

Histological and Lectin Histochemical Studies on the Main and Accessory Olfactory Bulbs in the Japanese Striped Snake, *Elaphe quadrivirgata*

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ABSTRACT. The main and accessory olfactory bulbs were examined by histological methods and lectin histochemistry in the Japanese striped snake. As the results, the histological properties are similar between the main olfactory bulb and the accessory olfactory bulb. In lectin histochemistry, 21 lectins used in this study showed similar binding patterns in the main olfactory bulb and the accessory olfactory bulb. In detail, 15 lectins stained these olfactory bulbs with similar manner, and 6 lectins did not stain them at all. Two lectins, *Lycopersicon esculentum* lectin (LEL) and *Solanum tuberosum* lectin (STL), stained the nerve and glomerular layers and did not stain any other layers in both olfactory bulbs. Four lectins, Soybean agglutinin (SBA), *Vicia villosa* agglutinin (VVA), Peanut agglutinin (PNA) and *Phaseolus vulgaris* agglutinin-L (PHA-L) stained the nerve and glomerular layers more intensely than other layers in both olfactory bulbs. In addition, VVA showed the dot-like stainings in the glomeruli of both olfactory bulbs. These findings suggest that the degree of development and the properties of glycoconjugates are similar between the main olfactory bulb and the accessory olfactory bulb in the Japanese striped snake.

KEY WORDS: histology, nervous system, reptiles, Squamates, vomeronasal system.

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The olfactory system receives and detects chemical substances in the external environment. This system is divided into 2 independent systems: the main olfactory system and the vomeronasal system. In the main olfactory system, the receptor cells in the olfactory epithelium project their axons to the glomeruli in the main olfactory bulb to form synapse with output neurons and intermediate neurons. On the other hand, in the vomeronasal system, the receptor cells in the vomeronasal epithelium project their axons to the glomeruli in the accessory olfactory bulb [14]. Although the main olfactory system exists in all vertebrate species, the vomeronasal system first appears in amphibians, is lost in several species such as crocodiles, birds, whales and humans, and has various morphological and histological features among animal species [4]. The localization, size and laminar structure of the main and accessory olfactory bulbs vary among species [25, 29] and appear to relate with behavioral patterns and living environment of each species.

Among all tetrapods, snakes and some lizards have the most developed vomeronasal system [13], i.e. the vomeronasal system of snakes and lizards mediates not only species-specific communications by pheromones, such as courtship and aggregative behaviors [10, 21], but also non-species-specific behaviors by odoriferous molecules, such as predatory and defensive behaviors [22, 27, 36]. Snakes sample

environmental substances by the tongue-flicking and deliver concentrated chemicals to the vomeronasal epithelium, and the information acquired with the tongue-flicking is mediated by both the main olfactory system and the vomeronasal system [13, 36]. Topographically, the size of the accessory olfactory bulb is as large as that of the main olfactory bulb in snakes [15, 16], although the size of the accessory olfactory bulb is much smaller than that of the main olfactory bulb in many other vertebrate species. Histologically, both the main and accessory olfactory bulbs in snakes are divided into 6 layers (the nerve, glomerular, mitral cell, internal plexiform, granule cell and ependymal cell layers), and the histological properties of the constituent cells are similar between these olfactory bulbs [15, 16]. However, there are few detailed reports on the sublamination and cell distribution in these layers of the main and accessory olfactory bulbs in snakes.

