Chem. Senses 26: 645–652, 2001

# Histochemical Identification of Carbohydrate Moieties in the Accessory Olfactory Bulb of the Mouse Using a Panel of Lectins

**Ignacio Salazar, Pablo Sánchez Quinteiro, Matilde Lombardero and José Manuel Cifuentes**

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, E-27002 Lugo, Spain

*Correspondence to be sent to: Ignacio Salazar, Department of Anatomy and Embryology, Faculty of Veterinary Medicine, E-27002 Lugo, Spain. e-mail: anigsabe@lugo.usc.es*

## Abstract

Lectin binding patterns in the olfactory bulb of the mouse were investigated using 12 biotinylated lectins. Three, with specificities for galactose, *N*-acetylgalactosamine and L-fucose, stained only the nervous and glomerular layers of the accessory olfactory bulb; four, with specificities for galactose or *N*-acetylglucosamine, stained these layers in both the accessory and the main olfactory bulbs; three, with specificities for *N*-acetylgalactosamine or L-fucose, effected general staining with little contrast between the background and the accessory olfactory bulb or other structures; the remaining two, both of them specific for mannose, stained no part of the tissue studied. In the nervous and glomerular layers of the accessory olfactory bulb six lectins stained the anterior and posterior halves with different intensities and two of these six similarly differentiated between rostral and caudal regions of the posterior half. We conclude that: (i) three lectins binding to different monosaccharides are specific stains for the vomeronasal system when used in this area of the mouse brain; (ii) it may be appropriate to distinguish three parts in the mouse accessory olfactory bulb, instead of the hitherto generally accepted two.

## Introduction

Cell surface carbohydrates mediate a variety of cell interactions and functionally significant sets of sensory neurons can be identified on the basis of the carbohydrate-containing molecules they bear. Since lectins are specific for particular carbohydrate moieties they can be used to survey the distribution of glycoconjugates in tissues (Cook, 1986; Jessell *et al.*, 1990) and in recent years they have not infrequently been employed for this purpose in neurobiology [see articles in *Acta Anat.*, 161, Special Issue *Glycosciences*, especially Sharon (Sharon, 1998)]. Their use in this field has helped to clarify such topics as the subdivision of the accessory olfactory bulb (AOB) (Ichikawa *et al.*, 1992; Taniguchi *et al.*, 1993; Shapiro *et al.*, 1995) and has also shown that there is considerable between-species variation in the cell surface carbohydrate moieties expressed by homologous structures, the AOB being a case in point (Halpern, 1987; Halpern *et al.*, 1998a). Lectins have also begun to be used in an exciting new methodology with immense potential: the introduction of a plant lectin gene into the genome of an animal species, followed by its controlled expression in tissues of interest, allows the use of the lectin as a trans-neuronal tracer, a procedure that has already begun to afford valuable information on the development of connectivity (Horowitz *et al.*, 1999; Yoshihara *et al.*, 1999).

As far as we know, the only previous studies of lectin binding by the mouse olfactory system have been those of Key and Giorgi and Plendl and Schmahl (Key and Giorgi, 1986; Plendl and Schmahl, 1988). Here we report the findings of a descriptive study of the distribution of carbohydrate moieties in the olfactory bulb of the mouse as determined using a battery of 12 different lectins. We found that three lectins with different monosaccharide specificities bind only to the AOB and that it may be appropriate to distinguish three parts in the mouse AOB, instead of the hitherto generally accepted two.

## Materials and methods

Adult, singly housed female BALB/c mice bred in the Animal House of the University of Santiago de Compostela (Registry no. 15003AE) were placed under deep anaesthesia and trans-cardially perfused with phosphate-buffered saline (PBS) followed by Bouin's fixative (both pH 7.3). Series of 10 μm sagittal and transverse paraffin sections of the whole olfactory bulbs and frontal cortex were cut and, after elimination of endogenous peroxidase activity with $H_2O_2$, were incubated overnight at room temperature with one of the following biotinylated lectins, the monosaccharide specificities of which are listed, together with suppliers and concentrations, in Table 1: BSI-$B_4$ (from *Bandeiraea simplicifolia*), DBA (from *Dolichos biflorus*), ECA (from *Erythrina cristagalli*), SBA (from *Glycine max*), LCA (from *Lens cunicularis*), LTA (from *Lotus tetragonolobus*), LEA (from *Lycopersicum esculentum*), PSA (from *Pisum sativum*),

