

Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism

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SUMMARY

L-14 is a divalent, lactosamine-binding lectin expressed in many vertebrate tissues. In the rat nervous system, L-14 expression has been observed previously in restricted neuronal subsets within the dorsal root ganglia and spinal cord. In this study we report that L-14 is expressed by non-neuronal cells in the rat olfactory nerve. We demonstrate that L-14 binds and co-localizes with two ligands in the rat olfactory system: a β -lactosamine-containing glycolipid, and a putative member of the laminin family. The former is expressed on the surfaces of nascent olfactory axons originating from neuron cell bodies in the olfactory epithelium. The latter is present in the extracellular matrix of the axonal path leading to synaptic targets in the olfactory bulb.

In vitro, we find that recombinant L-14 promotes primary olfactory neuron adhesion to two laminin family members, and promotes intercellular adhesion. Both activities are dose-dependent, and are independent of integrin-mediated mechanisms. We have thus found that L-14 can serve two distinct adhesive functions *in vitro*, and propose that L-14 *in vivo* can promote olfactory axon fasciculation by crosslinking adjacent axons and promote axonal adhesion to the extracellular matrix.

Key words: adhesion, carbohydrates, extracellular matrix, glycolipids, L-14, laminin, merosin, olfactory neurons

INTRODUCTION

Complex carbohydrates are expressed in the vertebrate nervous system with varying degrees of spatiotemporal specificity, but the functions of these substances on neural cell surfaces remain largely unknown (Yamamoto and Schwarting, 1992). Rat dorsal root ganglion (DRG) neurons and their projections to the dorsal horn of the spinal cord constitute a system in which the co-expression of specific glycoconjugates and complementary binding proteins (lectins) has been characterized (Dodd and Jessell, 1985; Regan et al., 1986; Hynes et al., 1990). One observed pairing of carbohydrate and lectin is that of the lectin, L-14, and a terminal lactosamine epitope recognized by the monoclonal antibodies (mAbs), 1B2 and A5 (Regan et al., 1986). Since L-14 is a homodimer and thus divalent (Leffler and Barondes, 1986), Hynes et al. (1990) suggested that L-14 might promote fasciculation of functional classes of sensory axons.

L-14, also known as L-14-I (Gitt et al., 1992), has particular affinity for poly-lactosamine chains (Zhou and Cummings, 1990; Cooper et al., 1991), such as those expressed by 'prototypical' laminin (Arumham et al., 1986; Knibbs et al., 1989). Prototypical laminin purified from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma is a heterotrimer composed of A-, B1-, and B2-chains (reviewed by Martin and Timpl, 1987).

Isoforms of the B1-chain known as the S-chain, and of the A-chain known as the M-chain (which forms merosin in conjunction with the B-chains) have also been identified (Hunter et al., 1989; Ehrig et al., 1990). All five chains are observed in nervous tissue (Sanes et al., 1990). Many neuronal receptors for laminin-family members, primarily members of the integrin family, have been described to regulate axon outgrowth (reviewed by Reichardt et al., 1989, and Reichardt and Tomaselli, 1991). L-14, through its potential to crosslink poly-lactosamine in the extracellular matrix and lactosamine antigens on neuron cell surfaces, might also serve as a receptor that regulates axonal outgrowth. The plausibility of such a mechanism is supported by the report of Zhou and Cummings (1993) that L-14 can promote the adhesion of two cell lines to EHS laminin. A role for L-14 in neuronal axon fasciculation or matrix binding, however, has not been directly tested.

We have demonstrated, by *in situ* hybridization, that L-14 is produced by non-neuronal cells that populate the olfactory nerve pathway. By immunocytochemistry we have examined the expression of L-14, laminin family members, and the 1B2-epitope in the olfactory system. Within the lamina propria of the olfactory epithelium and the outer nerve layer of the olfactory bulb, we find that L-14 immunoreactivity co-localizes with both laminin-immunoreactivity and the axon-associated 1B2-epitope. Furthermore, *in vitro*, L-14 binds

laminin, merosin, a laminin-related molecule present in olfactory homogenates, and a 1B2-immunoreactive olfactory glycolipid.

Hypothesizing that L-14 may mediate olfactory axon fasciculation by crosslinking adjacent axonal membranes that express the 1B2-epitope, and may serve a guidance function by crosslinking the surface of growing axons to olfactory laminin(s), primary olfactory neurons were cultured on a variety of extracellular matrix molecules and the effects of L-14 on neuronal behavior assayed. We report here that (i) L-14 promotes olfactory neuron adhesion to laminin and merosin, but not fibronectin, in a dose-dependent manner; and (ii) L-14-mediated adhesion appears to be independent of integrin-mediated adhesion.

MATERIALS AND METHODS

Generation of L-14 RNA probes and in situ hybridization

Poly(A)⁺ mRNA was isolated from rat olfactory tissue using total RNA separator and mRNA separator kits from Clontech Laboratories. Reverse transcription-polymerase chain reactions (PCR) were performed using a Perkin Elmer Cetus kit. L-14 PCR primers were synthesized that correspond to bases 12-31 (primer A2) and 451-471 (primer B1) of the sequence reported by Clerch et al. (1988) (plus flanking sequences containing *Pst*I and *Xba*I sites, respectively): A2 = 5' GGCTGCAGTCGCAGGAATCTCTCGCTT 3', and B1 = 5' GGTCTAGAGGGGCTACGGGCTGGGGCTGG 3'. PCR proceeded through 35 cycles of 1 minute at 95°C and 1 minute at 60°C, and resulted in a single amplified message that was cloned into the pBI30 vector (International Biotechnologies). The insert was sequenced by the dideoxy chain termination technique using a 'Sequenase' kit (United States Biochemical), and the olfactory clone determined to be identical to previously cloned L-14.

In situ hybridizations were carried out by the method of Sassoon et al. (1988) as modified by Ressler et al. (1993) on 8 µm paraffin sections prepared from embryonic day 19 and postnatal day 2 rats. Amplified, purified, and linearized plasmid was transcribed using T3 and T7 RNA-polymerases to generate sense and antisense ³⁵S-RNA probes. Sections were hybridized at 50°C for 16 hours, washed, dehydrated, and dipped in NTB-2 autoradiography emulsion (Kodak). Slides were developed after exposure for 9 days at 4°C and counterstained with toluidine blue or cresyl violet.

Immunocytochemistry

Immunocytochemistry was performed as detailed in Schwarting et al. (1992a). Staining was performed with polyclonal anti-rat L-14 (Cooper et al., 1991), polyclonal anti-EHS laminin (Sigma), a mAb directed against the rat laminin B1-chain (C21) (Sanes and Chiu, 1983; Green et al., 1992) (gift of A. Y. Chiu, City of Hope), and the 1B2 mAb (Young et al., 1981).

Tissue homogenization for protein analysis

Homogenates of olfactory tissue (ectoturbinate, caudal nasal septum, cribriform plate, and olfactory bulbs) were prepared using a sequential homogenization technique described by Hunter et al. (1992). Briefly, olfactory tissue was dissected from 5 day old rats and stored at -20°C. Tissue was first homogenized using a Polytron tissue homogenizer in 25 mM Tris-HCl, pH 7.5 containing 0.2 mg/ml α-2 macroglobulin as a protease inhibitor. The homogenate was centrifuged at 15,000 g for 20 minutes, the supernatant discarded, and the pellet homogenized again in 20 mM EDTA and 25 mM Tris-HCl, pH 7.4, with 0.2 mM phenylmethyl-sulfonyl fluoride, 0.25% aprotinin, and 60 mM leupeptin (Sigma) as protease inhibitors. The homogenate was centrifuged as above, and the insoluble material was sequentially

homogenized and centrifuged in 2 M MgCl₂ in 25 mM Tris (pH 7.5); 2% Triton X-100 in 25 mM Tris (pH 7.5); 6 M guanidine-HCl in 25 mM Tris (pH 7.5); and 6 M guanidine-HCl with 10 mM dithiothreitol in 25 mM Tris (pH 7.5). Most of the laminin was extracted from the olfactory tissue in the 6 M guanidine-HCl in 25 mM Tris fraction. This was dialyzed into PBS, precipitated in 40% saturated ammonium sulfate, dissolved in PBS and dialyzed again, then frozen at -80°C.

