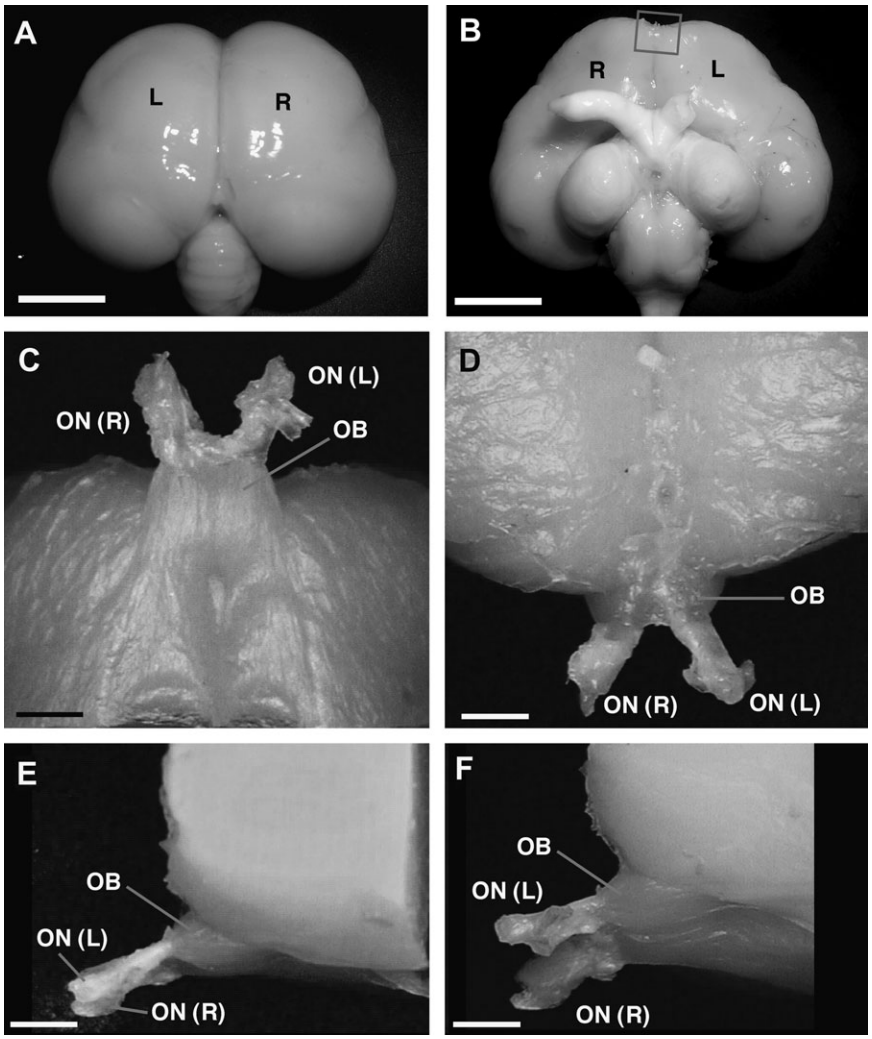

Figure 2 Brain and fused OB of the jungle crow. **(A)** Dorsal view of the crow brain. The cerebral hemisphere of the crow is swollen towards the periphery. **(B)** Ventral view of the brain. The OB is in close proximity to the rostroventral aspects of the cerebral hemispheres (inside the square). Bars in (A and B) = 10 mm. **(C)** High-magnification photographs of the ventral surface of the fused OB indicated in the square in (B). **(D)** Semifrontal view of the OB. **(E)** Lateral view of the OB. **(F)** Lateral view from a different angle from that in (E). Although the projections from the 2 olfactory nerve bundles (ONs) separately joined to the anterior aspects of the olfactory lobes, the right and left OBs seem to be almost completely fused in the midline. ON, olfactory nerve bundle. L, left, R, right. Bars in (C–F) = 1 mm.

The lectin histochemical examinations confirmed the complete fusion of all layers of the left and right OBs (Figure 7A,B). Furthermore, the examinations distinguished the ONL and GL from the other layers of the OB, and hence, individual glomeruli in the GL could be clearly visualized. Each glomerulus was approximately 100 μm in diameter and incompletely surrounded by juxtaglomerular (JG) cells, which were scattered around the glomeruli (Figure 7C vs. D). The EPL and IPL, which are present above and below the MCL in the mammalian main OB (MOB), were difficult to distinguish in the crow OB by using lectin histochemical analysis.

Projection of the olfactory nerve bundles to the fused OB

Although the left and right olfactory nerve bundles in the crows projected to the fused OB, the projections remained distinct (Figures 1B and 2). Further, it was unclear how these bundles projected to the fused GL in the OB. Hence, we next examined the projection pattern of the olfactory nerve bundles to the GL by injecting DiI into one of the olfactory nerve bundles (Figure 8A–C). We found that the left and right olfactory nerve bundles projected to the ONL and GL of the left and right halves, respectively, of the OB. This projection pattern was observed throughout the anteroposterior region of the OB (Figure 8D–G).

