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ORIGINAL ARTICLE

Valeria Franceschini · Maurizio Lazzari
Franco Ciani

Lectin cytochemical localisation of glycoconjugates in the olfactory system of the lizards *Lacerta viridis* and *Podarcis sicula*

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Abstract To investigate the presence of defined carbohydrate moieties on the cell surface of the olfactory and vomeronasal receptor cells and the projections of the latter into the olfactory bulbs, a lectin binding study was performed on the olfactory system of the lizards: *Lacerta viridis* and *Podarcis sicula*. Both lizards showed a high lectin binding for *N*-acetyl-glucosamine in the sensory neurons. The lectin binding patterns in *Lacerta* indicated that the main olfactory system possessed a moderate density of *N*-acetyl-galactosamine residues and detectable levels of galactose ones. The vomeronasal system on the other hand contained a high density of *N*-acetyl-galactosamine moieties and a moderate density of glucosamine ones. In *Podarcis* the main olfactory system and vomeronasal organ contained respectively detectable and moderate levels of galactose residues. The expression of specific glycoconjugates may be associated with outgrowth, guidance and fasciculation of olfactory and vomeronasal axons.

Key words Glycoconjugates · *Reptilia* · *Lacertidae* · Olfactory receptor cells · Vomeronasal receptor cells · Olfactory bulbs

Introduction

The olfactory system of lizards, as in the majority of tetrapods, possesses two well-segregated populations of primary olfactory neurons: (1) the receptor cells of the olfactory epithelium that project their axons into the main olfactory bulb; (2) the receptors of the vomeronasal organ that terminate in the glomerular layer of the accessory olfactory bulb (Allison 1953; Scalia and Winans 1975; Halpern 1987).

V. Franceschini (✉) · M. Lazzari · F. Ciani
Department of Biology, University of Bologna, Via Selmi 3, 40126 Bologna, Italy
e-mail: vfrance@alma.unibo.it

The presence and the development of a vomeronasal organ varies considerably among reptiles (Bertmar 1981; Negus 1958; Saint Girons 1975). The vomeronasal organ disappears in adult crocodiles (Parsons 1970), in birds (Negus 1958) and in some mammals (Wysocki 1979). In squamates (snakes and lizards) the vomeronasal organ is separated from the nose by the secondary palate and the organ does not directly communicate with the nasal cavity (Labate et al. 1982). In general, it is well-developed in all genera of snakes. Among lizards the development and use of the nasal chemical senses varies and this variation appears related to the taxonomic group and to the role of the tongue in delivery of nonvolatile substances to the vomeronasal organ, as demonstrated by Graves and Halpern (1989) in the lizard *Chalcides ocellatus*. The olfactory epithelium and the vomeronasal organ are extremely reduced in *Chamaeleo chamaeleo* (Haas 1937). Otherwise the vomeronasal organ is relatively small in the Agamidae, well-developed in Gekkonidae and Scincoidea, and very well-developed in the Lacertidae (Gabe and Saint Girons 1976).

Several studies have reported that the olfactory and vomeronasal receptor cells possess a high density of cell surface glycoconjugates selectively labelled by some lectins (see Plendl and Sinowatz 1998, for recent review). In this study we report the lectin-binding pattern in the receptor cells of the olfactory mucosa and vomeronasal organ and the projections of the latter into the olfactory bulb in *Lacerta viridis* and *Podarcis sicula*. Both lizard species possess an excellent nasal chemosensory apparatus and a well-developed vomeronasal organ.

Materials and methods

Five specimens of *Lacerta viridis* (Laurenti 1768) and seven adult specimens of *Podarcis sicula* (Rafinesque-Schmaltz 1810) were used for this study. All procedures were in accordance with the guidelines of the European Communities Council Directive (86/609/CEE), the current Italian legislation for the use and care of animals, and conform to NIH guidelines. This study was also approved by the Ethic-Scientific Committee of the University of Bologna. The animals were anaesthetised by packing them in ice

Table 1 Lectin binding to the main olfactory system (MOS) and vomeronasal system (VNS) in *Lacerta viridis* and *Podarcis sicula*. Carbohydrate binding specificities listed are from Van Damme et al. (1998). The staining intensity is based on a subjectively estimated scale ranging from unstained (–), weak (±), detectable (+), moderate (++) and strong (+++)

