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Heterogeneous Expression of Glycoconjugates among Individual Glomeruli of the Hamster Main Olfactory Bulb

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Abstract

Glomeruli within the main olfactory bulb (MOB) are known as areas of synapse formation between axon terminals of olfactory neurons in the olfactory epithelium and dendrites of the first relay neurons (mitral and tufted cells) in the MOB, so that they serve as functional units in olfaction. We examined expression patterns of glycoconjugates in the glomeruli of the hamster MOB by lectin histochemistry using 21 biotinylated lectins. Thirteen lectins, WGA, s-WGA, DSL, DBA, SBA, VVA, SJA, RCA-I, PNA, ECL, UEA-I, PSA and LCA, showed differential binding patterns among the glomeruli. To evaluate these differential binding patterns of lectins, we analysed staining intensity of each of the 13 lectins on the level of individual glomeruli by image analysis, and classified staining intensity into five grades (negative, 1+, 2+, 3+, 4+) on the basis of results obtained. This classification enables us to make detailed comparison among individual glomeruli. We further analysed the grade of staining intensity of each of the 13 lectins in the same glomerulus in adjacent serial sections by image analysis, and found that individual glomeruli varied in combination of grades of staining intensity and kinds of lectins. These results indicate that glycoconjugates are expressed heterogeneously in individual glomeruli, and that heterogeneous expression may contribute to the topographic organization of the primary olfactory pathway.

Introduction

Glomeruli of the main olfactory bulb (MOB) are the first relay sites of olfactory information from the peripheral olfactory receptor neurons to the central olfactory cortex (Halász and Shepherd, 1983; Macrides and Davis, 1983; Mori, 1987; Shepherd, 1992). Axons of bipolar receptor neurons in the olfactory epithelium (OE) lining the nasal cavity converge to the glomeruli to form synapses with dendrites of the first relay neurons (mitral and tufted cells) in the MOB. Since the number of glomeruli is by far less than that of olfactory receptor neurons, each glomerulus within the MOB receives a massive convergence of axons of olfactory receptor neurons (Allison and Warwick, 1949; Mori, 1987). Thus, clear anatomical topography, such as point-to-point topography in the visual system, is not constructed between the olfactory receptor neurons and the glomeruli. The complicated relationship between them has made it difficult to study initial processing of olfactory information in the olfactory system.

Immunohistochemical studies using monoclonal antibodies raised against neuronal membrane and lectin histochemical studies to detect specific glycoconjugates expressed on the membrane surface have been performed to resolve the topographic relationship between the OE and the MOB (Fujita *et al*., 1985; Mori *et al*., 1985; Hoffmann and Meyer, 1991; Schwarting and Crandall, 1991; Key and Akeson, 1993; Riddle *et al.*, 1993; Ichikawa *et al*., 1994). These histochemical studies could classify olfactory receptor neurons and their termination glomeruli into subsets by detecting glycoconjugates expressed specificically in the glomeruli, and revealed that olfactory receptor neurons within a specific OE zone project their axons to glomeruli within a corresponding zone of the MOB (Fujita *et al*., 1985; Mori *et al*., 1985; Hoffmann and Meyer, 1991; Schwarting and Crandall, 1991; Key and Akeson, 1993). These findings suggest that glycoconjugates appear to play an important role in primary olfactory organization, although their detailed expression patterns in the MOB still remain unclear. In the present study, therefore, expression patterns of glycoconjugates were examined on the level of individual glomeruli by lectin histochemistry using 21 lectins, and were evaluated on the basis of lectin staining intensity quantified by image analysis in the hamster MOB.