Lectins are proteins binding nonimmunologically with glycoconjugates [3] and are extensively used for the differentiation of cell types on histological sections based on the staining regions and the staining intensities [23]. In lizards with well-developed vomeronasal system, the lectin binding patterns are similar between the main olfactory bulb and the accessory olfactory bulb [6], although the lectin binding patterns are different between these olfactory bulbs in many other species, such as amphibians [32–34] and mammals [26, 28, 31]. According to these reports on the lizards and many other species, the glycoconjugate moieties appear to be similar between the main olfactory bulb and the accessory bulb in the species with well-developed vomeronasal system, such as some lizards. Although snakes are equipped with the most developed vomeronasal system [13, 14] and belong to Squamata as well as lizards, there is no report on the lectin histochemistry on the main olfactory bulb and ac-

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cessory olfactory bulb in snakes. Squamata and mammals have evolved separately from primitive reptiles, and it is possible that the histochemical features of olfactory system are different between these two groups. In this study, we examined the main olfactory bulb and the accessory olfactory bulb of the Japanese striped snake, *Elaphe quadrivirgata*, by histological methods and 21 lectins extensively-used for screening the differentiation of the glycoconjugate moieties between the main olfactory system and the vomeronasal system in many species to detect possible similarities between these olfactory bulbs in snakes.

MATERIALS AND METHODS

Animals: Six snakes in the reproductive season (June) were studied (Table 1). They were kept in near-natural conditions in the Japan Snake Institute (Ota, Japan) and purchased. Based on an age estimated by the body length

correlation [9], all snakes were sexually mature. This study was approved and conducted according to the Guideline for Animal Experiment of Iwate University. All procedures were approved by the local animal ethical committee of Iwate University.

Histology: The animals were anesthetized by intraperitoneal injection of pentobarbital (0.13–0.20 mg/g body weight) and were sacrificed by cardiac perfusion with Ringer's solution followed by Zamboni's fixative. After decapitation, brains were removed from heads, fixed in the same fixative for 3–4 hr, routinely embedded in paraffin and cut frontally or horizontally at 5 μ m thickness. Some of these sections were stained with luxol fast blue/cresyl violet (staining of Klüver-Barrera), and other sections were processed for lectin histochemistry.

Lectin histochemistry: Several sections were processed for histochemistry with ABC method using 21 biotinylated lectins (Table 2) in the lectin screening kit I-III (Vector Laboratories, Burlingame, CA, U. S. A.). After deparaffinization and rehydration, sections were incubated with 0.3% H_2O_2 in methanol to eliminate endogenous peroxidase. Sections were rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and incubated with 1% bovine serum albumin to block nonspecific reactions. After rinsing, sections were incubated with biotinylated lectins at 4°C overnight, reacted with ABC reagent (Vector) at room temperature for 30 min and colorized with 0.05 M Tris-HCl (pH 7.4) containing 0.006% H_2O_2 and 0.02% 3–3'-diaminobenzidine tetrahydrochloride. Staining intensities were judged as 5 grades: intense, moderate, weak, faint and negative staining. Control stainings

Table 1. Details of animals used in the present study

	Date	TL (cm)	W(g)	Sex
No. 22	Jun-09	116	202.1	Male
No. 23	Jun-09	119	225.7	Female
No. 24	Jun-09	114	202.9	Male
No. 25	Jun-09	124	458.7	Male
No. 36	Jun-10	130	341.1	Male
No. 37	Jun-10	89	226.8	Female

TL, Total length; W, Weight

Table 2. Concentrations and binding specificities of lectins used in the present study