**Table 1** The 12 lectins used in this study, with their reported monosaccharide specificities (Sharon and Lis, 1989), the concentrations at which they were used and the supplier

| Lectin | Sugar specificity | Concentration (%) | Supplier |
|---|---|---|---|
| BSI-$B_4$ | Galactose | 0.001–0.003 | Sigma |
| ECA | Galactose | 0.002–0.004 | Sigma |
| DBA | N-acetylgalactosamine | 0.003 | Sigma |
| SBA | N-acetylgalactosamine | 0.002–0.004 | Sigma |
| VVA | N-acetylgalactosamine | 0.00025–0.0005 | Sigma |
| UEA-I | L-Fucose | 0.001 | Sigma |
| LTA | L-Fucose | 0.0005–0.002 | Vector Laboratories |
| LCA | Mannose | 0.002–0.003 | Sigma |
| PSA | Mannose | 0.001–0.003 | Vector Laboratories |
| LEA | N-acetylglucosamine[a] | 0.003 | Sigma |
| WGA | N-acetylglucosamine | 0.0001–0.00025 | Sigma |
| WGAs | N-acetylglucosamine | 0.0001–0.00025 | Vector Laboratories |

[a]Oligomers (Nachbar *et al*., 1980).

WGA (from *Triticum vulgaris*), UEA-I (from *Ulex europaeus*), VVA (from *Vicia villosa*) and WGAs (succinylated WGA). The lectin-labeled sections were then incubated with avidin–biotin–horseradish peroxidase complexes, after which fixed peroxidase was visualized by incubation with 3,3'-diaminobenzidine (0.05%) and $H_2O_2$ (0.03%) in 0.2 M Tris–HCl buffer. Two types of control were used: (i) sections processed without any lectin; (ii) sections processed with lectins pre-saturated with the corresponding monosaccharide.

All experiments were performed in accordance with the norms of the Declaration of Helsinki and the *Guiding Principles in the Care and Use of Animals* (DHEW, NIH 86-23). All efforts were made to minimize animal suffering and limit the number of animals used.

## Results

The arrangement and general morphological characteristics of the mouse AOB are shown in selected sagittal and transverse sections in Figure 1. In neither plane is it possible to distinguish mitral from tufted cells. The internal plexiform layer of the AOB is enclosed in the lateral olfactory tract (LOT) (Figure 1A, B).

The patterns of reactivity for each lectin are summarized below.

DBA (Figure 2A–C), BSI-$B_4$ (Figure 2D) and UEA-I (Figure 2E–H) stained only the AOB. Of the three, UEA-I stained most densely and DBA least densely. More importantly, whereas BSI-$B_4$ stained the whole AOB uniformly DBA stained the posterior part more intensely than the anterior, while UEA-I stained the anterior part more intensely than the posterior. UEA-I furthermore stained a particular rostral region of the posterior part much less intensely than the rest of this part of the AOB (Figure 2F, arrowhead).

ECA (Figure 3A), WGA (Figure 3B), WGAs (Figure 3C) and LEA (Figure 3D–F) stained both the AOB and the main olfactory bulb (MOB), with intensities increasing in the order ECA < WGA < WGAs < LEA. All these lectins also effected diffuse background staining. Like UEA-I and DBA, ECA and LEA differentiated between the anterior and posterior parts of the AOB; the difference was particularly marked with LEA, which also, like UEA-I, differentiated a poorly stained rostral region of the posterior AOB from the rest of this region (Figure 3E, arrowhead).

SBA (Figure 4A), VVA (Figure 4B) and LTA (Figure 4C) effected general staining of the whole section, with little contrast between the background and the AOB or other structures (e.g. the LOT, arrowhead in Figure 4B, C), although SBA and VVA nevertheless differentiated the anterior AOB from the posterior. These results were less reproducible than those obtained with the other lectins.

LCA (Figure 5A, C) and PSA (Figure 5B, D) failed to stain any part of the tissue studied.

In all cases in which the AOB or MOB bound lectins the only strata affected were the nervous layer (NL) and the glomerular layer (GL).

All lectin-less control sections were unstained and no specific binding was observed in controls with pre-saturated lectins.

## Discussion

The above results show that the 12 lectins used in this study are classifiable into three groups on the basis of their binding to the NL and GL of the mouse olfactory bulb (OB): Group I, binders (BSI-$B_4$, DBA, UEA-I, ECA, WGA, WGAs and LEA, the first three of which bound only to the AOB and the others to both the AOB and MOB); Group II, non-binders (PSA and LCA); Group III, unconfirmed or dubious binders that significantly stained the background as well as the OB and/or other structures (SBA, VVA and LTA).

