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Differential Effects of Glycosaminoglycans on Neurite Growth on Laminin and L1 Substrates

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Glycosaminoglycans (GAGs), the carbohydrate moieties of proteoglycans, are thought to be positive and negative regulators of axonal growth. The physiological role of GAGs is controversial as some studies have shown that GAGs inhibit cell adhesion and neurite elongation (Exp Neurol 109:111, 1990) whereas other studies have reported a growth stimulatory effect of GAGs (Development 114:17, 1992). These and other studies have examined the effects of GAGs using different types of neurons and different substrate conditions thereby making a direct comparison of the experimental data difficult. To resolve the controversy concerning the ability of exogenous GAGs to modulate neurite growth, we examined the effects of a panel of structurally different GAGs on the growth of postnatal rat cerebellar granule neurons and embryonic rat dorsal root ganglia (DRG) neurons on substrates of either laminin or the L1 glycoprotein. Here we show that chondroitin 4-sulfate (CS4), chondroitin 6-sulfate (CS6), and keratan sulfate (KS) inhibit neurite growth from both cerebellar and DRG neurons on laminin-coated surfaces. On L1 surfaces, however, these GAGs are either extremely weak inhibitors of neurite extension or, in the case of CS4, a modest stimulator of neurite growth. Heparan sulfate (HS) and dermatan sulfate (DS) inhibited the growth of cerebellar neurons but not the growth of DRG neurons on L1-coated surfaces. On laminin surfaces, DS and HS had no effect on neurite growth from both cerebellar and DRG neurons. These results demonstrate a cellular and a substrate specificity to the effects of exogenous GAGs on neurite extension *in vitro*. They suggest that while CS and KS GAGs may not exert strong negative influences over axonal growth in regions of the developing CNS where the L1 glycoprotein is abundant, these GAGs are capable of inhibiting the growth of axons that extend within an environment rich in laminin.

[Key words: glycosaminoglycan, proteoglycan, cell adhesion molecule, axonal growth, laminin, L1]

To find their appropriate target cells, the axons of developing neurons navigate through a complex terrain composed of other neurons, glial cells, and extracellular matrix (ECM) components. This complex terrain is likely to contain the information that guides growing axons to their appropriate postsynaptic targets (for review, see Goodman and Shatz, 1993). At least some of this information is encoded by (1) target-derived chemoattractants (Kennedy et al., 1994; Serafini et al., 1994) and repellents (Fitzgerald et al., 1993; Pini, 1993; Colamarino and Tessier-Lavigne, 1995), (2) cell adhesion molecules and ECM components that are permissive for axon growth (Rutishauser and Jessell, 1988; Sanes, 1989), and (3) extracellular and cell surface molecules that inhibit neurite growth (reviewed by Patterson, 1988; Keynes and Cook, 1995). By integrating these diverse environmental signals, growth cones are able to find their appropriate target cells with precision and reproducibility.

A large number of molecules can provide a positive substrate for neurite extension *in vitro*. These neurite growth-promoting molecules include ECM-associated molecules such as laminin (Sanes, 1989) and multiple members of the immunoglobulin super family of cell adhesion molecules or CAMs such as L1(NILE), G4, and TAG1 (Lemmon et al., 1989; Furley et al., 1990; Kuhn et al., 1990). CAMs such as L1 are thought to act primarily through homophilic binding mechanisms (Lemmon et al., 1989), although heterophilic interactions among different CAMs can also occur (Kadmon et al., 1990a,b; Kuhn et al., 1990; Felsenfeld et al., 1994). CAMs and the cellular receptors for ECM components are linked to intracellular second messenger systems whose activation is prerequisite for neurite extension (Bixby, 1989; Schuch et al., 1989; Walsh and Doherty, 1992). While CAMs have a potent ability to promote neurite elongation *in vitro*, these molecules, with the exception of TAG1 (Dodd et al., 1988), are not distributed in a manner that delineates specific axonal pathways *in vivo* (Faissner et al., 1984; Stallcup et al., 1985; Rutishauser and Jessell, 1988). Thus, ECM-associated molecules and CAMs can be considered as generally permissive for axonal extension (Lemmon et al., 1992) and these molecules alone cannot explain fully the specificity with which growing axons find their target cells.

Molecules with the ability to inhibit axonal extension *in vitro* include numerous proteins (Lochter et al., 1991; Kolodkin et al., 1993; Luo et al., 1993), glycoproteins (Caroni and Schwab, 1988; Davies et al., 1990) and proteoglycans (Snow et al., 1990a; Cole and McCabe, 1991; Oohira et al., 1991; Geisert and Bidanset, 1993; Dou and Levine, 1994; Friedlander et al., 1994). As is the case with CAMs, some of these growth inhibitory molecules share structural features and comprise a molecular family known as semaphorins (Kolodkin et al., 1993; Luo et al., 1993). These molecules participate in the guidance of growing axons to their targets by defining areas of the nervous system that are nonpermissive for axonal growth (Patterson, 1988).

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Proteoglycans are multifunctional macromolecules comprising a core polypeptide and covalently attached glycosaminoglycan (GAG) chains (for review, see Jackson et al., 1991; Hardingham and Fosang, 1992). The physiological functions of GAGs in the developing nervous system are controversial. The observations that exogenous GAGs inhibit cell attachment and neurite growth (Carbonetto et al., 1983; Akeson and Warren, 1986; Snow et al., 1990a) and that GAGs are responsible for the neurite growth inhibitory effects of some brain proteoglycans (Cole and McCabe, 1991; Geisert and Bidanset, 1993) suggest that these carbohydrate polymers may be endogenous inhibitors of axonal extension. Other studies, however, have shown that the GAG chains are not required for the inhibitory actions of other proteoglycans of the nervous system (Oohira et al., 1991; Grumet et al., 1993; Dou and Levine, 1994). The physiological relevance of the negative effects of GAGs on neurite extension *in vitro* is further brought into question by the observation that thalamocortical axons extend within the chondroitin sulfate rich subplate of the developing telencephalon (Bicknese et al., 1994).

Variations in the types of neurons and the types of substrates tested in the studies cited above make a direct comparison of the experimental results difficult. Therefore, we studied the effects of a panel of different GAGs on axonal growth from two populations of developing neurons, cerebellar granule cells and dorsal root ganglia (DRG) neurons. The effects of individual GAGs were tested in the presence of either laminin or the L1 glycoprotein. The results presented here demonstrate that the effects of GAGs on neurite extension are complex and vary according to other components of the substrate and according to neuronal type. Our data infer that GAGs, with the exception of HS, may play only a minor role as inhibitors of neurite growth within those areas of the developing CNS where the L1 glycoprotein is abundant (Faissner et al., 1984; Stallcup et al., 1985); however, GAGs may exert strong inhibitory influences over the growth of neurons in an environment where laminin predominates.

Materials and Methods

Materials. Monoclonal antibody 74-5H7 against L1 was generously provided by Dr. V. Lemmon (Lemmon et al., 1989). Poly-L-lysine (PLL), phenyl methyl-sulfonylfluoride (PMSF), *N*-ethyl maleimide (NEM), leupeptin, chondroitin 6-sulfate (chondroitin sulfate C), heparan sulfate, dermatan sulfate, monoclonal antibody CS-56 (Avnur and Geiger, 1984), Sigma 104 phosphatase substrate, normal rabbit serum, and rabbit anti-mouse IgG antiserum were bought from Sigma (St. Louis, MO). Alkaline phosphatase conjugated goat anti-mouse IgG was purchased from Fisher (Pittsburgh, PA); Pansorbin cells and chondroitin 4-sulfate (chondroitin sulfate A) from Calbiochem (San Diego, CA). Protease-free chondroitinase ABC and quick spin protein columns were procured from Boehringer Mannheim (Indianapolis, IN); keratan sulfate and keratanase from Seikagaku America Inc. (Rockville, MD); collagenase from GIBCO (Grand Island, NY) and trypsin from Worthington (Freehold, NJ). Laminin, basic fibroblast growth factor (bFGF), and monoclonal anti-laminin B1 chain antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY); lithium 3,5-diiodosalicylate (LIS) from Eastman Kodak Company (Rochester, NY); protein assay kit (Bradford) and Tween 20 from Bio-Rad (Richmond, CA); and 48- and 96-well tissue culture plates from Costar (Cambridge, MA). Sprague–Dawley rats were maintained and bred in the University animal facility.

Immunoaffinity purification of L1. The L1 glycoprotein was purified from postnatal day 6 rat brain membranes using a solid phase immunoabsorbent technique (MacSween and Eastwood, 1978) as described previously (Dou and Levine, 1994). For each preparation, the purified material was subjected to SDS-PAGE under reducing conditions to monitor the purity and yield of the L1 glycoprotein.

Substrate preparation and characterization. Forty-eight well tissue culture plates were coated with 25 μg/ml of PLL overnight followed by either L1 or laminin, both at 2 μg/ml, or a mixture of the same amount of L1 or laminin and 1–1000 μg/ml of various GAGs for 3 hr at 37°C. The surfaces were washed with PBS before seeding of the neurons. Previous studies (Dou and Levine, 1994) employing ^{125}I-labeled laminin or L1 established that 65% of the input laminin and 80% of the input L1 bound to the PLL-coated surfaces. A previous dose response analysis (Dou and Levine, 1994) also showed that when surfaces are coated with laminin or L1 at a concentration of 2 μg/ml, the mean neurite length was 90% of the maximal length obtained with higher concentrations of these proteins.