Lectins	Abbreviation	Concentration (mg ml ⁻¹)	Binding specificity
Wheat germ agglutinin	WGA	1.0×10 ⁻²	GlcNAc, NeuAc
Succinylated-wheat germ agglutinin	s-WGA	1.0×10 ⁻²	(GlcNAc) _n
<i>Lycopersicon esculentum</i> lectin	LEL	2.0×10 ⁻³	(GlcNAc) ₂₋₄
<i>Solanum tuberosum</i> lectin	STL	1.0×10 ⁻²	(GlcNAc) ₂₋₄
<i>Datura stramonium</i> lectin	DSL	4.0×10 ⁻³	(GlcNAc) ₂₋₄
<i>Bandeiraea simplicifolia</i> lectin-II	BSL-II	5.0×10 ⁻²	GlcNAc
<i>Dolichos biflorus</i> agglutinin	DBA	5.0×10 ⁻²	Gal, GalNAc
Soybean agglutinin	SBA	1.0×10 ⁻²	Gal, GalNAc
<i>Bandeiraea simplicifolia</i> lectin-I	BSL-I	5.0×10 ⁻³	Gal, GalNAc
<i>Vicia villosa</i> agglutinin	VVA	1.0×10 ⁻²	Gal, GalNAc
<i>Sophora japonica</i> agglutinin	SJA	5.0×10 ⁻²	Gal, GalNAc
<i>Ricinus communis</i> agglutinin-I	RCA-120	2.0×10 ⁻³	Gal, GalNAc
Jacalin		5.0×10 ⁻⁴	Gal, GalNAc
Peanut agglutinin	PNA	4.0×10 ⁻³	Gal
<i>Erythrina cristagalli</i> lectin	ECL	2.0×10 ⁻²	Gal, GalNAc
<i>Ulex europaeus</i> agglutinin-I	UEA-I	5.0×10 ⁻²	Fuc
Concanavalin A	ConA	3.3×10 ⁻³	Man, Glc
<i>Pisum sativum</i> agglutinin	PSA	4.0×10 ⁻³	Man, Glc
<i>Lens culinaris</i> agglutinin	LCA	4.0×10 ⁻³	Man, Glc
<i>Phaseolus vulgaris</i> agglutinin-E	PHA-E	5.0×10 ⁻³	Oligosaccharide
<i>Phaseolus vulgaris</i> agglutinin-L	PHA-L	2.5×10 ⁻³	Oligosaccharide

Fuc: Fucose; Gal: Galactose; GalNAc: N-acetylgalactosamine; Glc: Glucose; GlcNAc: N-acetylglucosamine; Man: Mannose; NeuAc: N-acetylneuraminic acid.

were performed (a) by the preabsorption of lectins with each specific sugar residue (Table 2) or (b) by the use of PBS to replace ABC reagent.

RESULTS

Topographical and histological features of the main and accessory olfactory bulbs: The olfactory bulb was located on the rostral surface of the telencephalon as a pair of elongated structures and was divided into 2 structures, rostrally located main olfactory bulb and caudally located accessory olfactory bulb (Fig. 1A). The size of the accessory olfactory bulb was as large as that of the main olfactory bulb (Fig. 1A). The main olfactory bulb was a round structure and had centrally situated olfactory ventricle (Fig. 1B). The glomeruli of the main olfactory bulb were laid at rostral to lateral region uniformly to receive many thin olfactory nerves (Fig. 1B). On the other hand, the accessory olfactory bulb was a semicircular structure and had laterally situated olfactory ventricle (Fig. 1C). The glomeruli of the accessory olfactory bulb were restricted to the medial region to receive a single thick vomeronasal nerve in the medial region (Fig. 1C).

Histologically, in both the main and accessory olfactory bulbs, round or oval glomeruli were formed at the terminals of the olfactory or vomeronasal nerves and were surrounded by many small periglomerular cells with round nuclei (Fig. 1D, E). According to the staining of Klüver-Barrera, both the main (Fig. 1D) and accessory olfactory bulbs (Fig. 1E) were divided into six layers: the nerve layer, glomerular layer, mitral cell layer, internal plexiform layer, granule cell layer and ependymal cell layer. The internal plexiform layer of the main and accessory olfactory bulbs was divided into 3 sublaminae: the outer, middle and inner sublaminae (Fig. 1D, E, a-c). These sublaminae were identifiable according to the middle sublamina containing thick bundles of myelin sheaths. In both the main and accessory olfactory bulbs, the neurons in the mitral cell layer were classified into two types, i.e. the cells with large cell bodies (Fig. 1D, E, arrowheads) and the cells with small cell bodies (Fig. 1D, E, arrows), and these cells were scattered in the whole mitral cell layer.