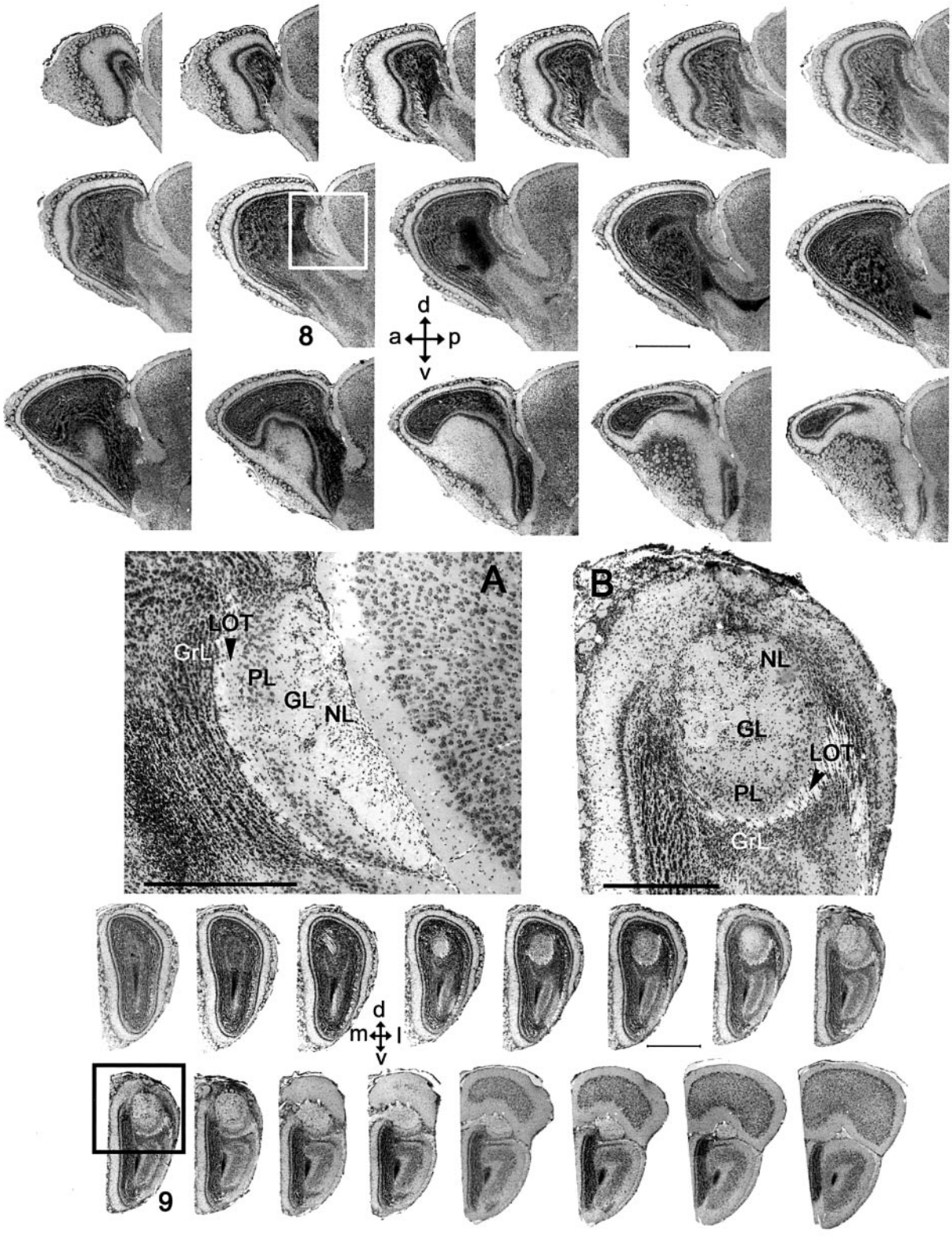


**Figure 1** The anterior part of the mouse brain, showing the topography of the AOB in serial sagittal sections ordered from medial to lateral (top) and serial transverse sections ordered from anterior to posterior (bottom). **(A, B)** Enlargements of the boxes in sagittal section 8 **(A)** and transverse section 9 **(B)**, showing the laminae and cell layers of the AOB. GL, glomerular layer; GrL, granular layer; LOT, lateral olfactory tract; NL, nervous layer; PL, plexiform layer; a, anterior; d, dorsal; l, lateral; m, medial; p, posterior; v, ventral. Scale bars: 1 mm for serial sections; (A, B) 500 μm.