Materials and methods

Subjects and preparation of tissues

Eight adult golden hamsters (140–200 g body wt) of either sex were deeply anesthetized by i.p. injection of sodium

Table 1 Binding specificities of the 21 lectins used in this study

Lectin	Specific sugar
Wheat germ agglutinin (WGA)	β-GlcNAc>-α-NeuAc
Succinylated wheat germ agglutinin (s-WGA)	β-GlcNAc
Lycopersicon esculentum lectin (LEL)	β-GlcNAc
Soranum tuberosum lectin (STL)	β-GlcNAc
Datura stramonium lectin (DSL)	β-GlcNAc
Bandeiraea simplicifolia lectin-II (BSL-II)	α,β-GlcNAc
Dolichos biflorus agglutinin (DBA)	α-GalNAc
Soybean agglutinin (SBA)	α,β-GalNAc
Bandeiraea simplicifolia lectin-I (BSL-I)	α-GalNAc
Vicia villosa agglutinin (VVA)	α,β-GalNAc
Sophora japonica agglutinin (SJA)	β- GalNAc
Ricinus communis agglutinin-I (RCA-I)	β- GalNAc
Jacalin	galactosyl-β-GalNAc
Peanut agglutinin (PNA)	galactosyl-β-GalNAc
Erythrina cristagalli lectin (ECL)	galactosyl-β-GlcNAc
Ulex europaeus agglutinin-I (UEA-I)	α-Fuc
Concanavalin A (Con A)	α-Man
Pisum sativum agglutinin (PSA)	α-Man
Lens culinaris agglutinin (LCA)	α-Man
Phaseolus vulgaris agglutinin-E (PHA-E)	oligosaccharide
Phaseolus vulgaris agglutinin-L (PHA-L)	oligosaccharide

Abbreviations: Fuc, fucose; Gal, D-galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; NeuAc, *N*-acetylneuraminic acid.

pentobarbital (60 mg/kg body wt) (Abbott, North Chicago, IL) and perfused with physiological saline, followed by Bouin's solution without acetic acid. The olfactory bulbs were removed, immersed in the same fixative for 24 h, and routinely embedded in paraffin. Some olfactory bulbs were cut coronally or parasagittally at 5 μm to make sections for staining with 21 lectins. The other olfactory bulbs were cut coronally at 5 μm to make serial paraffin sections. Individual serial sections were placed on separate slides to make a series consisting of 13 slides. A total of 36 series of 13 adjacent serial sections was used to observe differential binding of lectins in the same glomerulus.

Lectin histochemistry

The sections were deparaffinized with xylene, and processed for lectin histochemistry by the avidin–biotin complex (ABC) method with 21 biotinylated lectins (Table 1) in commercial lectin screening kits (Vector, Burlingame, CA, USA). Lectin histochemistry involved the following steps: (i) incubation with 1% bovine serum albumin (BSA) at 32°C for 30 min; (ii) rinsing in 0.02 M phosphate buffered saline (PBS; pH 7.25) for 15 min; (iii) incubation with a biotinylated lectin at 4°C for 48 h; (iv) rinsing in PBS for 15 min; (v) incubation with ABC at 32°C for 30 min; (vi) rinsing in PBS for 15 min; (vii) incubation with 0.05 M Tris–HCl (pH 7.6) containing 0.01% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.003% hydrogen peroxide for 30 min; (viii)

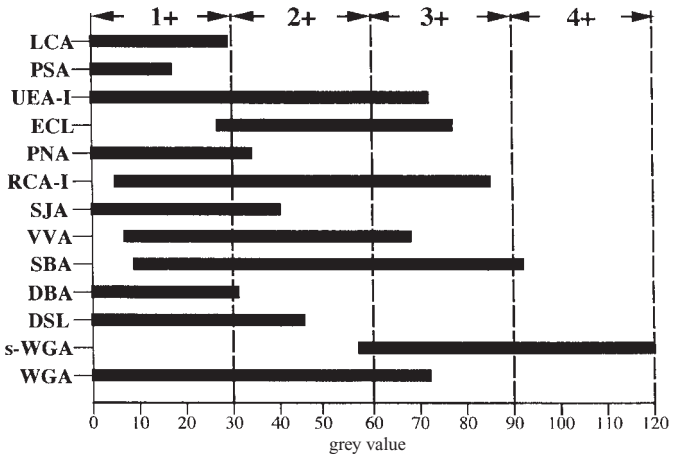

Figure 1 Ranges of staining intensities of 13 lectins. The abcissa represents grey values indicating staining intensities of lectins in individual glomeruli. Increases in grey value correspond to increases in staining intensity of lectins.