Lectin histochemistry in the main and accessory olfactory bulbs: No specific staining was observed in the control staining (Figs. 2D, H, L, 4C, F), and no difference based on sex or estimated age was observed. In the following sentences, we used abbreviations of lectins as shown in Table 2.

All the lectins used in this study showed similar binding patterns between the main olfactory bulb and the accessory olfactory bulb (Fig. 2). Although 5 lectins among the 21 lectins, LEL, STL, RCA-120, PHA-E and PHA-L, stained the endothelial cells in the whole brain intensely, these stainings were excluded from results described below because the endothelial cells were not the neural components of the olfactory bulb.

Two lectins, LEL and STL, stained the nerve and glomerular layers intensely and did not stain other layers at all in the main olfactory bulb (Figs. 2A, 3A). These 2 lectins stained the nerve and glomerular layers moderately and did not stain other layers at all in the accessory olfactory bulb (Figs. 2E,

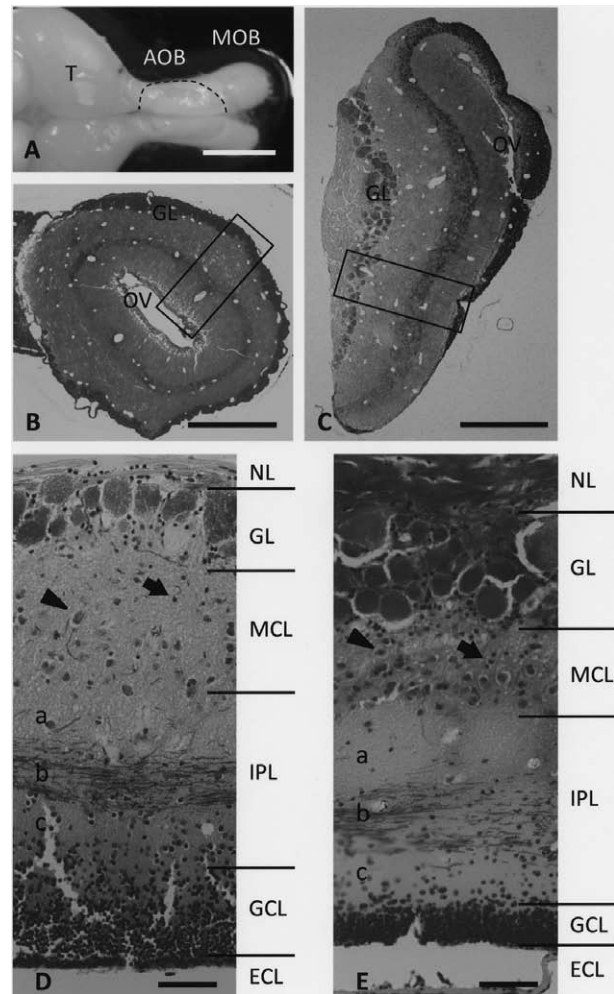


Fig. 1. Topographical (A) and histological features stained with the staining of Klüver-Barrera (B to E) of the main and accessory olfactory bulbs in the Japanese striped snake. A: Dorsal aspect of the brain. The right of the figure is rostral. Bar=3 mm. B: Frontal section of the main olfactory bulb. The left sides of the figures are medial, and the uppers are dorsal in (B) and (C). The box indicates the region shown in (D). Bar=300 μ m. C: Frontal section of the accessory olfactory bulb. The box indicates the region shown in (E). Bar=300 μ m. D: Histological structure of the main olfactory bulb. Character (a) indicates the outer sublamina, (b) indicates the middle sublamina and (c) indicates the inner sublamina of the internal plexiform layer in (D) and (E). Arrow and arrowhead indicate the cells with small cell bodies and the cells with large cell bodies in the mitral cell layer, respectively, in (D) and (E). Bar=50 μ m. E: Histological structure of the accessory olfactory bulb. Bar=50 μ m. AOB, accessory olfactory bulb; ECL, ependymal cell layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer; MOB, main olfactory bulb; NL, nerve layer; OV, olfactory ventricle; T, telencephalon.