To determine the amount of chondroitin sulfate GAGs, laminin and the L1 glycoprotein that had bound to the substrates, we used solid phase ELISA assays. Ninety-six well culture plates were coated with PLL (25 μg/ml) overnight followed by either pure GAGs, the neurite-promoting proteins or mixtures of the GAGs and the neurite-promoting proteins for 3 hr at 37°C. The surfaces were then blocked with blotto (5% nonfat dry milk, 0.05% Tween 20 in PBS) for 1 hr at room temperature (RT) followed by incubation with the antibodies described below. After three washes with PBS plus 0.5% Tween 20, the plates were incubated with alkaline phosphatase-linked goat anti-mouse IgG (1:500) for 1 hr. After 3 more washes, 50 μl of freshly prepared substrate mixture (1 mg/ml of Sigma 104 phosphatase substrate in 100 mMM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was added to each well for 5–40 min depending on the speed of color reaction. The color development was stopped by adding 50 μl/well of 0.1 M EDTA, pH 7.5, and the plate was read at 405 nm using an ELISA reader. PLL wells without antigen served as blanks and the reading from the no antigen wells incubated with antibodies was subtracted to exclude any nonspecific binding of antibodies to the tissue culture surfaces.

To measure substrate-bound CS4 and CS6, we used monoclonal antibody CS-56 which recognizes both CS4 and CS6 (Avnur and Geiger, 1984). Since the antibody has a higher avidity for CS6 than for CS4 (Avnur and Geiger, 1984), we used it at 4 μg/ml for CS4 and 400 ng/ml for CS6. The L1 glycoprotein was detected with monoclonal antibody 74-5H7 (Lemmon et al., 1989), at a concentration of 200 ng/ml, and laminin was detected with a monoclonal antibody directed against the B1 chains used at 1 μg/ml. In all cases, the antibodies were tested against their respective antigens over a wide range of concentrations to ensure that the antibody responses were in the linear range. All experiments comprised duplicate or tetraplicate wells and were repeated two or three times each.

Enzymatic treatment of the glycosaminoglycans. The chondroitin sulfates were digested with protease-free chondroitinase ABC in 40 mM Tris, pH 8.0, 40 mM sodium acetate, 0.1 mg/ml BSA. Typically, 2.5 μg of either CS4 or CS6 was incubated with 0.02 unit of chondroitinase ABC at 37°C for 1 hr. The reaction mixture was cooled on ice and CaCl2 was added to a final concentration of 1 mM to inactivate the enzyme. KS was treated with keratanase (0.1 unit/μg KS) in PBS (pH 7.4) containing 0.1 mg/ml BSA and protease inhibitors (2 mM PMSF and 0.1 mg/ml leupeptin) at 37°C for 1 hr. In control experiments, GAGs were treated with inappropriate GAG lyases. For example, CS4 was treated with keratanase and KS was treated with chondroitinase ABC. The reaction mixtures were mixed with either laminin or L1 and adsorbed to PLL-coated tissue culture wells as described above.

Primary cell cultures. Cerebellar granule neurons were purified from trypsin dissociates of postnatal day 5 or 6 rat cerebella on discontinuous Percoll gradients as described previously (Hatten, 1985). Neurons were seeded onto 48-well plates at 20,000 cells/well (250 cells/mm^2) in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal bovine serum (FBS), 25 mM KCl, and 20 ng/ml bFGF. After 24 hr, the cultures were washed in PBS and fixed in PBS containing 2% glutaraldehyde.

Dorsal root ganglia were isolated from embryonic day 15–16 rats and digested with 0.25% trypsin, 0.05% collagenase for 20 min at 37°C. Following neutralization and washing with serum containing medium, the ganglia were mechanically dissociated with flame-narrowed Pasteur pipettes. The cells were washed and preplated onto petri dishes in serum containing medium for 2 hr at 37°C to remove fibroblasts and other non-neuronal cells. The DRG neurons were seeded at 3000 cells/well in DMEM containing 10% FBS and 60 ng/ml NGF (NGF was a gift from Dr. S. Halegoua). After 24 hr, the cultures were washed in PBS and fixed in 2% glutaraldehyde in PBS.

Quantitization of neurite length and cell attachment. The quantitation and analysis of cell attachment and neurite length was carried out as described previously (Dou and Levine, 1994). Each experiment com-

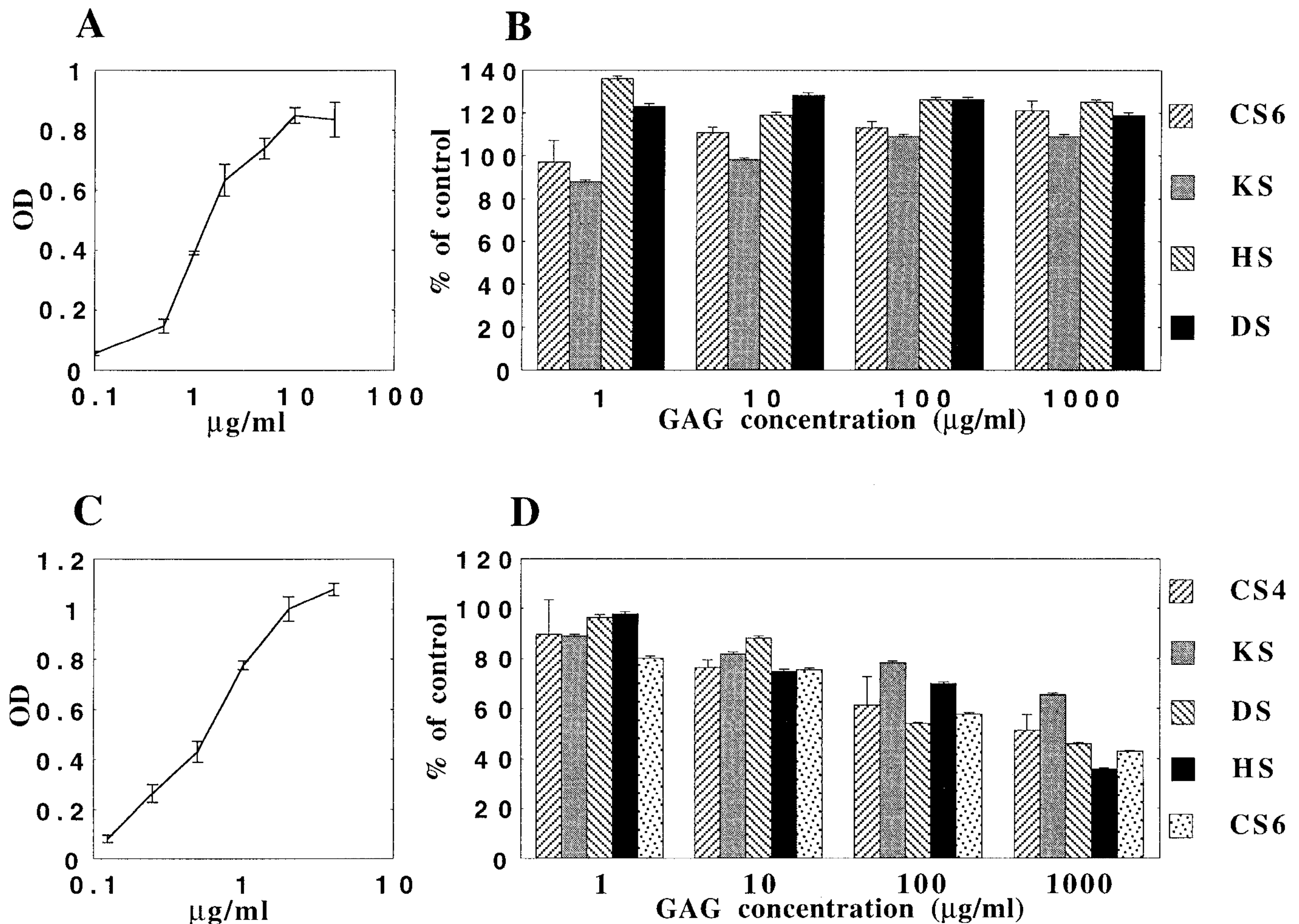

Figure 1. ELISA assays of substrate-bound laminin and L1. *A*, 96-well plates were coated with PLL followed by laminin at 0.1, 0.5, 1, 2, 5, 10, 25 μg/ml. After treatment with a monoclonal antibody directed against the laminin B1 chain and an alkaline phosphatase conjugated goat anti-mouse antibody, the wells were reacted with Sigma alkaline phosphatase substrate and optical density (*OD*) at 405 nm determined as detailed under Materials and Methods. The mean OD value and SD were determined from tetraplicate wells. Optical density is a function of the amount of laminin used to coat the wells. *B*, 96-well plates were coated with laminin at 2 μg/ml or mixtures of laminin and the indicated GAGs at the indicated concentrations. OD was determined as in *A* and the percent of control OD (i.e., OD measurements from wells coated with laminin alone) is plotted versus the concentration of the GAGs used. *C*, 96-well plates were coated with PLL followed by L1 at 0.125, 0.25, 0.5, 1, 2, and 4 μg/ml and OD determined as described above using monoclonal antibody 74-5H7 as a probe for substrate-bound L1 (Lemmon, et al., 1989). As was the case with laminin-coated wells, OD is a function of the amount of L1 added to the wells. *D*, 96-well plates were coated with mixtures of L1 and the indicated GAGs and optical densities determined as described above. The percent of control OD is plotted versus the concentrations of the GAG used.

prised duplicate wells and most experiments were repeated 3–10 times. A neurite was defined as a process extending from neuronal cell body by more than one cell diameter. A minimum of 50 neurites from duplicate wells of each substrate condition were measured for every experiment, except for a few strongly inhibitory substrates on which very few cells grew out neurites. In those cases, we scored all the neurites we could find. For cells that had more than one neurite, only the longest neurite was measured. Only those neurites that did not contact other neurites or cell bodies were measured. The mean neurite length for each individual experiment was calculated and the means were pooled to create the summary data shown in the tables. The percentage of inhibition of neurite growth is defined as $[1 - (\text{mean}^{\text{expt}}/\text{mean}^{\text{control}})] \times 100$ where $\text{mean}^{\text{expt}}$ is the mean length under the experimental conditions and $\text{mean}^{\text{control}}$ is the mean of the appropriate controls.