rinsing in distilled water. Working dilutions of 21 lectins were the same as in our previous report (Taniguchi *et al*., 1993). If the concentrations of lectins were higher than those in working dilutions as reported in our previous report, lectins bound non-specifically to all the histological structures in sections. In contrast, if their concentrations were lower, lectins bound to no histological structures at all. Therefore, the working dilutions were settled within ranges of concentrations providing specific staining of lectins. Their specific stainings were stable within these ranges of concentrations. Control lectin stainings were performed by the preabsorption of lectins with excess amounts of respective specific sugar residues or by the use of PBS to replace the biotinylated lectins or ABC. No specific lectin bindings were observed in these control stainings.

Image analysis

Image analysis was performed to measure the staining intensity of lectins in individual glomeruli as follows. A CCD-X1 camera (Shimadzu Scientific Instrument Inc., Kyoto, Japan) attached to a microscope (Olympus, Tokyo, Japan) was connected to a Power Macintosh 7100/66AV (Apple Computer Inc., Cupertino, CA) to obtain color images of lectin-stained sections. Color images were obtained under the same conditions, including magnification and voltage, to stabilize the brightness. A commercial software package (Adobe Photoshop 2.0J4.1, Adobe Systems Inc., Mountain View, CA) was used to extract only the images of individual glomeruli from images obtained and to divide the extracted images into 256 grades of grey scale. Each image of an individual glomerulus was analysed using NIH Image 1.56 software to obtain an average grey value of the pixels (AOP) in each image. The AOP represents the sum of the grey value of all the pixels divided by the number of pixels in the image. Each AOP was in proportion to the

