Role of Galectin-1 in the Developing Mouse Olfactory System

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Primary sensory olfactory neurons reside in a neuroepithelium lining the nasal cavity and project topographically onto the surface of the olfactory bulb, a rostral extension of the telencephalon. Galectin-1, a bivalent galactose-binding vertebrate lectin, is expressed in the developing rodent olfactory system. In the present study, the mouse olfactory neuron cell line 4.4.2 was used to examine the role of galectin-1 in neurite outgrowth in vitro. Recombinant galectin-1 has neurite outgrowth-promoting activity when used as a substrate for 4.4.2 cells. When either galectin-1 or lactose was added to the culture media, the neurite outgrowth-promoting activity was abolished. These results demonstrate that galectin-1 can modulate neurite growth in vitro. The in vivo role of galectin-1 was investigated by examining the topographical organization of the olfactory pathway in mice carrying a null mutation for galectin-1. Using Dolichos biflorus agglutinin as a convenient histochemical marker of a subpopulation of primary sensory olfactory neurons which project topographically to the dorsomedial olfactory bulb, we show an aberrant topography of olfactory axons in the null mutants. A subset of primary sensory olfactory axons failed to project to their correct target sites in the caudal olfactory bulb. These data indicate that galectin-1 is involved in the growth and/or guidance of primary sensory olfactory axons between the nasal cavity and the olfactory bulb. This is the first demonstration that a lectin has neurite outgrowth-promoting activity and plays a role in neuronal pathfinding in the mammalian nervous system.

INTRODUCTION

Galectins are a family of animal lectins previously referred to as S-type lectins (Drickamer, 1988) which share conserved amino acid sequences and have binding specificity for carbohydrates containing β-galactoside (Barondes et al., 1994). To date, eight members of the galectin family have been identified (Hadari et al., 1995). Although the functions of these molecules are not well understood, evidence from localization studies indicates that galectins are widely expressed and might be involved in a variety of physiological functions. Galectin function is most certainly related to the bivalent or multivalent nature of these molecules and to their ability to agglutinate cells or bind cells to the extracelluar matrix (Barondes et al., 1994). These lectins are soluble and secreted extracellularly via nonclassical pathways (Cooper and Barondes 1990; Sato et al., 1993) where their functional activity is dependent on binding to cell surface or substrate ligands (Cho and Cummings, 1995a,b). Galectin-1, the ~14-kDa lactose-binding protein which forms homodimers, is the best characterized member of the galectin family. Numerous cellular functions have been ascribed to galectin-1 in vitro but the role of this molecule in vivo has remained elusive (Barondes, 1984; Drickamer and Taylor, 1993). While a role for galectin-1 in cell growth (Wells and Mallucci, 1991) and apoptosis (Perillo et al., 1995) is novel, its role in cell-cell and cell-matrix interactions in many tissues is well established (Cooper et al., 1991; Hughes, 1992; Gu et al., 1994; Lotan et al., 1994; Mahanthappa et al., 1994; Baum et al., 1995).

Galectin-1 is widely expressed in tissues of mesodermal origin and in select regions of the nervous system during development and in adults (Barondes, 1984; Regan et al., 1986; Poirier et al., 1992). In the somatosensory nervous system, galectin-1 is expressed at high levels by all dorsal root ganglion neurons (Hynes et al., 1990). These neurons consist of sub-
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classes which express different lactoseries carbohydrates, putative ligands for galectin-1 (Jessell et al., 1990). Thus, this lectin may function to fasciculate distinct subclasses of dorsal root axons as they project into the spinal cord.

We are interested in the role of galectin-1 in the olfactory system. Galectin-1 is expressed by primary sensory olfactory neurons, olfactory nerve glial cells, and second-order neurons in the rat olfactory bulb (Mahanthappa et al., 1994; Puche and Key, 1995). Because galectin-1 can mediate cell-substrate and cell-cell adhesion in the olfactory system in vitro, it might be involved in selective fasciculation and growth of olfactory axons to their target in vivo (Mahanthappa et al., 1994; Puche and Key, 1996). Primary sensory olfactory neurons, located in a neuroepithelial sheet lining the surface of the nasal cavity, project to the olfactory bulb where they synapse on dendrites of second-order olfactory neurons in specialized globules of neuropil referred to as glomeruli. In contrast to the point-to-point topographical map of the retinotectal projection in the visual system, the primary olfactory pathway has a mosaic organization. That is, subpopulations of primary sensory olfactory neurons which are dispersed throughout the nasal cavity project axons that sort out on route and terminate in discrete glomeruli within the olfactory bulb (Key and Akeson, 1993). The molecular mechanisms governing axon growth, sorting, and targeting in the olfactory pathway remain unknown (Buck, 1995).

