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Original Article

Expression of galectin-1 in the mouse olfactory system

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ABSTRACT Primary sensory olfactory axons arise from the olfactory neuroepithelium that lines the nasal cavity and then project via the olfactory nerve into the olfactory bulb. The β-galactoside binding lectin, galectin-1, and its laminin ligand have been implicated in the growth of these axons along this pathway. In galectin-1 null mutant mice, a subpopulation of primary sensory olfactory axons fails to reach its targets in the olfactory bulb. In the present study we examined the spatiotemporal expression pattern of galectin-1 in normal mice in order to understand its role in the development of the olfactory nerve pathway. At E15.5, when olfactory axons have already contacted the olfactory bulb, galectin-1 was expressed in the cartilage and mesenchyme surrounding the nasal cavity but was absent from the olfactory neuroepithelium, nerve and bulb. Between E16.5 and birth galectin-1 began to be expressed by olfactory nerve ensheathing cells in the lamina propria of the neuroepithelium and nerve fibre layer. Galectin-1 was neither expressed by primary sensory neurons in the olfactory neuroepithelium nor by their axons in the olfactory nerve. Laminin, a galectin-1 ligand, also exhibited a similar expression pattern in the embryonic olfactory nerve pathway. Our results reveal that galectin-1 is dynamically expressed by glial elements within the nerve fibre layer during a discrete period in the developing olfactory nerve pathway. Previous studies have reported galectin-1 acts as a substrate adhesion molecule by cross-linking primary sensory olfactory neurons to laminin. Thus, the coordinate expression of galectin-1 and laminin in the embryonic nerve fibre layer suggests that these molecules support the adhesion and fasciculation of axons en route to their glomerular targets.

KEY WORDS: *galectin-1, olfactory system, laminin, lectin*

Introduction

Primary sensory olfactory neurons are unique in the mammalian nervous system because they continually turn over throughout life. These neurons have a limited lifespan of approximately 30 days and are replaced by stem cells present in the basal layer of the olfactory neuroepithelium (Farbman, 1990). The newly generated sensory neurons project axons along the olfactory nerve to the olfactory bulb where they form synaptic connections within specific target sites called glomeruli. Despite this continual renewal of sensory neurons, the olfactory pathway maintains a stable topographic organization between the olfactory neuroepithelium and the olfactory bulb (Moulton, 1976; Kauer, 1987). At a gross level, this pathway is organized about orthogonal axes, for instance, neurons in the dorsal portion of the nasal cavity project to glomeruli on the dorsal surface of the olfactory bulb (Astic and Saucier, 1986; Saucier and Astic, 1986). However, near-neighbor relations in the olfactory epithelium are not preserved in the pathway. Each glomerulus is innervated by sensory neurons that are widely dispersed in the topographically appropriate region of the nasal cavity (Vassar *et al.*, 1994).

Spatial resolution of the sensory environment is encoded in neural maps involving point to point projections between the periphery and the central nervous system in both the visual and somatosensory systems. In contrast, the olfactory system processes a largely non-spatial modality and thus does not require olfactory space to be mapped onto the olfactory neuroepithelium. Instead, the neural organization of the olfactory epithelial sheet and the neuronal sheets of the olfactory bulb and cortices appear to be used to encode chemical diversity present in olfactory stimuli. Ngai

Abbreviations used in this paper: OE, olfactory neuroepithelium; OB, olfactory bulb; MC, mitral cell; GL, glomerular layer; DBA, *Dolichos biflorus* agglutinin; NFL, nerve fibre layer; CART, cartilage; MES, mesenchyme; GCL, granule cell layer; AOB, accessory olfactory bulb; SSEA, stage specific embryonic antigen; DRG, dorsal root ganglion; EC, ensheathing cell; IPTG, isopropylthio-β-D-galactoside; PMSF, phenylmethylsulfonyl fluoride; TBS, tris buffered saline.

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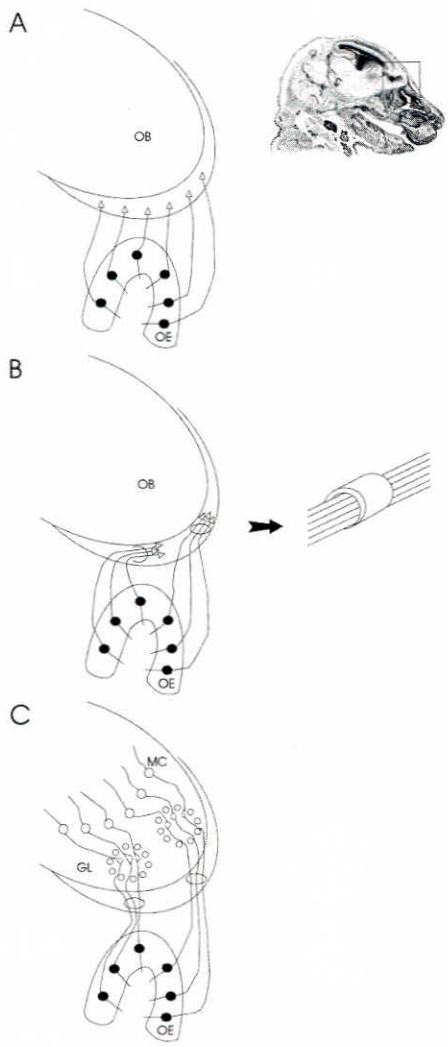

Fig. 1. Schematic representation of the three principal stages in the development of the olfactory nerve pathway. (A) *A photomicrograph of a parasagittal section of a mouse embryonic head is presented in the right panel. The boxed area encloses the nasal cavity and olfactory bulb, a rostral outgrowth of the forebrain, and is diagrammatically enlarged in the left panel. During the first stage of development, primary olfactory axons exit the olfactory neuroepithelium (OE) lining the nasal cavity and project over the surface of the olfactory bulb (OB) to form the nerve fiber layer.* **(B)** *Between E15.5 and E18.5 primary olfactory axons continue to enter the nerve fiber layer and sort out into bundles expressing similar cell surface carbohydrates. These bundles are surrounded by the processes of ensheathing cells (right hand panel).* **(C)** *In early postnatal life olfactory axons form terminal arbors on the dendrites of second-order olfactory neurons (mitral cells, MC) in specialized globules of neuropil called glomeruli (GL). Glomeruli are demarcated by the perikarya of periglomerular neurons.*

et al. (1993) showed by *in situ* hybridization that neurons expressing specific odorant receptors were widely distributed across the olfactory epithelium in catfish. These results suggested that individual sensory neurons probably express only one or a few different odor receptor genes. Although similar results have been reported in both rat (Vassar *et al.*, 1994) and mouse (Sullivan *et al.*, 1996) olfactory epithelium, individual types of receptor mRNA are selectively restricted in their expression to one of four spatially segregated zones of the olfactory epithelium in these animals. Within each zone, sensory neurons expressing a particular receptor mRNA are not clustered, but are rather randomly dispersed within the epithelium (Ressler *et al.*, 1993; Vassar *et al.*, 1993). Consequently, each epithelial zone forms a unique mosaic of sensory neurons expressing different odor receptor proteins. Those neurons expressing a given receptor type probably project onto one or a few defined glomerular targets within the olfactory bulb (Vassar *et al.*, 1994).