Purification and radioiodination of recombinant L-14

Recombinant rat L-14 was produced and purified as described by Cooper et al. (1991). Radioiodination was performed as described by Leffler and Baronides (1986); for each reaction approximately 200 µg of L-14 was labeled using 250 µCi of Bolton-Hunter reagent (New England Nuclear). Unreacted and hydrolyzed Bolton-Hunter reagent was removed using a 5 ml 'Presto' de-salting column (Pierce Chemical) pre-equilibrated in 1 mg/ml radioimmunoassay-grade BSA (RIA-BSA, Sigma) in buffer A (see below). To eliminate lectin inactivated by radioiodination, labeled protein was repurified by lactose affinity chromatography with 1 ml (packed volume) of lactosyl-agarose beads (Pierce Chemical) with rocking, overnight, at 4°C. Beads were washed 5 times with 10 volumes of buffer A. ¹²⁵I-L-14 was eluted with 100 mM lactose in buffer A, and concentrated and dialyzed out of lactose by ultrafiltration in a Centricon-10 (Amicon). Buffer A consists of 75 mM KH₂PO₄/Na₂HPO₄ (mixed to pH 7.2), 75 mM NaCl, 4 mM β-mercaptoethanol, and 2 mM EDTA (Leffler and Baronides, 1986).

Quantitative assay of L-14 binding to proteins

Proteins assayed for binding with ¹²⁵I-L-14 were BSA (Sigma), bovine serum fibronectin (Sigma), EHS laminin (gift of A. Mercurio, Harvard Medical School), human merosin (initial studies were performed using material that was a gift of E. Engvall, La Jolla Cancer Research Foundation; subsequent studies used material from Chemicon), and olfactory homogenate. All proteins were applied to wells of a 96-well assay plate (Falcon) at 1 µg/well in a volume of 100 µl of PBS, except olfactory homogenate which was applied at 10 µg/well. After incubation at 37°C for 2 hours, wells were aspirated and blocked with 200 µl of 1 mg/ml RIA-BSA at 37°C for 1 hour. Wells were again aspirated, and incubated overnight at 4°C with 100 µl containing 10⁶ cts per minute of ¹²⁵I-L-14 in buffer A and 1 mg/ml RIA-BSA, with or without 100 mM lactose. The wells were aspirated, washed 3 times with 200 µl buffer A, dried, cut apart, and counted individually in a γ-radiation counter.

Antibody and L-14 binding to blotted proteins

EHS sarcoma laminin, human placental merosin, and olfactory homogenate were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970), transferred to nitrocellulose paper in a wet electroblotter (Bio-Rad Scientific Plastics) in the buffer of Haid and Suissa (1983), and blocked in 1% BSA in phosphate-buffered saline (PBS). Nitrocellulose was then incubated for 1 hour in rabbit anti-EHS laminin (Sigma) diluted 1:1000 in 0.05% Tween-20 in PBS (Tween/PBS), or two different rabbit anti-merosin antisera (see below) diluted 1:500 in Tween/PBS. After 3 washes in Tween/PBS, nitrocellulose was incubated for 1 hour in HRP-coupled, goat anti-rabbit immunoglobulin G (Boehringer Mannheim) diluted 1:200 in Tween/PBS, washed 3 times, and developed with 4-chloro-naphthol. Anti-merosin antisera were a gift of E. Engvall and were made against whole human placental merosin (thus recognizing M- and B-chains), or against a synthetic peptide corresponding to a unique sequence in the second repeat of the G-like domain of the M-chain (Ehrig et al., 1990).

Alternatively, the nitrocellulose was incubated overnight at 4°C in 20 µg/ml of L-14 in Tween/PBS. In control studies, 100 mM lactose was included in the overnight incubation with L-14. After washing, the nitrocellulose was incubated with affinity purified anti-L-14

(1:200) for 8 hours at 4°C, followed by peroxidase conjugated goat anti-rabbit IgG as above and developed with 4-chloro-naphthol.

Detection of 1B2-, and L-14-binding glycolipids

Neutral glycolipids were extracted from the olfactory tissue of postnatal day 7 Sprague-Dawley rats as previously described (Suchy et al., 1988; Schwarting and Crandall, 1991). Lacto-*N*-neotetraosylceramide (LNnT) was prepared from human erythrocytes in the same manner. To assay 1B2 binding, the glycolipid preparations were separated by thin layer chromatography (TLC) and prepared as described by Schwarting and Crandall (1991). For binding studies with ¹²⁵I-L-14 lectin, approximately 2 ml of 2.5×10⁵ cts per minute/ml labeled lectin (1 μg) in 1% RIA-BSA was incubated with the TLC plates at 4°C overnight. The plates were rinsed with PBS, dried, and exposed to X-Omat-AR film (Kodak) for 12-48 hours at -70°C. Control binding studies were performed under the same conditions except that 100 mM lactose was added to the binding buffer.

Cell culture

Primary cultures of fully dissociated olfactory neurons were prepared from postnatal day 1-3 rats as previously described (brain-derived neurotrophic factor was a gift of Amgen) (Mahanthappa and Schwarting, 1993). Substrates were prepared by cleaning 22 mm² glass coverslips (Corning) as previously described, and coated with 100 μl of 25 μg/ml fibronectin, merosin, or laminin. Coverslips were incubated overnight at 37°C, blocked with 100 μl of 1 mg/ml RIA-grade BSA (Sigma) in Hank's buffered salt solution (HBSS; Gibco) for 1 hour at 37°C, and rinsed three times with HBSS prior to cell plating. It is important that batches of laminin be tested for their ability to support cell attachment; we previously reported that rat olfactory neurons do not adhere to laminin, but lack of attachment appears to have been due to batch inactivity. Cells were plated in a volume of 100 μl/coverslip, and maintained in a humidified chamber within a 37°C, 5% CO₂ incubator. Plating efficiencies were approximately 80%; cell density varied with the amount of starting tissue.

Coated and blocked coverslips were preincubated in the indicated quantities of L-14 in a volume of 100 μl for 2-4 hours at 4°C, and the unbound material rinsed off prior to final plating. By including ¹²⁵I-L-14 in the preincubation mixture, the amounts of L-14 bound to the substrate after preincubation, and at the end of the culture period could be assessed (as in Table 1). Other additions made to the culture medium at the indicated concentrations were thiodigalactoside (Sigma), Arg-Gly-Asp-Ser peptide (RGDS; Sigma), and a rat antiserum directed against the β1 integrin subunit (Albelda et al., 1989) (gift of Dr C. Buck, Wistar Institute).

Immunostaining and cell counting

We have previously demonstrated that the anti-neural cell adhesion molecule mAb, AG1 (DiFiglia et al., 1989), specifically stains olfactory neurons in culture (Mahanthappa and Schwarting, 1993). Neuron cultures were fixed 14-18 hours after plating, and stained as previously described with the following modifications: 1:100 horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim) was used as the secondary antibody, and the positive cells developed using 0.05% 3,3'-diaminobenzidine with 0.2% nickel ammonium sulfate in Tris-buffered saline.

All cell counting and photography was performed using bright-field microscopy at a total magnification of 100×. Randomly selected fields were scored for number of stained cells, and number of stained aggregates containing greater than 4 cells. Values presented for the number of adherent cells were normalized to the number adhering to laminin under control conditions on the given date. In the dose-response studies, each data point indicates the average value (± s.e.m.) derived from 18 fields (3 fields each from 6 separate cultures established on 2 different dates). Values presented in the studies comparing substrate effects were derived from 12 fields/condition (3 fields each from 4 cultures established on 2 dates), and those presented on the effects of

integrin-perturbing reagents were derived from 6 fields/condition (3 fields each from 2 cultures established on the same day). Absolute numbers of adherent AG1⁺ cells ranged from 50-100 cells/field.

RESULTS

L-14 is synthesized by non-neuronal cells of the olfactory nerve, and co-localizes with laminin-immunoreactivity

In situ hybridization of an antisense RNA probe, corresponding to the entire coding region of rat L-14, to sections of rat olfactory tissue reveals that L-14 message is abundant throughout the olfactory nerve and lamina propria of the olfactory epithelium (Fig. 1A-B). L-14 message is completely absent from the neuroepithelium and thus olfactory neurons are not responsible for L-14 gene expression (Fig. 1E-F). It is thus likely that either glial cells or other mesenchymal cell types within the olfactory nerve are expressing the L-14 gene. Signal specificity is demonstrated by the lack of specific hybridization with a corresponding sense probe (Fig. 1C-D). Furthermore, hybridization patterns in rat DRG and spinal cord using the above anti-sense probe replicate previously observed L-14 hybridization patterns (Hynes et al., 1990; Poirier et al., 1992; Fig. 1G).