To address the role of galectin-1 in the olfactory system, we first examined neurite outgrowth by the 4.4.2 olfactory neuron cell line when cultured on a substrate of recombinant galectin-1. We report here that substrate-bound recombinant galectin-1, and the galectin-1 ligand lactose, are strong and specific neurite outgrowth-promoting molecules. Primary sensory olfactory neurons were then examined in galectin-1 null mutant mice; these animals are viable and fertile and do not exhibit any obvious phenotype (Poirier and Robertson, 1993). We analyzed a defined subpopulation of primary sensory olfactory axons, taking advantage of the convenient Dolichos biflorus agglutinin (DBA) histochemical marker which recognizes a cell surface oligosaccharide containing N-acetylgalactosamine. DBA binds to a subpopulation of primary sensory olfactory neurons which are widely scattered in the nasal cavity (Key and Akeson, 1993; Treolar et al., 1996a). The DBA-reactive axons are dispersed in the olfactory nerve but sort out and selectively fasciculate upon entering the nerve fiber layer of the olfactory bulb (Key and Akeson, 1993). These axons then terminate in glomeruli present predominantly in the dorso-medial olfactory bulb. We observed in mice lacking the galectin-1 gene that few DBA-reactive axons grew into caudal regions of the olfactory bulb, while the rostral projection appeared unaffected. These results demonstrate that galectin-1 is involved in axon growth and/or guidance in the mammalian nervous system in vivo.

MATERIALS AND METHODS

Tissue Preparation

Tissue was collected from mice carrying a homozygous null mutation in the gene encoding galectin-1 (Poirier and Robertson, 1993). Neonatal heads from five mutant mice and three wild-type control mice were fixed in 4% paraformaldehyde in isotonic phosphate buffer (PBS) for 24 hr at 4°C. All tissue was processed and embedded in paraffin following standard procedures. Coronal sections (5 μm) were cut at 100-μm intervals throughout the nasal cavity and serially throughout the olfactory bulbs and collected on 2% aminopropyltriethoxysilane (Sigma Chemical Corp., St. Louis, MO)-coated slides.

Lectin Histochemistry, Immunohistochemistry, and In Situ Hybridization

Lectin histochemistry was performed as previously described (Key and Akeson, 1993). Briefly, sections were sequentially incubated in buffered BSA (2% bovine serum albumin and 0.3% Triton X-100) in Tris-buffered saline, TBS, pH 7.4) for 30 min, biotinylated DBA (Vector Laboratories, Burlington, CA) or biotinylated Erythrina cristagalli (ECA; Sigma Chemical Corp.) at 20 μg/ml in buffered BSA containing 1 mM MgCl2, 1 mM MnCl2, and 1 mM CoCl2 for 2 hr, TBS (three washes of 5 min duration), avidin–biotin–horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories), and then reacted with diaminobenzidine (0.5 mg/ml) and H2O2 (1.2% v/v) following standard procedures for visualization of horseradish peroxidase. All incubations were performed at 22°C.

For immunohistochemistry, sections were washed in buffered BSA and incubated with either polyclonal goat antiserum against olfactory marker protein (OMP, 1:500; Hartman and Margolis, 1975), polyclonal rabbit 162 antiserum against neural cell adhesion molecule (N-CAM; 1:200; Akeson et al., 1988), or polyclonal rabbit antiserum against laminin (1:200; Sigma Chemical Corp.) for 15 hr at 4°C. Sections were then washed with TBS, incubated for 60 min in biotinylated rabbit anti-goat antibodies (1:200; Sigma Chemical Corp.) for OMP staining and goat anti-rabbit antibodies (1:200; Sigma Chemical Corp.) for N-CAM and laminin staining, washed with TBS, and finally incubated with avidin–biotin–horseradish peroxidase and developed as above. In control sections, primary antiserum was replaced by normal goat serum which eliminated all staining of primary sensory olfactory neurons.

In situ hybridization was performed as previously described (Puche and Key, 1995). Briefly, sections were incubated sequentially with 0.1 M glycine in PBS (5 min), 0.3% Triton X-100 in PBS (15 min), 0.25% acetic anhydride in 0.1 M Tris, pH 7.0 (10 min), PBS wash (5 min), prehybridization mixture (50% deionized formamide, 15 mM Tris, pH 7.0, 1 M NaCl, 0.5% SDS, 0.25% polyvinylpyrrolidone, 0.02% Ficoll 400, 1 mg/ml hRNA, 0.25 M Tris, pH 7.0, and 1 U/ml RNase), and then hybridized at 37°C. Hybridization was performed in hybridization buffer (50% formamide, 2 × SSC, 10% polyethylene glycol, 0.3% BSA, 0.5% SDS, 0.25% polyvinylpyrrolidone, 0.02% Ficoll 400, 1 mg/ml hRNA, 0.25 M Tris, pH 7.0, and 1 U/ml RNase), and then washed in 2 × SSC (2 × 10 min at 37°C) and then PBS (2 × 10 min at 37°C). Nonspecific protein binding sites were subsequently blocked with buffered BSA for 30 min before incubation with avidin–biotin–horseradish peroxidase (Vectastain Elite ABC kit) and color visualization of horseradish peroxidase as above.