How do olfactory sensory axons arriving from widely scattered neuronal perikarya in the olfactory neuroepithelium specifically terminate in the same area within the bulb? It has been hypothesized that cell surface carbohydrates expressed on olfactory neurons may be involved in targeting axons to specific glomeruli (Key and Akeson, 1993; Puche and Key, 1995; Puche and Key, 1996; Puche *et al.*, 1997). Primary sensory olfactory neurons consist of a number of subpopulations that express different cell surface carbohydrates. One of these subpopulations in mice is identified by reactivity with the lectin *Dolichos biflorus agglutinin (DBA)*, which recognizes terminal N-acetyl-D-galactosamine residues (Etzler and Kabat, 1970). These neurons are widely dispersed within the olfactory epithelium and their axons course randomly within the olfactory nerve. At the junction of the olfactory nerve and olfactory bulb there is considerable rearrangement of axon trajectories (Key and Akeson, 1993). In the nerve fiber layer of the olfactory bulb, axons coalesce into discrete fascicles before terminating in select glomeruli. These results suggest that cell surface galactose derivatives may be ligands for endogenously expressed lectins and that lectin-carbohydrate interactions may mediate selective fasciculation of axon subpopulations (Key and Akeson, 1993). Galectin-1, a member of a family of β-galactose-binding proteins (Barondes *et al.*, 1994), is expressed in the rat olfactory system (Mahanthappa *et al.*, 1994; Puche and Key, 1995) where it appears to mediate cell adhesion to laminin (Mahanthappa *et al.*, 1994) and stimulates neurite outgrowth (Puche *et al.*, 1997). Moreover, a subpopulation of olfactory axons expressing DBA ligands fail to form fascicles and innervate glomeruli in the dorsocaudal olfactory bulb in galectin-1 null mutant mice (Puche *et al.*, 1997). These results led us to conclude that galectin-1 was involved in axon guidance in the olfactory nerve pathway. In the present study we describe the expression pattern of galectin-1 in the developing mouse olfactory system. We postulate that galectin-1, expressed by olfactory nerve ensheathing cells, directs the sorting of discrete subpopulations of primary sensory olfactory axons in the nerve fiber layer of the bulb during embryonic development.

Results

Expression of galectin-1 during development of the olfactory nerve

The olfactory nerve pathway develops in a series of distinct steps (Fig. 1; Key and Puche, 1997). First, primary sensory olfactory axons and neuroglial cells migrate from the olfactory neuroepithelium and form the outer nerve fiber layer of the olfactory bulb between E11.5 and 15.5 (Fig. 1A). Between E15.5 and E18.5 olfactory axons continue to enter the nerve fiber layer and sort out into distinct fascicles (Fig. 1B). These fascicles are surrounded by

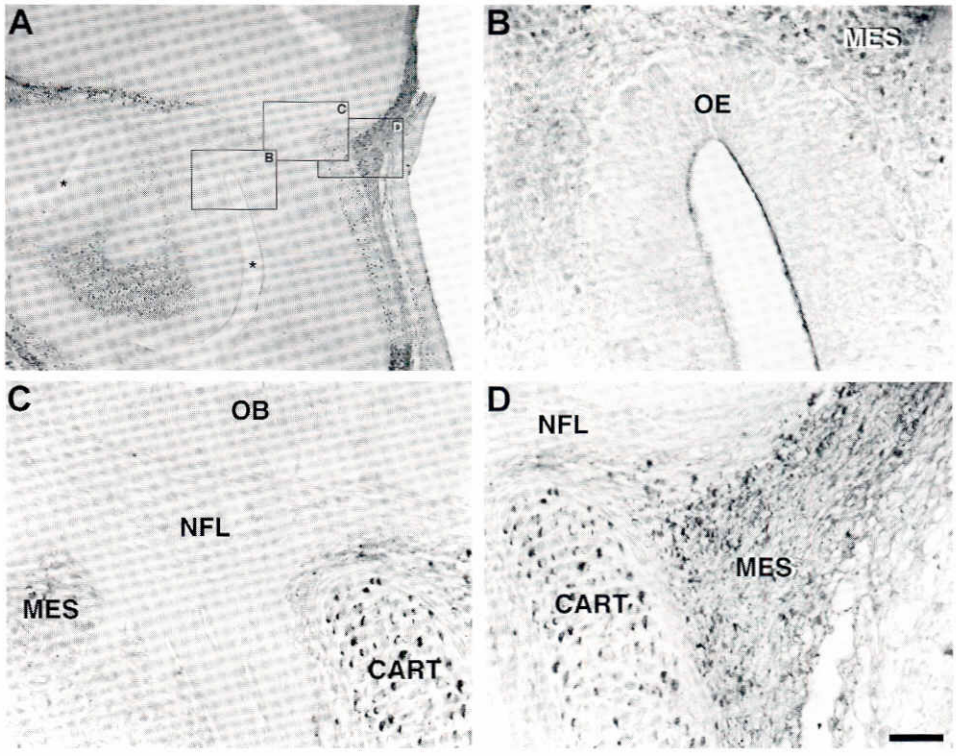

Fig. 2. Galectin-1 immunoreactivity in sagittal sections of mouse head at E15.5. (A) *Low magnification micrograph showing the nasal cavities (asterisks) which are located ventral to the rostral brain. Galectin-1 staining is present in the cartilage and mesenchyme surrounding the olfactory neuroepithelial lining of the nasal cavities and in the dermis of the skin. Boxes demarcate higher magnification images presented in panels B, C and D.* **(B)** *Staining is absent form the olfactory neuroepithelium (OE) but is present in cells in the surrounding mesenchyme (MES).* **(C)** *Galectin-1 immunoreactivity is neither detected in the nerve fiber layer (NFL) nor in deeper layers of the olfactory bulb (OB). Galectin-1 is present in chondrocytes in the nasal cartilage (CART) and in the mesenchyme.* **(D)** *Immunoreactivity is present in the mesencyhme between the nasal cartilage and the skin of the head. Rostral is to the right and dorsal to the top in all panels. Bar in A, 250 μm. Bar in B,C and D, 50 μm.*

processes of olfactory nerve ensheathing cells. Finally, around birth olfactory axons separate from the nerve fiber layer and form terminations on the dendrites of second-order mitral/tufted cells in globules referred to as glomeruli (Fig. 1C). In the present study, the distribution of galectin-1 was examined at each of these three different stages in the developing olfactory system in mouse.