Results

Substrate characterization

To assay the effects of different GAGs on neurite growth on substrates comprising laminin and the L1 glycoprotein, it was necessary to first characterize the molecular nature of the substrates being tested. As described under Materials and Methods, we used ELISA assays to determine the amount of laminin and L1 bound to surfaces in the presence of the various GAGs and to determine the amount of CS4 and CS6 bound to the laminin- and L1-coated surfaces. In the case of laminin, we used a monoclonal antibody directed against the B1 chain. As shown in Figure 1*A*, optical density is a direct measure of the input amount of laminin over a concentration range of 0.5–10 μg/ml. When PLL-coated surfaces were treated with solutions containing 2 μg/ml of laminin plus either CS6, KS, DS, or HS at concentrations ranging from 1–1000 μg/ml, there was no reduction in the amount of laminin bound to the substrate as compared to surfaces coated with laminin alone (Fig. 1*B*). A similar assay using monoclonal antibody 74-5H7 (Lemmon et al., 1989) was performed to measure substrate bound L1. As was the case with the assays designed to detect laminin, optical density is a function of the input amount of L1 (Fig. 1*C*). Mixing CS4, CS6, DS, KS, or HS with L1 resulted in a concentration-dependent reduction in the amount of L1 bound to the substrate (Fig. 1*D*).

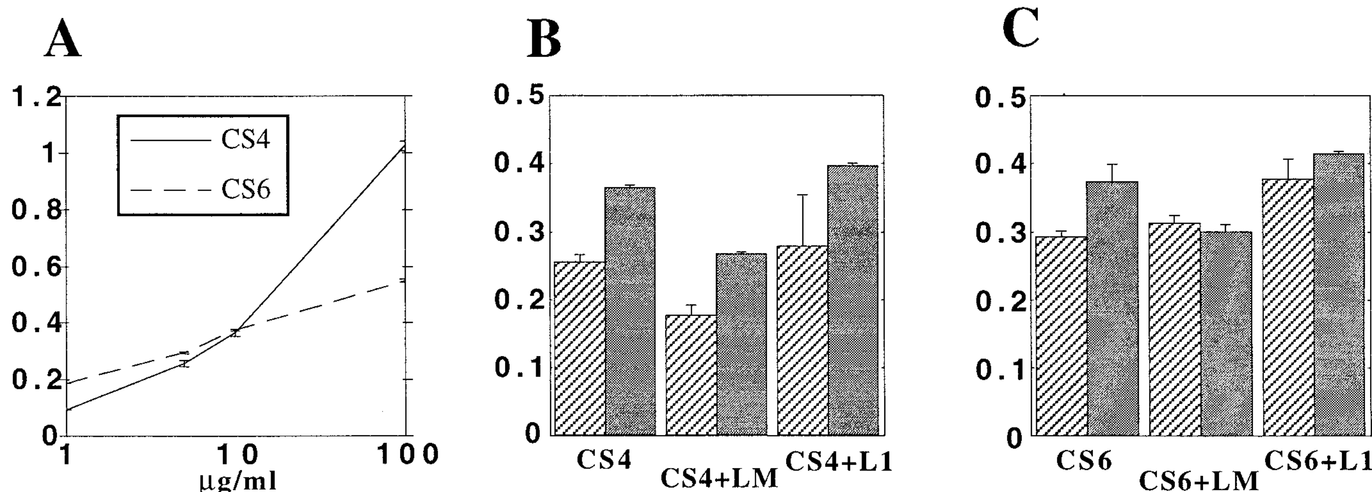

Figure 2. ELISA assay of substrate-bound chondroitin sulfates. *A*, 96-well plates were coated with PLL followed by either CS4 or CS6 at 1–100 μg/ml as detailed under Materials and Methods. The optical density (OD) values from duplicate wells are plotted versus the concentration of GAGs used to coat the substrate. The OD values increase as the concentration of the GAGs increases. The anti-CS antibody (CS-56) is more reactive against CS6 than CS4 (Avnur and Geiger, 1984); hence it was used at 1:500 (4 μg/ml) to detect CS4 and 1:5000 (400 ng/ml) to detect CS6. *B*, Optical density values for wells coated with CS4 alone or CS4 mixed with laminin (LM) or the L1 glycoprotein. The presence of laminin reduces the amount of CS4 bound to the substrates by 25–30%. The L1 glycoprotein does not alter the amount of GAGs bound to the substrates. *C*, Optical density values for CS6 alone and for CS6 mixed with either LM or L1. The cell adhesion proteins do not reduce the amount of CS6 bound to the substrates. In *B* and *C*, the *hatched bars* represent experiments in which the GAGs were used at 5 μg/ml and the *solid bars* represent experiments in which the GAGs were used at 10 μg/ml. The data shown are the means and SDs.

When each of the GAGs was used at 1 μg/ml, only CS6 caused a significant reduction in the amount of L1 bound to the substrate. As the concentration of CS6, HS, and DS was increased from 10–1000 μg/ml, the reduction in L1 binding to the surfaces also increased reaching a maximum reduction of 50–60% relative to control substrates. Mixing L1 with CS4 at 1 mg/ml, however, lead to only a 20% reduction in substrate bound L1. Thus, while GAGs, over a 1000-fold concentration range, do not reduce laminin binding to the substrate, they can reduce the amount of substrate bound L1.

We employed an ELISA assay to determine (1) whether exogenous CS GAGs bind to the tissue culture surfaces used and (2) whether laminin or L1 reduce the amount of CS GAG bound to the substrate. Figure 2*A* shows that the optical density values increase as the concentration of CS GAGs increases indicating that there are more molecules bound when higher concentrations of GAGs are used to coat the culture substrates. As shown in Figure 2*C*, when CS6 was mixed with either laminin or the L1 glycoprotein, the amount of the GAG bound to the surfaces did not differ significantly from the amount that was bound in the absence of the cell adhesion proteins. Thus, the presence of cell adhesion proteins does not interfere with the binding of CS6 to PLL-coated tissue culture surfaces. In the case of CS4, however, the amount of the GAG bound to the PLL-coated surfaces in the presence of laminin was 25–30% less than the amount bound in the absence of protein (Fig. 2*B*). The L1 glycoprotein did not reduce the amount of CS4 bound (Fig. 2*B*). Although laminin causes a reduction in the amount of CS4 bound to the tissue culture surfaces, as shown below, these reduced amounts of GAG were capable of neutralizing the neurite promoting properties of laminin.

GAGs inhibit neurite growth from CNS neurons on laminin- but not on L1-coated surfaces

To determine whether substrate bound GAGs modulate neurite outgrowth from CNS neurons, we plated postnatal day 5 cerebellar granule neurons on surfaces coated with either laminin or the L1 glycoprotein (both at 2 μg/ml) or with these same molecules mixed with different GAGs. Our previous studies have shown that when both laminin and L1 are used at a concentration of 2 μg/ml, neurite outgrowth from cerebellar granule neurons is 90% of the maximum lengths achieved at higher concentrations of the cell adhesion molecules (Dou and Levine, 1994). When used at this concentration, between 65–80% of the input molecules bound to the PLL-coated tissue culture surfaces (Dou and Levine, 1994).