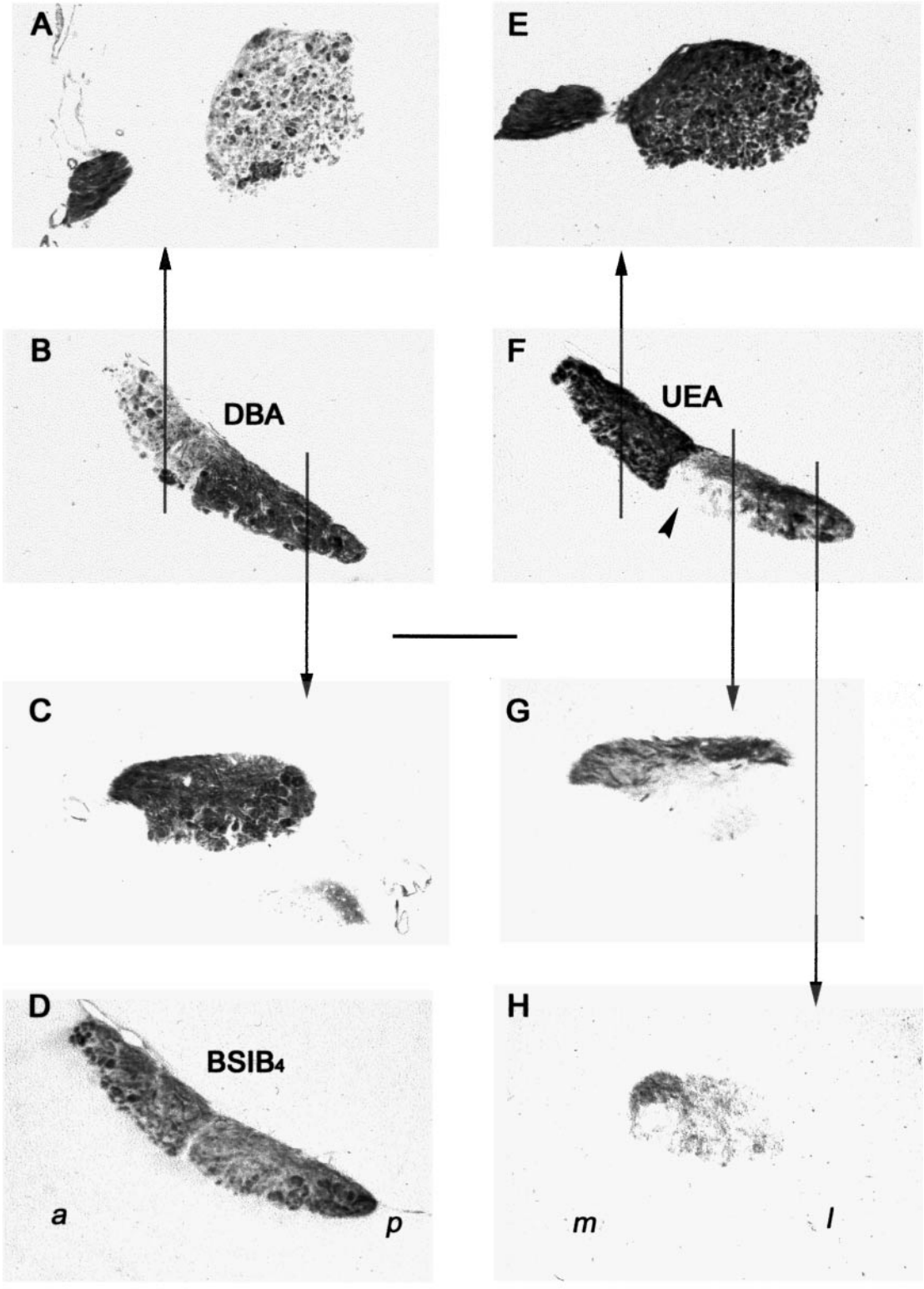


**Figure 2** **(A, C)** Transverse sections of the DBA-stained AOB at the levels shown in sagittal section **(B)**. **(D)** Sagittal section of the BSI-B4 stained AOB. **(E, G, H)** Transverse sections of the UEA-I stained AOB at the levels shown in sagittal section **(F)**; the arrowhead indicates the more weakly stained area in the posterior part. a, anterior; l, lateral; m, medial; p, posterior. a and p for sagittal sections; l and m for transverse sections. Scale bar: 380 μm for all sections.