Parasagittal sections of embryonic (E15.5, E16.5 and E18.5) and neonatal (P0.5) mouse heads were immunostained with rabbit polyclonal antiserum against recombinant galectin-1. At E15.5, the olfactory neuroepithelium, olfactory nerve bundles and nerve fiber layer were clearly identified in parasagittal sections of the mouse head (Fig. 2A). Immunostaining of these sections revealed that galectin-1 was absent from both the olfactory neuroepithelium (Fig. 2B) and the nerve fiber layer (Fig. 2C). However, galectin-1 was present in mesenchymal and cartilage cells surrounding both of these tissues (Fig 2B-D). The mesenchymal cells underlying the epidermis covering the head strongly expressed galectin-1 (Fig. 2D). At E16.5, galectin-1 exhibited a similar expression pattern as at E15.5 (Fig. 3A). Although the nerve fiber layer appeared to be devoid of galectin-1 at this age (Fig. 3A), some weakly expressing cells were observed at higher magnification (arrows, Fig. 3B). Again no galectin-1 immunoreactivity was present in the olfactory neuroepithelium, however, it was still detected, albeit weakly, in cells in the underlying lamina propria (Fig. 3B).

At E18.5, ensheathing cells surrounding nerve bundles in the olfactory nerve fiber layer clearly expressed galectin-1 (Fig. 3C,D). In addition to the expression in individual cells (arrows; Fig. 3d), galectin-1 was also localized to long fibrous bands in the nerve fiber layer (arrowheads; Fig. 3D). In contrast with earlier ages, chondrocytes only weakly expressed galectin-1 at E18.5 (Fig. 3C) while high levels of galectin-1 persisted in the perichondrial layer (arrowheads; Fig. 3C). The distribution of galectin-1 at P0.5 (Fig. 3E,F) was similar to that observed at E18.5, although staining of cells in the nerve fiber layer was considerably weaker (arrows; Fig. 3F). Galectin-1 continued to be present in ensheathing cells throughout the nerve fiber layer and in fibrous bands between axon bundles in the olfactory nerve (arrowheads; Fig. 3E). Galectin-1 was not detected in the olfactory neuroepithelium, chondrocytes or in cells deep to the nerve fiber layer of the olfactory bulb at this age.

Coordinate expression of laminin and galectin-1 in the olfactory nerve pathway during development

Galectin-1 mediates adhesion of primary olfactory neurons to substrate-bound laminin by cross-linking poly-N-lactosamine sequences in carbohydrate chains of laminin to cell surface carbohydrate ligands (Zhou and Cummings, 1993; Mahanthappa *et al.*, 1994). Laminin was previously shown to be expressed by glial cells in the olfactory nerve fiber layer of the adult rat olfactory bulb (Liesi, 1985) and to co-localize with galectin-1 in the early postnatal rat olfactory bulb (Mahanthappa *et al.*, 1994; Raabe *et al.*, 1997). This raised the possibility that galectin-1 was mediating its effects on axon guidance *in vivo* (Puche *et al.*, 1997) by binding to laminin in the nerve fiber layer during embryogenesis. In order to begin to address this question we examined the distribution of laminin in parasagittal sections of the olfactory nerve pathway in embryonic and neonatal mice. At both E15.5 and E16.5, laminin, like galectin-1, was distributed throughout the head mesenchyme (Fig. 4A,B). No laminin was detected in the olfactory neuroepithelium, nerve fiber bundles or in the nerve fiber layer at these ages. At E18.5, laminin was strongly expressed by ensheathing cells within the olfactory nerve fiber layer and by cells in the lamina propria of the olfactory neuroepithelium (Fig. 4C). This was in stark contrast to the weak immunoreactivity observed in these tissues two days earlier. Interestingly, this upregulation in laminin expression by

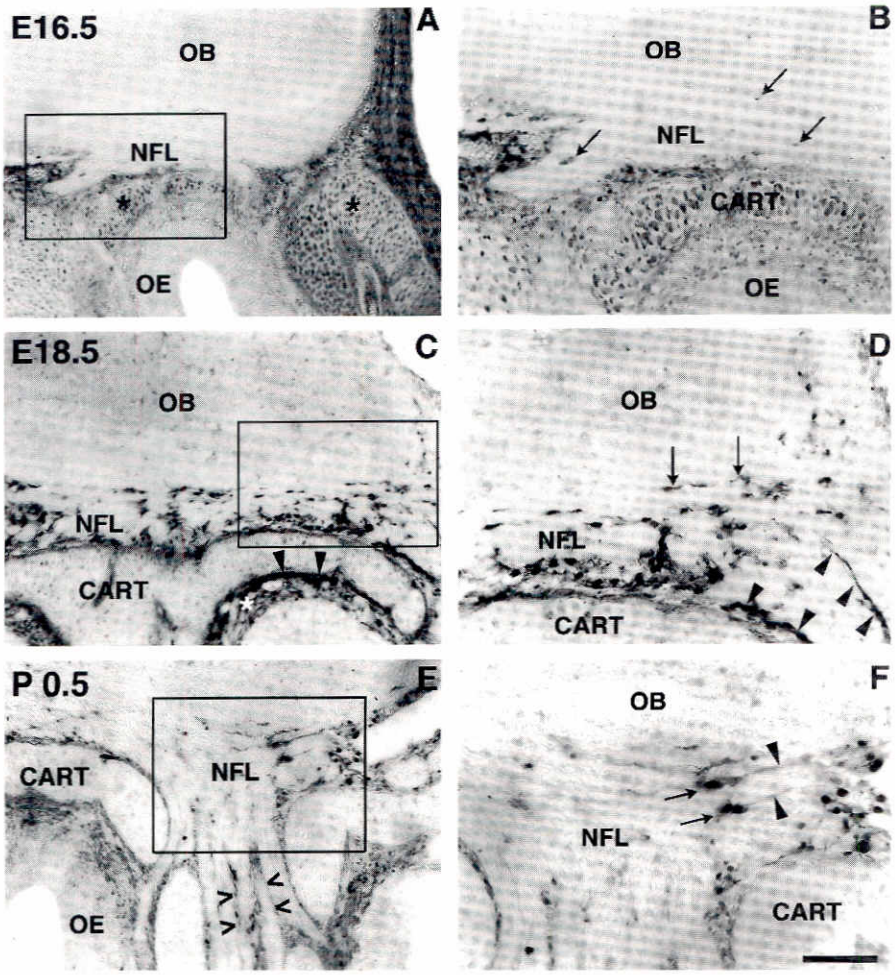

Fig. 3. Galectin-1 immunoreactivity in sagittal sections of mouse head at E16.5 (A, B), E18.5 (C, D) and P0.5 (E, F). (A) *Strong immunoreactivity is present in the mesenchyme, and nasal cartilage (asterisk) at E16.5. Galectin-1 is not detected in the deep layers of the olfactory bulb or in the olfactory neuroepithelium. The boxed area is depicted in panel B.* **(B)** *Weak staining of cells is observed in the nerve fiber layer (arrows). The nerve fiber layer contains olfactory nerve Schwann cells which ensheathe bundles of olfactory axons.* **(C)** *Galectin-1 expression is upregulated in the olfactory nerve Schwann cells in the olfactory nerve fiber layer at E18.5. Immunoreactivity is present in the mesenchyme underlying the olfactory neuroepithelium (asterisk). Galectin-1 staining of chondrocytes in the nasal cartilage is decreased at this age while strong staining remains in the perichondrium (arrowheads). The boxed area is depicted in panel D.* **(D)** *Galectin-1 is localized to individual cells (arrows) and fibers (arrowheads) in the nerve fiber layer.* **(E)** *Galectin-1 continues to be expressed in the mesenchyme underlying the olfactory neuroepithelium and within the nerve fiber layer at P0.5. Expression is decreased in the chondrocytes and in the nerve fiber layer. Galectin-1 is observed in fibers that appear to be demarcating axon fascicles in the olfactory nerve (arrowheads). The boxed area is depicted in panel F.* **(F)** *Although galectin-1 is weakly expressed by most cells throughout the nerve fiber layer, some cells (arrows) and fibrous bands (arrowheads) have stronger staining. Rostral is to the right and dorsal to the top in all panels. Bar in A,C and E, 50 μm. Bar in B,D and F, 25 μm.*

ensheathing cells between E16.5 and E18.5 paralleled a similar rise in expression of galectin-1 by these cells during this same period. Laminin continued to be present in the olfactory nerve fiber layer in the neonatal bulb, although at lower levels than at E18.5.