As shown in Figure 3*A–D*, cerebellar granule neurons attached equally well to the laminin-coated surfaces and the surfaces coated with laminin mixed with the different GAGs. This demonstrates that the GAGs do not inhibit cell attachment in this assay. Among the five GAGs tested, CS4, CS6 and KS were potent inhibitors of axonal extension and initiation. As shown in Table 1, maximum growth inhibition occurred when CS6 and KS were used at 10 μg/ml. In the case of CS4, 10 μg/ml gave approximately 75% of the maximum inhibitory effect. As shown in Figure 4*A*, on laminin alone, 50% of all neurites measured

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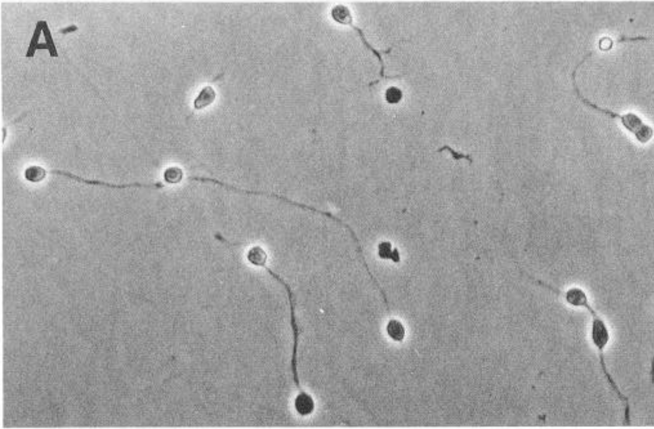
Figure 3. GAGs inhibit neurite outgrowth from cerebellar neurons on laminin but not on L1-coated surfaces. Cerebellar granule neurons were isolated from postnatal day 5 rats and grown on different substrates for 24 hr as described in Materials and Methods. The substrates are: *A*, laminin (2 μg/ml); *B*, laminin plus CS4 (10 μg/ml); *C*, laminin plus KS (10 μg/ml); *D*, laminin plus HS (10 μg/ml); *E*, L1 (2 μg/ml); *F*, L1 plus CS6 (100 μg/ml); *G*, L1 plus KS (100 μg/ml); *H*, L1 plus HS (10 μg/ml). Note that fewer cells extended neurites and the neurites were shorter on laminin plus CS or KS relative to on laminin alone. Neurite extension on L1 and CS or KS was indistinguishable from that on L1 control, however, both the number of cells growing neurites and the neurite length were reduced on L1 plus HS. Scale bar, 50 μm.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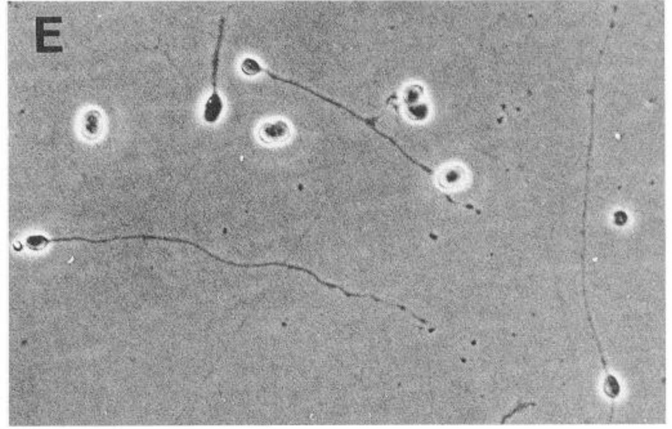
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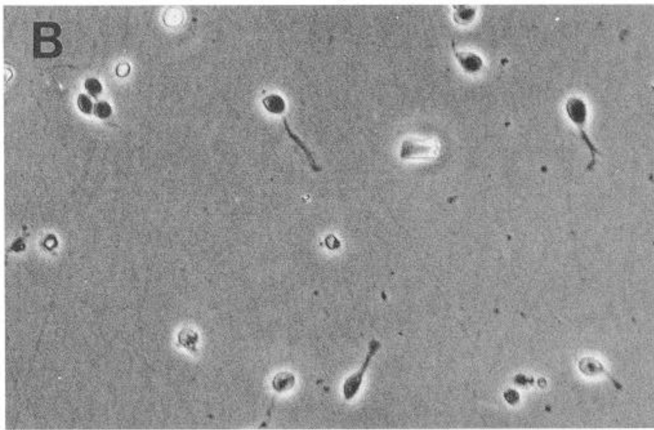
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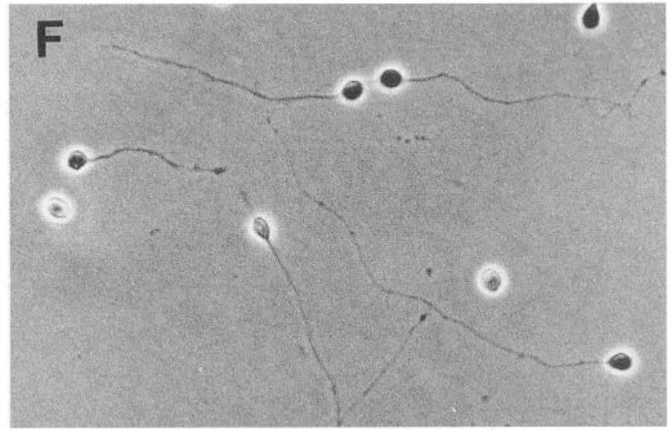
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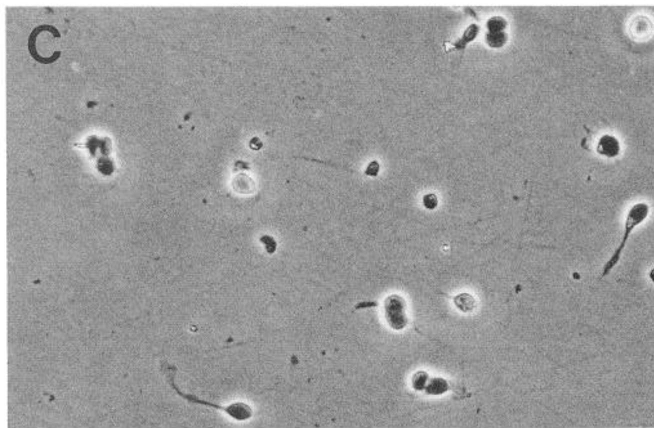
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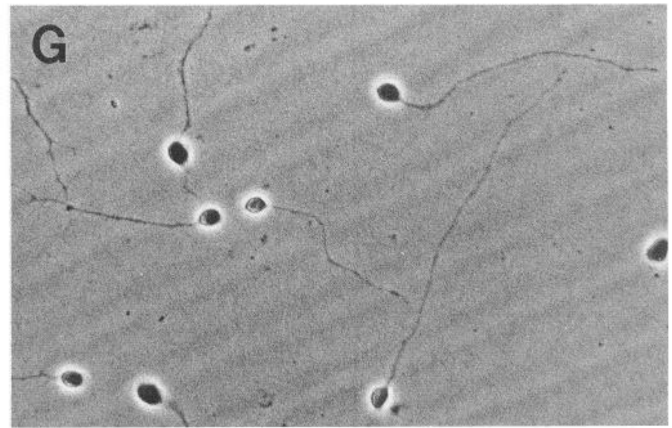
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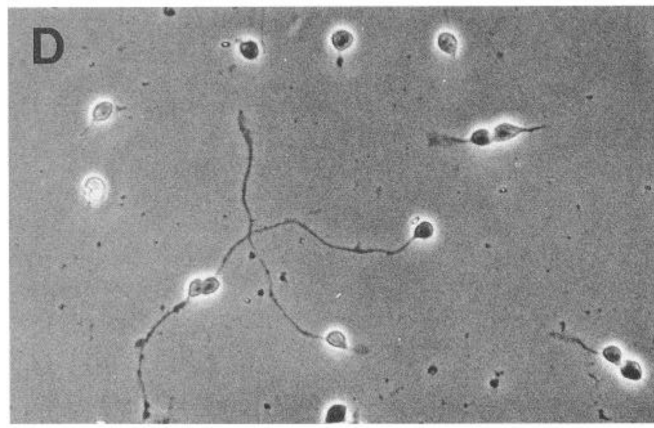
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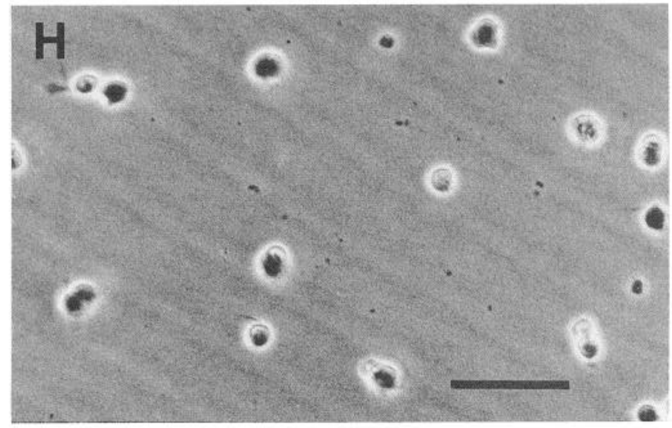
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Table 1. Effect of GAGs on neurite growth from cerebellar granule neurons on laminin-coated substrates