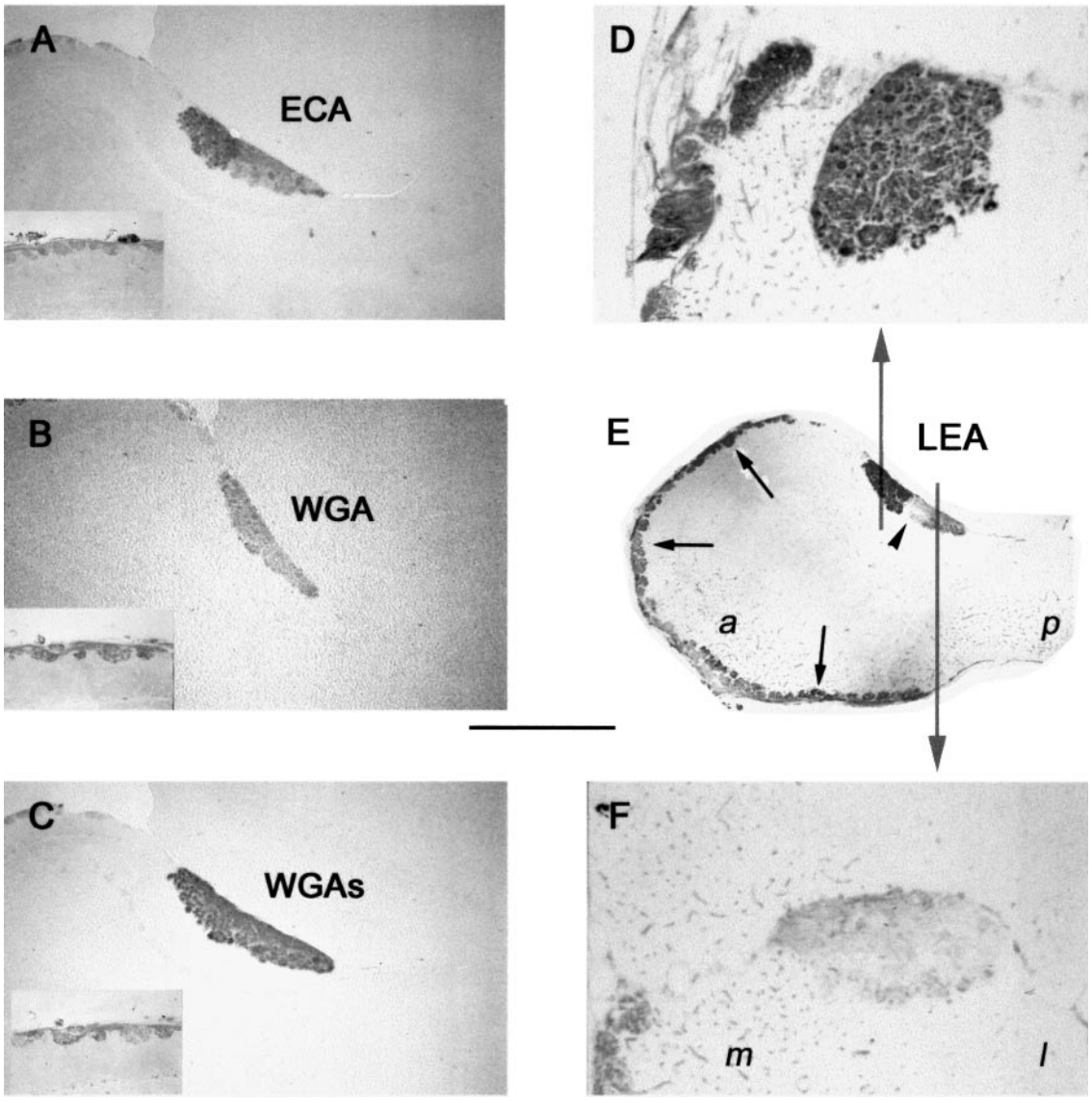


**Figure 3** **(A–C)** Sagittal sections of the AOB stained by ECA **(A)**, WGA **(B)** and WGAs **(C)**; insets show the MOB of the these sections. **(D, F)** Transverse sections of the LEA stained AOB at the levels shown in sagittal section **(E)**. Note the weakly stained area in the posterior part (arrowhead) and staining of the surface layers of the MOB. a, anterior; l, lateral; m, medial; p, posterior. a and p for sagittal sections; l and m for transverse sections. Scale bars: (A–C) 770 μm; insets, 875 μm; (D, F) 380 μm; (E) 1220 μm.

The finding that DBA binds to the AOB agrees with the results of the only other study in which lectins of Group I have been used to study the olfactory system of the adult mouse (Plendl and Schmahl, 1988). For the lectins of Group II no data have previously been published on their binding behaviour in the mouse. The Group III lectin SBA was considered by Key and Giorgi to bind specifically to the AOB and, hence, to the vomeronasal system, in this area of the mouse brain (Key and Giorgi, 1986).

The discrepancy between Key and Giorgi's findings and our own with respect to the behaviour of SBA may be at least partially due to Key and Giorgi having used coronal sections and frozen tissue, but also raises the question of what objective criteria there may be for evaluation of background staining (Key and Giorgi, 1986). Background stain can of course be largely avoided by diluting the lectin, but if this procedure also significantly reduces stain density in the putatively positively stained structure then it is difficult to distinguish among the following three possibilities: (i) the target structure has only a low density of molecules bearing the carbohydrate for which the lectin is specific; (ii) the affinity of these molecules for the lectin is low; (iii) staining of both the background and the target structure is non-specific, even though the target structure may have greater non-specific affinity than the background. In this study attempts to remove background staining by SBA, VVA and LTA caused a significant reduction in OB staining; these lectins not only stained the background, but also