Galectin-1 expression in the adult mouse olfactory bulb

In the adult olfactory bulb, primary sensory olfactory axons engulf the entire outer surface of the olfactory bulb and form an olfactory nerve fiber layer. Beneath this fiber layer lie spherical regions of neuropil, referred to as glomeruli (GL; Fig. 5A), which contain dense synaptic connections between the terminal arbors of primary sensory olfactory axons and the dendrites of mitral cells, external plexiform tufted neurons and periglomerular cells. The external plexiform layer (EPL; Fig. 5A) lies deep to the glomerular layer and consists of the neuropil between the dendrites of the mitral/tufted cells and the granule cells. The granule cell layer is the innermost cellular layer of the bulb and contains densely packed granule cells (GCL; Fig. 5A). Immunostaining with galectin-1 antiserum revealed the punctate distribution of galectin-1 in the perikarya of a subpopulation of cells dispersed throughout the depth of the olfactory bulb (Fig. 5A). These cells were concentrated in the periglomerular layer that surrounds the glomeruli and were diffusely distributed in both the external plexiform and granule cell layers. Galectin-1 was not detected in mitral cells of the adult olfactory bulb. In addition to the perikaryal staining of a subpopulation of dispersed cells, there was some fibrous staining of the dendrites of these cells within the granule and external plexiform cell layers. Moreover, there was a discrete subpopulation of cells within the granule cell layer that exhibited dense cytoplasmic immunostaining of their dendrites and axons (Fig. 5B). Axons emanating from these cells were traced into the subependymal layer of the olfactory bulb (Fig. 5C). In all cases, control sections that were incubated with pre-immune serum exhibited negligible background staining.

Discussion

Our previous studies of galectin-1 null mutant mice revealed that this lectin plays an important role in the development of the olfactory system (Puche *et al.*, 1996). In the absence of galectin-1, a subpopulation of primary olfactory axons failed to innervate their normal target in the dorsocaudal olfactory bulb, suggesting that galectin-1 was involved in axon guidance. In the present study we examined the cellular localization of galectin-1 in the normal mouse olfactory nerve pathway in order to begin to understand its role in this region of the nervous system.

The olfactory nerve pathway develops in a series of distinct stages. Initially olfactory axons and neural cells grow out from the olfactory neuroepithelium and form a tissue aggregate, referred to as the migratory mass, which lies ventral to the rostral pole of the telencephalon. This mass fuses with the olfactory bulb to form the nerve fiber layer. During this stage, galectin-1 was present in the mesenchyme surrounding the olfactory nerve and migratory mass. This distribution is consistent with previous reports that galectin-1

is expressed by mesenchymal cells in numerous tissues (Poirier *et al.*, 1992). Although galectin-1 is in the mesenchyme surrounding the nerve fiber bundles, this tissue also expresses chondroitin sulfate proteoglycans, a chemorepulsive molecule which probably inhibits olfactory axons from growing into this region and interacting with galectin-1 (Treloar *et al.*, 1996). Since galectin-1 was not detected in either the mouse olfactory neuroepithelium or the olfactory nerve pathway at this stage it is unlikely to be involved in the initial outgrowth of axons from the nasal cavity to the telencephalon.

During the second stage of development in the olfactory nerve pathway (between E15.5 and birth), the nerve fiber layer grows and axons begin to sort out into discrete chemically-distinct bundles. During this stage, galectin-1 began to be expressed by ensheathing cells within both the olfactory nerve and olfactory nerve fiber layer in the bulb. The expression of galectin-1 was also accompanied by an upregulation in the expression of laminin by these cells. Why should ensheathing cells begin expressing galactin-1 and laminin specifically between E16.5 and 18.5 during development of the olfactory nerve pathway? This dynamic temporal regulation of galectin-1 expression is consistent with previous reports that the level of galectin-1 increases dramatically following differentiation in several cell types (Ohannesian *et al.*, 1994; Gillenwater *et al.*, 1998). Moreover, elevated levels of both galectin-1 and its cell surface ligands occur in some cell lines following differentiation and they are believed to promote cell adhesion and migration (Ohannesian *et al.*, 1994). Because of its bivalent nature galectin-1 acts both as a cell adhesion molecule, by binding to specific cell surface ligands (Lotan *et al.*, 1994; Baum *et al.*, 1995), and as a substrate adhesion molecule by binding to generic ligands such as laminin in the extracellular matrix (Zhou and Cummings, 1993; van den Brule *et al.*, 1995). We have previously shown that the nerve fiber layer is a major site for the sorting out of axons that express specific cell surface carbohydrates (Key and Akeson, 1993; Puche *et al.*, 1996; Dowsing *et al.*, 1997). Galectin-1 could cross-link axons, expressing appropriate cell surface ligands, to laminin in the extracellular matrix (Fig. 6A). Laminin is one of a number of ligands for galectin-1 in the olfactory system and galactin-1 promotes adhesion of primary sensory olfactory neurons to laminin independently of integrins (Mahanthappa *et al.*, 1994). Although primary sensory olfactory axons in rat express lactosamine, a galactin-1 ligand (Mahanthappa *et al.*, 1994; Puche and Key, 1996), the identity of specific axonal ligands in mice remains to be determined.

Galectin-1 secreted from ensheathing cells may also act as a catalyst in cross-linking axons that express appropriate cell surface carbohydrate ligands and hence facilitate sorting of axons into discrete bundles (Fig. 6B). Mahanthappa *et al.* (1994) have previously shown *in vitro* that galectin-1 can dissociate from laminin substrates and cross-link olfactory neurons to create neuronal aggregates. We propose that a similar mechanism is occurring *in vivo* as galectin-1 dissociates from laminin and selectively cross-links axons expressing specific ligands to form fascicles. These axon fascicles would be widely dispersed in the nerve fiber layer since ensheathing cells expressing galectin-1 are not spatially restricted in the nerve fiber layer. This is consistent with our previous observations that bundles of axons expressing distinct cell surface carbohydrates are dispersed in the nerve fiber layer (Key and Akeson, 1993; Puche and Key, 1996; Dowsing *et al.*, 1997). Moreover, the role of galectin-1 in selective fasciculation is also consistent with the failure of a discrete subpopulation of primary olfactory to fasciculate in the nerve fiber layer of the dorsocaudal olfactory bulb in galectin-1 null mutant mice (Puche *et al.*, 1996). We believe that once axons have sorted out into discrete bundles, they respond to other signals that direct them to innervate specific glomeruli in the olfactory bulb. Interestingly, we predict that loss of function of galectin-1 in rat would produce a severe phenotype in axon sorting since a large subpopulation of primary sensory neurons express the lactosamine ligand (Puche and Key, 1996) and because all of these neurons also express galactin-1 (Puche and Key, 1995). We have also shown that substrate-bound lactosamine stimulates neurite outgrowth by rat primary sensory olfactory *in vitro* (Puche and Key, 1996). Thus, at least in rat, galactin-1 may have a dual function of promoting axon growth and of sorting axons in the nerve fiber layer.