Discussion

In this paper, we describe the olfactory system of the jungle crow and identify several notable points: 1) the "scrolled" MC of the crow occupies the center of the nasal cavity

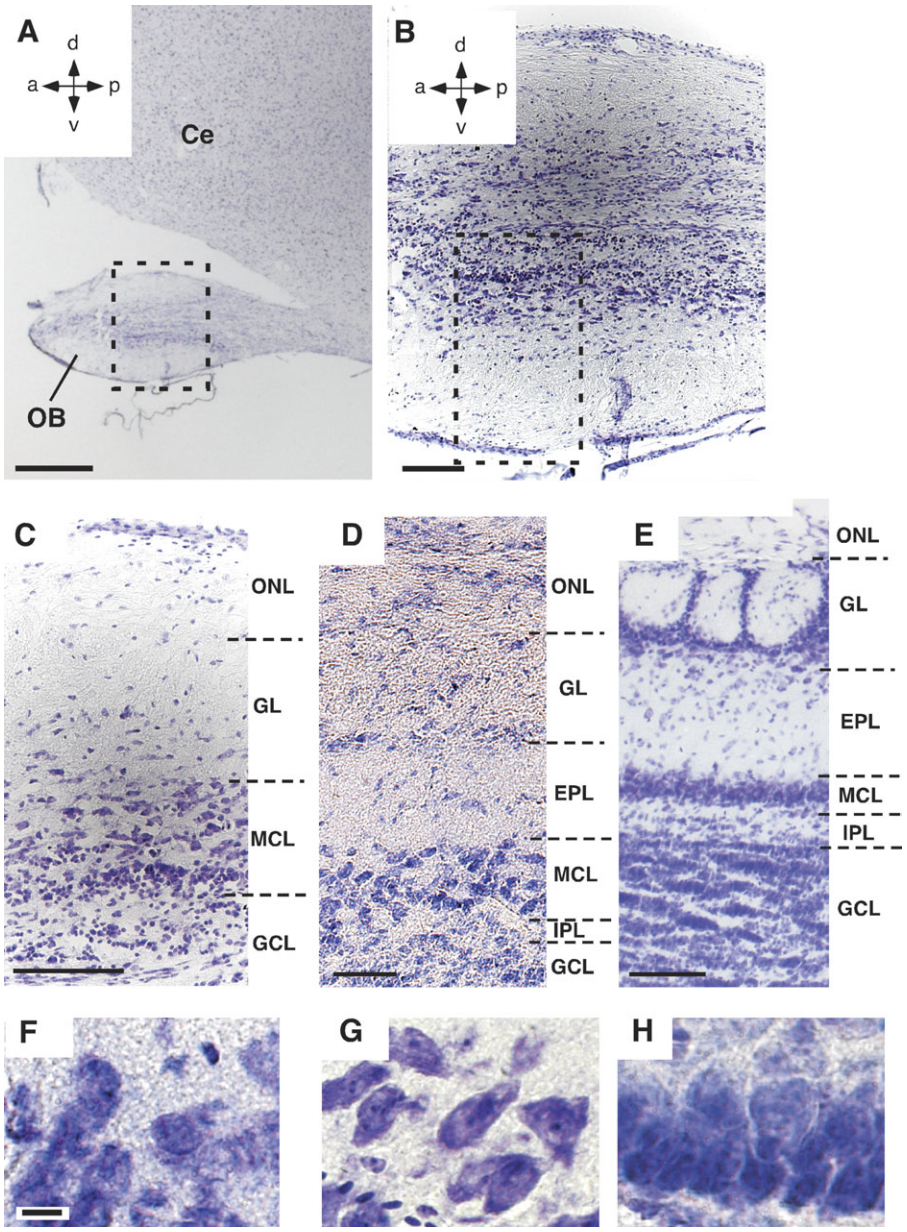

Figure 3 Histological organization of the OB of the crow. **(A)** Nissl-stained (cresyl violet staining) anterior portion of the cerebrum (Ce) and OB of the jungle crow (sagittal section). **(B)** High-magnification photograph of the OB in the dotted square in (A). **(C)** High-magnification photograph of the dotted square in (B). The OB is extremely small, but its histological organization is similar to that in other birds (vs. **D**: Japanese quail). a, Anterior, p, posterior, d, dorsal, v, ventral. Bar in (A) = 1000 μm; bars in (B and C) = 100 μm. **(D** and **E)** Layers of the quail (D) and mouse (E) OBs. Bars = 100 μm. **(F–H)** High-magnification photographs of the MCL in the OBs of the crow (F), quail **(G)**, and mouse (H), respectively. In the MCL, large cells (diameter ≥ 10 μm), which were presumed to be the principal neurons of the OB (mitral or tufted cells), were irregularly distributed. Bars in (F–H) = 10 μm. Note: In the OB of the crow (C), 4 layers can be clearly distinguished after cresyl violet staining: the ONL, GL, MCL, and GCL. The EPL and IPL, which are present above and below the MCL in the quail and mouse OBs, are indistinct in the crow OB.

and the posterior concha is obscure; 2) the OB is very small relative to the entire cerebral hemisphere (OBBR = 6.13) and the right and left OBs are completely fused, as observed on both gross anatomical and histological examinations, 3) the left and right olfactory nerve bundles project to the ONL and GL of the left and right halves, respectively, of the fused OB, and 4) ECL, LEL, STL, and sWGA clearly bind with the ONL and GL of the OB.

On the basis of these anatomical and histological findings, we evaluated the olfactory system and olfaction of the jungle crow as follows.

The MC and obscure posterior concha

The structure of the avian nasal cavity varies widely among species, but it is known to be divided by 3 conchae, namely,