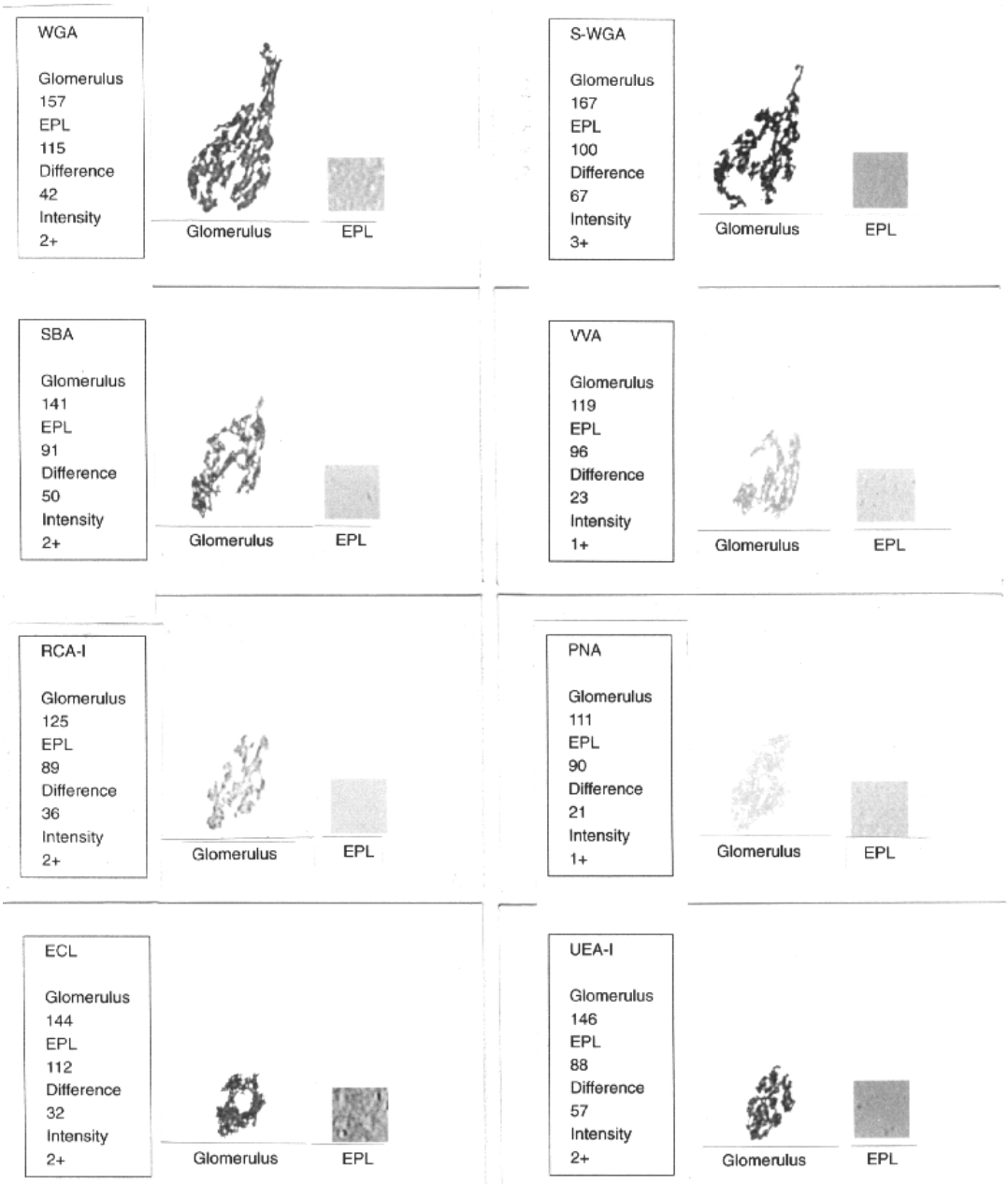

Figure 2 Digital images of the grade of staining intensities of lectins in one glomerulus. This glomerulus is arbitrarily extracted from a series of 13 adjacent sections of the MOB. The external plexiform layer (EPL) lying under this glomerulus is used to measure the background staining of lectins. This glomerulus reacts to eight out of 13 lectins, i.e. WGA, s-WGA, SBA, VVA, RCA-I, PNA, ECL and UEA-I. Numerals under 'Glomerulus' and 'EPL' represent their respective average grey value of pixels (AOP). A numeral under 'Difference' represents the remainder of AOP after the subtraction of AOP of EPL from that of glomerulus. The grade of staining intensity of the lectin in this glomerulus is estimated by the value of 'Difference'.

intensity of lectin staining in each glomerulus. The AOP of the external plexiform layer (EPL) located under the glomerular layer was also obtained in each of the analysed sections. The AOP of the EPL was deduced from the AOP of each individual glomerulus obtained from the same section, and the remainder of the AOP was determined as the staining intensity of a lectin of each of individual glomeruli. If the AOP of one glomerulus was less than that