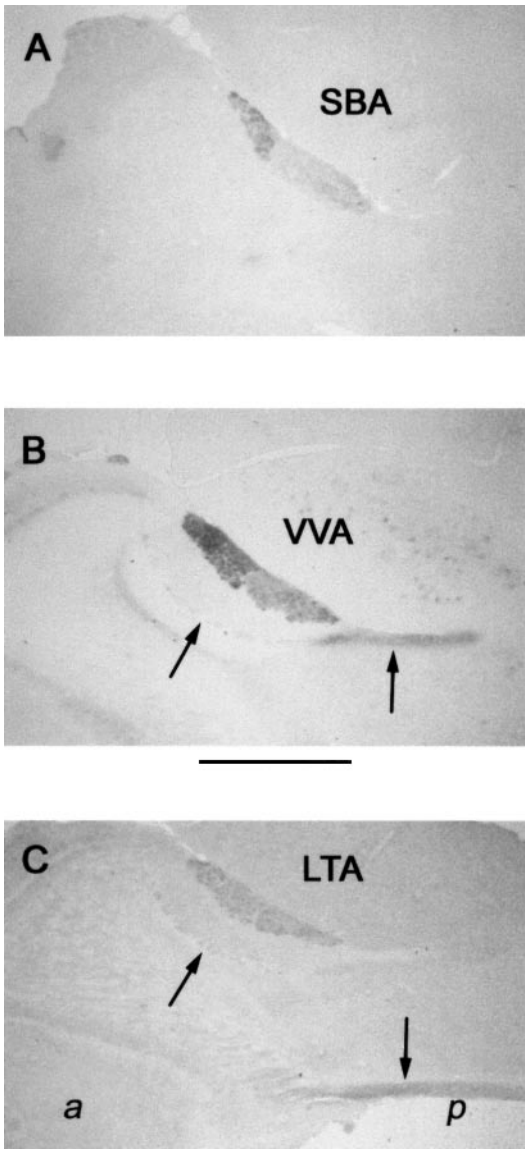


**Figure 4** Sagittal sections of the AOB stained by SBA **(A)**, VVA **(B)** and LTA **(C)**. Note the staining of the lateral olfactory tract (arrows). a, anterior; p, posterior. Scale bar: 770 μm for all sections.

non-OB structures such as the LOT. The results obtained with these lectins were less reproducible than those obtained with the others. We therefore regard their binding specifically to the OB as unconfirmed or dubious and group them accordingly.

The two mannose-specific lectins, LCA and PSA, both failed to stain anything and the three *N*-acetylglucosamine-specific lectins, LEA, WGA and WGAs, all stained both the AOB and the MOB. In contrast, the *N*-acetylgalactosamine-specific group (DBA, SBA and VVA), the galactose-specific group (ECA and BSI-$B_4$) and the fucose-specific group (UEA-I and LTA) were all heterogeneous as regards the staining behaviour of their members (in particular, ECA stained both the AOB and the MOB but BSI-$B_4$ only the former). These results suggest, subject to the limitations of the lectin battery used, that in neither the AOB nor the MOB are there cells bearing lectin-bindable mannose, show that in both structures there are cells bearing lectin-bindable *N*-acetylglucosamine and that BSI-$B_4$ binds to only a subset of the galactose-bearing molecules bound by ECA. The behaviour of DBA, ECA, UEA-I and LEA in the AOB also shows that the anterior and posterior AOB differ as regards the density and/or nature of their lectin-bindable *N*-acetylgalactosamine, galactose, fucose and *N*-acetylglucosamine and that the rostroventral part of the posterior AOB differs from the rest of the posterior AOB as regards the density and/or nature of lectin-bindable fucose and *N*-acetylglucosamine. It was pointed out by Sharon and Lis that some lectins are specific for just one anomer while others bind both, and many other factors may also influence the affinity of a monosaccharide-bearing molecule for a lectin that in principle is specific for that monosaccharide (Sharon and Lis, 1989).

As noted above, the mouse AOB has previously been studied with lectins by Key and Giorgi and Plendl and Schmahl (Key and Giorgi, 1986; Plendl and Schmahl, 1988). Lectins have also been used to study the AOB and other areas of the vomeronasal system in the rat (Barber, 1989; Ichikawa *et al*., 1992; Salazar and Sánchez Quinteiro, 1998), dog (Salazar *et al*., 1992), hamster (Taniguchi *et al*., 1993), sheep (Salazar *et al*., 2000), pig (Salazar *et al*., 2000) and opossum (Shapiro *et al*., 1995). These studies show both similarities and differences among different species as regards the distribution of lectin-bindable carbohydrate moieties. Unfortunately, the available data are an insufficient basis for any convincing explanation of these differences. Like Shapiro *et al.* (Shapiro *et al.*, 1995), we can only point out that care must be taken in comparing results obtained for different systems in different species using different lectins.