Photographs were taken on an Olympus BH2 microscope fitted with either differential interference contrast or phase contrast optics. Color 35-mm slides were scanned on a Polaroid SpectraScan 35 scanner and converted into digital form. These images were color balanced using Adobe Photoshop 3.0 (Adobe Systems Inc., CA), assembled into montages using CorelDraw (Coral Corp., Ltd, Dublin, Ireland) and printed on a Fuji X Pictrography 3000 digital image printer (Fuji Photo Film Co., Tokyo, Japan).
TUNEL Histochemistry

The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling method (TUNEL; Kasagi et al., 1994; Wijmker et al., 1993; Gavrieli et al., 1992) was used to identify apoptotic cells. Tissue sections were dewaxed, blocked as above, and incubated sequentially with 100 mM Tris (pH 7.2) for 10 min; 20 µg/ml proteinase K in 100 mM Tris (pH 8.0) and 50 mM EDTA (pH 8.0) for 15 min at 37°C; three 100 mM Tris (pH 7.2) washes each of 5 min duration; 1% H2O2 in methanol for 5 min; three 100 mM Tris (pH 7.2) washes each of 5 min duration; terminal transferase reaction buffer consisting of 30 mM Tris (pH 7.2), 140 mM sodium cacodylate, and 1 mM CoCl2 for 10 min; 0.3 U/µl of terminal transferase (Boehringer-Ingeheim, Mannheim, Germany) with 10 mM biotinylated 16-dUTP (Boehringer) in terminal transferase reaction buffer for 2 hr at 37°C; three 100 mM Tris (pH 7.2) washes each of 5 min duration; one wash in 2% bovine serum albumin (Sigma Chemical Corp.) in TBS for 15 min; and finally avidin-biotin–horseradish peroxidase and color development as above. All incubations were at 22°C unless otherwise stated. Control sections incubated in the absence of terminal transferase demonstrated negligible background staining. For positive control sections, reactions were performed as above except chromosomal DNA was prefragmented with 1 U/µl DNase RQ1 (Promega, Sydney, Australia) in 30 mM Tris (pH 7.2), 140 mM sodium cacodylate, 0.1 mM DTT and 1 mM CoCl2 for 15 min at 22°C prior to treatment for endogenous peroxidase.

Olfactory Neuron Cell Line Cultures

Clonal cell lines were generated by n-myc immortalization of dissociated olfactory neuroepithelial cells from adult mice as previously described (Bernard et al., 1988; Bartlett et al., 1988). These cells were immortalized from progenitor and precursor cells in the epithelium and differentiate into bipolar neurite-containing possessing cells with voltage-dependent sodium currents following serum starvation (Cuffel et al., 1994). The olfactory neuroepithelial cell line 4.4.2 expresses olfactory marker protein and olfactory receptor genes (Dowsing et al., 1995). Full details regarding the generation of the 4.4.2 cell line and expression of odorant receptors will be published elsewhere. Dissociated cells from the 4.4.2 mouse olfactory neuron cell line were plated onto sterile coverslips at a density of 200 cells/coverslip. These cells were grown for 24 hr at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemical Corp.) containing 10% fetal calf serum (FCS; Gibco). Cultures were grown for 24 hr in the presence of the reducing agent 14 mM sodium solubile fucose, (3) 50 mM soluble asialofetuin (Sigma Chemical Corp.), and (4) 10 µM soluble recombinant galectin-1. Slides were washed in fresh medium, fixed in 4% paraformaldehyde in PBS for 30 min, and either mounted immediately in Faramount aqueous mounting medium (DAKO, Carpinteria, CA), or stained with the plant lectins ECA or DBA as described above.

 Cultures were digitally imaged under phase contrast using a WV-FL600 low-light level video camera (Panasonic, Melbourne, Australia) and MD 30 Plus image capture/analysis software (Leading Edge Software, Adelaide, Australia). Total neurite length was measured from individual neurons using the MD30 PLUS image capture/analysis software. Only those processes >1 cell diameter in length and growing from neurons not in contact with other cells were measured (Dotti et al., 1988). The Mann–Whitney nonparametric statistical test was used to assess differences in neurite lengths between control and experimental substrates. All experiments were performed in duplicate and repeated at least three times.