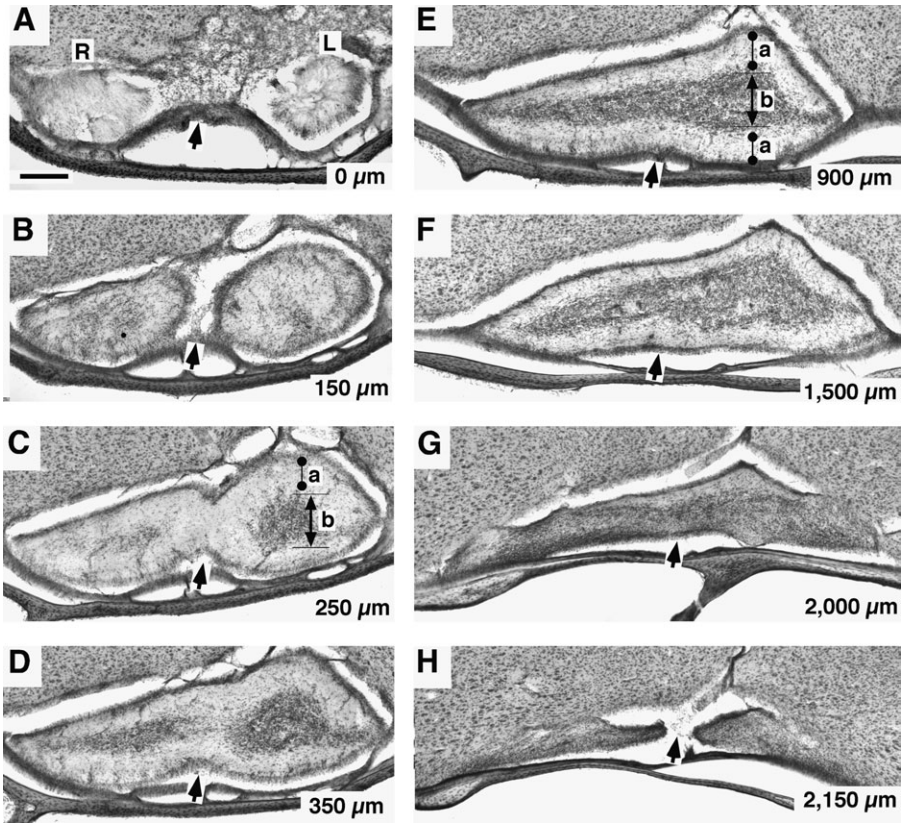

Figure 4 Histological organization of the fused OB. **(A–H)** Nissl-stained transverse sections through several levels from the rostral (A) to the caudal (H) portions of the fused OB. (A) The left (L) and right (R) olfactory nerve bundles independently project to the brain. **(B)** At the rostral end of the OB, the left and right bulbs are distinct. **(C** and **D)** In the rostral portion of the OB (except for the end), both sides of the bulb are fused in the center of the brain. **(E** and **F)** All layers of the OB join in the midline and form a one-loop structure in the main body of the OB. (H) This one-loop structure divides the caudal portion of the OB into 2 lobes. Layers a and b correspond to the ONL–GL and MCL–GCL, respectively. The arrows indicate the midline of the fused OB. Distances from the first section (0 μm) are indicated in parentheses. L, left, R, right. Bar in (A) = 200 μm.

the AC, MC, and posterior concha (or olfactory concha) (Bang 1971; Bang and Wenzel 1985). Of these 3 conchae, the MC occupies a large part of the nasal cavity, regardless of the bird species. Species differences are observed in the internal structure of the MC, and the MC structure is complex in birds with well-developed olfactory abilities, such as the brown kiwi (*Apteryx australis*). In contrast, birds whose olfactory senses are mediocre or poorly developed, such as the domestic chicken (*Gallus gallus*), show scrolled MCs (Bang and Wenzel 1985). In the avian nasal cavity, the olfactory epithelium, in which olfactory cells are distributed, is known to be situated on the surface of the posterior concha. This is why the posterior concha is also called the "olfactory concha." Indeed, it has been reported that the posterior (or olfactory) concha tends to be well developed in birds with excellent olfactory abilities (Bang 1971; Bang and Wenzel 1985). We found that in the jungle crow, the scrolled MC occupies the center of the nasal cavity, but we could not confirm the existence of the posterior concha. However, because the olfactory nerves were clearly observed in the crow, olfactory receptor cells must be present somewhere in the nasal cavity. However, the distribution of olfactory receptor cells in the crow remains unknown. Bang and Wenzel (1985) reported that in the white-bellied swiftlet (*Collacalia esculenta*), in which no posterior concha is observed, the olfactory epithelium forms an arch in the ceiling of the nasal cavity. Olfactory epithelium has also been identified in the upper half or on the ceiling of the nasal cavity in other animals (Clerico et al. 2003; Ding and Dahl 2003; Menco and Morrison 2003). Therefore, in the jungle crow, the olfactory epithelium, from which the right and left olfactory nerves originate, is expected to be distributed on the ceiling of the nasal cavity. Unfortunately, however, we were unable to identify the olfactory epithelium in the ceiling of the nasal cavity of the crow on histological examination. Further investigations involving tracing of the olfactory nerve, electron microscopy, and molecular biological studies are underway.

The small and fused OB

In birds, the olfactory ability is reflected by the OBBR, which is the ratio of the size of the OB to that of the cerebral