This study is the first in which the distinction between the anterior and posterior regions of the NL and GL of the AOB in the mouse has been observed using lectin histochemistry, but this distinction has previously been observed in the mouse at the molecular level (Belluscio *et al.*, 1999; Malnic *et al.*, 1999; Rodríguez *et al.*, 1999) and has been seen using a variety of methods in a number of other species [for a review see Halpern *et al.* (Halpern *et al.*, 1998a,b)]. Similarly, our observation that with UEA-I and LEA the rostral part of the posterior AOB stained differently from the caudal part has a precedent in Sugai *et al.*'s finding that in rat the *Ricinus communis* agglutinin RCA120 bound strongly to the anterior AOB NL/GL, weakly to the rostral two-thirds of the posterior AOB GL and not at all to the caudal third of the posterior AOB NL/GL (Sugai *et al.*, 2000). Again, there seem to be between-species differences as regards the identity of the lectin-bindable carbohydrate, since RCA120 is specific for galactose but neither of the galactose-specific lectins used in

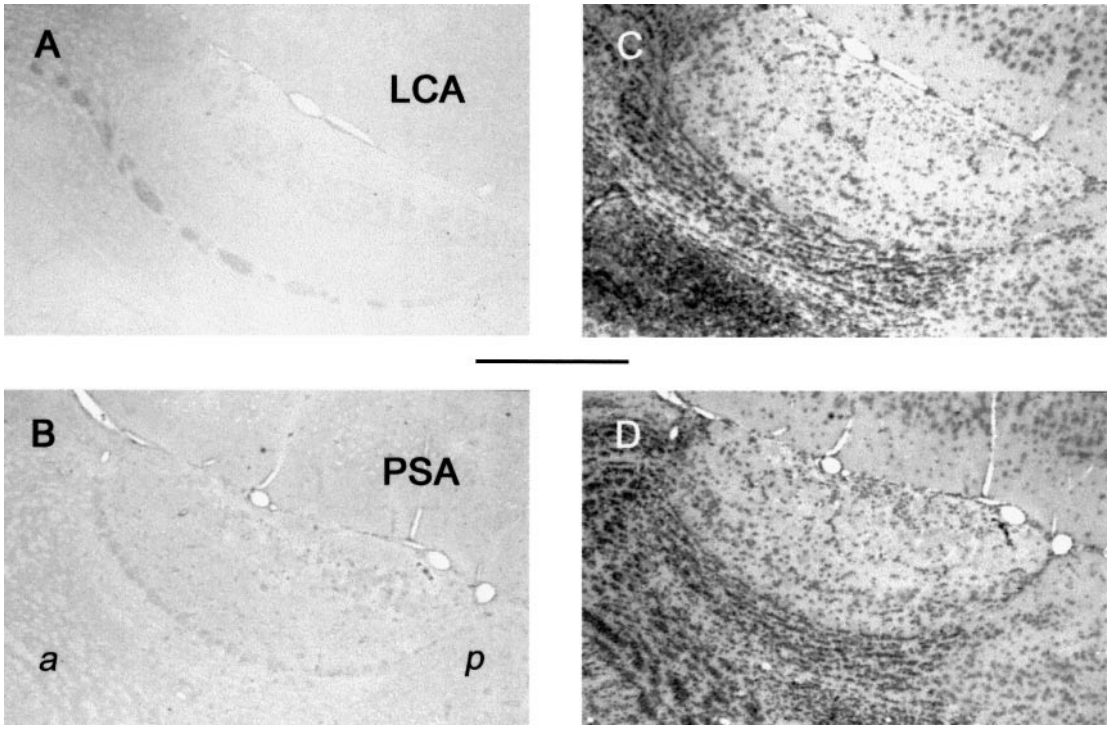


**Figure 5** Sagittal sections of the AOB stained by LCA **(A)** and PSA **(B)**, with the corresponding counterstained sections, **(C)** and **(D)**, respectively. a, anterior; p, posterior. Scale bar: 380 μm for all sections.

this study, BSI-$B_4$ and ECA, differentiated between rostral and caudal regions in the posterior AOB. Nevertheless, the binding pattern of the galactose-specific RCA120 in rat is the same as those of the fucose-specific UEA-I and poly(*N*-acetylglucosamine)-specific LEA in mouse and, following Sugai *et al.*'s reasoning for rat (Sugai *et al.*, 2000), we suggest that in mouse, too, this pattern probably reflects a functional division of the AOB into three parts (anterior, rostral posterior and caudal posterior) that may correspond to three input/output routes.

On a more technical level, we stress that the three region organization of the AOB described above might well have gone unnoticed if we had not systematically stained and examined whole series of sections in both transverse and sagittal planes [see also Salazar and Sánchez-Quinteiro for other aspects of the use of lectins in histochemistry (Salazar and Sánchez-Quinteiro, 1998)].

In conclusion, the results of this study show that in the region of the mouse brain studied the lectins BSI-$B_4$, DBA and UEA-I stain only the AOB, that six lectins (ECA, DBA, SBA, VVA, UEA-I and LEA) stain the anterior and posterior halves of the AOB with different intensities and that two of these six (UEA-I and LEA) stain the caudal part of the posterior AOB more intensely than the rostral part. The latter two findings suggest that the AOB comprises three distinct regions. The functional significance of this should be a fruitful subject for future research.