Substrate	% Cells with neurites	Mean neurite length, μm (*n*)	% Inhibition
Laminin (2 μg/ml)	60 ± 10	84 ± 12 (783)	
Laminin + CS4 (1 μg/ml)	56 ± 10	77 ± 28 (69)	8
Laminin + CS4 (10 μg/ml)	33 ± 11*	58 ± 7 (270)**	31
Laminin + CS4 (10 μg/ml, C'ase digested)	48 ± 12	77 ± 2 (162)	8
Laminin + CS4 (100 μg/ml)	18 ± 7*	49 ± 5 (145)*	42
Laminin + CS4 (1 mg/ml)	13 ± 5*	48 ± 11 (86)*	43
Laminin + CS6 (1 μg/ml)	48 ± 11	74 ± 30 (70)	12
Laminin + CS6 (10 μg/ml)	25 ± 5*	50 ± 2 (101)*	40
Laminin + CS6 (100 μg/ml)	24 ± 7*	50 ± 1 (125)*	40
Laminin + CS6 (1 mg/ml)	31 ± 9*	49 ± 2 (93)*	42
Laminin + KS (1 μg/ml)	48 ± 10	75 ± 10 (101)	11
Laminin + KS (10 μg/ml)	25 ± 6*	49 ± 4 (244)*	42
Laminin + KS (10 μg/ml, K'ase digested)	54 ± 6	82 ± 3 (122)	2
Laminin + KS (100 μg/ml)	24 ± 5*	48 ± 3 (230)*	43
Laminin + KS (1 mg/ml)	33 ± 7*	50 ± 2 (88)*	40
Laminin + HS (1 μg/ml)	54 ± 8	81 ± 21 (74)	4
Laminin + HS (10 μg/ml)	39 ± 10**	72 ± 4 (137)	14
Laminin + HS (100 μg/ml)	34 ± 7*	72 ± 5 (130)	14
Laminin + HS (1 g/ml)	44 ± 14	63 ± 17 (56)**	25
Laminin + DS (1 μg/ml)	60 ± 9	84 ± 33 (70)	0
Laminin + DS (10 μg/ml)	64 ± 7	75 ± 8 (191)	11
Laminin + DS (100 μg/ml)	45 ± 8	57 ± 3 (235)**	32
Laminin + DS (1 mg/ml)	20 ± 3*	38 ± 12 (23)*	55

Cerebellar granule neurons were seeded onto the indicated substrates. After 24 hr, the percentages of cells with neurites and neurite lengths were determined as described in Materials and Methods. Data shown are the percentage of attached cells with neurites (mean ± SD) and the mean neurite length (±SD) from 1–12 separate experiments. The numbers in parentheses (*n*) are the total numbers of neurites measured for each substrate condition. There was no difference in the total number of attached cells per unit area under all conditions tested. C'ase, Chondroitinase ABC; K'ase, keratanase.

*, $p < 0.001$; **, $0.001 < p < 0.01$ (Student's *t* test); where not indicated, the numbers are not statistically different ($p > 0.15$) from the control values on laminin alone.

were greater than 85 μm. On mixtures of laminin and CS4, 50% of all neurites were greater than 55 μm, on KS and laminin, 50% were greater than 48 μm and on CS6 and laminin, the median length was 35 μm. The percentage of the attached cells that extended neurites was also reduced from a value of about 60% on laminin alone to between 25–33% on the GAG-containing surfaces (Table 1). Dermatan sulfate, which differs from CS4 and CS6 in that it contains α-L-iduronic acid rather than β-D-glucoronic acid residues, was much less effective as an inhibitor of axonal growth (Table 1). Although high levels of inhibition could be achieved with DS, this inhibition required 10–100-fold higher concentrations of DS than of CS4, CS6, or KS. Heparan sulfate was also a weak inhibitor of axonal growth from cerebellar neurons on laminin-coated surfaces and statistically significant inhibition was achieved only when HS was used at a concentration of 1 mg/ml (Table 1). These growth inhibitory effects were due to the GAGs themselves and not any contaminating material in the commercially available preparations used since digestion with the appropriate GAG lyases destroyed the growth inhibitory activity (Table 1). Moreover, digestion with inappropriate GAG lyases, that is, digestion of CS4 with keratanase or digestion of KS with chondroitinase ABC, had no effect on the ability of the GAGs to modify neurite growth. For example, the mean neurite length on laminin and KS (10 μg/ml) that had been treated with chondroitinase ABC was 43 μm and the mean length on laminin and CS4 (10 μg/ml) that had been treated with keratanase was 58 μm, values that are comparable to the mean neurite lengths on laminin and the respective GAGs without any enzyme treatment. As shown in Figure 3*A–D*, there were no obvious morphological differences among the cells grown on these various substrates except that the neurites were shorter on laminin plus CS or KS. Thus, with the exception of HS, all GAGs tested were able to inhibit the potent growth-promoting effects of laminin.

Cerebellar granule neurons extend their axons *in vivo* within areas rich in the L1 glycoprotein (Faissner et al., 1984; Stallcup et al., 1985). To determine whether GAGs can inhibit neurite growth in an assay that may better approximate physiological conditions, we evaluated the abilities of GAGs to influence neurite growth on L1-coated surfaces.

As shown in Table 2, CS4, CS6, and KS at concentrations of up to 100 μg/ml did not inhibit neurite growth from cerebellar neurons on L1-coated surfaces. Both the percentages of attached cells that extended neurites and the mean neurite lengths on

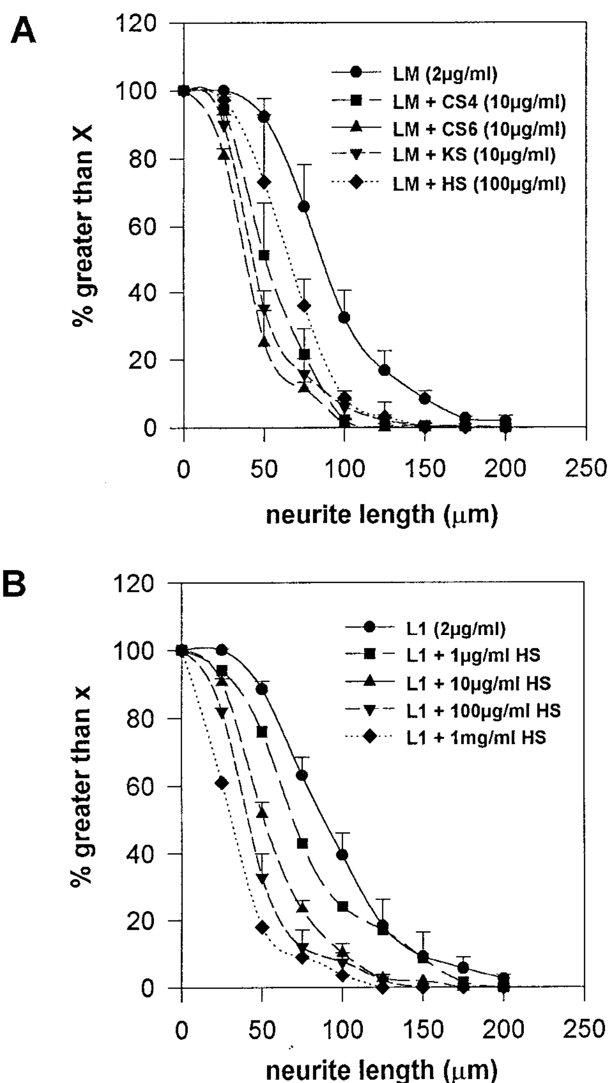

Figure 4. Quantitative analysis of neurite growth from cerebellar neurons on different substrates. *A*, Cumulative neurite length histograms showing the distribution of neurites according to length on laminin alone, laminin plus CS4, laminin plus CS6, laminin plus KS, and laminin plus HS. Data were pooled from 12 separate experiments and distribution was plotted as percentage of neurons with neurites (y-axis) longer than a given length (x-axis). These GAGs caused a shift of neurite distribution curve towards the left (median neurite length reduced). *B*, Distribution of neurite lengths on L1 alone, L1 plus 1, 10, 100, 1000 µg/ml of HS. HS inhibits the growth of cerebellar neurons on the L1-coated surfaces in a dose-dependent manner.

surfaces coated with L1 and each of these three GAGs were statistically identical to those on surfaces coated with L1 alone (Table 2, Fig. 3*E–G*). No inhibition by KS at concentrations up to 1 mg/ml was observed and statistically significant inhibition by the chondroitin sulfates was seen only when they were used at 1 mg/ml (Table 2). In contrast to the lack of significant effects of KS and the chondroitin sulfates, HS was a potent inhibitor of neurite extension on L1-coated surfaces (Table 2). As shown in Figure 4*B*, HS, at 1 µg/ml, reduced the median neurite length from 96 µm to 75 µm. The median length was further reduced as the concentration of HS was increased reaching a maximum of 62% inhibition of growth when HS was used at 1 mg/ml. Dermatan sulfate, at concentrations greater than 50 µg/ml, also inhibited both the initiation and the extension of neurites (Table

Table 2. Effects of GAGs on the growth of cerebellar neurons on L1-coated substrates