Preparation of Recombinant Protein

Biotinylated recombinant galectin-1 fusion protein was produced using the PinPoint Protein Purification system (Promega) and the generation of a fusion protein in E. coli cells. Briefly, PCR was used to generate a cDNA containing the full coding region of galectin-1 (forward primer 5’-CTC-GTCGAC-TTCAACCATGGCGCTGTGGT-3’, reverse primer 5’-CTC-GGATCC-TTACACTCAAAGGCCACACACTAG-3’). The galectin-1 PCR product was ligated into the XA-2 PinPoint expression vector (Promega), fully sequenced twice to confirm that the cDNA construct was without error, and transformed into JM109 Escherichia coli cells (Promega). Galectin-1-positive colonies were grown and processed according to manufacturer’s instructions. Cho and Cummings (1995) reported that galectin-1 is only stable for long periods of time when in the presence of the reducing agent 14 mM BME. Thus, all binding, washes, and elutions involving galectin-1 were performed in a stabilizing buffer containing 6.7 mM KH2PO4, 150 mM NaCl, 14 mM BME, 0.2% sodium azide, and the protease inhibitors 10 mM EDTA (pH 8.0) and 0.1 mM phenylmethylsulfonyl fluoride (PM SF). A crude protein fraction from lysed bacteria was incubated with lactose-4% beaded agarose resin (Pierce Research Products, Rockford, IL), washed with stabilization buffer, and eluted with 100 mM lactose. The major protein eluted from this lactose resin was a 27-kDa fusion protein, consisting of galectin-1 (14.5 kDa) and the fusion protein tag (13 kDa). Proteins eluted from the lactose resin were further purified by binding to a SoftLink Soft Release avidin–poly-methacrylate resin (Promega), washed in stabilization buffer, and eluted with 5 mM biotin. This eluent contained a single protein of 27 kDa which was dialyzed against 0.67 mM KH2PO4, 15 mM NaCl, and 14 mM BME for 24 hr at 4°C and then freeze dried and

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stored at −20°C. The carbohydrate binding specificity of reconstituted recombinant galectin-1 was confirmed by dot blot analysis.

RESULTS

Galectin-1 Is a Neurite Outgrowth-Promoting Molecule

Galectin-1 mediates cell–substrate adhesion between N-acetyllactosamine residues present on laminin and cell surface carbohydrate ligands on primary sensory olfactory neurons in vitro (Mahanathappa et al., 1994). We were interested in determining whether galectin-1 or simple disaccharides were capable of promoting neurite-outgrowth in addition to cell–substrate adhesion. The 4.4.2 olfactory neuroepithelial cell line (Cuffel et al., 1994; Dowling et al., 1995) was used to assess the neurite outgrowth-promoting ability of galectin-1 and neoglycoproteins. We show here by in situ hybridization (Figs. 1A and 1B) and Northern blot analysis that the differentiated 4.4.2 cell line strongly expresses galectin-1 mRNA. Histochemical staining with E. cristaagalli agglutinin (Fig. 1C) revealed that these cells express cell surface oligosaccharides containing N-acetyllactosamine, which are putative ligands for galectin-1 (Cerra et al., 1985; Leffler and Barondes, 1986; Leffler et al., 1989). The 4.4.2 cells were also stained with DBA (Fig. 1D), which is a histochemical marker of a subpopulation of primary sensory olfactory neurons in vivo (Key and Akeson, 1993). Thus, this cell line expresses a phenotype which corresponds to a distinct subpopulation of primary sensory olfactory neurons in vivo.

The 4.4.2 cells were induced to extend neurites on substrates of lactose–BSA neoglycoproteins, BSA, and monomeric recombinant galectin-1 (Fig. 2). Poly-L-lysine, a synthetic and nonspecific adhesive molecule, was present in all substrates to facilitate uniform plating of cells. The derivatized neoglycoprotein consisted of lactose conjugated to BSA and ensured that lactose, a galectin-1 ligand, was present in the absence of other carbohydrates or core proteins which may have endogenous neurite outgrowth-promoting activity. The recombinant galectin-1 proteins had the same carbohydrate binding specificity as wild-type ga-
FIG. 2. Quantitation of neurite outgrowth from the 4.4.2 olfactory neuron cell line when grown on a substrate of lactose-derivatized BSA (A) or a substrate of recombinant galectin-1 (B). The ordinate shows the percentage on neurons with total neurite length greater than the value shown on the abscissa. Both plots represent the results of at least three separate experiments, each performed in duplicate.

Galectin-1 even though it contained a fusion peptide which inhibited the formation of dimers. This monomeric recombinant galectin-1 prevented spurious effects of cross-linking of cells and substrate as occurs with wild-type bivalent galectin-1 dimers. We observed no significant difference in the number of 4.4.2 cells attached to any of the substrates.
and their respective controls. However, there was significantly greater neurite outgrowth by 4.4.2 cells when they were plated on a substrate of lactose-BSA compared to a substrate of BSA alone (Fig. 2A; P < 0.0001). Neurite outgrowth on lactose-BSA was reduced to control levels by addition of either 50 mM soluble lactose or 10 μg/ml soluble monomeric recombinant galectin-1 to the culture medium, but was not affected by the addition of 50 mM soluble fucose (Fig. 2A). These results suggested that substrate-bound lactose specifically bound to a carbohydrate receptor, possibly a lectin, present on the olfactory neuron cell line and that this interaction stimulated neurite outgrowth. Because addition of exogenous monomeric recombinant galectin-1 successfully competed interactions between substrate-bound lactose and its cell surface receptor, it is feasible that galectin-1 mediates the lactose neurite outgrowth activity.