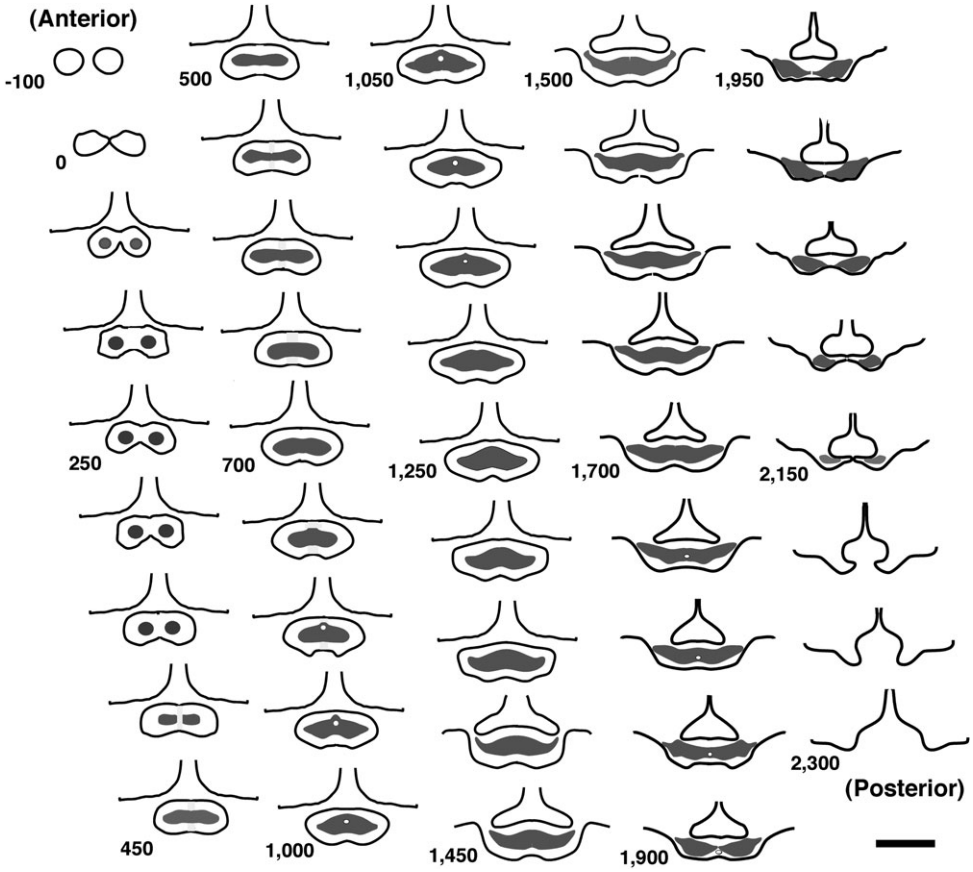

Figure 5 Overview of the fused OB. Diagrams showing serial sections through the rostral and caudal ends of the OB of the crow. In the rostral-most portion of the OB, all layers maintain their bilateral structure but join in the midline and form a one-loop structure in the main body of the OB. Moreover, this one-loop structure divides the caudal portion of the OB into 2 lobes. The gray layers in the maps indicate the MCL and GCL. Distances from the first section (0 μm) are indicated in parentheses. Bar = 1000 μm.

hemisphere (Bang and Cobb 1968; Bang 1971). For instance, the OBBR of brown kiwis (*A. australis*), which have the most developed olfactory sense of all birds, is 34.0, whereas the OBBRs of canaries and sparrows, in which olfaction has not yet been documented, are 6.0–4.0 (Bang 1971). Indeed, Steiger et al. (2008) found that the estimated number of avian olfactory receptors is positively correlated with the OBBR. Therefore, the OBBR of the jungle crow (6.13), as determined in this study, strongly suggests that compared with other birds, crows have a poorly developed sense of smell.

It has long been known that the OBs are fused in the American crow; the raven (*Corvus corax*), which belongs to the same family as the crow; and *Passeriformes* species such as the sparrow (Crosby and Humphrey 1939; Bang 1971). This fusion was observed using both gross anatomical and histological examinations. However, whereas research on olfaction has rapidly progressed after the discovery of olfactory receptors by Buck and Axel (1991), this unique fused OB has not been investigated by researchers of olfaction and neuroscience. In this study, we also confirmed the complete fusion of the OBs in the crow species inhabiting Japan (Figures 2, 4, 5, 7, and 8). Thus, we confirmed that fused OBs are a characteristic of crows. However, it is not yet known if the OBs develop in a fused state or if they fuse at a subsequent stage of development. Therefore, it is difficult to discuss to what extent the fused OB reflects olfaction in crows and/or sparrows.

Within the OB of the crow, the scattered distribution of the principal neurons (mitral or tufted cells) prevented the ready distinction of the EPL and IPL, which are present above and below the MCL in the mammalian MOB (Figure 3E). In addition, the small number of JG cells surrounding the glomeruli rendered the identification of individual glomeruli by using routine neurostaining methods such as Nissl staining and PI nuclear staining difficult (Figures 3 and 7). The JG cells are well developed in mammals, which are more evolutionarily advanced, 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 poor olfaction, the JG cells have been shown to play an important role in processing the information transmitted by the olfactory receptors in mammals (Wachowiak and Shipley 2006) as well as in the Drosophilidae (Root et al. 2008). In some birds such as ducks (e.g., wood duck), which