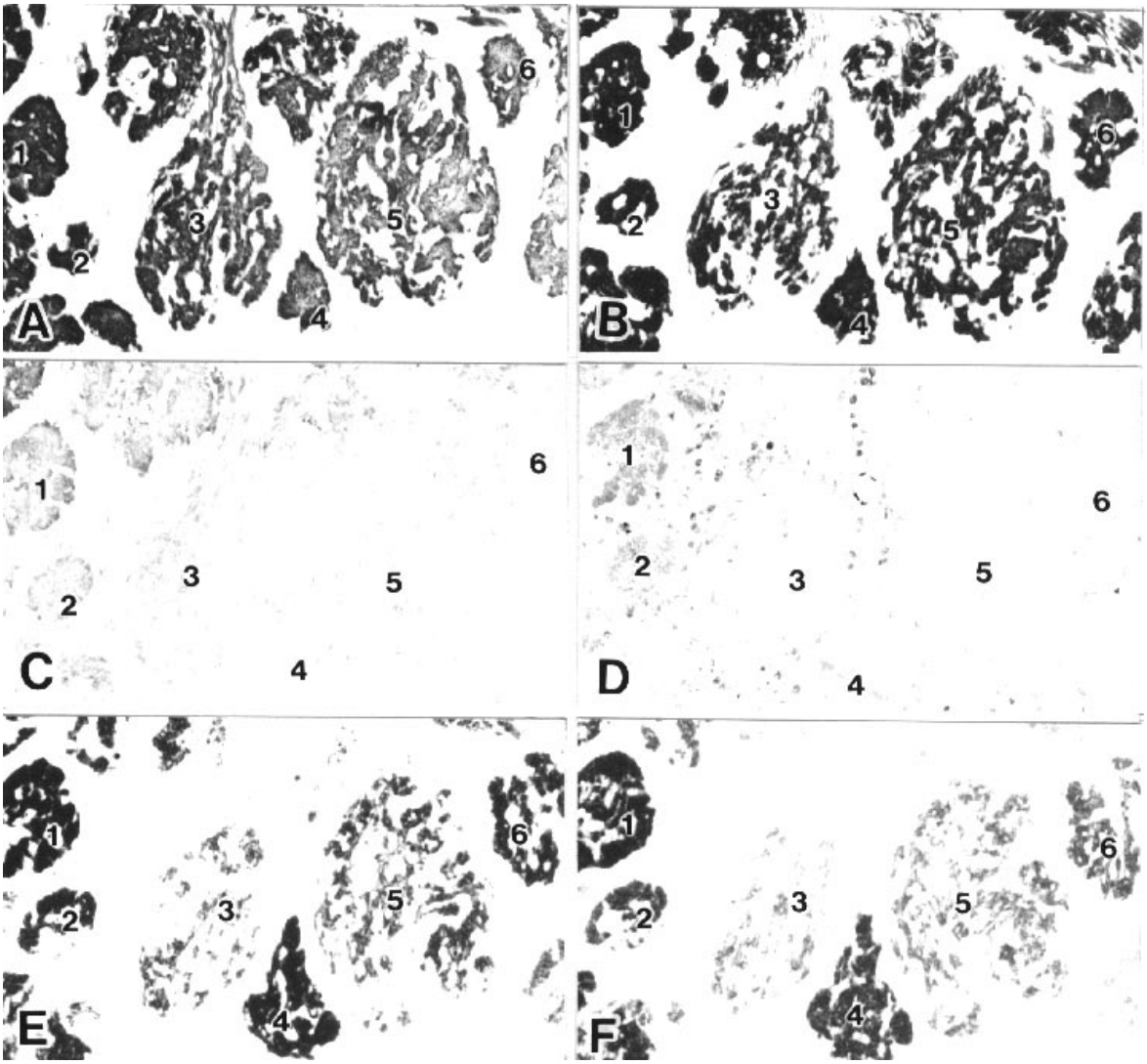

Figure 3 A series of 13 adjacent sections of the MOB showing the differential binding patterns of lectins WGA (A), s-WGA (B), DSL (C), DBA (D), SBA (E) and VVA (F) in the six glomeruli (1–6). Sections stained with PSA and LCA are not shown because of their negative stainings. Scale bar = 100 μm.

of the EPL, the staining intensity of this glomerulus was regarded as negative.

Results

We used 21 lectins to examine expression patterns of glycoconjugates in the glomeruli of the hamster MOB (Table 1). The olfactory nerve layer and glomeruli were stained by 16 out of 21 lectins. These 16 lectins bound specifically with axons of OE receptor neurons, but not with dendrites or axons of the mitral and tufted cells or any other interneurons. Among the 16 lectins, three lectins, LEL, STL and Con A, stained the olfactory nerve layer and glomeruli uniformly, while the other 13 lectins, WGA, s-WGA, DSL, DBA, SBA, VVA, SJA, RCA-I, PNA, ECL, UEA-I, PSA and LCA showed differential binding patterns in the olfactory nerve layer and/or glomeruli, and divided the axons of OE receptor neurons into subsets. In the olfactory nerve layer, differential binding patterns of lectins were not always ubiquitous, but observed in several regions on the level of bundles of axons of OE receptor neurons. Within a single glomerulus, however, all axons of OE receptor neurons were almost uniformly stained by each lectin and thus belonged to the same subset. Therefore, differential binding patterns of lectins were observed on the level of individual glomeruli.