Lectin	Acronym	Group Damjanov 1987	Carbohydrate specifity	Inhibitory sugar (molarity)	*L. viridis*		*P. sicula*	
					MOS	VNS	MOS	VNS
Glycine max agglutinin	SBA	III	D-galNAc >>D-gal	GalNAc (0.2 M)	++	+++	+	++
Dolichos biflorus agglutinin	DBA	III	α-D-galNAc	GalNAc (0.2 M)	++	+++	–	–
Bandeiraea simplicifolia agglutinin I	BSA-I	III	α-D-gal >α-D-galNAc	Gal (0.2 M)	+	++	+	++
Bandeiraea simplicifolia (isolectin B_4) agglutinin I	BSA-I-B_4	III	α-D-gal	Gal (0.2 M)	+	++	+	++
Lycopersicon esculentum agglutinin	LEA	II	D-glcNAc	GlcNAc (0.2 M)	+++	+++	+++	+++

prior to killing by decapitation. Brains, olfactory and vomeronasal epithelium were dissected and fixed in 6% $HgCl_2$, 1% sodium acetate, and 0.1% glutaraldehyde for 3 h at 4°C (Schulte and Spicer 1983). After dehydration, the specimens were embedded in Paraplast plus and cut in serial 7-µm sections.

For this investigation we used four horseradish peroxidase-conjugated lectins (Sigma): *Glycine max* agglutinin (SBA), *Bandeiraea simplicifolia* agglutinin (BSA-I) and its isomer BSA-I-B_4, *Dolichos biflorus* agglutinin (DBA), and one biotinylated lectin (Vector Laboratoires) – *Lycopersicon esculentum* agglutinin (LEA). These lectins were chosen as representative of groups II and III according to Damjanov (1987) and, for comparative reasons, with the findings obtained in our previous investigations on the tetrapod olfactory and vomeronasal system. (Franceschini et al. 1994b; 1996)

The sections, mounted on slides coated with poly-L-lysine (100 µg/ml), were deparaffinised and treated for 10 min with Lugol's solution to remove the mercury. Then, to quench the endogenous peroxidases, the slides were treated for 20 min with 1% H_2O_2 in 0.1 M TRIS buffer (pH 7.2). After washing in TRIS buffer, the slides were incubated at room temperature and in a moist chamber in the same buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and $MnCl_2$ (TBS) and each 5–10 µg/ml of the conjugated lectin. After 30 min incubation in the biotinylated lectin, the sections were washed in TBS and then incubated in the avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratoires) for 1 h in a moist chamber at room temperature. The time of incubation for the HRP-conjugated lectins was 3 h. The peroxidase was revealed by using the diaminobenzidine (DAB) method modified by Adams (1981). The slides were then rinsed, dehydrated, and mounted in Permount.

Controls for the lectin staining procedure included: (a) omission of lectin from the medium; (b) preincubation of the lectins with the appropriate inhibitory sugars (0.2–0.3 M in TBS) for 1 h at room temperature (Table 1).

Results

Histochemical lectin binding in *Lacerta viridis*

Soybean agglutinin strongly stained the vomeronasal receptor cells (Fig. 1A) and the accessory olfactory bulb (AOB) (Fig. 1B). The reaction product was present in the Golgi region as well as in the dendrite of vomeronasal receptor cells, in the vomeronasal nerve fibres and the glomerular layer of the accessory olfactory bulbs. The nerve fibres of the main olfactory system and their projections in the main olfactory bulb (MOB) were only moderately labelled (Fig. 1C).

Also the *Dolichos biflorus* agglutinin showed strong affinity to the vomeronasal epithelium. The sensory epithelium showed the typical bipolar receptor cells intensely stained (Fig. 1D). The reaction product was also present in the luminal surface region of vomeronasal projections into the glomerular layer of AOB were strongly stained by lectin binding (Fig. 1E). The MOB was less labelled by this lectin binding (Fig. 1F).

The incubation with *Bandeiraea simplicifolia* agglutinin I and its B_4 isomer moderately stained the olfactory receptor cells and their projections in the MOB (Fig. 1G) and more intensely the vomeronasal receptor cells and the glomeruli of the AOB (Fig. 1H).