Since L-14 has been previously determined to bind laminin, and to co-localize with it in muscle basement membranes (Zhou and Cummings, 1990; Cooper et al., 1991), the expression of laminin in olfactory extracellular matrix was examined. As detected with a polyclonal antiserum to the A-B1-B2 form, laminin is observed in the nerve layer of the olfactory bulb from E18 through adulthood (data not shown). Comparison of the localization of L-14 and laminin by immunofluorescence in neonatal rats (Fig. 2) demonstrates the overlapping expression of L-14 and laminin epitopes in the nerve layer of the olfactory bulb and in the lamina propria adjacent to the olfactory epithelium. L-14-immunoreactivity on the luminal surface of the olfactory epithelium is inconsistent and may be due to nonspecific interactions with mucosal oligosaccharides. Olfactory homogenate separated by reducing SDS-PAGE, and immunoblotted with an antiserum directed against recombinant L-14, reveals a single band of *M_r* 14.5×10³ that co-migrates with recombinant L-14 (data not shown). Thus between E18 and P2, when the pattern of olfactory system connections is still developing, the L-14 gene is transcribed and translated by non-neuronal cells of the primary olfactory pathway, and the protein deposited in proximity to laminin immunoreactivity.

L-14 binds laminin family members and to merosin-immunoreactive peptides derived from olfactory tissue

Co-localization of L-14 and laminin-immunoreactivity in the olfactory nerve raises the possibility that L-14 might functionally interact with olfactory forms of laminin. Thus the binding of ¹²⁵I-L-14 to various purified proteins and olfactory homogenate was measured in 96-well plates. Binding was measured in the presence and absence of 100 mM lactose in order to distinguish sugar-dependent binding, and sugar-independent binding (i.e. non-specific binding). ¹²⁵I-L-14 displays significant binding to laminin purified from EHS sarcoma

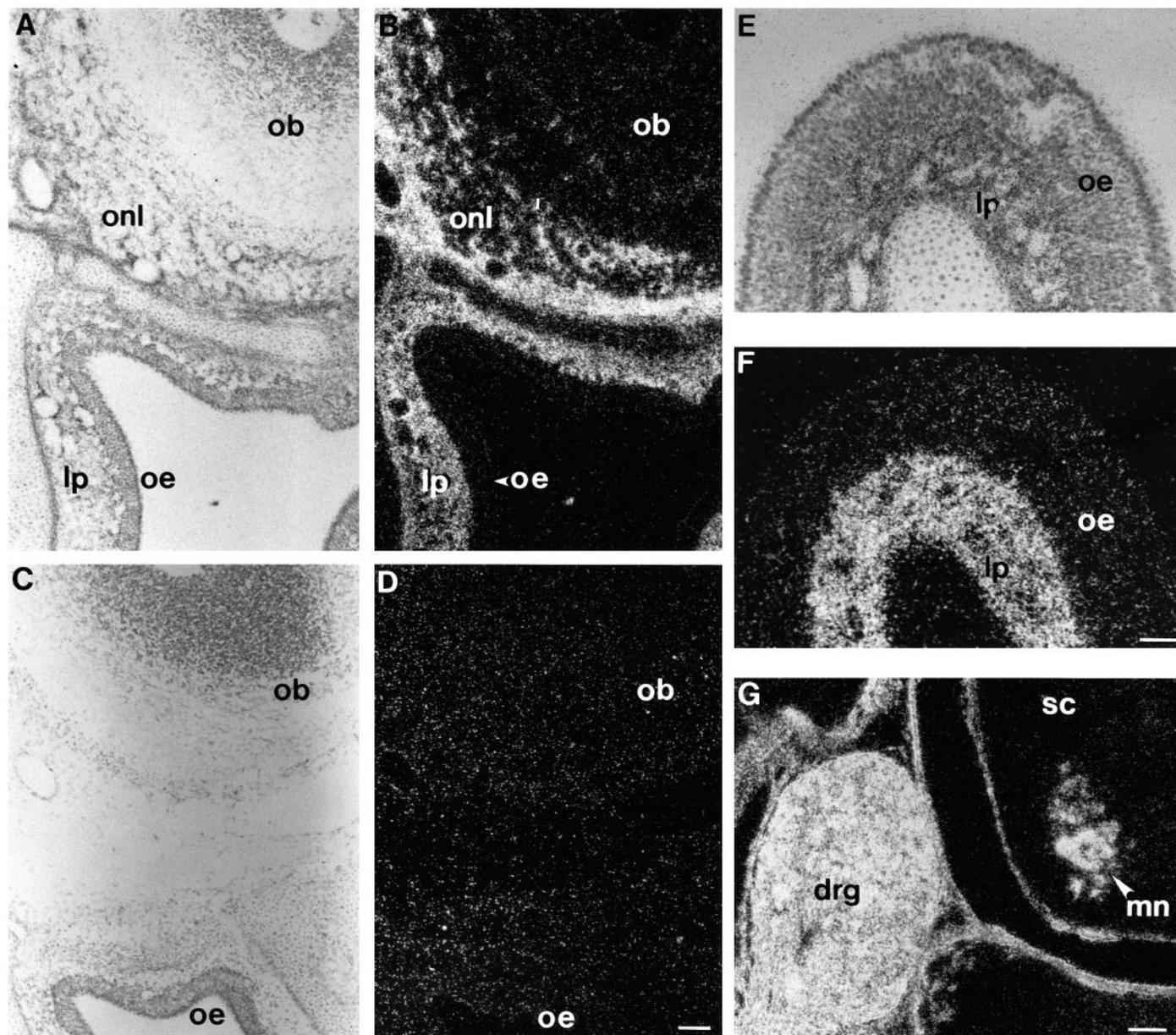


Fig. 1. In situ hybridization of L-14 in the rat. Coronal sections (8 μm) cut through the rat nasal cavity and olfactory bulb were hybridized with ^{35}S -labeled RNA probes transcribed from a cDNA containing the entire coding region of L-14 (see text for details). A and C are counterstained sections of E19 olfactory epithelium (oe) and olfactory bulb (ob) viewed in bright-field illumination. Dark-field illumination (B) of the section shown in A displays specific hybridization of anti-sense L-14 probe over the lamina propria (lp) adjacent to the oe, and the olfactory nerve layer (onl) surrounding the ob. Sense L-14 probe (D) shows no specific hybridization to the section shown in C. Higher magnification of a section from the P2 rat hybridized with anti-sense L-14 probe further demonstrates the restriction of L-14 expression to the lp, and its complete lack of expression within the oe proper (E,F). Hybridization of antisense L-14 probe to the dorsal root ganglia (drg) and motoneurons (mn) of the spinal cord (sc) replicates previously reported L-14 RNA localization and supports the specificity of binding observed in the olfactory system (G). Scale bar, 100 μm (A-D); 50 μm (E-G).

(composed of A-, B1-, and B2-chains), and merosin purified from human placenta (composed of M-, and B-chains) (Fig. 3). The level of ^{125}I -L-14 binding to all proteins tested is reduced to background in the presence of lactose; fibronectin and BSA show no specific binding. The presence of an L-14 ligand in total olfactory homogenate is demonstrated by a significant level of lactose-inhibitable binding (Fig. 3). Thus two members of the laminin family bind significant quantities of L-14, and olfactory protein homogenate contains an L-14 ligand.