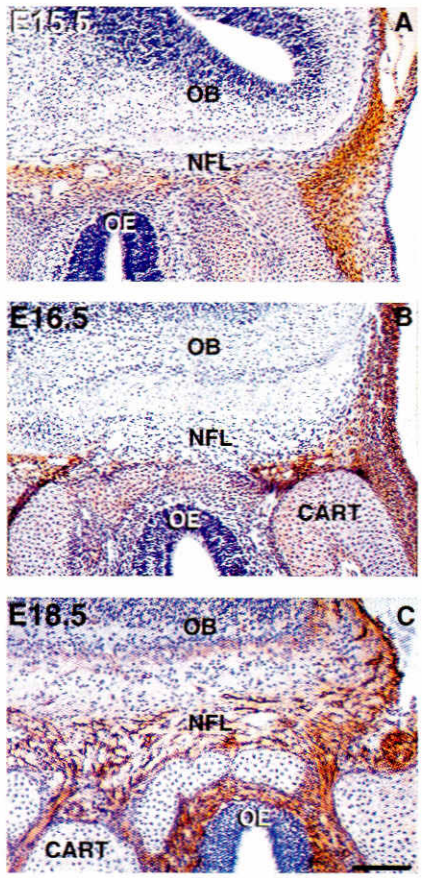

Fig. 4. Laminin immunoreactivity in sagittal sections of mouse head at E15.5 (A), E16.5 (B) and E18.5 (C). *All sections were counterstained with haematoxylin.* **(A)** *Brown laminin staining is present in the mesenchyme underlying the skin and surrounding the olfactory neuroepithelium (OE) and nerve fiber layer (NFL) of the olfactory bulb (OB). Weak staining is also detected in the nasal cartilage.* **(B)** *Strong laminin immunoreactivity is detected in the nasal cartilage and mesenchyme at E16.5.* **(C)** *Laminin expression becomes detectable in cells in the nerve fiber layer and in the lamina propria of the olfactory neuroepithelium at E18.5. Galectin-1 is no longer expressed in the nasal cartilage at this age. Weak immunoreactivity is observed in some cells in deep layers of the olfactory bulb. Rostral is to the right and dorsal to the top in all panels. Bar, 50 μm.*

The olfactory system is not unique in its expression of carbohydrate-binding proteins and specific cell surface carbohydrates. Discrete subpopulations of sensory neurons in the gustatory, auditory and somatosensory systems also express distinct cell surface carbohydrates (Dodd *et al.*, 1984; Dodd and Jessell, 1985,1986; Astic *et al.*, 1989). In the somatosensory system, neurons of the dorsal root ganglion (DRG) can be divided into ~15 classes on the basis of anatomical projection and neurochemistry. Each of these classes of neurons project to specific domains and laminar divisions in the spinal cord (Dodd and Jessell, 1986). Subpopulations of DRG neurons express specific cell surface

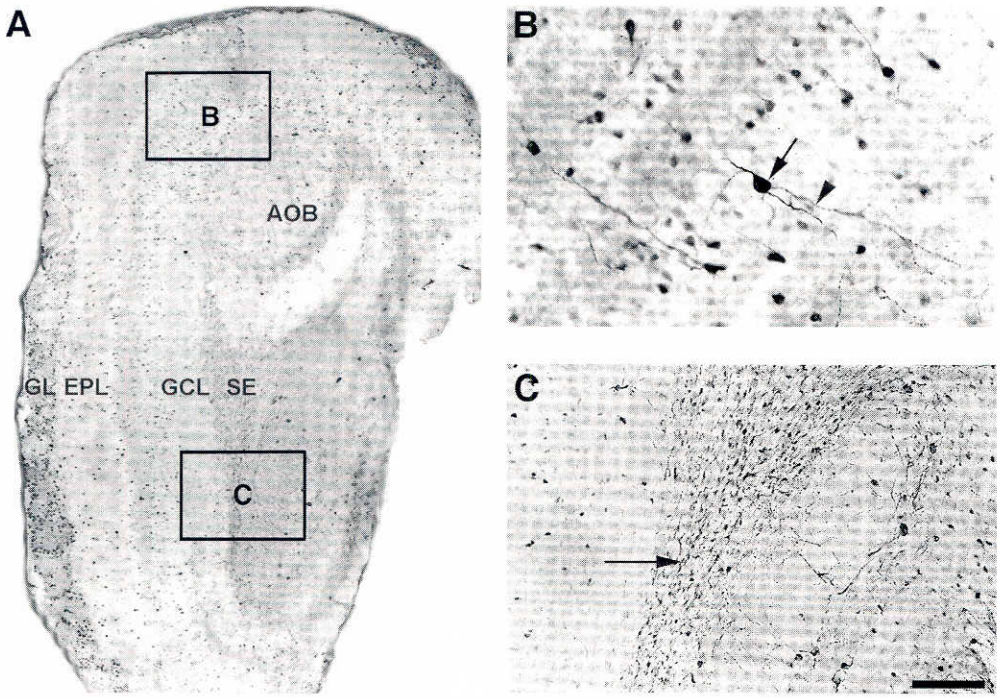

Fig. 5. Galectin-1 immunoreactivity in a vibratome section from the caudal olfactory bulb. (A) *Punctate staining of cell perikarya was observed in the glomerular layer (GL), external plexiform layer (EPL) and granule cell layer (GCL). These thick sections revealed a subpopulation of cells in the granule cell layer (GCL) that contained galectin-1 in both their axons and dendrites. The boxed areas are depicted in panels B and C.* **(B)** *The arrow demarcates a densely stained cell expressing galectin-1 throughout its processes (arrowhead).* **(C)** *The axons (arrow) of those densely stained cells exit the olfactory bulb via the intermediate olfactory tract in the subependymal layer (SE) of the olfactory bulb. AOB, accessory olfactory bulb. Bar in A and C, 100 μm. Bar in B, 20 μm.*