Lycopersicon esculentum agglutinin exhibited strong binding to the receptor cells both in the main olfactory system (Fig. 1I) and in the vomeronasal organ (Fig. 1L). The lectin also labelled intensely the olfactory terminals in the MOB (Fig. 1M) and AOB (Fig. 1N).

These findings indicated that the olfactory receptor cells and their projections into the MOB of *Lacerta* possessed high density of *N*-acetyl-D-glucosamine and α-*N*-acetyl-galactosamine residues, as revealed respectively by LEA and DBA binding. Moreover the main olfactory system presented a low level of α-galactose moieties. SBA and BSA-I binding further confirmed the presence of α-*N*-acetyl-galactosamine and α-D-galactose residues on the saccharidic chains of the cell surface glycoproteins of the MOB. The vomeronasal neurons, their axons and the projections into the AOB also possessed a high density of α-*N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine residues, showed respectively by the DBA and LEA binding. Besides, lectin histochemistry revealed the presence of α-galactose residues in the vomeronasal system evidenced by the moderate staining of the BSA-I-B_4.

Histochemical lectin binding in *Podarcis sicula*

Soybean agglutinin and *Bandeiraea simplicifolia* agglutinin I (BSA-I and BSA-I-B_4) showed a detectable binding in the olfactory receptor cells and their projections into MOB and a moderate staining in the vomeronasal