I, 3D). In both the main and accessory olfactory bulbs, 3 lectins, SBA, PNA and PHA-L, stained the nerve and glomerular layers moderately and stained other layers weakly

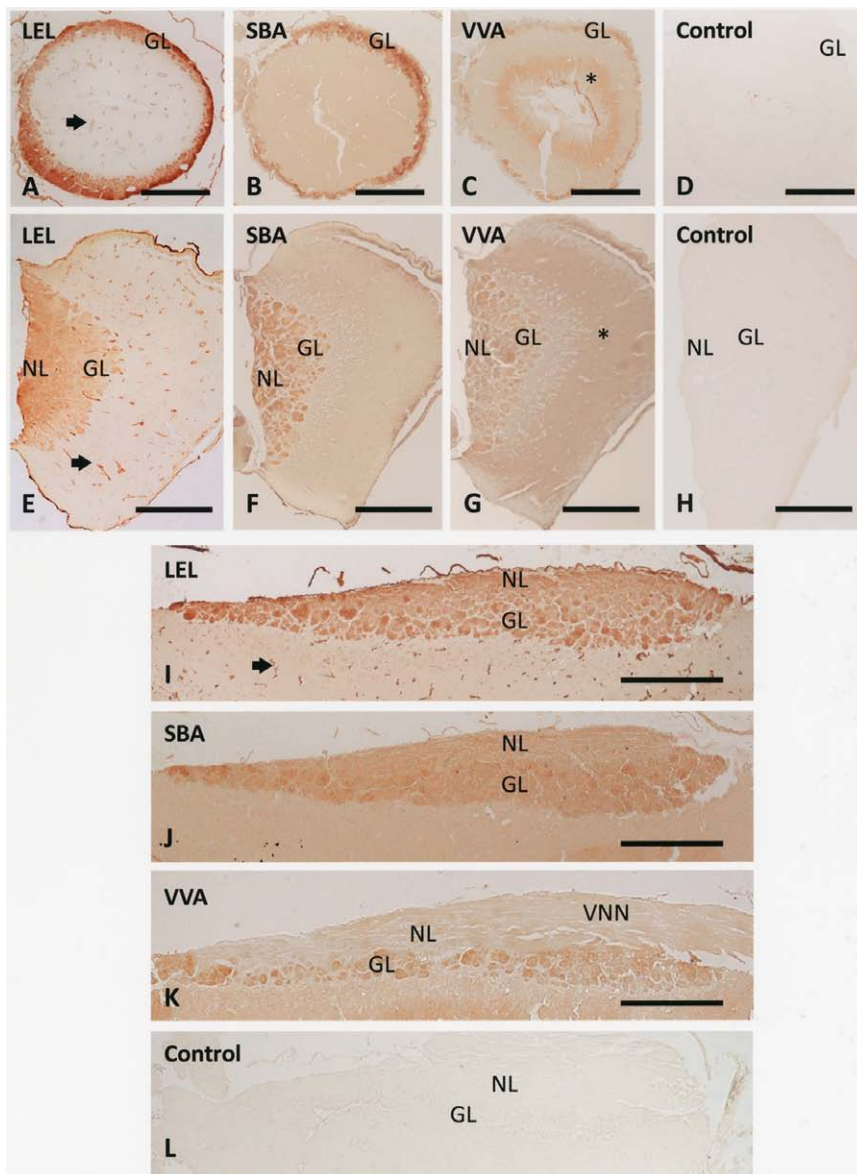


Fig. 2. Binding patterns of lectins in the main olfactory bulb and the accessory olfactory bulb in the Japanese striped snake. A–H: Frontal section of the main (A to D) and accessory (E to H) olfactory bulbs labeled by LEL (A and E), SBA (B and F) and VVA (C and G) stainings and control staining by the use of PBS to replace ABC reagent (D and H). The left side of the figure is medial, and the upper, dorsal in (A) to (H). Arrows in (A) and (E) indicate the endothelial cells. Asterisks in (C) and (G) indicate the internal plexiform layer. I–L: Horizontal section of the accessory olfactory bulb labeled by LEL (I), SBA (J) and VVA (K) stainings and control staining by the use of PBS to replace ABC reagent (L). Arrow in (I) indicates the endothelial cells. Bars=300 μ m. GL, glomerular layer; NL, nerve layer; VNN, vomeronasal nerve.