To evaluate differential binding patterns of 13 lectins on the level of individual glomeruli, we measured the intensity of lectin staining of more than 100 individual glomeruli by image analysis for each of 13 lectins. Image analysis applied in this study enabled us to measure the intensity of lectin staining of individual glomeruli to express by grey value. Figure 1 shows the range of staining intensity of each of 13 lectins in more than 100 glomeruli. A single glomerulus

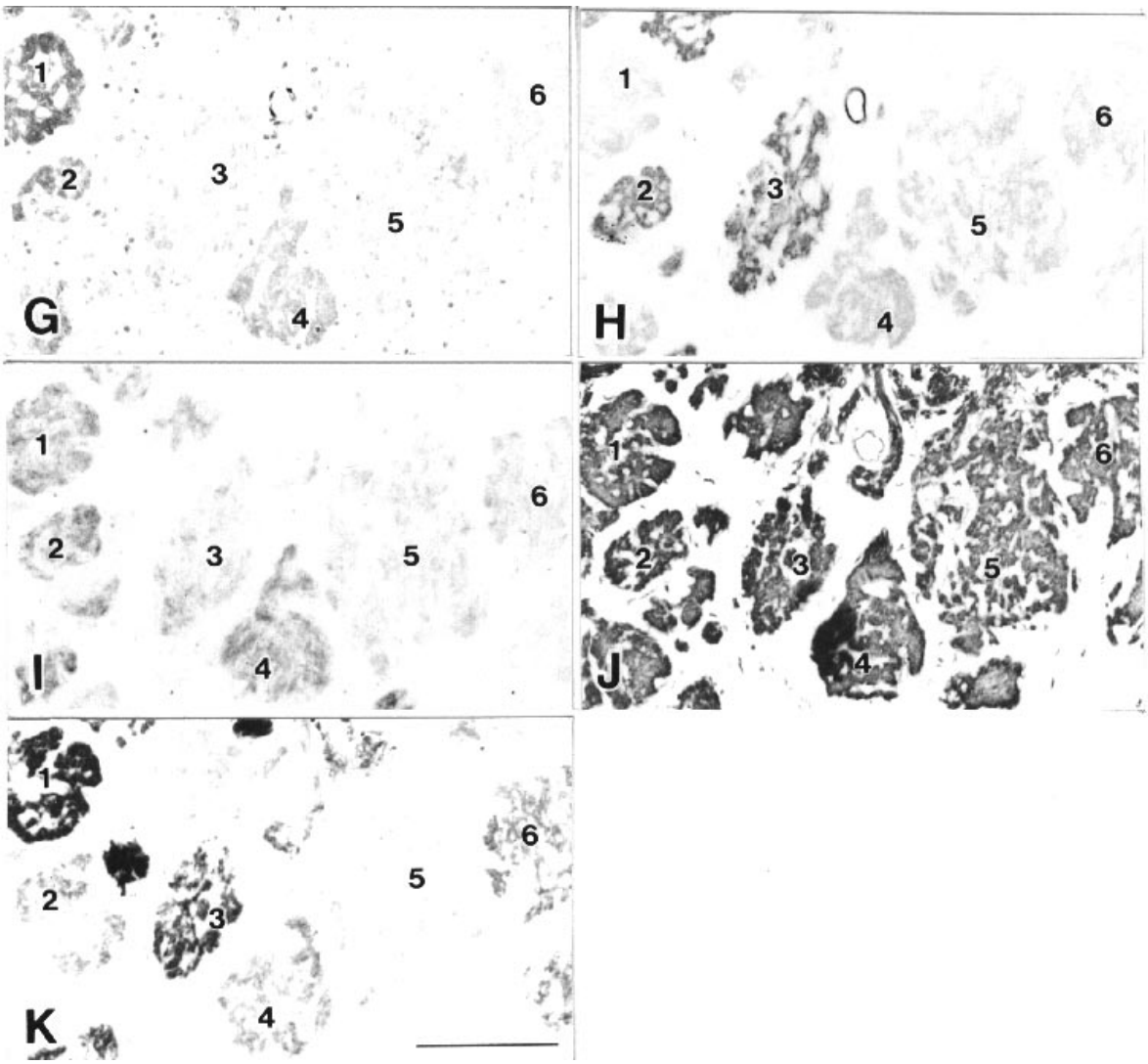

Figure 4 Continuation of Figure 3 showing the differential binding of lectins SJA (G), RCA-I (H), PNA (I), ECL (J), and UEA-I (K). Scale bar = 100 μm.