Substrate	% Cells with neurites	Neurite length, µm (*n*)	% Inhibition
L1 (2 µg/ml)	55 ± 12	96 ± 7 (608)	
L1 + CS4 (1 µg/ml)	63 ± 6	95 ± 27 (117)	1
L1 + CS4 (10 µg/ml)	62 ± 11	94 ± 9 (132)	2
L1 + CS4 (100 µg/ml)	62 ± 14	97 ± 11 (159)	
L1 + CS4 (1 mg/ml)	17 ± 4*	47 ± 4 (70)*	51
L1 + CS6 (1 µg/ml)	55 ± 10	91 ± 33 (52)	5
L1 + CS6 (10 µg/ml)	65 ± 10	103 ± 2 (154)	
L1 + CS6 (100 µg/ml)	64 ± 4	95 ± 10 (138)	1
L1 + CS6 (1 mg/ml)	27 ± 4*	50 ± 4 (87)*	48
L1 + KS (10 µg/ml)	51 ± 14	95 ± 4 (243)	1
L1 + KS (100 µg/ml)	59 ± 12	95 ± 5 (393)	1
L1 + KS (1 mg/ml)	55 ± 5	87 ± 20 (97)	9
L1 + HS (1 µg/ml)	53 ± 10	78 ± 5 (135)	19
L1 + HS (10 µg/ml)	28 ± 8*	58 ± 1 (254)*	40
L1 + HS (100 µg/ml)	21 ± 5*	46 ± 8 (205)*	52
L1 + HS (1 mg/ml)	30 ± 5*	36 ± 7 (56)*	62
L1 + DS (1 µg/ml)	60 ± 16	95 ± 37 (54)	1
L1 + DS (10 µg/ml)	55 ± 13	105 ± 4 (168)	
L1 + DS (50 µg/ml)	39 ± 2**	67 ± 22 (48)**	30
L1 + DS (100 µg/ml)	24 ± 6*	61 ± 5 (125)*	36
L1 + DS (1 mg/ml)	16 ± 14*	42 ± 11 (28)*	56

Cerebellar granule neurons were seeded onto the indicated substrates. After 24 hr, the percentages of cells with neurites and neurite lengths were determined as described in Materials and Methods. Data shown are the percentage of attached cells with neurites (mean ± SD) and the mean neurite length (±SD) from one to nine separate experiments for each condition. *n* indicates the total number of neurites measured for each substrate condition. There was no difference in the total number of attached cells per unit area under all conditions tested.

*, $p < 0.001$; **, $0.001 < p < 0.01$ (Student's *t* test); where not indicated, the numbers are not statistically different ($p > 0.2$) from the control values on laminin alone.

2). Thus, while HS and DS inhibit the growth of cerebellar neurons on L1-coated surfaces, other GAGs (CS4, CS6, and KS) do not inhibit neurite elongation on the L1 substrates when used at concentrations sufficient to cause robust inhibition on laminin substrates.

Effects of GAGs on the growth of embryonic rat DRG neurons

The studies described above demonstrate that CS and KS GAGs inhibit the growth of a population of rat CNS neurons on laminin-coated surfaces but not on L1-coated surfaces and that HS and DS inhibit neurite growth from these neurons on L1-but not on laminin-coated substrates. To determine whether this growth inhibition is specific to CNS neurons or is a general property of all neurons, we tested the effects of these GAGs on axonal growth from rat DRG neurons. As DRG neurons extend their axons in an environment containing both abundant laminin (Sanes, 1989) and the L1 glycoprotein (Stallcup et al., 1985), we evaluated the growth modulatory effects of the panel of GAGs on substrates containing either laminin or the L1 glycoprotein.

When DRG neurons were plated onto surfaces that had been coated with PLL followed by laminin, 65% of the attached cells elaborated long (mean length = 191 µm) neurites after 24 hr

Table 3. Effects of GAGs on the growth of DRG neurons on laminin-coated substrates

Substrate	% Cells with neurites	Neurite length, μm (*n*)	% Inhibition
Laminin (2 μg/ml)	65 ± 9	191 ± 36 (320)	
Laminin + CS4 (1 μg/ml)	59 ± 4	180 ± 45 (55)	6
Laminin + CS4 (10 μg/ml)	42 ± 11*	76 ± 10 (83)*	60
Laminin + CS4 (100 μg/ml)	38 ± 9*	83 ± 12 (88)*	57
Laminin + CS6 (1 μg/ml)	65 ± 10	170 ± 55 (50)	11
Laminin + CS6 (10 μg/ml)	39 ± 11*	85 ± 10 (92)*	55
Laminin + CS6 (100 μg/ml)	41 ± 8*	73 ± 4 (60)*	62
Laminin + KS (1 μg/ml)	68 ± 4	145 ± 49 (56)	24
Laminin + KS (10 μg/ml)	35 ± 7*	62 ± 13 (85)*	68
Laminin + KS (100 μg/ml)	35 ± 12*	63 ± 3 (44)*	67
Laminin + HS (10 μg/ml)	58 ± 18	181 ± 49 (171)	5
Laminin + HS (100 μg/ml)	56 ± 9	166 ± 40 (174)	13
Laminin + HS (1 mg/ml)	56 ± 16	149 ± 44 (48)	22
Laminin + DS (10 μg/ml)	48 ± 14	154 ± 53 (137)	19
Laminin + DS (100 μg/ml)	53 ± 12	159 ± 45 (143)	17
Laminin + DS (1 mg/ml)	56 ± 9	161 ± 57 (52)	16

Embryonic day 15 rat DRG neurons were seeded onto the indicated substrates. After 24 hr, the percentages of cells with neurites and neurite lengths were determined as described in Materials and Methods. Data shown are the means and SDs from one to five separate experiments. *n* indicates the total number of neurites measured for each substrate condition.

*, $p < 0.001$ (Student's *t* test); where not indicated, the numbers are not statistically different ($p > 0.2$) from the control values on laminin alone.

growth (Table 3). The majority of neurons were either unipolar or bipolar in appearance and the growth cones often had long filopodia (Fig. 5*A*). When grown on surfaces comprising laminin and either CS4, CS6, or KS, the DRG neurons gave rise to shorter neurites (Fig. 5*B,C*, Table 3). Chondroitin 4-sulfate, CS6, and KS, at 10 μg/ml, inhibited neurite growth by 55 to 68% relative to growth on laminin alone (Fig. 6, Table 3). Each of these three GAGs was an equally potent inhibitor of neurite growth and the growth inhibitory effects appeared to be maximal at a concentration of 10 μg/ml (Table 3). As shown in Figure 5*B*, growth inhibition by GAGs often resulted in growth cones with a "stunted" appearance characterized by numerous short filopodia. Heparan sulfate and dermatan sulfate, on the other hand, did not have significant inhibitory effects on the growth of these neurons as the mean neurite lengths on HS- or DS-containing surfaces were not statistically different from that on laminin control surfaces (Table 3, Fig. 5*D*). These data demonstrate that the growth inhibitory activities of CS4, CS6, and KS on laminin-containing substrates are equivalent whether assayed on central or peripheral neurons.

When embryonic DRG neurons were grown on the L1-coated substrates, most neurons extended a single, short neurite (Fig. 5*E*). The morphology of growth cones on L1 substrate differed from that on laminin-coated surfaces. On L1-coated surfaces, growth cones were large and characterized by lamellipodia, whereas on laminin-coated surfaces, small growth cones with long filopodia predominated (compare Fig. 5*A,E*). These differences in growth cone morphology, which were not analyzed quantitatively, closely resemble those reported by Payne and colleagues (1992) for chick DRG neurons. With the exception of DS at a concentration of 1 mg/ml, none of the GAGs tested inhibited the growth of DRG neurons on the L1-coated surfaces (Table 4). Chondroitin 4-sulfate stimulated neurite growth by 30–40% (Table 4). The neurons grown on surfaces containing CS4, KS, or HS were morphologically indistinguishable from those grown on L1 alone (Fig. 5*E–H*). Thus, in contrast to their growth inhibitory effects on laminin substrates, GAGs do not exert negative influences over DRG neurite elongation on L1-containing surfaces.

Discussion

Proteoglycans and their covalently attached GAG chains have attracted much attention recently as molecules that may encode both positive and negative growth cone guidance cues (Snow et al., 1990a; Cole and McCabe, 1991; Oohira et al., 1991; Lafont et al., 1992, 1994; Grumet et al., 1993, Faissner et al., 1994). The molecular diversity of proteoglycans and GAGs, their multifunctional structure, and their ability to form stable complexes