To examine further the expression of laminin family members in the olfactory system, laminin, merosin, and guanidine-HCl solubilized olfactory proteins were subjected to reducing, 4-20% gradient SDS-PAGE, and transferred to nitrocellulose. Parallel strips of nitrocellulose were exposed to antisera directed against EHS laminin, human merosin, or incubated with L-14 followed by anti-L-14. In EHS laminin, anti-laminin recognizes bands of M_r appropriate for the A-chain (approximately 400×10^3) and the B-chains (approxi-

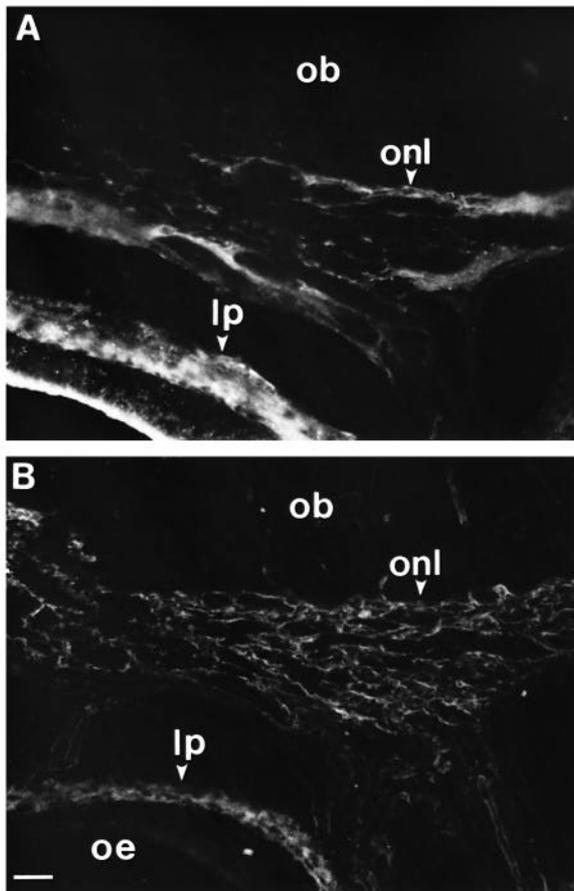


Fig. 2. L-14 and laminin are both concentrated in the lamina propria and olfactory nerve layer of the P2 olfactory system. Immunocytochemistry of a coronal section through the rat nasal cavity and olfactory bulb shows expression of L-14 (A) in the lamina propria (lp) and olfactory nerve layer (onl). Little or no reactivity is seen on cells within the olfactory epithelium (oe) itself or in neuronal regions of the olfactory bulb (ob). Laminin expression (B) is also present throughout the lp and onl, but is absent from the oe and ob. Scale bar, 50 μ m.

mately 200×10^3) (Fig. 4, left). B-chains could not be separated from each other on such gradient gels. In merosin, a heterotrimeric complex of M- and B-chains (Ehrig et al., 1990), anti-laminin stains solely B-chains; the bands above $400 \times 10^3 M_r$ are likely to be unreduced or aggregated merosin. The olfactory homogenate contains peptides that react with anti-laminin and co-migrate with the B-chains of merosin. The olfactory homogenate does not contain intact A-chain.

The anti-merosin blots were probed with an antiserum directed against a synthetic peptide corresponding to a unique region within the M-chain. As described by Ehrig et al. (1990), this antiserum does not recognize any peptides in EHS laminin. It does, however, bind very strongly to a $300 \times 10^3 M_r$ M-chain in placental merosin and to several apparent breakdown products between 180 and $240 \times 10^3 M_r$ (Fig. 4, center). Again, the presumed merosin aggregate at the top of the blot is also stained. In olfactory homogenate, this antiserum recognizes multiple bands: the most intensely stained band appears at approximately at the position of $240 \times 10^3 M_r$ adjacent to one of the major merosin peptides, while fainter bands appear between

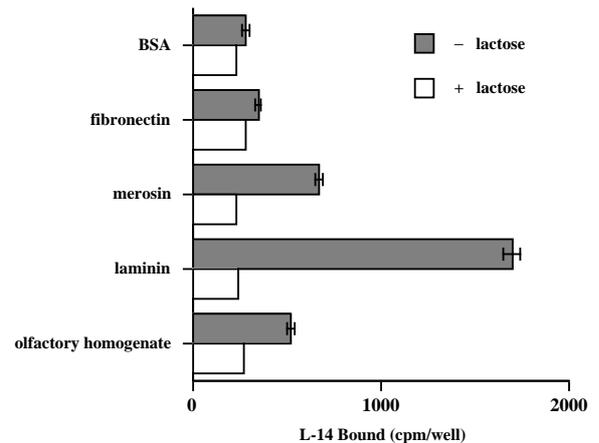


Fig. 3. L-14 binds to laminin, merosin, and olfactory protein in a lactose-inhibitable manner. BSA, bovine serum albumin; fibronectin, EHS sarcoma laminin, human placental merosin, and rat olfactory homogenate were applied to a microassay plate (1 μ g protein/well, except olfactory homogenate at 10 μ g/well) and incubated with 125 I-L-14 +/- 100 mM lactose. Identical levels of L-14 bind to all proteins tested in the presence of lactose.

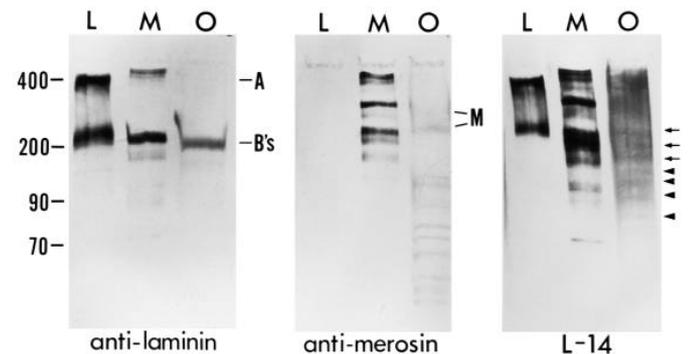


Fig. 4. Olfactory homogenates contain laminin-related glycoproteins. L, EHS sarcoma laminin; M, human placental merosin; and O, rat olfactory homogenate, were subjected to 4-20% SDS-PAGE, transferred to nitrocellulose, and probed. Anti-laminin antiserum (left) stains A, A-chain of laminin; B's, B-chains of laminin; the B-chains of merosin; and olfactory peptides that co-migrate with the B-chain of merosin. In addition, the human merosin preparation contains antibody-reactive material migrating above the A-chain that is probably unreduced or aggregated merosin. Anti-merosin antiserum (center) stains M, intact M-chain of merosin, having a M_r of 300×10^3 and 3 bands between M_r of 180 and 240×10^3 . The merosin aggregate is also labeled. Anti-merosin reacts with a ladder of bands in the olfactory homogenate, most prominently a band of approximately $240 \times 10^3 M_r$, which is adjacent to one of the major merosin peptides. L-14 (right) bound to all chains of laminin, all chains of merosin, and 3 bands in the olfactory homogenate (arrows) that migrate near the $200 \times 10^3 M_r$ band, in the region of B-chains and M-chain peptides. In addition, L-14 binds to 4 smaller olfactory peptides (arrowheads) of between M_r 80 and 150×10^3 .

40 and $150 \times 10^3 M_r$ (Fig. 4, center). Blots using different anti-merosin antisera generate similar patterns with prominent bands of identical mobility (data not shown). Thus these bands are not likely to be the result of non-specific binding, and probably represent proteolytic products of M-chain.

Nitrocellulose blots probed with L-14 followed by anti-L-14 show lower background than those probed with ^{125}I -L-14. L-14 binds all subunits of purified laminin and merosin (Fig. 4, right). In olfactory homogenate, L-14 binds 3 distinct bands in the area of $200 \times 10^3 M_r$ (arrows) that correspond to L-14-reactive bands in the merosin lane. These L-14 binding peptides are likely to be either B-chains or proteolytic products of the M-chain as both migrate in this vicinity. L-14 binds to four additional bands (arrowheads) of between 80 and $150 \times 10^3 M_r$, but does not bind to the 6 anti-merosin reactive bands below M_r of 80×10^3 . These smaller peptides presumably do not contain carbohydrate epitopes recognized by L-14. In the presence of 100 mM lactose, there is no detectable L-14 binding to any of the proteins tested (data not shown). These data suggest that an L-14-binding laminin isoform is present in olfactory tissue, but due to proteolysis similar to that observed for placental M-chain (Leivo and Engvall, 1988), the olfactory proteins remain to be definitively identified.