To further examine the role of galectin-1 in neurite outgrowth, 4.4.2 cells were plated on a substrate of monomeric recombinant galectin-1. Neurite outgrowth was significantly longer on recombinant galectin-1 than on control BSA substrate alone (Fig. 2B; P < 0.0001). This neurite outgrowth was inhibited by the addition of 50 mM soluble lactose to the culture media (Fig. 2B). Similarly, the asialofetuin glycoprotein, a potent ligand for galectin-1 (Leffler and Barondes, 1986; Leffler et al., 1989), when added to the culture medium (50 μg/ml; Fig. 2B) also specifically inhibited neurite outgrowth on substrate-bound monomeric recombinant galectin-1. In contrast, addition of 50 mM soluble fucose, which is not a galectin-1 ligand but is expressed on the surface of primary sensory olfactory neurons (Barber, 1989), to the culture medium did not affect neurite outgrowth on a substrate of monomeric recombinant galectin-1 (Fig. 2B). Thus, the results of these experiments demonstrated that both substrate-bound lactose and monomeric recombinant galectin-1 were capable of promoting neurite outgrowth in vitro. Galectin-1 and its putative ligands are also present in the olfactory nerve pathway (Mahanthappa et al., 1994; Puche and Key, 1995, 1996); therefore, we next assessed the in vivo role of galectin-1 in the development of the olfactory system.

Normal Peripheral Olfactory Pathway in Galectin-1 Null Mice

We examined in detail the primary olfactory pathway in transgenic mice carrying a null mutation of the gene encoding galectin-1 (Poirier and Robertson, 1993). Primary sensory olfactory neurons reside in the olfactory neuroepithelium lining the nasal cavities. Axons arising from these neurons course caudally to the olfactory neuroepithelium in large axon fascicles located in the lamina propria underlying the olfactory neuroepithelium. These axon fascicles form a series of nerve bundles en route to the bulb and are collectively termed the olfactory nerve. Immunohistochemistry for OMP, an intracellular protein expressed by all mature primary sensory olfactory neurons (Hartman and Margolis, 1975), revealed a normal overall distribution of these neurons in the olfactory neuroepithelium lining the nasal cavities of galectin-1 mutants. We observed that in mutant mice the axons of these neurons project normally to the olfactory bulb where they terminate in glomeruli which were indistinguishable from glomeruli in control wild-type animals (Figs. 3C–D, 4, and 5A–D). Hematoxylin counterstaining further revealed that cellular layers in the olfactory bulb also appeared normal.

We have previously shown that DBA is a convenient histochemical marker which can be used to label a subpopulation of primary sensory olfactory neurons. These DBA-stained neurons exhibit a mosaic distribution in the olfactory neuroepithelium (Key and Akeson, 1993). We used DBA staining of coronal sections of galectin-1 null mutants mice heads as a simple way of assessing the topography of the olfactory pathway in these animals. The spatial distribution of the subpopulation of primary sensory olfactory neurons stained by DBA within the olfactory neuroepithelium was unaffected by the loss of galectin-1 in null mutant mice. Neurons stained by DBA were scattered throughout the neuroepithelium and axons from these neurons projected caudally to the olfactory bulb within the olfactory nerve. As in wild-type mice, the DBA-stained axons were randomly distributed throughout fascicles of the olfactory nerve. Upon reaching the olfactory bulb these axons sorted into homogeneous bundles and terminated in discrete glomeruli.

Primary sensory olfactory axons course in large fascicles between the nasal cavity and the olfactory bulb. These fascicles are encapsulated by olfactory nerve Schwann cells which express galectin-1 (Mahanthappa et al., 1994; Puche and Key, 1995). These cells stimulate the growth of primary

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**FIG. 3.** Topography of primary sensory olfactory axons in coronal sections of the caudal olfactory bulb. (A, C, and E) are galectin-1 null mutant mice and (B, D, and F) are wild-type mice. Sections pass through the rostral AOB nuclear layers which are outlined by the dashed line in A–D. Sections in A, B, E, and F were reacted with DBA. Sections in C and D were reacted with OMP antiserum. (A) DBA-reactive primary sensory olfactory axons are absent from the dorsocaudal region of mutant mice (arrows). (B) DBA-reactive axons are present in the dorsocaudal olfactory bulb in wild-type mice (arrows). (C) OMP expression in a section adjacent to A demonstrates the presence of primary sensory olfactory axons within this region of the olfactory bulb (arrows) as well as along its medial surface (arrowheads). (D) OMP expression in wild-type animals reveals that primary sensory olfactory axons are present throughout the dorsal (arrows) and medial (arrowheads) surfaces of the olfactory bulb. (E) High-power micrograph of DBA labeling in mutant mice. The dashed line depicts the outer surface of the olfactory bulb. Arrows mark the glomerular and nerve fiber layers which are largely devoid of DBA-stained axons, although a few DBA-reactive fascicles are present in the nerve fiber layer. Nonspecific brown reaction product is present in erythrocytes in blood vessels (BV). (F) High-power micrograph of DBA-reactive axons in the dorsocaudal olfactory bulb of a wild-type mouse demonstrating stained glomeruli and axon fascicles (arrows). Scale bar in A, 500 μm, applies to A–D. Scale bar in E, 100 μm, applies to E and F.
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FIG. 4. Camera lucida drawings of coronal sections of the olfactory bulb at four representative rostrocaudal levels of neonatal mice. Dorsal is to the top of the page, medial to the left in all drawings. Cross-hatching depicts DBA reactivity or OMP immunostaining within the nerve fiber and glomerular layers. In wild-type mice, DBA-stained axons terminate widely in the medial half of the rostral olfactory bulb. These axons gradually become restricted to the dorsal surface in the caudal olfactory bulb. In the most caudal regions of the olfactory bulb (level of the accessory olfactory bulb, AOB, depicted in Fig. 1) DBA-reactive axons are restricted to the dorsal surface only. In mutants, DBA-reactive axons exhibit a wild-type-like distribution at the rostral and middle levels of the olfactory bulb. However, in the caudal olfactory bulb very few (asterisks) or no DBA-stained axons (filled arrow) are present. OMP immunostaining of mutants revealed that the overall distribution of primary olfactory axons was identical to wild-type animals (not shown). Despite the absence of DBA-stained axons in the caudal olfactory bulb of mutant mice, other primary sensory olfactory axons terminated normally over the dorsal (open arrow) and medial (arrowheads) surfaces of the olfactory bulb. The AOB was equally stained in both mutant and wild-type mice by DBA and OMP.