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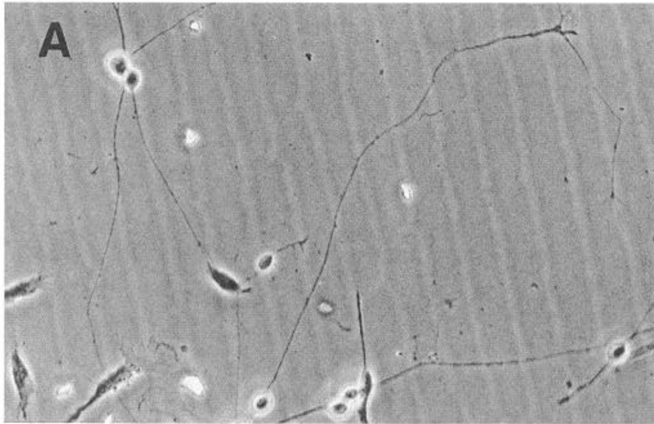
Figure 5. GAGs inhibit neurite outgrowth from DRG neurons on laminin- but not on L1-coated surfaces. DRG neurons were isolated from embryonic day 15 rats and plated onto PLL-coated tissue culture wells containing various substrates as described in Materials and Methods. The cells were fixed after 24 hr growth and photographed. The substrates are: *A*, laminin (2 μg/ml); *B*, laminin plus CS4 (10 μg/ml); *C*, laminin plus KS (10 μg/ml); *D*, laminin plus HS (10 μg/ml); *E*, L1 (2 μg/ml); *F*, L1 plus CS4 (100 μg/ml); *G*, L1 plus KS (100 μg/ml); *H*, L1 plus HS (10 μg/ml). Neurite growth on surfaces containing laminin and either CS4 or KS was reduced relative to growth on laminin alone. The GAGs did not inhibit neurite growth on the L1-coated surfaces. Chondroitin 4-sulfate stimulated neurite growth from DRG neurons on L1-coated surfaces. Scale bar, 50 μm.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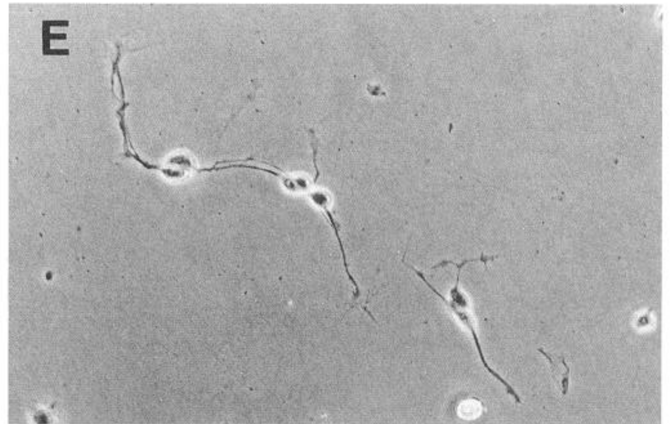
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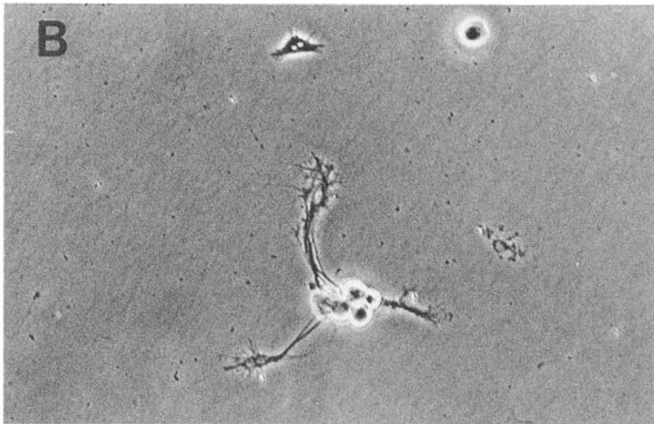
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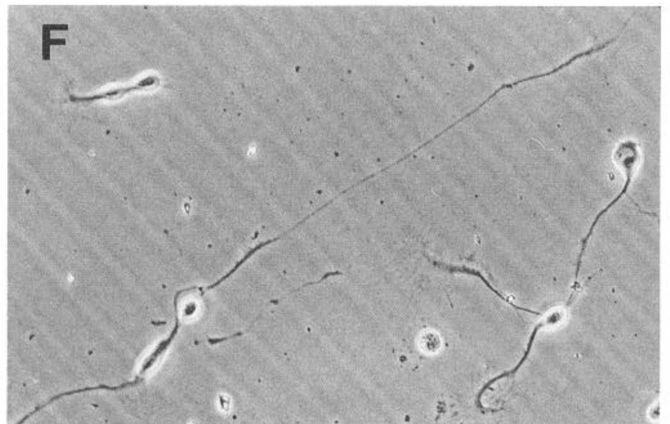
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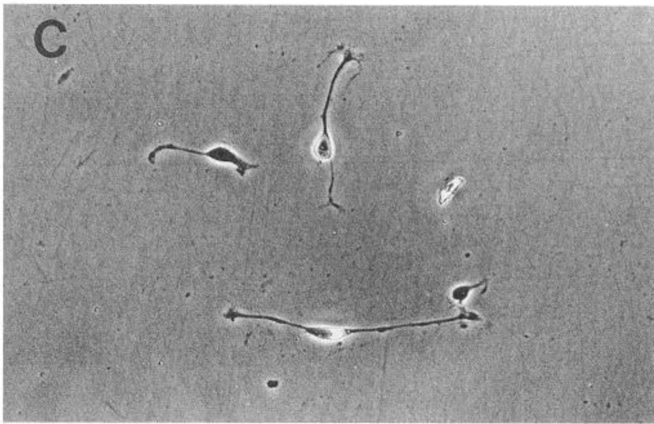
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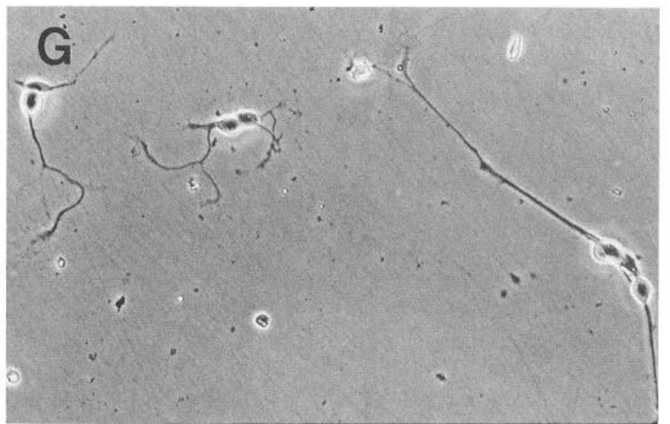
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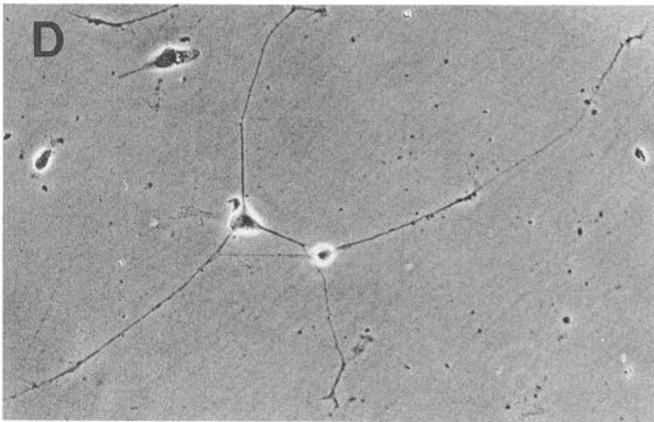
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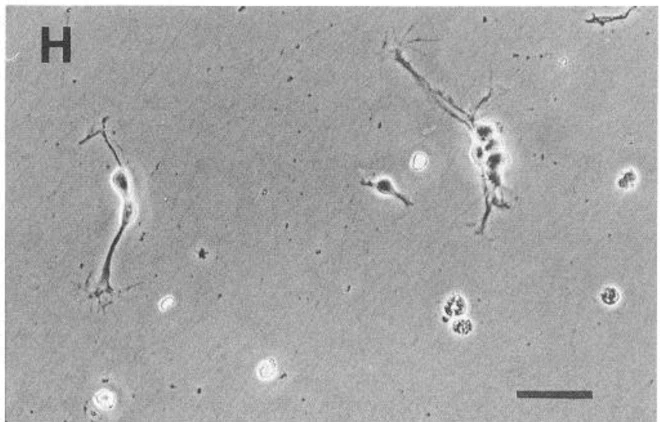
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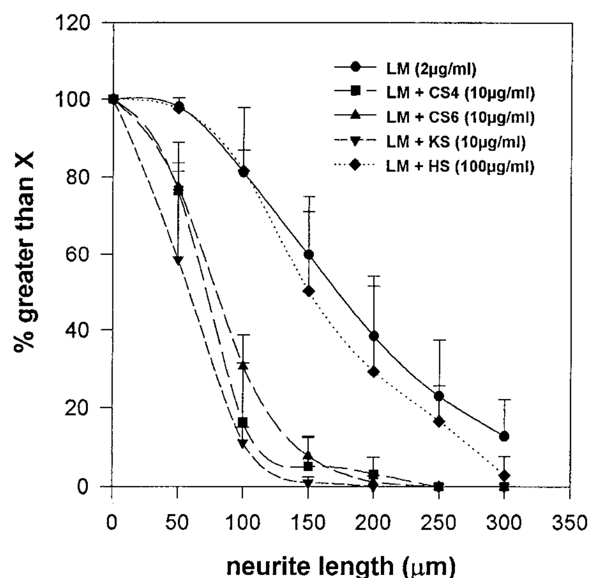

Figure 6. Quantitative analysis of neurite growth from DRG neurons on various substrates. Cumulative neurite length histograms showing the distribution of neurites according to length on laminin alone, laminin plus CS4, laminin plus CS6, laminin plus KS, and laminin plus HS. Data were pooled from five separate experiments and distribution was plotted as percentage of neurons with neurites (y-axis) longer than a given length (x-axis). CS4, CS6, and KS all reduced significantly the median neurite length while HS at 100 μg/ml did not shift the neurite distribution curve.