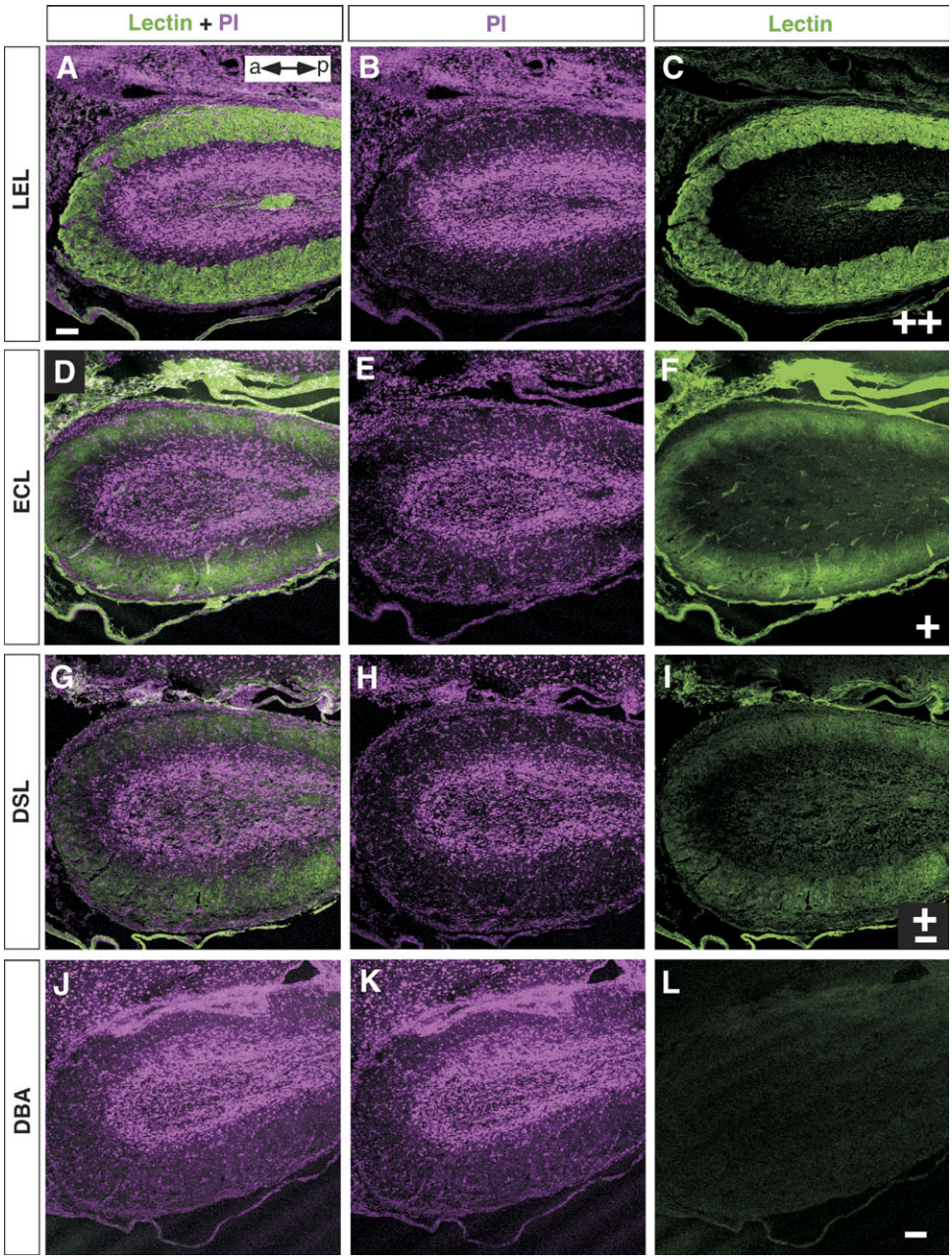

Figure 6 Lectin-binding patterns in the OB. Binding patterns of fluorescence-labeled lectins (light green), LEL **(A**, **E**, and **I)**, and ECL **(B**, **F**, and **J)**. *Datura stramonium* lectin (DSL) **(C**, **G**, and **K)** and *Dolichos biflorus* agglutinin (DBA) **(D**, **H**, and **L)** in the ONL and GL of the OB of the crow (sagittal sections). The extent of lectin binding varied from intense to negative. (E–H) Nuclear staining with PI (purple) was performed for the identification of OB structures in each section. Photographs in the lectin + PI column (A–D) are images of PI-stained sections merged with the images obtained using lectin histochemical analysis (in the lectin column). The levels of lectin binding are indicated in the bottom right corners of the images in the lectin column (I–L). ++, Intense staining; +, moderate staining; ±, faint staining; –, negative staining. a, Anterior, p, posterior. Bar in (A) = 100 μm. The abbreviations for the lectins are defined in Table 1.

have a well-developed sense of smell, large OBs are observed (OBBR, 25–30) (Bang 1971), and many JG cells are found to be distributed in the GL of the OB (Rebiere et al. 1983). Thus, individual glomerulus units can be easily identified in the duck OB by using routine neurostaining methods. In comparison, differentiation of the crow OB, which was shown in this study to be the center for olfactory control, indicates that olfaction is less well developed in crows than in mammals and other birds with a well-developed sense of smell.

Projection pattern of the olfactory nerve bundles

The left and right olfactory nerve bundles were found to project to the ONL and GL of the left and right halves, respectively, of the fused OB (Figure 8). This finding, which

Table 1 Qualitatively judged lectin-binding patterns in the jungle crow OB

Lectins	Abbreviation	Binding to olfactory nerve and GL	
		ONL	GL
Bandeiraea simplicifolia lectin-I	BSL-I	–	–
B. simplicifolia lection	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	–	–
P. vulgaris agglutinin-L	PHA-L	–	–
Peanut agglutinin	PNA	–	–
Pisum sativum agglutinin	PSA	–	–
Ricinus communis agglutinin-I	RCA-I (RCA 120)	–	–
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	–	–

The extent of lectin binding varied from intense to negative in the olfactory nerve and GL of the OB. –, negative staining; ±, faint staining; +, positive staining; ++, intense staining.

indicates that the left and right olfactory nerves separately project to the OB (Figures 1B and 2) in the crow as in other vertebrates, is one of the most important discoveries of this study. Whether or not this distinction is preserved in the OB and the stage of projection from the OB to the cerebrum warrant further evaluation; however, at present, left and right olfactory signals are believed to be processed independently, even in animals with a fused OB. Clarification of the embryological molecular mechanism that determines this nerve-projection pattern is also considered important. In mammals, the axons of olfactory receptor cells expressing the same type of olfactory receptors are known to project to particular glomeruli in the OB; further, the relative positions of these glomeruli do not show any interindividual differences (Mombaerts et al. 1996). The establishment of the axonal projections of the olfactory nerves and the laminar structure of the OB have been shown to progress in an interdependent manner (Kauer and White 2001; Hirata et al. 2006). 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 the factor that determines the laminar structure of the OB may provide important information to help clarify the mechanisms leading to the development and regression of the olfactory system.