(Figs. 2B, F, J, 3B, E). VVA stained the glomerular layer moderately, the internal plexiform layer weakly and other layers faintly (Figs. 2C, G, K, 3C, F). Four lectins, DSL, Jacalin, ConA and PHA-E, stained all layers moderately. Two lectins, WGA and RCA-120, stained all layers weakly, and 3 lectins, ECL, PSA and LCA, stained all layers faintly. The

remaining 6 lectins did not stain the layers at all.

In detail, 6 lectins, LEL, STL, SBA, VVA, PNA and PHA-L, stained some glomeruli intensely and others weakly, and the glomeruli stained intensely and those stained weakly were distributed as tessellation with no reference to the region of the main and accessory olfactory bulbs (Fig. 2).

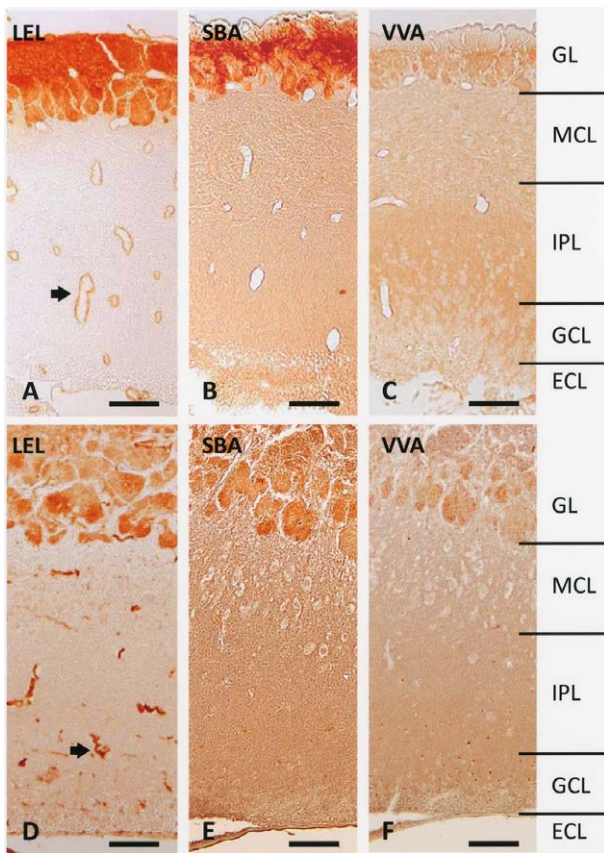


Fig. 3. Higher magnifications of the main olfactory bulb (A to C) and the accessory olfactory bulb (D to F) labeled by LEL (A and D), SBA (B and E) and VVA (C and F) stainings in the Japanese striped snake. Arrows in (A) and (D) indicate the endothelial cells. Bar=50 μ m. ECL, ependymal cell layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer.

Among the 15 lectins staining the glomeruli, VVA showed the dot-like staining in the glomeruli of both the main and accessory olfactory bulbs (Fig. 4B, E), although the remaining 14 lectins stained the whole region of the glomeruli equally (Fig. 4A, D).

These findings are summarized in Table 3.