stained by one lectin had one grey value within the range of the lectin staining intensity shown in Figure 1. The staining intensity of one glomerulus was distinguished adequately from that of another glomerulus at a difference of 30 grey values, so that the range of staining intensity was divided at intervals of 30 grey values. Figure 1 does not include negative values, because the grey value of a glomerulus with negative staining of a lectin is expressed as >0 by this image analysis. Therefore, the intensity of lectin staining was divided into five grades, i.e. negative, 1+, 2+, 3+ and 4+. It was demonstrated by this analysis that each lectin could stain glomeruli within a range of 2–4 grades of intensity.

On the basis of the results obtained, we further investigated the grades of staining intensity of each of 13 lectins in a single glomerulus. Serial paraffin sections consisting of 13 slides were stained by 13 different lectins, and the staining intensity of each of the 13 lectins was determined by image analysis, as described above, in the same glomerulus in the series of 13 adjacent sections. Figure 2 shows the staining pattern of one of the glomeruli analysed in the serial sections. This glomerulus reacts to eight out of 13 lectins: WGA, s-WGA, SBA, VVA, RCA-I, PNA, ECL and UEA-I. We classified the staining intensity of each of the lectins in this glomerulus into five grades on the basis of results obtained, and decided a binding pattern of 13 lectins in this glomerulus. This glomerulus showed staining intensities of 3+ with s-WGA, 2+ with WGA, SBA, RCA-I and ECL, and 1+ with VVA, PNA and UEA-I. In the same way, we decided binding patterns of the 13 lectins for each of the glomeruli in 36 series of 13 adjacent serial sections. In comparison with binding patterns of the 13 lectins among individual glomeruli, no glomerulus showed the same combination of the grade of staining intensity and the kind of lectin as that of any other glomerulus. Evidential data are

Table 2 Binding patterns of lectins in individual glomeruli in Figures 2 and 3 to show the combination of grades of staining intensities and kinds of lectins

Lectin	Glomerulus					+
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
WGA	++	+++	++	++	++	++
s-WGA	++++	++++	+++	+++	+++	+++
DSL	+	+	–	–	–	–
DBA	++	+	–	+	+	+
SBA	++++	+++	++	++++	++	+++
VVA	+++	++	+	+++	++	+++
SJA	++	++	–	+	–	+
RCA-I	+	++	++	+	+	+
PNA	++	++	+	++	+	+
ECL	++	++	++	++	++	++
UEA-I	+++	+	++	+	–	+
PSA	–	–	–	–	–	–
LCA	–	–	–	–	–	–

+, staining intensity 1+ in Figure 1; ++, staining intensity 2+ in Figure 1; +++, staining intensity 3+ in Figure 1; ++++, staining intensity 4+ in Figure 1; –, negative staining.

given in Figures 3 and 4 and Table 2. No sex differences were observed in the binding patterns of lectins in the MOB.

Discussion

Many investigators have examined expression patterns of glycoconjugates in the olfactory bulb of various species by immunohistochemistry or lectin histochemistry. For example, Key and Akeson (Key and Akeson, 1993) identified three types of glomeruli in the mouse MOB on the basis of expression levels of lectin DBA (DBA+, weakly DBA+, DBA–), and revealed that DBA+ neurons are located preferentially in the rostromedial and caudodorsal regions of the OE and project their axons predominantly to the ventral, medial and dorsal portions of the rostral MOB and to the dorsomedial portion of the caudal MOB. They thought that the weak DBA+ glomeruli did not appear to represent selective innervation by axons with low expression levels of glycoconjugate liganded for DBA, but two very distinct subsets of olfactory receptor neurons (DBA+ and DBA–) might terminate together within the same glomeruli. If this is true, the differential expression of glycoconjugates exists among axons arising from neurons expressing the same odorant receptor, because recent experiments on olfactory receptor genes suggest that one glomerulus corresponds to a given odorant receptor (Vassar *et al.*, 1994; Monbaerts *et al.*, 1996; Wang *et al.*, 1998). Alternatively, it is possible that the differential expression of levels and kinds of glycoconjugates exists among neurons expressing different odorant receptors. In this case, weak DBA+ glomeruli represent the selective innervation by weak DBA+ axons.