Lectin-binding pattern

The biochemical properties of the main olfactory system and the vomeronasal system are often studied using lectin histochemical analysis, and this type of study has been conducted in newts (Franceschini and Ciani 1993; Saito et al. 2003), toads (*Bufo* species) (Saito et al. 2006), frogs (*Xenopus* species) (Key and Giorgi 1986; Hofmann and Meyer 1991, Saito and Taniguchi 2000), lizards (Franceschini et al. 2000), opossums (Shapiro et al. 1995), rats (Ichikawa et al. 1992; Takami et al. 1994), common marmosets (Nakajima et al. 1998), and goats (Mogi et al. 2007). The findings of the above studies suggest that differences in the lectin-binding patterns in the olfactory and vomeronasal systems reflect functional differences between the 2 systems. In the crow OB, we observed intense binding of LEL, STL, and sWGA and positive binding of ECL to the ONL and GL. Of these lectins, LEL, STL, and ECL also strongly bind to the olfactory receptor cells and/or olfactory nerve in newts, toads (*Bufo* species), frogs (*Xenopus* species), and rats. Positive binding of sWGA to the olfactory and/or vomeronasal receptor cells and nerves is seen in newts, toads (*Bufo* species), and frogs (*Xenopus* species), but nonspecific or negative binding to the GL of the accessory OB (AOB) and MOB is seen in rats (Ichikawa et al. 1992; Saito and Taniguchi 2000; Saito et al. 2003, 2006). In contrast, sWGA strongly binds to the vomeronasal nerve layer and GL of the AOB in goats (Mogi et al. 2007). These results suggest that LEL, STL, and ECL binding can be used as basic markers for the presence of a vertebrate olfactory sensory nerve. However, the pattern of sWGA binding to the olfactory sensory nerves differs between animal species. Moreover, 7–12 lectins can bind to the olfactory neurons and/or ONL and GL of the OB of animals that have a well-developed sense of smell (e.g., newts, frogs, and rats). In contrast, in this study, only 4 lectins bound to the OBs of the crows. As lectins are sugar-binding proteins or glycoproteins of nonimmune origin, our results suggest that the expression of surface glycoconjugate molecules, which can bind to LEL, STL, and ECL, may reflect the basic properties of the olfactory nerves in animals. However, fewer lectins intensely react with the olfactory system of the crow than with that of other animals. The relationship between the degree of olfactory development and the number of lectins that bind to the olfactory nerve remains unclear. Because

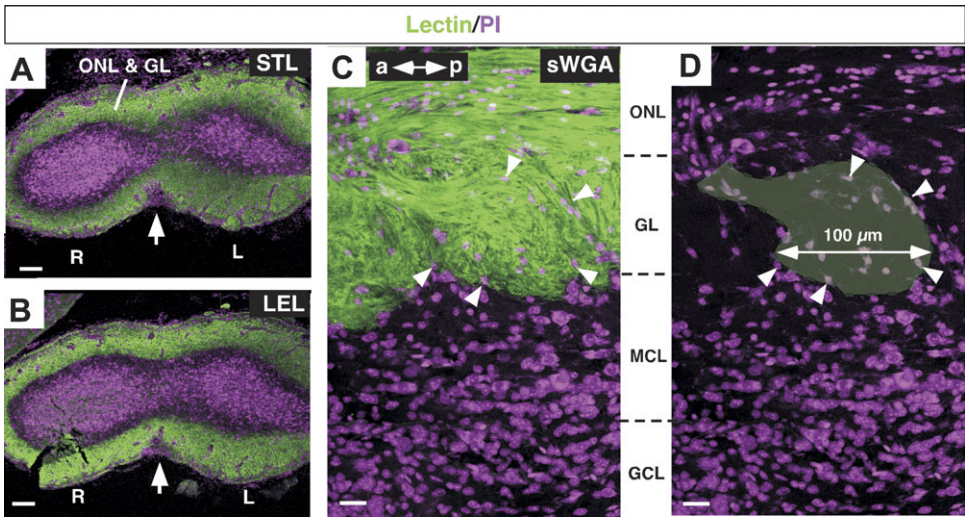

Figure 7 Layered structure of the OB, as observed on lectin histochemical analysis. To analyze the layered structure of the OB in greater detail, we examined the binding patterns of fluorescence-labeled lectins. Three lectins **(A**: STL, **B**: LEL, and **C**: sWGA**)** strongly bound to the olfactory nerve bundle and glomeruli. (A and B) The GLs of the OBs fuse in the midline. The arrows in the coronal sections indicate the midline. Bars in (A and B) = 100 μm. **(C** and **D)** A glomerulus with a diameter of approximately 100 μm can be seen clearly. This glomerulus is surrounded by a few JG cells (arrowheads). a, Anterior, p, posterior. Bars in (C and D) = 20 μm. The abbreviations for the lectins are defined in Table 1.