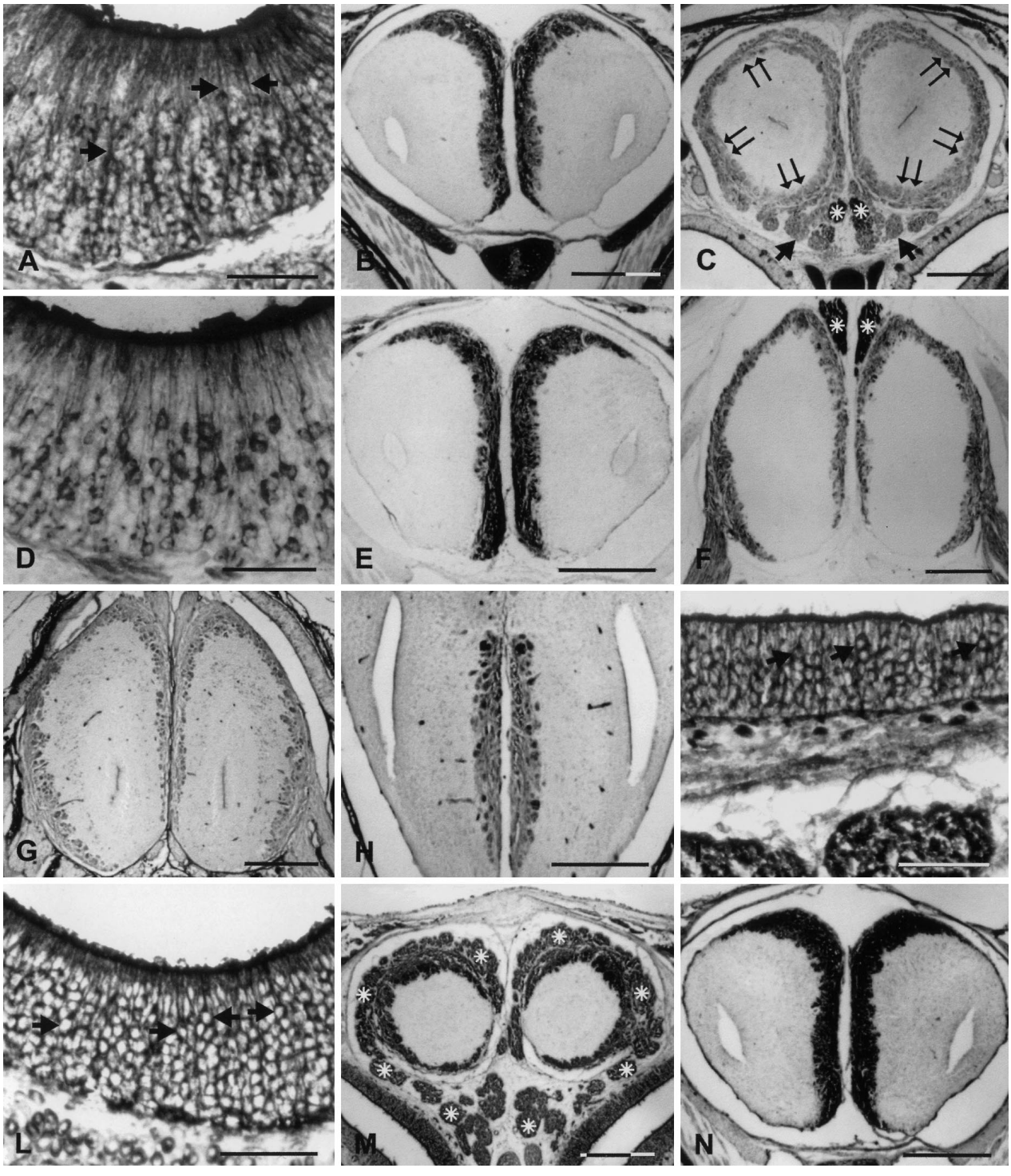

Fig. 1A–N Lectin histochemical characterisation of the olfactory system of *Lacerta viridis*. **A** Vomeronasal epithelium strongly labelled by SBA. The reaction product is present in the dendritic region of the vomeronasal receptor cells (*arrows*). *Bar* 50 µm. **B** Cross section of the AOB stained by SBA. *Bar* 250 µm. **C** The bundles of olfactory axons (*arrows*) projecting in the MOB (*double arrows*) show only a moderate staining after SBA binding. The vomeronasal nerves, in contrast, display an intense staining (*asterisks*). *Bar* 250 µm. **D** Vomeronasal receptor cells strongly stained by DBA; the mucus layer covering the luminal surface of the epithelium is also labelled. *Bar* 50 µm. **E** Vomeronasal projections into the AOB are strongly labelled by DBA. *Bar* 250 µm. **F** DBA labels the olfactory projections in the MOB less than the axon bundles of the vomeronasal organ (*asterisks*). *Bar* 250 µm. **G** Olfactory projections in the MOB are slightly stained by BSA-I-B_4. *Bar* 250 µm. **H** Coronal section at the level of the AOB shows intense staining of the glomeruli by BSA-I-B_4. *Bar* 250 µm. **I** Receptor cells (*arrows*) of the main olfactory system strongly stained by LEA. *Bar* 50 µm. **L** LEA intensely labelled also the olfactory neurons in the vomeronasal epithelium (*arrows*). *Bar* 50 µm. **M** Axon bundles (*asterisks*) of the olfactory nerve and their projections into the MOB stained by LEA. *Bar* 250 µm. **N** Cross section of the AOB stained by LEA. *Bar* 250 µm