carbohydrates and project to specific spinal cord laminae (Dodd *et al.*, 1984; Dodd and Jessell, 1985; Chou *et al.*, 1989; Jessell *et al.*, 1990). For example, DRG neurons expressing the stage specific embryonic antigen-3 (SSEA-3) project predominantly to lamina III and the medial aspect of lamina IV (Dodd *et al.*, 1984). The carbohydrate antigen recognized by the monoclonal antibody 1B2, which has been identified as N-acetyl-lactosamine, is expressed by ~50% of DRG neurons which terminate in lamina I and II (Dodd and Jessell, 1985). Other subpopulations of DRG neurons express cell surface carbohydrates recognized by monoclonal antibodies A5 (Dodd and Jessell, 1985), LD2 (Chou *et al.*, 1989) and anti-SSEA-4 (Dodd *et al.*, 1984). These carbohydrate antigens are expressed during the time when sensory neurons extend axons into the spinal cord and establish specific synapses. Thus, the cell surface carbohydrates of DRG neurons may be acting as ligands for endogenous lectins in the spinal cord during development. Regan *et al.* (1986) first identified the expression of galectin-1 and galectin-3 in ~80% of neurons in the spinal cord and DRG. Galectin-1 was expressed soon after differentiation of these neurons at E14, while galectin-3 was not detected until E16 in rat (Regan *et al.*, 1986; Hynes *et al.*, 1989). This suggests that galectin-1 and/or galectin-3 may cross-link DRG axons expressing N-acetyl-lactoseries cell surface carbohydrates with target cells in the spinal cord (Dodd and Jessell, 1986), or to extracellular matrix components (Jessell *et al.*, 1990). The roles of galectins in the spinal cord and DRG have yet to be experimentally tested. The best evidence for a function of galectin-1-ligand interactions in the developing vertebrate nervous system comes from our own studies of the olfactory nerve pathway in galectin-1 null mutant animals (Puche *et al.*, 1996). However, there is considerable evidence for the role of other carbohydrate-binding proteins and lectins in cellular interactions and neurite outgrowth in the nervous system (Marschal *et al.*, 1989; Begovac and Shur, 1990; Thomas *et al.*, 1990; Begovac *et al.*, 1991; Riopelle and Dow, 1991; Babiarz and Cullen, 1992; Zanetta *et al.*, 1992)

No studies have yet examined the role of galectin-1 in the mature nervous system. We showed here that galectin-1 was highly upregulated in the deeper layers of the olfactory bulb in adult mice. In particular, galectin-1 was strongly expressed by a subpopulation of cells in the granule cell layer. These cells were obviously larger than the typically small-bodied granule cells. They also possessed an axon that projected into the white matter surrounding the subependymal layer of the olfactory bulb. The identity of these cells and the termination sites of their axons remains to be determined. One possibility is that they may be short axon cells that have been infrequently described in the olfactory bulb (Price and Powell, 1970). The function of these cells is not clear, but it has been proposed that they may act as modulators since they appear to contain numerous neuroactive substances (Halasz, 1990). Thus, expression of galactin-1 may be a fortuitous marker of these cells that can be used in functional studies. The significance of galectin-1 expression by a subpopulation of cells dispersed throughout the periglomerular, external plexiform and granule cell layers of the adult olfactory bulb remains to be determined. However, it is possible that this molecule may play a role in synaptic maintenance, either in turnover or stabilization. Galectin-1 may facilitate cell-cell contacts by cross-linking ligands or alternatively, it may regulate the adhesiveness of cell-cell or cell-matrix interactions as it does in other tissues (Zhou and Cummings, 1993; Gu *et al.*, 1994; Woynarowska *et al.*, 1994; Ozeki *et al.*, 1995; van den Brule *et al.*, 1995). Nonetheless, our results clearly demonstrate that galectin-1 expression is tightly regulated both during embryonic development and postnatal maturation. Galectin-1 only begins to be expressed in the deeper layers of the olfactory bulb in postnatal animals and it is then only expressed by a subpopulation of

neurons spread throughout the glomerular, external plexiform and granule cell layers. Thus, the olfactory bulb may be an interesting model for understanding mechanisms that restrict the expression of galectin-1 to a specific region of the nervous system and then to a subpopulation of cells in that region.

In summary, galectin-1 has been localized to the developing olfactory nerve fiber layer during a discrete period in development. The olfactory nerve fiber layer is considered a major site of axon sorting in the nerve pathway. Previous analysis of galectin-1 null mutant mice (Puche *et al.*, 1997) revealed that galectin-1 is involved in guidance of, at least, a subpopulation of primary sensory olfactory axons to the dorsocaudal olfactory bulb. Thus, the results of the present study suggest that expression of galactin-1 by ensheathing cells during a restricted period of embryonic development may underlie the guidance of a specific subpopulation of primary sensory olfactory axons. We are now in the position to directly test the hypothesis that interactions between galectin-1 and its ligands mediate axon sorting and fasciculation in the nerve fiber layer. Future studies will also examine the role of ensheathing cell in the nerve fiber layer during the late embryonic period in galectin-1 null mutant animals.

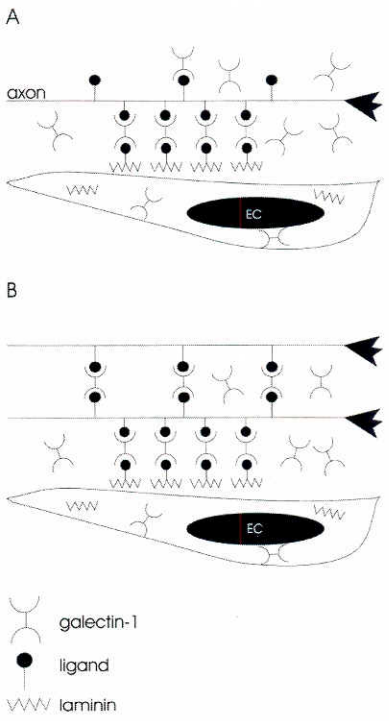

Fig. 6. Schematic representation of a model for the role of galectin-1 in axon fasciculation in the olfactory nerve fiber layer. (A) *Ensheathing cells (EC) in the nerve fiber layer express galectin-1 and laminin. Secreted galectin-1 binds to laminin in the extracellular matrix surrounding ensheathing cells and provides a substrate for axons expressing appropriate cell surface ligands.* **(B)** *Other axons that express similar cell surface ligands then fasciculate with these axons by using galectin-1 as a cross-linking molecule. Galectin-1 dissociates from the substrate as previously reported (Mahanthappa et al., 1994) and mediates the aggregation of further axons into larger fascicles.*

Materials and Methods

Tissue preparation

Adult male C57/BL6 mice were anaesthetized with Nembutal (0.08 ml/100 g body weight; Boehringer Ingelheim, Sydney, Australia) by intraperitoneal injection. The olfactory bulbs were dissected from three animals and fixed in 4% paraformaldehyde for 15 h at 4°C. These bulbs were processed and embedded within the same paraffin wax block. Coronal sections (7 μm) were cut and collected on 2% aminopropyltriethoxysilane-coated slides (Sigma Chemical Company, St. Louis, Missouri). Serial sections were collected from 12 rostrocaudal levels approximately 140 μm apart from both olfactory bulbs in each animal.

Pregnant C57/BL6 mice at different developmental stages were sacrificed by cervical dislocation. Heads were decapitated and placed into 4% paraformaldehyde for 15 h at 4°C followed by 30% sucrose for 15 h at 4°C. Serial coronal sections (30 μm) were cryostat cut and either thaw mounted on gelatinized slides or kept as free floating sections. Free floating sections were also collected from adult C57/BL6 mice. Adult C57/BL6 mice were sacrificed by cervical dislocation and olfactory bulbs were removed and snap frozen in liquid nitrogen. Serial coronal sections (10 μm) of unfixed frozen tissue were cryostat cut and thaw mounted on 2% aminopropyltriethoxysilane-coated slides.