This analysis revealed a normal complement of olfactory nerve Schwann cells in the galectin-1 null mutants. Thus, in the absence of galectin-1 these cells successfully migrated from the olfactory neuroepithelium during em-
bryogenesis and ensheathed the bundles of primary sensory olfactory axons as in wild-type mice.

Primary sensory olfactory neurons have a limited life span and are replaced following apoptosis by a population of globose stem cells in the basal layer of the olfactory neuroepithelium (Graziadei, 1973; Carr and Farbman, 1993; Suzuki and Takeda, 1993; Caggiano et al., 1995; Holbrook et al., 1995; Holcomb et al., 1995). Recent evidence suggests that galectin-1 is capable of modulating apoptosis in human lymphocytes in vitro (Perrillo et al., 1995). To assess the level of programmed cell death in the olfactory neuroepithelium of mice carrying a null mutation for galectin-1, we used the terminal dUTP nick end labeling reaction (TUNEL; Gavrieli et al., 1992; Wijman et al., 1993; Kasagi et al., 1994). No differences were observed between galectin-1 null mutant and wild-type mice with respect to the level of apoptosis, or the location of apoptotic cell bodies in the olfactory neuroepithelium (Figs. 5G and 5H).

In summary, we have revealed that there were no detectable differences in either the gross morphology or spatial distribution of the examined subpopulation of primary sensory olfactory neurons in mice carrying a null mutation in the gene encoding galectin-1.

**Aberrant Olfactory Axon Targeting in Galectin-1 Null Mutants**

The bundles of primary sensory olfactory axons coursing from the nasal cavity pierce the cribriform plate of the skull and directly enter the olfactory bulb where they form the nerve fiber layer. Immunostaining of the olfactory pathway for the olfactory neuron-specific marker OMP revealed that the primary olfactory axons in galectin-1 null mutants formed normal olfactory nerve fiber and glomerular layers in the olfactory bulb (Figs. 3C and 3D). These results suggested that galectin-1 did not have an essential role either in the overall growth of axons between the nose and brain or in the formation of synaptic connections between primary axons and the dendrites of second-order mitral/tufted cells in glomeruli. There is considerable sorting and intermixing of subpopulations of axons within the nerve fiber layer as they course toward their target glomeruli. We have shown previously that the DBA-stained subpopulation of axons terminate preferentially in glomeruli lining the dorsomedial surface of the olfactory bulb (Key and Akeson, 1993; Treloar et al., 1996a). In order to determine whether loss of galectin-1 affected the topography of the primary olfactory projection, we compared the distribution of DBA-stained axons along the rostrocaudal axis of the olfactory bulb in both wild-type and galectin-1 null mutant mice. The subpopulation of primary sensory olfactory axons stained by DBA projected to the medianorostral and dorsocaudal surfaces of the olfactory bulb in wild-type mice (Fig. 4). In mice lacking galectin-1, DBA-stained axons projected normally to the mediorostral region of the bulb, but there was a grossly abnormal projection to the dorsocaudal bulb (arrows in Figs. 3A-3E; Fig. 4). Few DBA-stained axons were present in the dorsocaudal olfactory bulbs of galectin-1 null mutants and most of these axons did not terminate in discrete glomeruli (arrows in Figs. 3E and 3F). Olfactory marker protein immunostaining of the entire population of primary sensory olfactory axons clearly revealed that there was no general defect in the formation of glomeruli in the dorsocaudal olfactory bulb of mutant mice (arrows in Figs. 3C and 3D; Fig. 4). Aberrant growth was therefore limited to specific subpopulations of primary sensory olfactory neurons, one of which was identified by labeling with DBA. Although these observations were found in all null mutants examined, some variability in the distribution of DBA-stained axons in the olfactory nerve fiber layer of the dorsocaudal olfactory bulb was observed. Some animals had very few or no DBA-stained axons in this locality, whereas small DBA-reactive fascicles were observed in other animals. However, no DBA-stained axons were ever observed to terminate in glomeruli.