L-14 binds an axonal glycolipid that is expressed within the olfactory nerve

In previous studies it was demonstrated that the 1B2 mAb reacts with terminal lactosamine structures (Young et al., 1981), and recognizes a glycolipid expressed by nascent olfactory axons *in vivo* (Schwartz and Crandall, 1991) and *in vitro* (Mahanthappa and Schwartz, 1993). Double-label immunofluorescence studies in the postnatal day 14 olfactory bulb (Fig. 5A,B) reveal nearly absolute co-localization of L-14 and laminin B1-chain in axon tracks of the nerve layer. There is thus a continuous band of laminin and L-14 immunoreactivity encircling the olfactory bulb, and there are irregular branch points where these proteins are deposited in tracks leading toward the glomerular layer. These laminin-containing tracks never extend into the glomeruli, and often extend only through part of the width of the nerve layer. In an adjacent section, which is double-labeled with 1B2 and antibodies to L-14 (Fig. 5C,D), 1B2-immunoreactive axons (arrows) that extend through the nerve layer into 1B2-immunoreactive glomeruli (arrowhead) follow pathways that express L-14 (Fig. 5C) and B1-chain-immunoreactivity (Fig. 5B). Thus L-14, 1B2-glycolipid, and at

least one form of laminin, are co-expressed within the outer nerve layer of the olfactory bulb.

To test directly whether L-14 can bind 1B2-glycolipid in a manner similar to laminin isoforms, neutral glycolipids extracted from olfactory homogenate were separated by TLC, and compared with a standard glycolipid isolated from human

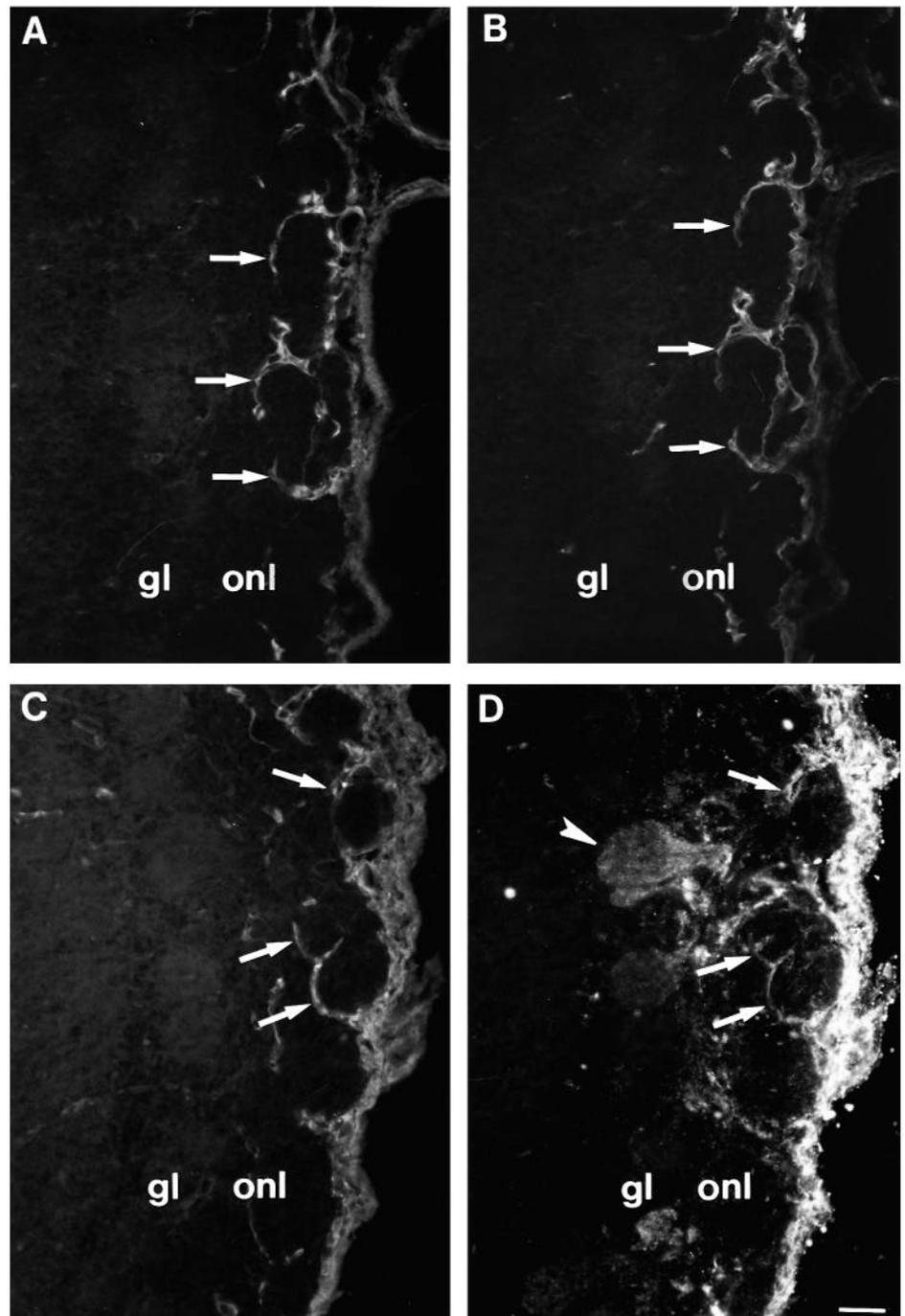


Fig. 5. L-14 immunoreactivity co-localizes with laminin deposition and with 1B2 antigens in the nerve layer of the P14 olfactory bulb. L-14 immunoreactivity (A) and laminin B1-chain immunoreactivity (B) are virtually identical (arrows) in the nerve layer of the olfactory bulb, using double-labeled immunofluorescence techniques. L-14 immunoreactivity (C) and 1B2 immunoreactivity (D) also co-localize (arrows) in tracks leading through the nerve layer to 1B2 immunoreactive glomeruli (arrowhead). gl, glomerular layer; onl, olfactory nerve layer. Scale bar, $50 \mu\text{m}$.

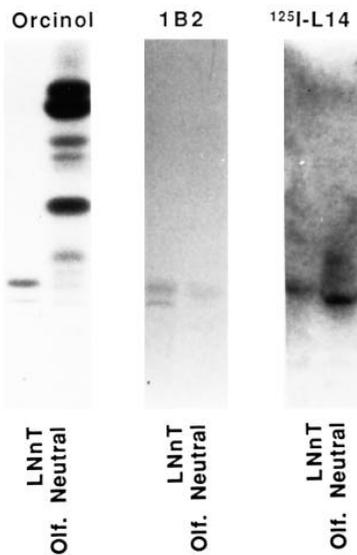


Fig. 6. A neutral glycolipid from olfactory tissue is bound by 1B2 and L-14. Total olfactory neutral glycolipids (Olf. Neutral) and lacto-N-neotetraosyl ceramide (LNnT), a positive control, were separated by thin layer chromatography (TLC) and stained with orcinol for total glycolipid (left). Identical chromatograms were reacted with 1B2 antibodies (center) and with ¹²⁵I-L-14 (right). A minor component of the olfactory glycolipid mixture migrates in the vicinity of standard LNnT on TLC and binds both 1B2 and ¹²⁵I-L-14.

erythrocytes, LNnT. It has previously been demonstrated that 1B2 antibodies react preferentially with LNnT and other glycolipids that terminate in galactose-(β 1 \rightarrow 4)-*N*-acetylglucosamine residues (Young et al., 1981). Orcinol staining of TLC plates for total glycolipids reveals a large number of bands in the olfactory preparation (Fig. 6, left). TLC-immunostaining with 1B2, however, reveals one minor immunoreactive glycolipid (Fig. 6, center). Binding studies using ¹²⁵I-L-14 on TLC plates demonstrate that both LNnT and the 1B2-immunoreactive olfactory glycolipid bind L-14 (Fig. 6, right). The binding of ¹²⁵I-L-14 to both glycolipids was inhibited by 100 mM lactose (data not shown). The fact that the olfactory glycolipid does not co-chromatograph exactly with LNnT from human erythrocytes is probably due to differences in ceramide composition.