Antibody production

Production of the biotinylated recombinant galectin-1 fusion protein has been described previously (Puche *et al.*, 1996). Start cultures of the host strain carrying the expression construct and the host strain carrying the pinpoint control DNA were incubated with 100 μg/ml ampicillin and 2 μM biotin overnight at 37°C. Cultures were diluted in LB broth with ampicillin for 1 h at 37°C and then isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 100 μM and incubated for 4-5 h at 37°C with shaking. Cells were harvested by centrifugation at 8000g for 10 min and resuspended in SPBE-azide stabilization buffer consisting of 6.7 mM KH2PO4, 150 mM NaCl, 14mM BME, 0.2% sodium azide, and the protease inhibitors 10 mM EDTA (pH 7.2), and 0.1 mM Phenylmethylsulfonyl fluoride (PMSF). Cells were then lysed by adding lysozyme to a final concentration of 1 mg/ml followed by stirring at 4°C for 20 min. Sodium deoxycholate was then added (0.1% final concentration) and stirred at 4°C for 5 min, followed by addition of 50U of DNase for 10 min to reduce viscosity. The crude lysate was then centrifuged for 15 min at 10,000g and the supernatant transferred to a clean tube.

Polyclonal rabbit antiserum was raised against recombinant galectin-1. Rabbits were immunized with an initial injection of 100 μg of purified galectin-1 in Freund's complete adjuvant, followed four, six and eight weeks later by booster injections of 60-80 μg in Freund's incomplete adjuvant. Animals were bled at 13 weeks and the specificity of the antiserum was confirmed with Western blots of recombinant galectin-1 and crude rat muscle extracts.

Immunohistochemistry

Mounted and free floating cryostat sections (30 μm) from embryonic, postnatal and adult olfactory bulb were pre-treated with 0.5% H_2O_2 in methanol for 5 min, blocked with 2% buffered BSA with 0.3% triton X-100 for 30 min and then incubated with either rabbit polyclonal antiserum against galectin-1 (#20; 1:1000) or rabbit polyclonal antiserum against laminin (Sigma;1:500) for 2 h at room temperature. Sections were subsequently washed with Tris buffered saline (TBS) (3x5 min), incubated for 60 min in biotinylated goat anti-rabbit antibodies, washed with TBS (3x5 min), incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) and then reacted with diaminobenzidine and H2O2 as above. To identify cellular layers of the olfactory bulb a series of reacted sections were subsequently counterstained with Mayers haematoxylin. Control sections incubated with either buffered BSA or pre-immune serum produced negligible background staining.

Photomicroscopy

Black and white 35 mm negatives were taken on an Olympus BH2 photomicroscope. Negatives were scanned on a polaroid sprintscan 35 scanner and images were color balanced using Adobe Photoshop 3.0 (Adobe Systems Inc., CA). Stained specimens were photographed with an Olympus BH2 photomicroscope with Kodak Ektachrome Elite 100 color slide film. Slides were then digitized by using a polaroid sprintscan 35 scanner and images were assembled with Adobe Photoshop 3.0.

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References

ASTIC, L. and SAUCIER, D. (1986). Anatomical mapping of the neuroepithelial projection to the olfactory bulb in the rat. *Brain Res. Bull. 16*: 445-454.

ASTIC, L., LE PERDU, J., MOLLICONE, R., SAUCIER, D. and ORIOL, R. (1989). Cellular expression of H and B antigens in the rat olfactory system during development. *J. Comp. Neurol. 289*: 386-394.

BABIARZ, B. and CULLEN, E. (1992). 3T3 cell surface galactosyltransferase is a calcium dependent adhesion molecule for collagen type IV. *Exp. Cell Res. 203*: 276-279.

BARONDES, S.H., CASTRONOVO, V., COOPER, D.N.W., CUMMINGS, R.D., DRICKAMER, K., FEIZI, T., GITT, M.A., HIRABAYASHI, J., HUGHES, C., KASAI, K-I., LEFFLER, H., LIU, F.-T., LATAN, R., MERCURIO, A.M., MONSIGNY, M., PILLAI, S., POIRIER, F., RAZ, A., RIGBY, P.W.J., RINI, J.M. and WANG, J.L. (1994). Galectins: A family of animal galactoside-binding lectins. *Cell 76*: 597-598.

BAUM, L.G., PANG, M., PERILLO, N.L., WU, T., DELEGEANE, A., UITTENBOGAART, C.H., FUKUDA, M. and SEILHAMER, J.J. (1995). Human thymic epithelial cells express an endogenous lectin, galectin-1, which binds to core 2 O-glycans on thymocytes and T lymphoblastoid cells. *J. Exp. Med. 181(3):* 877-887.

BEGOVAC, P.C. and SHUR, B.D. (1990). Cell surface galactosyltransferase mediates the initiation of neurite outgrowth from PC12 cells on laminin. *J. Cell Biol. 110:* 461-470.

BEGOVAC, P.C., HALL, D.E. and SHUR, B.D. (1991). Laminin fragment E8 mediates PC12 cell neurite outgrowth by binding to cell surface β1,Galactosyltransferases. *J. Cell Biol. 113*: 637-644.

CHOU, D.K., DODD, J., JESSELL, T.M., COSTELLO, C.E. and JUNGALWALA, F.B. (1989). Identification of α-galactose (α-fucose)-asialo GM1 glycolipid expressed by subsets of rat dorsal root ganglion neurons. *J. Biol. Chem. 264*: 3409-3415.

DODD, J. and JESSELL, T.M. (1985). Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to superficial dorsal horn of rat spinal cord. *J. Neurosci. 5*: 3278-3294.

DODD, J. and JESSELL, T.M. (1986). Cell surface glycoconjugates and carbohydrate-binding proteins: Possible recognition signals in sensory neuron development. *J. Exp. Biol. 129*: 225-238.

DODD, J., SOLTER, D. and JESSELL, T.M. (1984). Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurons. *Nature 311*: 469-472.

DOWSING, B., PUCHE, A.C., HEARN, G. and KEY, B. (1997). Presence of novel N-CAM glycoforms in the rat olfactory system. *J. Neurobiol. 32*: 659-670.

ETZLER, M.E. and KABAT, E.A. (1970). Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from *Dolichos biflorus*. *Biochemistry 9*: 869-877.

FARBMAN, A.I. (1990). Olfactory neurogenesis: genetic or environmental controls? *Trends Neurosci. 13*: 362-365.

GILLENWATER, A., XU, X.C., ESTROV, Y., SACKS, P.G., LOTAN, D. and LOTAN, R. (1998). Modulation of galectin-1 content in human head and neck squamous carcinoma cells by sodium butyrate. *Int. J. Cancer. 75(2):* 217-224.

GU, M., WANG, W., SONG, W.K., COOPER, D.N. and KAUFMAN, S.J. (1994). Selective modulation of alpha 7 beta 1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J. Cell Sci. 107*: 175-181.