DISCUSSION

In the Japanese striped snake, both the main and accessory olfactory bulbs were divided into 6 layers, and these laminar structures are similar between the main olfactory bulb and the accessory olfactory bulb. These histological properties conformed to previous reports by a Golgi study [15, 16]. In the glomerular layer, many periglomerular cells were observed in both the main and accessory olfactory bulbs. In mammals, the number of the periglomerular cells in the accessory olfactory bulb is much smaller than that in the main olfactory bulb [25]. In addition, the internal plexiform layer was divided into 3 sublaminae in both the main and accessory

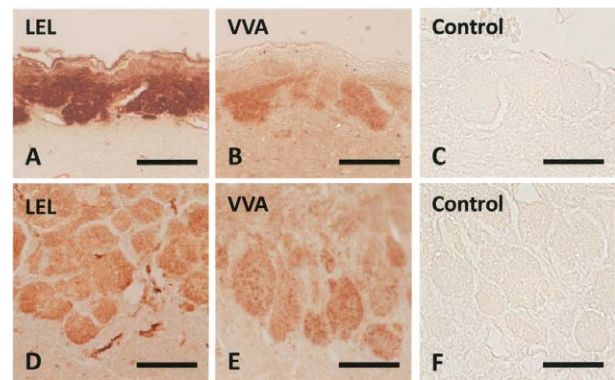


Fig. 4. Higher magnifications of the glomeruli in the main olfactory bulb (A to C) and the accessory olfactory bulb (D to F) labeled by LEL (A and D) and VVA (B and E) stainings and control staining by the use of PBS to replace ABC reagent (C and F) in the Japanese striped snake. Bar=50 μ m.

olfactory bulbs according to the staining of Klüver-Barrera, although it has been reported that this layer is divided into 3 sublaminae only in the accessory olfactory bulb according to toluidine blue staining or GABA-immunostaining [19]. The staining of Klüver-Barrera enabled us to show clearly the middle sublamina containing thick bundles of myelin sheaths in both the main and accessory olfactory bulbs (Fig. 1D, E). This finding appears to allow distinction of the middle sublamina from the outer and inner sublaminae. In the mitral cell layer of both the main and accessory olfactory bulbs, 2 types of neurons, the cells with large cell bodies and the cells with small cell bodies, were scattered. The cells with large cell bodies appear to correspond to the mitral cells as output neurons, and the cells with small cell bodies may correspond to the tufted cells, which are well-known as other cell type of output neurons in the main olfactory bulb of mammals [12], or the interneurons in the mitral cell layer, although we could not determine these correspondences. On these present findings, the histological properties are similar between the main olfactory bulb and the accessory olfactory bulb in the snake. The complexity of layer organization of the olfactory bulb is proportional to information-processing capability in the olfactory bulb and reflects the degree of development of the olfactory bulb [29]. Therefore, these present results indicate that the degree of development is similar between the main olfactory bulb and the accessory olfactory bulb in the snake and that the snake depends on both the main olfactory system and the vomeronasal system equally to detect information on external environments.

Fifteen lectins stained the nerve and glomerular layers in both the main and accessory olfactory bulbs. On the other hand, in the olfactory and vomeronasal epithelia of the Japanese striped snake, 4 lectins among these 15 lectins, SBA, PNA, ECL and PSA, stain the cell processes of the receptor cells only after sialic acid removal [18] and do not stain them before this treatment [17]. These reports suggest that several glycoconjugate moieties in receptor cells are capped by si-

Table 3. Lectin binding patterns in the olfactory bulb of the Japanese striped snake