These results indicate that galectin-1 is involved in the growth of some axons into the caudal olfactory bulb. Moreover, even when some of these axons managed to reach the dorsocaudal surface, they failed to target and terminate in specific glomeruli. Taken together, our in vitro demonstration of the role of galectin-1 in neurite outgrowth and our in vivo observation of abnormal neuronal targeting in null mutants suggests that galectin-1 is directly involved in axon growth and/or guidance in the olfactory pathway. Other adhesion molecules, such as N-CAM and laminin, have been implicated in the development of the olfactory system (Miraqall et al., 1989; Mahanathapa et al., 1994; Tomasiwicz et al., 1993; Treloar et al., 1996a,b). To examine the possibility of abnormal expression of N-CAM and laminin in galectin-1 null mutants, we analyzed the expression patterns of these molecules in both the peripheral and central regions of the olfactory nerve pathway. The expression of N-CAM was identical in mice carrying a targeted disruption to the gene encoding galectin-1 (Figs. 5A-5C) and wild-type mice (Figs. 5B-5D). Expression of N-CAM in the cellular layers of the olfactory bulb and in the olfactory neuroepithelium of the galectin-1 null mutants appeared normal (Figs. 5A-5D). N-CAM immunohistochemistry clearly demonstrated that normal glomeruli were formed in the absence of galectin-1 (arrows in Figs. 5C and 5D). There were also no differences in laminin expression between wild-type and mutant mice (Figs. 5E and 5F). Laminin was present around the olfactory axon fascicles within the submucosa of the olfactory neuroepithelium as well as on the surface of the olfactory bulb (Figs. 5E and 5F).

**DISCUSSION**

Galectin-1 is a 14.5-kDa galactose-binding protein which is expressed in lungs, heart, muscle, and brain of many ver-
tebrate species (Regan et al., 1986; Wasano, 1990; Hynes et al., 1990; Poirier et al., 1992). This S-type lectin, previously referred to as RL 14.5, has been implicated in cell adhesion, cell–cell recognition and apoptosis (Dodd and Jessell, 1986; Hynes et al., 1989; Cooper et al., 1991; Outenreath and Jones, 1992; Mahanathappa et al., 1994; Perillo et al., 1996). The in vivo role of galectin-1 has, however, remained elusive. We report here that galectin-1 is a neurite outgrowth-promoting molecule and is involved in axon growth and/or guidance in the mouse olfactory system. Mice carrying a targeted disruption in the gene encoding galectin-1 possess an apparently normal central nervous system, although they exhibit a specific defect in the topographical organization of the olfactory nerve pathway.

We have shown that the 4.4.2 mouse olfactory neuroepithelium cell line expresses galectin-1 as well as cell surface N-acetyllactosamine, a galectin-1 ligand. When 4.4.2 cells were plated on a substrate of monomeric recombinant galectin-1, these cells exhibited enhanced neurite outgrowth which could be specifically inhibited by soluble lactose in the medium. In complementary experiments, substrate-bound neoglycoproteins containing lactose were also shown to be specific neurite outgrowth-promoting molecules for 4.4.2 cells. Lactoseries neoglycoproteins also stimulate neurite outgrowth by primary sensory olfactory neurons in vitro (Puche and Key, 1996). We propose that substrate-bound galectin-1 stimulates neurite outgrowth from 4.4.2 cells by binding to cell surface N-acetyllactosamine or other carbohydrate ligands. This neurite outgrowth appears to be independent of the cell adhesiveness mediated by the galectin-1 substrate because there were no differences in the number of cells attached in cultures plated on galectin-1 or bovine serum albumin controls. The presence or absence of soluble galectin-1 ligands in the medium also did not alter the number of cells attached to the substrate. Thus, interactions between galectin-1 and cell surface ligands appear to have a role in stimulating neurite outgrowth. Given the specificity of this response, it seems likely that galectin-1 is involved in activating transmembrane signaling pathways required for promoting neurite formation.

In addition to stimulating neurite outgrowth, galectin-1 can mediate both self-aggregation of primary sensory olfactory neurons, by cross-linking carbohydrate ligands present on primary sensory olfactory neurons, and adhesion of these neurons to laminin sugar chains (Mahanathappa et al., 1994). Laminin was proposed to act as a substrate pathway for subpopulations of primary sensory olfactory axons in vivo. We have recently demonstrated that laminin is indeed present within the olfactory nerve pathway between the nose and brain during embryogenesis (Tetreau et al., 1996b). Thus, primary olfactory axons which express galectin-1 and the appropriate cell surface ligands may be specifically stimulated to sort out and fasciculate upon extracellular matrix laminin. We believe that the correct targeting of olfactory axons relies on two distinct events in the olfactory nerve pathway. First, discrete subpopulations of primary olfactory axons sort out and fasciculate into small bundles within the nerve fiber layer of the olfactory bulb (Key and Akeson, 1993; Puche and Key, 1996; Tetreau et al., 1996a) and, second, these bundles are guided to their specific target site by gradients of recognition molecules on the surface of the bulb. It is unlikely that galectin-1 mediates selective targeting of axons because it is uniformly expressed by olfactory Schwann cells and target cells in the olfactory bulb (Puche and Key, 1995). Galectin-1 is probably acting to stimulate self-fasciculation and growth of a unique subset of primary sensory olfactory axons in the nerve fiber layer.