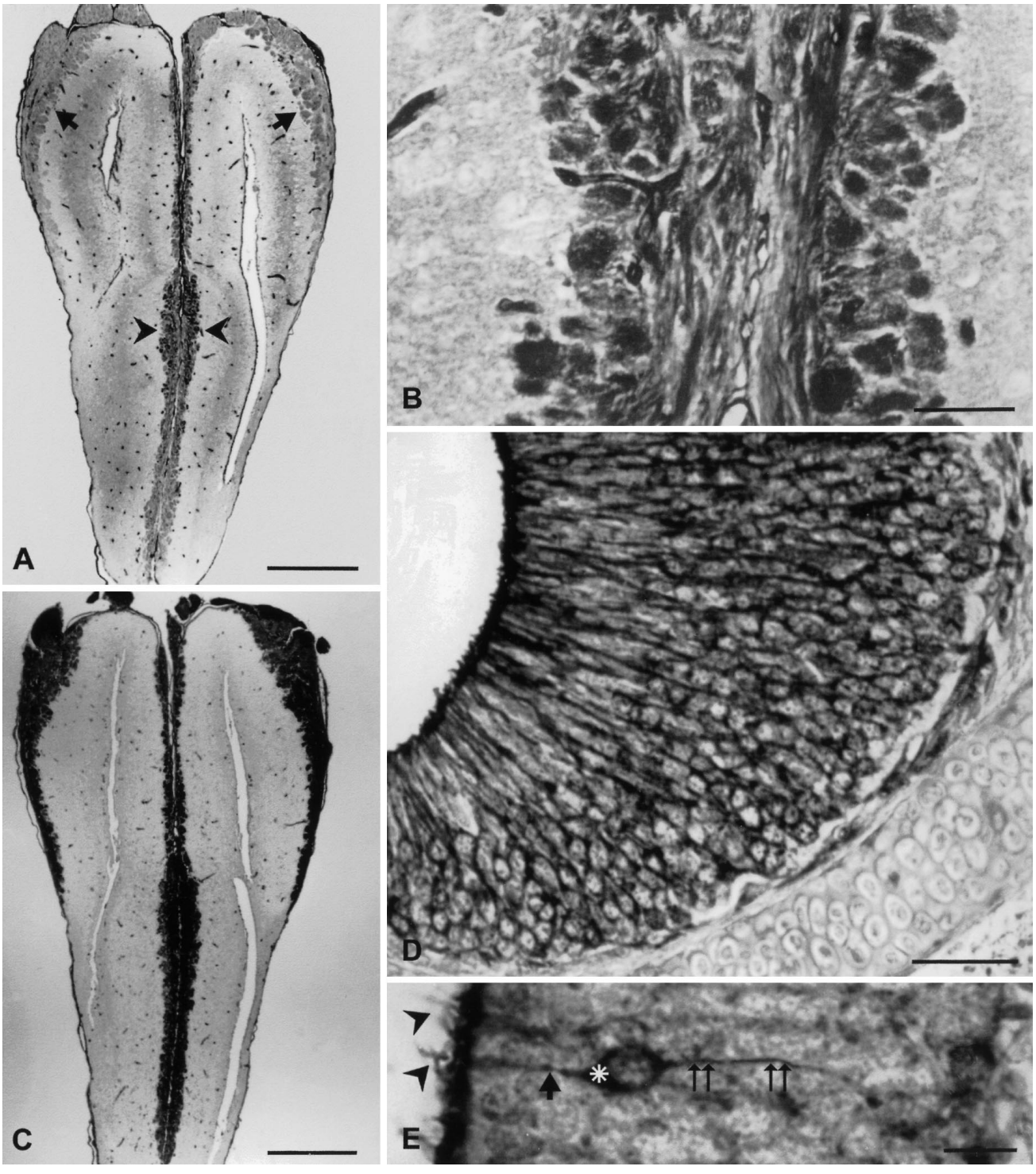

Fig. 2A–E Lectin histochemical characterisation of the olfactory system of *Podarcis sicula*. **A** Coronal section of the olfactory bulbs stained by BSA-I; the MOB (*arrows*) displays a detectable labelling, and the AOB (*arrowheads*) shows a moderate staining. *Bar* 500 µm. **B** Olfactory fibres and glomeruli in the AOB stained by BSA-I-B_4. *Bar* 50 µm. **C** Coronal section of olfactory bulbs with the MOB and AOB strongly labelled by LEA. *Bar* 500 µm. **D** Vomeronasal epithelium stained by LEA. *Bar* 20 µm. **E** Detail of a receptor cell in the olfactory epithelium stained by LEA. The reaction product is evident in the Golgi (*asterisk*), dendritic (*arrow*) and axonal region (*double arrows*). The staining is also evident on the mucous layer of the luminal surface and on the cilia (*arrowheads*). *Bar* 10 µm

receptor cells and AOB (Fig. 2A). At higher magnification it was possible to characterise the profiles of glomeruli that were localised in a thin layer between the unlabelled mitral cell layer on one side and the bundles of labelled nerve fibres on the other (Fig. 2B). The glomeruli were round or elliptical in shape and were always well-defined by the labelling.

The MOB and AOB were strongly stained by *Lycopersicon esculentum* agglutinin (LEA; Fig. 2C). In the pseudostratified columnar epithelium of the main and vomeronasal organ the sensory neurons were labelled. The reaction product was present in the Golgi region of both olfactory and vomeronasal receptor cells, as well as at the dendritic and axonal portions (Fig. 2D,E). The staining was also detectable on the cilia present in the apical region of the olfactory and vomeronasal neurons (Fig. 2E).

In contrast the incubation with DBA did not label any receptor cells and projections in the bulbs.