Lectin	Main olfactory bulb			Accessory olfactory bulb		
	Nerve layer	Glomerular layer	Mitral cell, internal plexiform, granule cell and ependymal cell layers	Nerve layer	Glomerular layer	Mitral cell, internal plexiform, granule cell and ependymal cell layers
WGA	+	+	+	+	+	+
s-WGA	—	—	—	—	—	—
LEL	+++	+++	—	++	++	—
STL	+++	+++	—	++	++	—
DSL	++	++	++	++	++	++
BSL-II	—	—	—	—	—	—
DBA	—	—	—	—	—	—
SBA	++	++	+	++	++	+
BSL-I	—	—	—	—	—	—
VVA	±	++	± or + ^{a)}	±	++	± or + ^{a)}
SJA	—	—	—	—	—	—
RCA -120	+	+	+	+	+	+
Jacalin	++	++	++	++	++	++
PNA	++	++	+	++	++	+
ECL	±	±	±	±	±	±
UEA-I	—	—	—	—	—	—
ConA	++	++	++	++	++	++
PSA	±	±	±	±	±	±
LCA	±	±	±	±	±	±
PHA-E	++	++	++	++	++	++
PHA-L	++	++	+	++	++	+

—: Negative staining; ±: Faint staining; +: Weak staining; ++: Moderate staining; +++: Intense staining.

a) The internal plexiform layer was stained weakly, and the mitral cell, granule cell and ependymal cell layer were stained faintly.

alic acid residues in the olfactory and vomeronasal epithelia and are not capped by sialic acid residues in the nerve and glomerular layers of the main and accessory olfactory bulbs. In general, sialoglycoproteins have many important roles in cell migration, axonal guidance and functional plasticity in the nervous system [1, 30]. It is considered that the olfactory and vomeronasal receptor cells have a high plasticity in the epithelia to relocate involved with turnover throughout life and that their axons have a low plasticity in the olfactory or vomeronasal nerves and the glomeruli to integrate and hold the axon terminals in a single glomerulus. Therefore, the glycoconjugates labeled by SBA, PNA, ECL and PSA appear to mediate functional plasticity in the receptor cells by sialic acid capping.

Six lectins, LEL, STL, SBA, VVA, PNA and PHA-L, stained individual glomeruli with various intensities in both the main and accessory olfactory bulbs. These results indicate that the amounts of several glycoconjugate moieties are different among individual glomeruli. In the olfactory system, glycoconjugates play several important roles in continuous regeneration of the olfactory neurons, i.e. neurite outgrowth and synapse formation [30]. Therefore, the different amounts of glycoconjugate moieties may reflect the processes of turnover stages of the receptor cells projecting their axons to individual glomeruli. In addition, VVA showed the dot-like staining in the glomeruli in both the main and accessory olfactory bulbs. As several neurotransmitter receptors, such as several muscarinic receptors, dopamine receptors

and GABA receptors, in the glomeruli show the dot-like staining by immunohistochemistry [2, 11, 20], VVA may stain some of the neurotransmitter receptors in the glomeruli of the main and accessory olfactory bulbs in the snake.

All the lectins used in this study showed similar binding patterns between the main olfactory bulb and the accessory olfactory bulb (Table 3). These results indicate that glycoconjugate moieties are similar between the main olfactory bulb and the accessory olfactory bulb in the snake. The binding patterns of several lectins are different between the main olfactory bulb and the accessory olfactory bulb in many other vertebrate species [5, 7, 8, 24, 26, 28, 32–35], although the binding patterns of lectins are similar between these olfactory bulbs in several lizards [6]. In particular, it is well-known that there are glycoconjugate moieties labeled by VVA in only the accessory olfactory bulb in several mammals [35]. Therefore, the findings from the present lectin histochemistry in the snake appear to be well accorded with the reports on the lectin histochemistry in several lizards [6]. As both snakes and lizards possess well-developed vomeronasal system, the present results support that glycoconjugate moieties are similar between the main olfactory bulb and the accessory olfactory bulb in the species with well-developed vomeronasal system. In addition, the lectin binding patterns in the receptor cells are reported to be similar between the main olfactory system and the vomeronasal system in the Japanese striped snake [17]. These similarities of lectin binding patterns between the main olfactory system and the

vomerolateral system may reflect that these systems mediate similar behaviors elicited by odoriferous molecules, such as predatory and defensive behaviors in snakes [21, 27, 36].

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