In mice carrying a null mutation in the gene encoding galectin-1, development proceeded normally with no gross abnormalities of the olfactory system. Considering the role observed for galectin-1 in neurite outgrowth in vitro, we decided to examine the topographical organization of the olfactory nerve pathway in these animals. In galectin-1 null mutant mice we observed misrouting of a subset of DBA-stained axons which normally terminate in the caudal olfactory bulb. The DBA-stained axons that terminate in the rostral olfactory bulb were unaffected by the loss of galectin-1. The aberrant topography of the olfactory pathway observed in the galectin-1 mutant mice is consistent with the loss of specific interactions between galectin-1 and ligands on the surface of a select subset of olfactory axons. Unfortunately, until the subpopulation of DBA-stained axons which normally terminate in the caudal olfactory bulb is identified by specific molecular markers, we will not be able to dis-
criminate between the effects of galectin-1 on axon growth, sorting out, and/or guidance in mutant mice.

Although our results are consistent with a direct role of galectin-1 in development of the olfactory system, it is possible that loss of galectin-1 indirectly affects other molecular interactions mediating the formation of topography. The expression of cell or substrate adhesion molecules may be either up- or down-regulated in the absence of galectin-1. N-CAM is involved in the development of the olfactory bulb (Tomasiewicz et al., 1993), and the correct formation of olfactory glomeruli (Treloar and Key, unpublished observations). However, in mice carrying a null mutation for galectin-1 the distribution of N-CAM was unaffected. The interactions between galectin-1 and laminin (Zho and Cummins, 1990, 1993; Mahanathappa et al., 1994) suggests that expression of this molecule could be important for the function of galectin-1. Immunohistochemistry for laminin in galectin-1 null mutant mice indicated that distribution of this molecule was unaffected by the absence of galectin-1. Thus, the normal expression of these major adhesion molecules and the specific effect of galectin-1 and lactose in vitro argue in favor of a direct role of galectin-1 in axon growth in vivo.

Axon growth, guidance, or adhesion may not be the only function of galectin-1 in the olfactory system. Novogrodsky and Ashwell (1977) first demonstrated that carbohydrate binding proteins could influence mitogenesis. Perillo et al. (1995) have recently shown that soluble galectin-1 can bind to an unidentified N-glycan on the glycoprotein CD45 and induce apoptosis in activated human T cells in vitro. Furthermore, galectin-1 is present on stromal cells of the thymus and lymph nodes at sites of T cell apoptosis during development (Baum et al., 1995). These data indicate that modulation of programmed cell death could be a function of galectin-1. Primary sensory olfactory neurons continually undergo apoptosis and are replaced by progenitor cells in the basal layer of the olfactory neuroepithelium (Graziadei, 1973; Carr and Farbman, 1993; Holcomb et al., 1995). We report here that galectin-1 does not appear to be involved in the normal regulation of cell number in the olfactory neuroepithelium because the number of apoptotic cells was identical in neonatal mice carrying a targeted disruption in the gene encoding galectin-1.

The results of the present study demonstrate that primary sensory olfactory neurons can use galectin-1 as a permissive substrate for neurite outgrowth in vitro, and that in vivo galectin-1 is involved in axon growth and/or guidance in the olfactory system. The mice carrying a null mutation in the gene encoding galectin-1 show a very clear phenotype in the olfactory system, and this is the first demonstration of an error in pathway formation in the mammalian nervous system. Although we observed no gross behavioral differences in galectin-1 null mutant mice so far, it does not preclude the possibility that they exhibit specific defects in olfactory perception. This can initially be addressed by examining the topography of subpopulations of neurons expressing known olfactory receptor genes (Vassar et al., 1994).

The role of carbohydrates and lectins in axon guidance has long been suspected but, to date, molecules clearly involved in this process include only members of the immunoglobulin superfamily, cadherins, semaphorins/collapsins, netrins, and receptor tyrosine kinases (Keynes and Cook, 1995). We now demonstrate that a member of the galectin family is directly contributing to mechanisms of axon growth and/or guidance in the mammalian nervous system. Transgenic mice in which glycosyltransferases have been overexpressed or misexpressed in inappropriate tissues are a novel way of assessing the role of specific oligosaccharides in morphogenesis (Shur, 1994). These animals will provide a further test of the in vivo role of oligosaccharides and galectin-1 in the development of the olfactory system.

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