In *Podarcis* the olfactory and vomeronasal receptor cells presented a high density of D-acetyl-glucosamine residues, as shown by LEA binding. Moreover, the BSA-I-B_4 binding showed the presence of α-D-galactose in the main and accessory olfactory system. SBA and BSA-I binding further confirmed the presence of α-D-galactose residues on the saccharidic chains of the cell surface glycoproteins of the receptor cells. The absence of labelling on the olfactory and vomeronasal receptor cells after incubation with DBA, which has prime specificity for α-*N*-acetyl-D-galactosamine and α-D-galactose, suggested that carbohydrate moieties identified by the SBA and BSA-I binding were only due to α-D-galactose.

The results of the lectin binding in *Lacerta viridis* and *Podarcis sicula* are summarised in Table 1. In both lizards, the lectin binding also labelled the mucus covering the apical surface of the vomeronasal organ, the olfactory epithelium and the Bowman's glands. Moreover when the lectin binding was present, no difference in intensity between the glomeruli in the main and accessory olfactory bulb was observed.

Discussion

The different molecular characteristic of cell surface glycoproteins of olfactory and vomeronasal receptor cells between *Lacerta* and *Podarcis* defies explanation. An analogous behaviour in the carbohydrate moieties of the olfactory system has been demonstrated both in amphibian (Franceschini et al. 1992; Franceschini and Ciani 1993) and some actinopterygian species (Franceschini et al. 1994a).

In the Australian lizard *Physignathus lesueurii* (Franceschini et al. 1999) we have demonstrated the presence of glycoconjugate-containing glycoproteins on the surface of the olfactory and vomeronasal receptor cells. In this lizard, as in *Podarcis sicula*, the olfactory receptor cells of the main olfactory system and of the vomeronasal organ are characterised by α-galactose and *N*-acetyl-D-glucosamine residues.

The lectin binding to the receptor cells of the olfactory system suggests that specific glycoconjugates have a role in development and growth. A correlation between development of the olfactory system and the lectin binding has been described by Plendl and Schmahl (1988) in the NMRI mouse and by Franceschini et al. (1994b) in the rat. Moreover it has been demonstrated that *Vicia villosa* agglutinin (VVA) influences the fasciculation of vomeronasal axons in organ culture (Ichikawa et al. 1994). This lectin binds specifically to *N*-acetyl-D-galactosamine, suggesting that the glycoconjugates bound to VVA are associated with fasciculation of the developing olfactory nerve.

The cell-cell recognition in the olfactory system occurs not only during development but also during all adult life. In fact, the neurons of the olfactory and vomeronasal epithelium are unique in being generated throughout the lifetime of the animal (Graziadei and Metcalf 1971). New neurons derive from cell populations along the base of the olfactory and vomeronasal epithelium.

The hypotheses that the membrane glycoconjugates of the receptor cells would be important in cell-cell recognition and adhesion is supported by Key and Akeson (1990a, b). These authors described unique N-CAM glycoforms in the frog olfactory system identifying specific subsets of olfactory pathways that selectively bind both SBA and MAb 9OE, a monoclonal antibody against hybridoma 9 possessing a high reactivity with the olfactory epithelium. Key and Akeson (1991) have characterised at least four distinct 200 kd N-CAMs in the olfactory system of the frog. Two of these isoforms selectively bind SBA as well as DBA. It is possible that these membrane glycoconjugates, identified by the lectin binding, play an important role in the histogenesis of the olfactory system. The N-CAM-similar expression pattern of other glycoproteins, as L1 (Whitesides and LaMantia 1996), OCAM (Yoshihara et al. 1997), Thy-1 (Xue et al. 1990) and LIG-1 (Suzuki et al. 1996), suggests that these immunoglobulins may also play a role in extension, guidance, fasciculation and selective zone-to-zone projection of the primary olfactory axons.

It is also possible that glycoproteins might be involved in chemoreception and transduction of the odorous message into a nervous signal. This hypothesis is supported by some reports indicating that the olfactory cilia membranes possess not only receptor sites able to bind the odours (Anholt 1991) but also glycoproteins involved in signal transduction (Chen et al. 1986; Fesenko et al. 1988; Anholt et al. 1990). The ciliary glycocalix of the main olfactory receptor cells in different vertebrates contains sialic acid (Foster et al. 1992) and *N*-acetyl-glucosamine residues (Getchell et al. 1993). Observations made by lectin binding suggest that glycoconjugates might represent a large part of the transmembrane proteins involved in olfaction (Menco 1992) and could be used for characterisation and isolation of specific odorant receptor cells (Hofmann and Meyer 1991).

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