L-14 in vitro can mediate olfactory neuron adhesion to laminin family members and neuron aggregation

To test the influence of L-14 on adhesion of olfactory neurons to laminin, laminin-coated coverslips were preincubated with quantities of L-14, ranging from 100 ng to 50 μ g. Prior to neuron plating, unbound L-14 was rinsed off. Cultures were fixed 14–18 hours after plating, and stained with the AG1 mAb to mark neurons. By including ¹²⁵I-L-14 in the preincubation solution, it was possible to determine the amount of L-14 bound to the substrate. Table 1 demonstrates that when ≤ 5 μ g of L-14 is applied to the substrate, approximately 20% of L-14 present is bound; higher concentrations appear to saturate available laminin binding sites. At the end of the culture period, 20–50% of the L-14 initially bound at the time of plating remained bound; thus a dynamic equilibrium exists between substrate-bound and unbound forms.

Table 1. Quantification of L-14 bound to laminin-coated substrates before and after the plating of olfactory neurons

ng L-14 applied to substrate	ng L-14 remaining after preincubations	ng L-14 remaining at end of culture period
100	23 \pm 0	5 \pm 1
500	93 \pm 1	46 \pm 2
1000	185 \pm 7	46 \pm 5
2000	312 \pm 8	164 \pm 25
5000	733 \pm 55	128 \pm 13
10000	813 \pm 12	330 \pm 14
50000	1867 \pm 214	700 \pm 36

Glass coverslips were prepared for tissue culture and laminin coated as described in the text. Coverslips were then preincubated in L-14 (to which had been added various amounts of ¹²⁵I-L-14) at 4°C for 4 hours. After rinsing, some were immediately dried, and the amount of L-14 bound calculated by measuring the amount of ¹²⁵I-L-14 bound in a γ -radiation counter ('after preincubations'). Others were placed in a 37°C, 5% CO₂ incubator for 18 hours with 100 μ l each of culture medium. These coverslips were then rinsed, dried and counted ('at end of culture period'). Each data point is followed by the s.e.m. ($n=3$).

At the time of staining, olfactory neurons are observed primarily as single cells, some of which have initiated axon outgrowth. Random fields in each culture condition were scored for number of adherent cells, and number of aggregates containing greater than four AG1⁺ cell bodies. Due to variability in plating density from date to date, adherent cell numbers were normalized in relation to the average number adhering in the control condition on the given date (control = 100). Aggregation is expressed as the average number of aggregates/100 adherent AG1⁺ cells. Neurite outgrowth was not observably affected by L-14 and was not scored after the initial experiments (data not shown).

As can be seen in Fig. 7A, the number of adherent cells increases as the amount of substrate-bound L-14 increases. This dose-dependent increase reaches a maximal value of 50% over control levels when the laminin substrate is preincubated in ≥ 5 μ g of L-14. As the amount of L-14 present on the substrate increases, the number of aggregates also increases approximately 8-fold when the substrate is preincubated with ≥ 10 μ g of L-14 (Fig. 7B). Effects are maximal in the range of L-14 concentrations known to be saturating available laminin binding sites (Table 1). Thus the effects of L-14 on substrate adhesion and aggregation are dose-dependent.

To test the substrate specificity of the observed L-14 effects, olfactory neurons were cultured on laminin, merosin, and fibronectin. Neurons were plated on either pure substrate or substrate that had been preincubated with 10 μ g of L-14 and rinsed. As can be seen in Fig. 8A, the control levels of neuron adhesion to all three substrates are similar. After substrate preincubation with L-14, however, levels of cell adhesion on merosin increase by approximately 50%, and on laminin increase by approximately 70%. Pre-incubation with L-14 had little effect on cell adhesion to fibronectin. The presence of 10 mM thiodigalactoside, a non-metabolizable competitive inhibitor of L-14 binding (Leffler and Barondes, 1986; Cooper et al., 1991), completely inhibits the ability of the lectin to promote substrate adhesion, but does not affect baseline levels of adhesion.

Interestingly, as observed in the dose-response study on laminin, increased adhesion to merosin is also accompanied by increased aggregation (Fig. 8B). Control levels of aggregation are higher on merosin than on either fibronectin or laminin. Nevertheless, the number of aggregates on merosin doubles in the presence of L-14, and increases 4-fold on laminin; aggregation induced by L-14 is inhibited by thiodigalactoside. Little aggregation activity is observed on fibronectin unless L-14 is included in the medium with the neurons (data not shown). Thus the ability of L-14 to mediate cell-substrate adhesion appears to be restricted to laminin-family members.

It has been reported that at least one glycolipid forms functional complexes with integrin-type receptors (Cheresh et al., 1987). Since the ligand of L-14 on olfactory neurons is a glycolipid, the possibility that L-14 acts as a modulator of integrin-mediated adhesion was tested. Substrates for this experiment were plain laminin and laminin preincubated with 10 μ g of L-14. Olfactory neurons were plated in control medium, 250 μ g/ml RGDS, or a 1:100 dilution of rat anti-integrin β 1 subunit. All perturbations were performed with and without 10 mM thiodigalactoside. In the absence of substrate-bound L-14, RGDS reduces the number of adherent cells by 20-30%, and the anti-integrin antiserum reduces adhesion by 30-40% (Fig. 9A). When plated on laminin preincubated with L-14, the number of adherent cells increases by 40-50 cells in all conditions, an effect inhibited by thiodigalactoside. Thus, despite varying degrees of inhibition of integrin-mediated adhesion by two completely different reagents, L-14-induced increases hold roughly constant and appear independent of integrin activity. Neither RGDS nor anti-integrin antiserum affect neuron aggregation in a consistent manner (Fig. 9B), thus L-14-mediated aggregation also occurs independent of integrin activity.

DISCUSSION

Carbohydrates and endogenous lectins in the nervous system

Though a variety of carbohydrates have been characterized in the nervous system of several animal species, few have been directly implicated in functional interactions. The studies of polysialic acid by Landmesser, Rutishauser, and their colleagues are notable for their direct demonstration of the role of this carbohydrate as a negative regulator of interactions requiring intercellular contact (Landmesser et al., 1990; Rutishauser and Landmesser, 1991). Zipser and Cole (1991) have demonstrated that a mannose-containing epitope on the leech axonal membrane protein, Lan3-2, is a required element in defasciculation that precedes arborization of Lan3-2⁺ axons within the synaptic neuropil.

Their model predicts the presence of a mannose-binding lectin activity in the leech nervous system that is yet to be identified. In the rat cerebellum, Zanetta and his colleagues have characterized two distinct mannose-binding lectins that may play roles in neuronal migration and myelination, but the ligands of these lectins have not been rigorously identified (Zanetta et al., 1992). There is also evidence that L-14 is expressed in a subset of DRG neurons that terminate in laminae I and II of the dorsal horn of the spinal cord. Undefined complementary lactoseries glycoconjugates recognized by 1B2 are specifically expressed within these laminae and the DRG axons innervating them. Thus L-14 has been suggested to play a role in axon guidance (Dodd and Jessell, 1985; Regan et al., 1986; Hynes et al., 1990).

Carbohydrate-dependent interactions involving laminin

Laminin itself has a lectin-like ability to bind sulfated carbohydrates such as sulfoglucuronyl glycolipids (Mohan et al., 1990), sulfate, and heparin (Taraboletti et al., 1990). In cerebellar explant cultures, each of these three sulfated glycoconjugates inhibit cell migration and neurite outgrowth, possibly via interactions with laminin (Kunemund et al., 1988).