HALASZ, N. (1990). The vertebrate olfactory system. *Chemical neuroanatomy, function and development.* Akademiai Kiado, Budapest, pp. 84-89.

HYNES, M.A., BUCK, L.B., GITT, M., BARONDES, S.H., DODD, J. and JESSELL, T.M. (1989). Carbohydrate recognition in neuronal development: structure and expression of surface oligosaccharides and β-galactoside-binding lectins. *Ciba Found. Symp. 145*: 189-210 discussion 210-218.

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.

KAUER, J.S. (1987). Coding in the olfactory system. In *Neurobiology of Taste and Smell.* (Eds. Finger T.E., and Silver W.L.). John Wiley and Sons, New York, pp. 205-229.

KEY, B. and AKESON, R.A. (1993). Distinct subsets of sensory olfactory neurons in mouse: Possible role in the formation of the mosaic olfactory projection. *J. Comp. Neurol. 335*: 355-368.

KEY, B. and PUCHE, A.C. (1997). Role of galectin-1 in the olfactory nervous system. *Trends Glycosci. Glycotechnol. 9(45):* 41-45.

LIESI, P. (1985). Laminin-immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones. *EMBO J. 4*: 2505-2511.

LOTAN, R., BELLONI, P.N., TRESSLER, R.J., LOTAN, D., XU, X.C. and NICOLSON, G.L. (1994). Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. *Glycoconjugate J. 11(5):* 462-468.

MAHANTHAPPA, N.K., COOPER, D.N.W., BARONDES, S.H. and SCHWARTING, G.A. (1994). Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. *Development 120*: 1373-1384.

MARSCHAL, P., REEBER, A., NEESER, J.R., VINCENDON, G. and ZANETTA, J.-P. (1989). Carbohydrate and glycoprotein specificity of two endogenous cerebellar lectins. *Biochimie 71*: 645-653.

MOULTON, D.G. (1976). Spatial patterning of response to odors in the peripheral olfactory system. *Curr. Top. Physiol. 56*: 578-593.

NGAI, J., DOWLING, M.M., BUCK L., AXEL, R. and CHESS, A. (1993). The family of genes encoding odorant receptors in the channel catfish. *Cell 72*: 657-666.

OHANNESIAN, D.W., LOTAN, D. and LOTAN, R. (1994). Concomitant increases in galectin-1 and its glycoconjugate ligands (carcinoembryonic antigen, lamp-1 and lamp-2) in cultured human colon carcinoma cells by sodium butyrate. *Cancer Res. 54(22):* 5992-6000.

OZEKI, Y., MATSUI, T., YAMAMOTO, Y., FUNAHASHI, M., HAMAKO, J. and TITANI, K. (1995). Tissue fibronectin is an endogenous ligand for galectin-1. *Glycobiology 5(2):* 255-261.

POIRIER, F., TIMMONS, P.M., CHAN, C.-T.J., GUENET, J.-L. and RIGBY, P.W.J. (1992). Expression of the L14 lectin during mouse embryogenesis suggests multiple like roles during pre- and post- implantation development. *Development 115*: 143-155.

PRICE, J.L. and POWELL, T.P.S. (1970). The mitral and short axon cells of the olfactory bulb. *J. Cell Sci. 7*: 631-651.

PUCHE, A.C. and KEY, B. (1995). Identification of cells expressing Galectin-1, a galactose-binding receptor, in the rat olfactory system. *J. Comp. Neurol. 357*: 513-523.

PUCHE, A.C. and KEY, B. (1996). N-acetyl-lactosamine in the rat olfactory system. Expression and potential role in neurite growth. *J. Comp. Neurol. 357*: 513-523.

PUCHE, A.C., BARTLETT, P.F. and KEY, B. (1997). Substrate-bound carbohydrates stimulate signal transduction in an olfactory neuron cell line. *NeuroReport 8*: 3183-3188.

PUCHE, A.C., POIRIER, F., HAIR, M., BARTLETT, P. F. and KEY, B. (1996). Role of galectin-1 in the developing mouse olfactory system. *Dev. Biol. 179*: 274-287.

RAABE, E.H., YOSHIDA, K. and SCHWARTING, G.A. (1997). Differential laminin isoform expression in the developing rat olfactory system. *Dev. Brain Res. 101*: 187-196.

REGAN, L.J., DODD, J., BARONDES, S.H. and JESSELL, T.M. (1986). Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. *Proc. Natl. Acad. Sci. USA 83*: 2248-2252.

RESSLER, K.J., SULLIVAN, S.L. and BUCK, L.B. (1993). A zonal organisation of odorant receptor gene expression in the olfactory epithelium. *Cell 73*: 597-609.

RIOPELLE, R.J. and DOW, R.J. (1991). Neurite formation on laminin: effects of a galactosyltransferase on primary sensory neurons. *Brain Res. 541*: 265-272.

SAUCIER, D. and ASTIC, L. (1986). Analysis of the topographical organization of olfactory epithelium projections in the rat. *Brain Res. Bull. 16*: 455-462.

SULLIVAN, S.L., ADAMSON, M.C., RESSLER, K.J., KOZAK, C.A. and BUCK, L.B. (1996). The chromosomal distribution of mouse odorant receptor genes. *Proc. Natl. Acad. Sci. USA 93*: 884-888.

THOMAS, W.A., SCHAEFER, A.W. and THREADWAY, R.J. Jr. (1990). Galactosyltransferase-dependence of neurite outgrowth on substrate bound laminin. *Development 110*: 1101-1114.

TRELOAR, H.B., NURCOMBE, V. and KEY, B. (1996). Expression of extracellular matrix molecules in embryonic rat olfactory pathway. *J. Neurobiol. 31*: 41-55.

VAN DEN BRULE, F.A., BUICU, C., BALDET, M., SOBEL, M.E., COOPER, D.N., MARSCHAL, P. and CASTRONOVO, V. (1995). Galectin-1 modulates human melanoma cell adhesion to laminin. *Biochem. Biophys. Res. Commun. 209(2):* 760-767.

VASSAR, R., CHAO, S.K., SITCHERAN, R., NUÑEZ, J.M., VOSSHALL, J.B. and AXEL, R. (1994). Topographic organisation of sensory projections to the olfactory bulb. *Cell 79*: 981-991.

VASSAR, R., NGAI, J. and AXEL, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell 74*: 309-318.

WOYNAROWSKA, B., SKRINCOSKY, D.M., HAAG, A., SHARMA, M., MATTA, K. and BERNACKI, R.J. (1994). Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. *J. Biol. Chem. 269(36)*: 22797-22803.

ZANETTA, J.-P., KUCHLER, S., LEHMANN, S., BADACHE, A., MASCHKE, S., MARSCHAL, P., DUFOURCQ, P. and VINCENDON, G. (1992). Cerebellar lectins. *Int. Rev. Cytol. 135:* 123-154.

ZHOU, Q. and CUMMINGS, R.D. (1993). L-14 lectin recognition of laminin and its promotion of *in vitro* cell adhesion. *Arch. Biochem. Biophys. 300(1)*: 6-17.

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