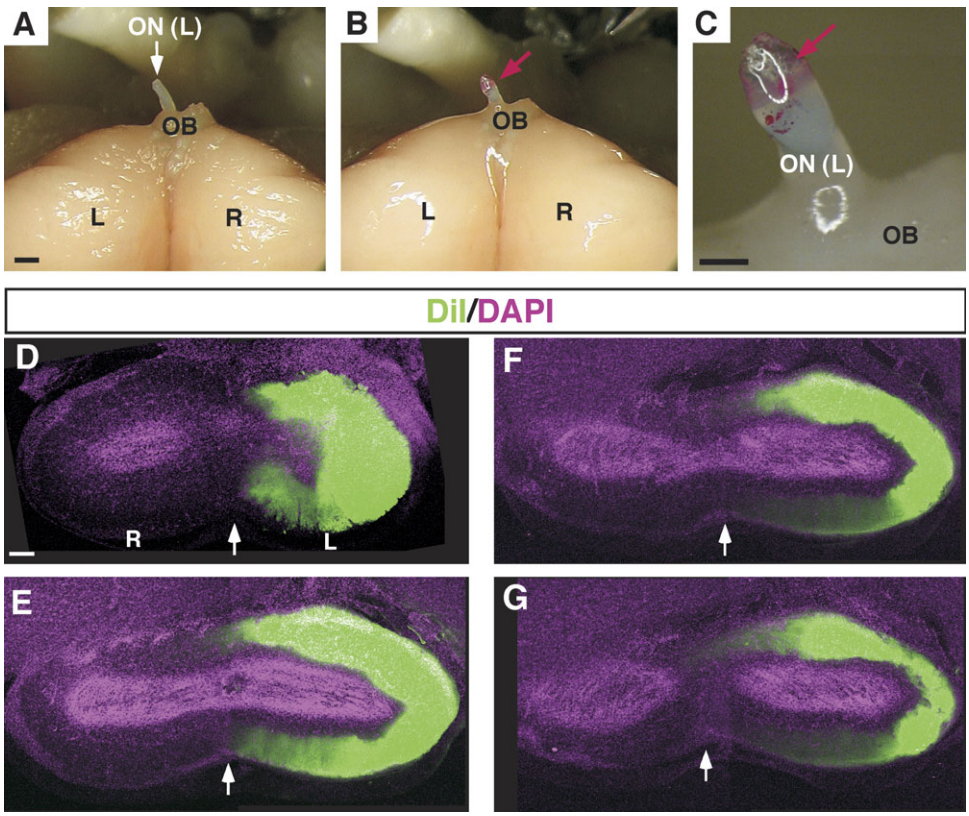

Figure 8 Projections of the olfactory nerves to the fused OB. **(A–C)** To determine whether the left and right olfactory nerves merge in the fused OB, we injected a neural tracer (DiI) into 1 olfactory nerve bundle. **(A** and **B)** Images obtained before (A) and after (B) DiI injection into the left olfactory bundle. (C) High-magnification photographs of the DiI-injected site in (B). L, left, R, right, ON (L), left olfactory nerve bundle. Bar in (A) = 1 mm and that in (C) = 0.5 mm. **(D–G)** Semiserial transverse sections through various levels from the rostral (D) to the caudal (G) regions in the fused OB. The images were obtained 14 days after the DiI injection. The green structure is the DiI-stained olfactory nerve terminal. The purple structures are 4′,6-diamidino-2-phenylindole (DAPI)–stained OB cells. The arrows indicate the midline of the fused OB. Note: The olfactory nerve bundles separately projected to the left and right halves of the fused OB. L, left, R, right. Bar in (D) = 100 μm.

very few lectins reacted with the olfactory nerves of the crows, the developmental level of the olfactory system of crows may be lower than that of the olfactory systems of other vertebrates, despite the fact that the basic molecular characteristics of the olfactory nerves of crows are similar to those of vertebrate animals.

Conclusion

The jungle crow, *C. macrorhynchos*, does not have a distinct posterior concha in the nasal cavity and has a very small OB(OBBR, 6.13). Moreover, consistent with the results of the pioneer study by Crosby and Humphrey (1939), we also confirmed that the crow has a fused OB. These anatomical results strongly suggest that the crow has a poorly developed olfactory system. Further, we found that the left and right olfactory nerve bundles project independently from the nasal cavity to the OB of the crow, and the histological structure of the OB resembled that of other avian species, which have an intermediate level of olfactory development. We also observed that some lectins, which commonly bind to the olfactory nerves and OB of animals with a sense of smell, bind to the ONL and GL of the crow OB. These observations indicate that although the crow does not have a well-developed sense of smell, it has a limited, but functional, sense of smell.

Recently, Steiger et al. (2008) analyzed avian olfactory receptors by using a molecular biological technique and suggested that the olfactory abilities of birds cannot be evaluated according to conventional indices such as the size of the OB and anatomical characteristics of the olfactory system alone. Therefore, the relationship between the anatomical findings of the crow olfactory system and the olfactory functions of the crow must be carefully evaluated. Our results may render the crow olfactory system an important research model not only for the reevaluation of the relationship between olfaction and specific morphological characteristics but also for the clarification of the biological significance of the existence of a pair of OBs in vertebrates and the molecular mechanisms preserving the independence of the left and right olfactory nerve circuits.

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References

Andres KH. 1970. Anatomy and ultrastructure of the olfactory bulb in fish, amphibia, reptiles, birds, and mammals. In: Wolstenholme GEW, Knight J, editors. Ciba Foundation Symposium on Taste and Smell in Vertebrates. London: Churchill. p. 177–194.

Bang BG. 1971. Functional anatomy of the olfactory system in 23 orders of birds. Acta Anat. 79:1–76.

Bang BG, Cobb S. 1968. The size of the olfactory bulb in 108 species of birds. Auk. 85:55–61.

Bang BG, Wenzel BM. 1985. Nasal cavity and olfactory system. In: King AS, McLelland J, editors. Form and function in birds. Vol. 3. London: Academic Press. p. 195–225.

Bonadonna F, Cunningham GB, Jouventin P, Hesters F, Nevitt GA. 2003. Evidence for nest-odour recognition in two species of diving petrel. J Exp Biol. 206:3719–3722.

Bonadonna F, Miguel E, Grosbois V, Jouventin P, Bessiere JM. 2007. Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? J Chem Ecol. 33:1819–1829.

Bonadonna F, Nevitt GA. 2004. Partner-specific odor recognition in an Antarctic seabird. Science. 306:835.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 65:175–187.

Burne TH, Rogers LI. 1996. Responses to odorants by the domestic chick. Physiol Behav. 60:1441–1447.

Clerico DM, To WC, Lanza DC. 2003. Anatomy of the human nasal passages. In: Doty RL, editor. Handbook of olfaction and gustation. 2nd ed. revised and expanded. New York: Marcel Dekker. p. 1–11.

Crosby EC, Humphrey T. 1939. Studies of the vertebrate telencephalon. J Comp Neurol. 71:121–213.

Cunningham GB, Van Buskirk RW, Bonadonna F, Weimerskirch H, Nevitt GA. 2003. A comparison of the olfactory abilities of three species of procellariiform chicks. J Exp Biol. 206:1615–1620.

Ding X, Dahl AR. 2003. Olfactory mucosa: composition, enzymatic localization, and metabolism. In: Doty RL, editor. Handbook of olfaction and gustation. 2nd ed. revised and expanded. New York: Marcel Dekker. p. 51–73.