Laminin is also likely to play important roles in nervous system development as a glycoconjugate ligand for other carbohydrate-binding proteins. In studies that utilized an unglycosylated form of EHS laminin, both a melanoma cell line and the PC12 cell line displayed normal substrate binding, but cell spreading and neurite outgrowth were impaired by the lack of laminin oligosaccharides (Dean et al., 1990). Cell surface galactosyltransferase has been implicated as an endogenous receptor mediating carbohydrate-dependent interactions with laminin. Galactosyltransferase has been demonstrated to

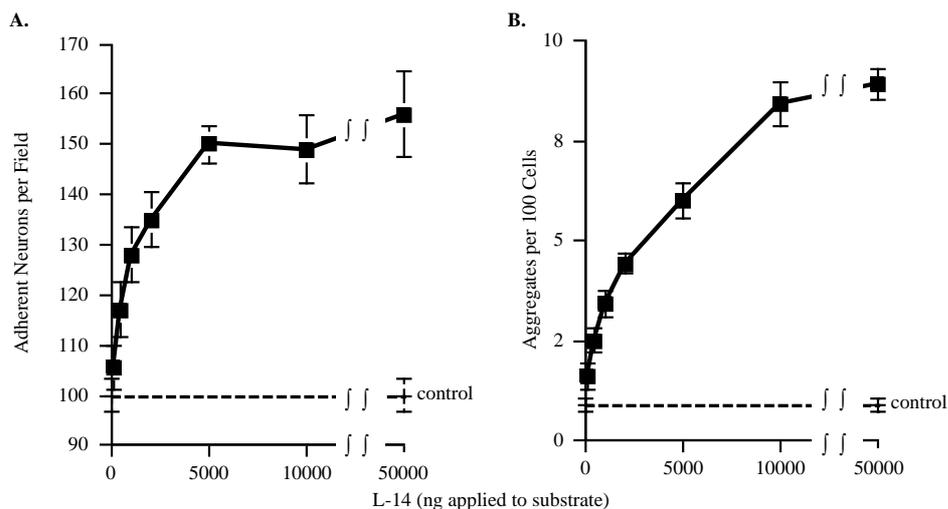


Fig. 7. The effects on cultured olfactory neurons of preincubating laminin substrates with L-14. Substrates were prepared by preincubating laminin-coated coverslips in the indicated quantities of L-14 for approximately 4 hours and then rinsing away unbound material. Fully dissociated olfactory neurons were cultured on the substrates 14-18 hours, stained for neural cell adhesion molecule, and the labeled cells scored at a total magnification of 100 \times . For each data point (\pm s.e.m.), $n=18$. The number of adherent cells in A was normalized so that control=100 (see text for details). Aggregates in B contained > 4 AG1⁺ cells.

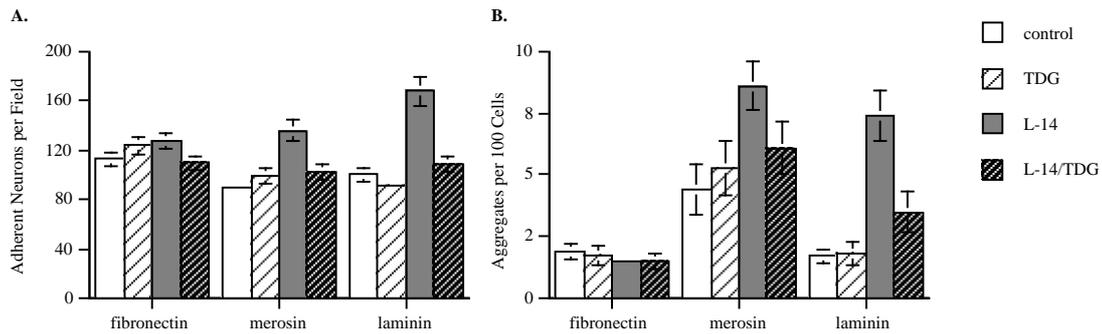


Fig. 8. Adhesion (A) and aggregation (B) effects on laminin, merosin, and fibronectin. Three different substrates were treated with and without 10 μg of L-14 as described in Fig. 7, and olfactory neurons plated in the presence or absence of 10 mM thiodigalactoside (TDG). 'Control', L-14⁻ and TDG⁻; 'TDG', L-14⁻ and TDG⁺; 'L-14', L-14⁺ and TDG⁻; and 'L-14/TDG', L-14⁺ and TDG⁺. In A, 100 = control level of adhesion on laminin. Error bars represent the s.e.m. ($n=12$).

mediate neural crest cell migration on laminin (Runyan et al., 1986; Hathaway and Shur, 1992), and to mediate neurite outgrowth from PC12 cells and DRG neurons cultured on laminin (Begovac and Shur, 1990; Thomas et al., 1990). Thus a variety of cell-matrix interactions depend on recognition of laminin-associated carbohydrates by specific binding proteins.

L-14 as a mediator of laminin interactions

L-14 has been suggested to interact with cell surfaces and extracellular matrix to modulate a variety of cell physiological events in vitro, including adhesion and proliferation (Wells and Mallucci, 1991). However, its abundance and localization in extracellular matrix of both developing muscle (Cooper and Barondes, 1990; Cooper et al., 1991) and the olfactory system suggests that the primary role of L-14 in vivo is to modulate interactions with the extracellular matrix. This hypothesis is supported by the observation that L-14 demonstrates high affinity binding to laminin, a major component of many basement membranes (Zhou and Cummings, 1990; Cooper et al., 1991).

In a direct test of the ability of L-14 to modulate interactions with laminin, Cooper et al. (1991) cultured the mouse myoblast line, C2C12, in the presence of L-14. It was found that L-14 in the culture medium inhibited myoblast adhesion to laminin, but not to fibronectin. This inhibitory effect was blocked by the presence of thiodigalactoside or by endo- β -galactosidase digestion of the laminin-linked poly-lactosamine. Laminin was the only L-14 ligand identified in this culture paradigm, and it was suggested that L-14 bound to the substrate prevented myoblast adhesion by sterically hindering interactions between laminin and cell surface laminin receptors (Cooper et al., 1991). More recently, Zhou and Cummings (1993) have shown that two cell lines, demonstrated to express cell surface poly-lactosamine, displayed enhanced adhesion to laminin

in the presence of L-14. This observation in conjunction with that of Cooper et al. (1991) suggests that the role of L-14 in cell adhesion to laminin is contingent on the relative expression of particular laminin receptors and L-14 ligands on the given cell surface.

L-14 in the rat olfactory system

By in situ hybridization it is clear that L-14 in the olfactory system is synthesized by the non-neuronal cells of the olfactory axon pathway. Furthermore, two L-14 ligands are also present in the olfactory axon pathway. One is a lactosamine-containing glycolipid that is expressed on a subset of olfactory neurons that are likely to be recently postmitotic and are in the process of extending axons into the olfactory bulb (Schwartz and Crandall, 1991; Schwartz et al., 1992b). The other ligand(s) appear to be members of the laminin family. Though prototypical laminin contains A- and B-chains (Timpl et al., 1979), A-chains could not be identified in olfactory homogenates. This observation is similar to the finding that forms of laminin lacking the A-chain are produced in culture by a Schwannoma

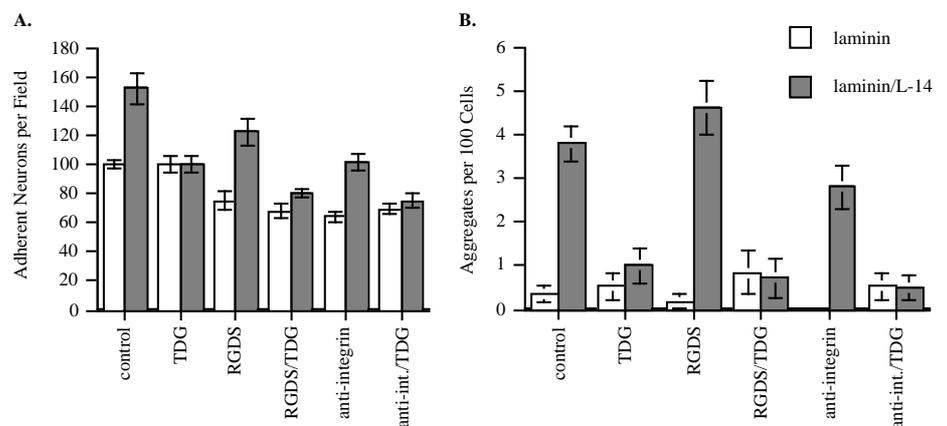


Fig. 9. The effects of integrin-perturbing reagents on L-14 mediated adhesion. Neurons were plated in 10 mM thiodigalactoside (TDG), 250 $\mu\text{g}/\text{ml}$ RGDS (RGDS), and/or 1:100 rat anti-integrin $\beta 1$ subunit (anti-integrin) as indicated. Substrates of untreated laminin (open bars), or laminin that had been preincubated in 10 μg of L-14 and rinsed prior to neuron plating (stippled bars) were used. Adherent cells (A) and aggregates (B) were scored as described in Fig. 7. Error bars represent the s.e.m. ($n=6$).

line (Davis et al., 1985; Edgar et al., 1988), and primary astrocytes (Liesi and Risteli, 1989). However, antisera directed against a homologue of the A-chain, known as the M-chain (Ehrig et al., 1990), stained a series of peptides in olfactory homogenate that may represent breakdown products of M-chain or a related protein (Fig. 4B). Whether olfactory laminin contains M-chain, or other A-chain-like variants (Marinkovich et al., 1992) is currently under investigation.