## Acknowledgements

The authors wish to thank Mr J. Castiñeiras for technical assistance and Mr I. C. Coleman for revising the English text. This work was supported in part by the Spanish Ministry of Education and Culture under project PB-0535.

## References

**Barber, P. C.** (1989) Ulex europeus *agglutinin I binds exclusively to primary olfactory neurons in the rat nervous system.* Neuroscience, 1, 1–9.

**Belluscio, L., Koentges, G., Axel, R.** and **Dulac, C.** (1999) *A map of pheromone receptor activation in the mammalian brain*. Cell, 97, 209–220.

**Cook, G. M. W.** (1986) *Cell surface carbohydrates: molecules in search of a function?* J. Cell Sci., 4 (suppl.), 45–70.

**Halpern, M.** (1987) *The organization and function of the vomeronasal system*. Annu. Rev. Neurosci., 10, 325–362.

**Halpern, M., Jia, C.** and **Shapiro, L. S.** (1998a) *Segregated pathways in the vomeronasal system*. Microsc. Res. Technol., 41, 519–529.

**Halpern, M., Shapiro, L. S.** and **Jia, C.** (1998b) *Hetereogeneity in the accessory olfactory system.* Chem. Senses, 23, 477–481.

**Horowitz, L. F., Montmayeur, J. P., Echelard, Y.** and **Buck, L. B.** (1999) *A genetic approach to trace neural c*ircuits. Proc. Natl Acad. Sci. USA, 96, 3194–3199.

**Ichikawa, M., Osada, T.** and **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.

**Jessell, T. M., Hynes, M. A.** and **Dodd, J.** (1990) *Carbohydrates and*

*carbohydrate-binding proteins in the nervous system*. Annu. Rev. Neurosci., 13, 227–255.

**Key, B.** and **Giorgi, P. P.** (1986) *Soybean agglutinin binding to the olfactory systems of the rat and mouse*. Neurosci. Lett., 69, 131–136.

**Malnic, B., Hirono, J., Sato, T.** and **Buck, L.** (1999) *Combinatorial receptor codes for odors*. Cell, 96, 713–723.

**Nachbar, M. S., Oppenheim, J. M.** and **Thomas, J. O.** (1980) *Lectins in the U.S. diet. Isolation and characterization of a lectin from the tomato (*Lycopersicon esculentum*)*. J. Biol. Chem., 255, 2056–2061.

**Plendl, J.** and **Schmahl, W.** (1988) Dolichos biflorus *agglutinin: a marker of the developing olfactory system in the NMRI-mouse strain.* Anat. Embryol., 177, 459–464.

**Rodriguez, I., Feinstein, P.** and **Mombaerts, P.** (1999) *Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system*. Cell, 97, 199–208.

**Salazar, I.** and **Sánchez Quinteiro, P.** (1998) *Lectin binding patterns in the vomeronasal organ and accessory olfactory bulb of the rat*. Anat. Embryol., 198, 331–339.

**Salazar, I., Barber, P. C.** and **Cifuentes, J. M.** (1992) *Anatomical and immunohistological demonstration of the primary neural connections of the vomeronasal organ in the dog.* Anat. Rec., 233, 309–313.

**Salazar, I., Sánchez Quinteiro, P., Lombardero, M.** and **Cifuentes, J. M.** (2000) *A descriptive and comparative lectin histochemical study of the vomeronasal system in pigs and sheep.* J. Anat., 196, 15–22.

**Shapiro, L. S., Ee, P. L.** and **Halpern, M.** (1995) *Lectin histochemical identification of carbohydrate moieties in opossum chemosensory systems during development, with spatial emphasis on VV-identified subdivisions in the accessory olfactory bulb*. J. Morphol., 224, 331–349.

**Sharon, N.** (1998) *Glycoproteins now and then: a personal account*. Acta Anat., 161, 7–17.

**Sharon, N.** and **Lis, H.** (1989) Lectins. Chapman & Hall, London, UK.

**Sugai, T., Sugitani, M.** and **Onoda, N.** (2000) *Novel subdivisions of the rat accessory olfactory bulb revealed by the combined method with lectin histochemistry, electrophysiological and optical recordings.* Neuroscience, 95, 23–32.

**Taniguchi, K., Nii, Y.** and **Ogawa, K.** (1993) *Subdivisions of the accessory olfactory bulb, as demonstrated by lectin-histochemistry in the golden hamster.* Neurosci. Lett., 158, 185–188.

**Yoshihara, Y., Mizuno, T., Nakahira, M., Kawasaki, M., Watanabe, Y., Kagamiyama, H., Jishage, K., Ueda, O., Suzuki, H., Tabuchi, K., Sawamoto, K., Okano, H., Noda, N.** and **Mori. K.** (1999) *A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene.* Neuron, 22, 33–41.

*Accepted March 6, 2001*