Franceschini V, Ciani F. 1993. Lectin histochemistry of cell-surface glycoconjugates in the primary olfactory projections of the newt. Cell Mol Biol. 39:651–658.

Franceschini V, Lazzari M, Ciani F. 2000. Lectin cytochemical localisation of glycoconjugates in the olfactory system of the lizards *Lacerta viridis* and *Podarcis sicula*. Anat Embryol. 202:49–54.

Harriman AE, Berger RH. 1986. Olfactory acuity in the common raven (*Corvus corax*). Physiol Behav. 36:257–262.

Hirata T, Nakazawa M, Muraoka O, Nakayama R, Suda Y, Hibi M. 2006. Zinc-finger genes Fez and Fez-like function in the establishment of diencephalon subdivisions. Development. 133:3993–4004.

Hofmann MH, Meyer DL. 1991. Functional subdivisions of the olfactory system correlate with lectin-binding properties in *Xenopus*. Brain Res. 564:344–347.

Hutchison LV, Wenzel BM. 1980. Olfactory guidance in foraging by procellariiformes. Condor. 82:314–319.

Ichikawa M, Osada T, Ikai A. 1992. Bandeiraea simplicifolia lectin I and Vicia villosa agglutinin bind specifically to the vomeronasal axons in the accessory olfactory bulb of the rat. Neurosci Res. 13:73–79.

Izawa E, Watanabe S. 2007. A stereotaxic atlas of the brain of the jungle crow (*Corvus macrorhynchos*). In: Watanabe S, Hofman MA, editors. Integration of comparative neuroanatomy and cognition. Tokyo: Keio University Press. p. 215–274.

Kauer JS, White J. 2001. Imaging and coding in the olfactory system. Annu Rev Neurosci. 24:963–979.

Key B, Giorgi PP. 1986. Selective binding of soybean agglutinin to the olfactory system of *Xenopus*. Neuroscience. 18:507–515.

Kitagawa T. 1980. For seasons of the Japanese jungle crow (*Corvus macrorhynchos*). Wild Birds. 45:416–421[Japanese].

Menco BPM, Morrison EE. 2003. Morphology of the mammalian olfactory epithelium: form, fine structure, function, and pathology. In: Doty RL, editor. Handbook of olfaction and gustation. 2nd ed. revised and expanded. New York: Marcel Dekker. p. 17–49.

Mogi K, Sakurai K, Ichimaru T, Ohkura S, Mori Y, Okamura H. 2007. Structure and chemical organization of the accessory olfactory bulb in the goat. Anat Rec. 290:301–310.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. 1996. Visualizing an olfactory sensory map. Cell. 87:675–686.

Nakajima T, Shiratori K, Ogawa K, Tanioka Y, Taniguchi K. 1998. Lectin-binding patterns in the olfactory epithelium and vomeronasal organ of the common marmoset. J Vet Med Sci. 60:1005–1011.

Rebiere A, Dainat J, Bisconte JC. 1983. Autoradiographic study of neurogenesis in the duck olfactory bulb. Dev Brain Res. 282:113–122.

Root CM, Masuyama K, Green DS, Enell LE, Nässel DR, Lee CH, Wang JW. 2008. A presynaptic gain control mechanism fine-tunes olfactory behavior. Neuron. 59:311–321.

Roper TJ. 1999. Olfaction in birds. In: Slater PJB, Rosenblatt JS, Snowden CT, Roper TJ, editors. Advances in the study of behavior. Vol. 28. New York: Academic Press. p. 247–332.

Saito S, Kobayashi N, Atoji Y. 2006. Subdivision of the accessory olfactory bulb in the Japanese common toad, *Bufo japonicus*, revealed by lectin histochemical analysis. Anat Embryol. 211:395–402.

Saito S, Matsui T, Kobayashi N, Wakisaka H, Mominoki K, Matsuda S, Taniguchi K. 2003. Lectin histochemical study on the olfactory organ of the newt, *Cynops pyrrhogaster*, revealed heterogeneous mucous environments in a single nasal cavity. Anat Embryol. 206:349–356.

Saito S, Taniguchi K. 2000. Expression patterns of glycoconjugates in the three distinctive olfactory pathways of the clawed frog, *Xenopus laevis*. J Vet Med Sci. 62:153–159.

Shapiro LS, Ee PL, Halpern M. 1995. Lectin histochemical identification of carbohydrate moieties in opossum chemosensory systems during development, with special emphasis on VVA-identified subdivisions in the accessory olfactory bulb. J Morphol. 224:331–349.

Steiger SS, Fidler AE, Valcu M, Kempenaers B. 2008. Avian olfactory receptor gene repertoires: evidence for a well-developed sense of smell in birds? Proc Biol Sci. 275:2309–2317.

Takami S, Getchell ML, Getchell TV. 1994. Lectin histochemical localization of galactose, N-acetylgalactosamine, and N-acetylglucosamine in glycoconjugates of the rat vomeronasal organ, with comparison to the olfactory and septal mucosae. Cell Tissue Res. 277:211–230.

Tamada K, Fukamatsu N. 1992. Seasonal changes in the number and age composition of crows captured by multi-traps. Jpn J Ornithol. 40: 79–82[Japanese].

Wachowiak M, Shipley MT. 2006. Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb. Semin Cell Dev Biol. 17:411–423.

Wallraff HG. 2004. Avian olfactory navigation: its empirical foundation and conceptual state. Anim Behav. 67:189–204.

Wenzel BM. 1968. Olfactory prowess of the kiwi. Nature. 220:1133–1134.

Wenzel BM. 2007. Avian olfaction: then and now. J Ornithol. 148: S191–S194.

Yokosuka M, Takagi S, Katou M, Pudcharaporn K, Gizurarson S, Ichikawa M, Saito TR. 2008. *p*-Chloroamphetamine-induced rat ejaculation is not associated with the preoptic nucleus or medial nucleus amygdala. Reprod Med Biol. 7:37–43.

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