According to our results, axons of OE receptor neurons converging onto the glomeruli displayed differential binding patterns of lectins in individual glomeruli of the hamster MOB. There was no relationship between the binding patterns of lectins and their specific sugar residues. Among or between the lectins binding to the same sugar residues (s-WGA, LEL, STL and DSL to β-*N*-acetylglucosamine; SJA and RCA-I to β-*N*-acetylgalactosamine and β-D-galactose; Jacalin and PNA to galactosyl-β-*N*-acetylgalactosamine; and ConA, PSA and LCA to α-mannose), no lectin showed the same binding pattern to the glomeruli as any of the other lectins. These differential binding patterns of lectins may indicate that lectins detecting the same monosaccharides recognize distinct glycoconjugates in the glomeruli, and may suggest that differential binding patterns of lectins in individual glomeruli reflect differences in the quality and quantity of specific sugar residues of glycolipids and glycoproteins among them.

The present data also suggest that individual glomeruli can be characterized by the combination of grades of staining intensity and kinds of lectins. Since each of 13 lectins showed 2–4 grades in its staining intensity in the present study, the combination of grades of staining intensities and kinds of lectins is calculated to be at least $>2^{13}$ (8192). On the other hand, the total number of glomeruli is estimated to be about 1900 in the rabbit (Allison and Warwick, 1949), and seems to be similar in the golden hamster. These values also indicate that virtually no glomerulus displays the same combination of grades of staining intensities and kinds of lectins as any other glomerulus in terms of probability. This suggestion agrees with the present results of the comparison of intensities of lectin stainings in individual glomeruli by 13 lectins in adjacent serial sections. No glomerulus showed the same combination of grades of staining intensities and kinds of lectins as that of any other glomerulus in this study. These results demonstrated the unique expression of levels and patterns of glycoconjugates in individual glomeruli. In addition, recent experiments on olfactory receptor genes suggest that neurons expressing a given odorant receptor project their axons to two of 1800 glomeruli in mice (Monbaerts *et al.*, 1996; Wang *et al.*, 1998). Although we have not examined whether expression patterns of glycoconjugates are different or not among glomeruli corresponding to the same odorant receptor, it is possible that the expression patterns of glycoconjugates are different among glomeruli corresponding to distinct odorant receptors.

Wang *et al*. (1998) demonstrated that axons of olfactory receptor neurons can project to the MOB, but cannot converge onto a given glomerulus by the deletion of olfactory receptor genes from the neurons. Judging from their report, olfactory receptor proteins may play a role as guidance molecules that enable the axons to converge onto appropriate glomeruli. Other guidance molecules may also take part in the topographic organization of the primary olfactory

projection, because the axons can project to the MOB without expression of the olfactory receptor protein. The guidance molecules involve members of neural cell adhesion molecule (NCAM), because some specific glycosylation forms of NCAM isoform have been identified in olfactory subsets (Key and Akeson, 1991; Pestean *et al*., 1995; Dowsing *et al*., 1997; Treloar *et al*., 1997). Various glycosylations in the NCAM appear to affect the ability of NCAM to result in the selective fasciation of the olfactory subsets. Since some of the lectins that we used recognize glycosylation forms of NCAM in the olfactory system (Key and Akeson, 1991; Pestean *et al*., 1995), it is possible that differential binding patterns of lectins on the level of individual glomeruli in this study reflect differential glycosylation of NCAM glycoforms and their differential expression levels in individual glomeruli.

In conclusion, our data suggest that individual glomeruli show unique expression patterns of glycoconjugates. The heterogeneous expression of glycoconjugates among individual glomeruli may underlie highly specific fasciation of olfactory axons onto appropriate glomeruli.

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