Both the glycolipid and glycoprotein ligands co-localize with L-14 in the axonal tract leading from the olfactory epithelium to the glomeruli of the olfactory bulb. It is thus hypothesized that L-14, as a divalent lectin, could crosslink 1B2-immunoreactive axons to each other to promote fasciculation, and crosslink axonal surfaces to molecules of the laminin-family as these axons elongate over extracellular matrix towards targets in olfactory bulb glomeruli. An attractive aspect of this model is the fact that 1B2-immunoreactive glycolipid is not expressed on the majority of immature olfactory neurons, nor is it present on the majority of mature olfactory axons or terminals. In postnatal rats, the 1B2-epitope is restricted to axons that have recently entered the olfactory bulb along the superficial surface of the nerve layer, where they send branches through the central nerve layer and terminate in dispersed glomeruli. The vast majority (80-90%) of axons and glomeruli in adult olfactory bulbs do not express the 1B2-epitope and we presume that the epitope is lost with maturation (Schwartz and Crandall, 1991; Schwartz et al., 1992b). Thus, in the proposed model, L-14 would only facilitate interactions involving axons that are still growing and/or actively forming synapses in the olfactory bulb.

In order to test such a mechanism, the behavior of primary cultured olfactory neurons in response to recombinant L-14 was studied. In the first series of experiments a laminin substrate was preincubated in L-14, the unbound lectin rinsed away, and the neurons plated on the L-14/laminin complex. In such a paradigm, neuron-substrate binding increased in proportion to the concentration of L-14 bound to the laminin substrate. Repeating this experiment on three different protein substrates, it was found that L-14 promotes adhesion to laminin, and merosin to a lesser degree, but not to fibronectin. This result agrees with the previous observation that L-14 binds laminin and merosin, but not fibronectin. That the effect was dependent on carbohydrate binding was demonstrated by its abrogation in the presence of 10 mM thiodigalactoside.

Increased cell attachment to the substrate was accompanied by increased intercellular adhesion. This aggregation did not merely increase in proportion to the number of adherent cells, but was apparent when expressed as a ratio of the number of aggregates to the number of adhering cells. There are two general classes of mechanisms by which the lectin might promote intercellular adhesion. One possibility is that L-14-mediated adhesion to the substrate activates an independent mechanism responsible for aggregation. The second possibility is that laminin-family members act as 'reservoirs' of L-14. In such a model, L-14 can mediate substrate adhesion, but by dissociating from the substrate, can also become available to mediate crosslinking of adjacent cells. The second mechanism is made likely by two observations: (i) during the assay, amounts of L-14 that dissociate from the laminin substrate (50-80% of that originally bound) are sufficient to mediate cell aggregation (Table 1); and (ii) if preincubated L-14 is not rinsed away, aggregation is observed regardless of substrate type (data not shown).

Using two distinct perturbations of integrin receptors, the RGDS synthetic peptide (Ruoslahti and Pierschbacher, 1987; Reichardt and Tomaselli, 1991) and an anti-serum directed against the integrin $\beta 1$ subunit (Albelda et al., 1989), olfactory neuron adhesion to laminin was partially blocked. Pretreatment of the substrate with L-14 gives rise to a consistent increase in

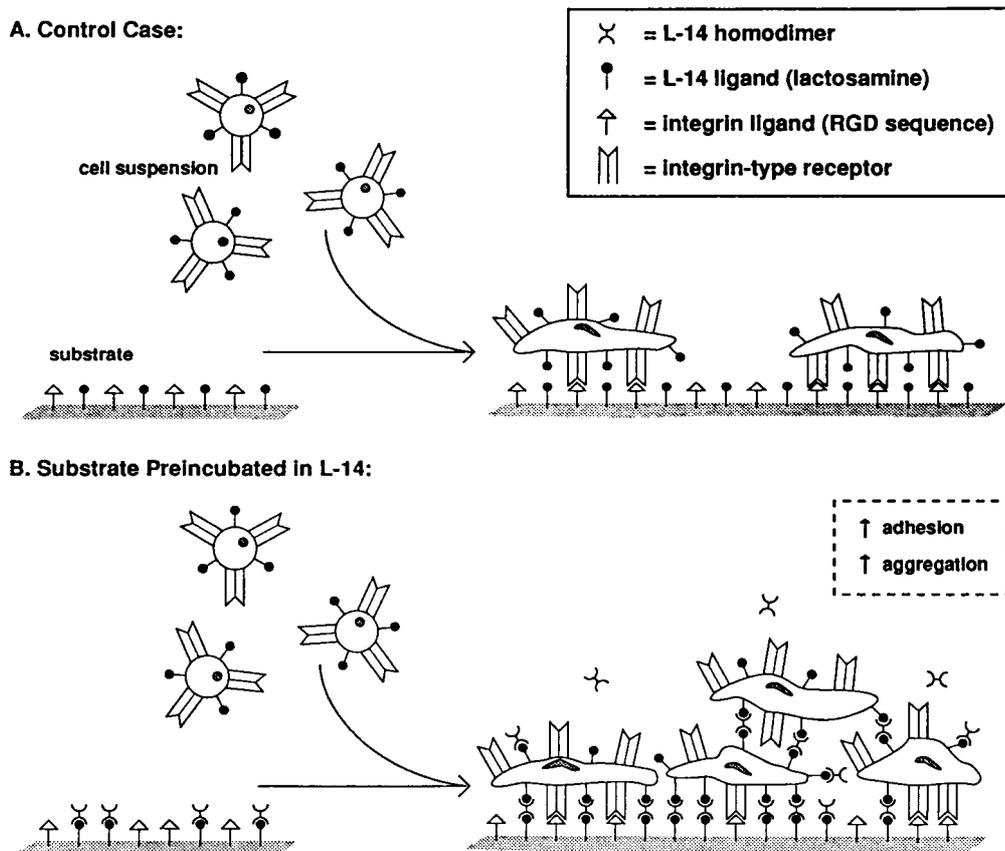


Fig. 10. Model of L-14 functions in vitro. In the control case (A), olfactory neurons adhere to the culture substrate through carbohydrate-independent mechanisms (e.g. integrin/RGDS). When the substrate has been preincubated in L-14 (B), both classical and L-14-mediated adhesion take place and adhesion is increased. Furthermore, L-14 that dissociates from the substrate becomes available to crosslink lactosamines on adjacent cells and thus increases aggregation as well.

cell adhesion however, and thus makes clear that L-14-mediated adhesion is a distinct mechanism from integrin-mediated adhesion. That L-14-mediated adhesion works in an additive fashion with integrin-type interactions is shown schematically in Fig. 10.

Conclusions

Adhesive interaction among neurons are mediated by a great variety of molecules (Jessell, 1988). Intercellular adhesive interactions mediated by members of the immunoglobulin gene superfamily (Salzer and Colman, 1989) and the cadherin family (Takeichi, 1988, 1990) are among the best characterized. Studies of neuronal interactions with the extracellular matrix have focused primarily on the integrin family of matrix receptors (Reichardt and Tomaselli, 1991). Despite the discovery of carbohydrate-dependent adhesive interactions in the circulatory system mediated by the selectins (Brandley et al., 1990; Bevilacqua et al., 1991), carbohydrates in the nervous system have been studied primarily as modulators of known neural adhesion systems (Landmesser et al., 1990; Rutishauser and Landmesser, 1991).

In this study we have demonstrated that not only are L-14 and its ligands co-localized in the rat olfactory nerve, but that L-14 can promote both cell-cell and cell-matrix binding by primary rat olfactory neurons cultured on laminin-family members. L-14 thus represents a novel class of adhesion molecule. The adhesion is neither homophilic nor heterophilic in a classic sense, but functions through the crosslinking of related carbohydrate structures on multiple glycoconjugates. These glycoconjugates may be glycolipids or glycoproteins, and may be expressed on the cell surface or the extracellular matrix. Thus the L-14 adhesion mechanism displays a versatility unknown among other adhesion molecules.

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Note added in proof

L-14, the β -galactose-binding lectin described in this manuscript, has been renamed, galectin-1, in accordance with suggestions in a February 25, 1994 letter to *Cell* **76**, 597-598.