Table 4. Effects of GAGs on the growth of DRG neuron on L1-coated substrates

Substrate	% Cells with neurites	Neurite length, μm (*n*)	% Inhibition
L1 (2 μg/ml)	68 ± 12	106 ± 6 (221)	
L1 + CS4 (1 μg/ml)	63 ± 9	155 ± 41 (51)*	
L1 + CS4 (10 μg/ml)	67 ± 7	150 ± 9 (94)*	
L1 + CS4 (100 μg/ml)	54 ± 14	139 ± 2 (94)*	
L1 + CS4 (1 mg/ml)	67 ± 7	138 ± 31 (51)*	
L1 + CS6 (10 μg/ml)	59 ± 18	110 ± 16 (106)	
L1 + CS6 (100 μg/ml)	48 ± 16	105 ± 2 (100)	1
L1 + CS6 (1 mg/ml)	47 ± 9	116 ± 36 (55)	
L1 + KS (10 μg/ml)	57 ± 9	103 ± 8 (105)	3
L1 + KS (100 μg/ml)	51 ± 9	101 ± 6 (108)	5
L1 + KS (1 mg/ml)	65 ± 9	118 ± 33 (53)	
L1 + HS (10 μg/ml)	57 ± 9	94 ± 17 (116)	11
L1 + HS (100 μg/ml)	56 ± 14	95 ± 16 (109)	10
L1 + HS (1 mg/ml)	55 ± 11	110 ± 29 (58)	
L1 + DS (10 μg/ml)	68 ± 8	106 ± 10 (128)	0
L1 + DS (100 μg/ml)	65 ± 6	117 ± 17 (154)	
L1 + DS (1 mg/ml)	37 ± 11*	56 ± 18 (37)*	47

Rat DRG neurons were seeded onto the indicated substrates. After 24 hr, the percentages of cells with neurites and neurite lengths were determined as described in Materials and Methods. Data shown are the means and SDs from one to four separate experiments for each condition. *n* is the total number of neurites measured.

*, $p < 0.001$ (Student's *t* test); where not indicated, the numbers are not statistically different ($p > 0.2$) from the control values on L1 alone.

with other ECM-associated molecules are properties of these molecules that can allow for the expression of the biological information needed to guide a growth cone to its appropriate target cell (for review, see Jackson et al., 1991; Hardingham and Fosang, 1992). Despite this high level of interest, the physiological functions of GAGs in neuronal development remain controversial and conflicting data from a number of *in vitro* studies have been reported (Carbonetto et al., 1983; Akeson and Warren, 1986; Verna et al., 1989; Snow et al., 1990a; McCabe and Cole, 1991; Lafont et al., 1992, 1994). We previously developed an assay to measure the effects of exogenous molecules such as proteoglycans and GAGs on the initiation and elaboration of neurites independent of their possible effects on cell substrate adhesion (Dou and Levine, 1994). Here we have used this sensitive assay to demonstrate that while most GAGs, with the exception of HS and DS, inhibit neurite growth from CNS neurons on surfaces comprising laminin, they are ineffective as growth inhibitors on substrates containing the L1 glycoprotein. In the case of DRG neurons, the effects of GAGs were more complex in that while CS4, CS6, and KS inhibited axon elongation on laminin, one of these GAGs, CS4, was able to enhance neurite growth on L1-coated surfaces. These data, which are summarized in Table 5, demonstrate that the effects of GAGs on neurite extension are dependent upon both the molecular context in which these carbohydrate polymers are presented to the neurons and the specific target neurons studied.

The chondroitin sulfates, in particular CS4, and hyaluronic acid (HA) are the predominant GAGs of the CNS (Margolis et al., 1975; Margolis and Margolis, 1989). Detailed studies of the composition and distribution of GAGs within the developing rat cerebellum have shown that the relative amount of CS4 GAG increases postnatally concurrent with a reduction in the amount of HA (Werz et al., 1985b). Heparan sulfate but not KS or DS has also been detected within the developing cerebellum (Werz et al., 1985a). Within the cerebellum, CS4 has been localized to the developing molecular layer where it has the potential to interact with the growth cones of elongating axons of granule neurons (Flaccus et al., 1991) as well as with the L1 glycoprotein (Faissner et al., 1984; Stallcup et al., 1985). The chondroitin

Table 5. Summary

Cell type substrate	Cerebellar neurons: Laminin	Cerebellar neurons: L1	DRG neurons: Laminin	DRG neurons: L1
Chondroitin 4-sulfate	Inhibitory	No effect	Inhibitory	Stimulatory
Chondroitin 6-sulfate	Inhibitory	No effect	Inhibitory	No effect
Keratan sulfate	Inhibitory	No effect	Inhibitory	No effect
Heparan sulfate	No effect	Inhibitory	No effect	No effect
Dermatan sulfate	No effect	Inhibitory	No effect	No effect

sulfates are also present in the subplate of the developing telencephalon where they may mark a preferential pathway for the growth of developing thalamocortical axons (Bicknese et al., 1994). Although KS accounts for only a small fraction of the total GAGs in the developing brain (Werz et al., 1985a; Oohira et al., 1986; Margolis and Margolis, 1989), it is enriched in several midline structures that are thought to act as barriers to growing axons (Steindler and Cooper, 1987; Snow et al., 1990b; Steindler et al., 1990; reviewed in Steindler, 1993). Similarly, in the developing PNS, CS6 and KS mark tissues such as the perinotochordal mesenchyme that act as barriers to the growth of motor and sensory axons (Oakley and Tosney, 1991; Perris et al., 1991). Thus, while KS may be associated with tissues that are nonpermissive for neurite extension, the chondroitin sulfates are associated with tissues through which axons grow as well as with tissues that axons avoid.

The observation that CS GAGs are associated with potential barrier tissues in the PNS but with growth permissive tissues in the CNS prompted us to evaluate the growth modulatory effects of GAGs under two different substrate conditions. Laminin is a major component of the ECM and is a potent promoter of neurite extension *in vitro* (Lander et al., 1983; Manthorpe et al., 1983; Edgar et al., 1984; Adler et al., 1985). Laminin is abundant in the peripheral tissues through which motor, sensory and sympathetic axons grow (Chui and Sanes, 1984; Rogers et al., 1986). Within the developing CNS, however, only small amounts of laminin have been detected. Laminin-like immunoreactivity is found transiently in the developing telencephalon (Hunter et al., 1992), optic nerve (McLoon et al., 1988), and hippocampus (Gordon-Weeks et al., 1989) as well as in some developing fiber tracts (Letourneau et al., 1988). This failure to find abundant laminin in the developing CNS may reflect limitations of the currently available reagents since several isoforms of laminin have been identified (Hunter et al., 1989; Ehrig et al., 1990), one of which, merosin, may be abundant in the CNS (Engvall et al., 1991). Both central and peripheral neurons bind to laminin via their heterodimeric integrin receptors (Gehlsen et al., 1988; Ignatius and Reichardt, 1988; Tomaselli et al., 1988; Hall et al., 1990; Sonnenberg et al., 1990). Integrin binding to laminin leads to an activation of protein kinase C whose enhanced activity is prerequisite for neurite extension (Bixby, 1989).

The L1 glycoprotein, a member of the immunoglobulin super family of cell adhesion molecules, is abundant in both the central and peripheral nervous systems. Like laminin, it is also a potent promoter of neurite growth *in vitro* (Lagenauer and Lemmon, 1987; Lemmon et al., 1989) and is thought to mediate axon–axon interactions such as fasciculation (Stallcup and Beasley, 1985; Rathjen et al., 1987). Although L1 was originally characterized as a homophilic cell adhesion molecule (Lemmon et al., 1989), heterophilic ligands for L1 have been identified (Kadmon et al., 1990a,b; Kuhn et al., 1990; Felsenfield et al., 1994). L1 binding also leads to an activation of cellular second messenger systems (Schuch et al., 1989; von Bohlen und Halbach et al., 1992; Walsh and Doherty, 1992). The L1-activated second messenger systems, which differ than those activated by integrin–laminin interactions, are complex and include changes in intracellular calcium levels, changes in cytoplasmic pH and the turnover of inositol phosphates (Schuch et al., 1989, von Bohlen und Halbach et al., 1992; Walsh and Doherty, 1992). Recently, a role for the *src* tyrosine kinase in the response of cerebellar granule neurons to L1 but not to laminin has been suggested (Ignelzi et al., 1994). By analyzing the effects of GAGs on the growth of neurites from populations of developing central and peripheral neurons on two different substrates, our studies have revealed previously unrealized complexities in the actions of GAGs on the growth of developing neurons. The ability of CS and KS GAGs to inhibit neurite growth on laminin but not on L1 surfaces is likely a reflection of the ability of these two neurite growth promoting molecules to activate different intracellular signaling systems.

Of the five GAGs tested, CS4, CS6, and KS inhibited the growth of both cerebellar and DRG neurons on laminin substrates. Inhibition on laminin-coated substrates was near maximal when these GAGs were used at a concentration of 10 μg/ml. Assuming that (1) the average molecular weight of the GAG polymers used is between 20–40,000 daltons and (2) the efficiency of the adsorption of the GAGs to the substrates was between 65–80% (see Materials and Methods), these GAGs are active at submicromolar concentrations. Although HS and DS were also capable of inhibiting neurite growth from cerebellar neurons on laminin, inhibition by HS required 100-fold higher concentrations. Dermatan sulfate was intermediate in the strength of its inhibitory action in that statistically significant inhibition occurred when this GAG was used at 50–100 μg/ml. At these concentrations, the high density of negative charges added to the substrates may contribute to the growth inhibition observed. The ability of CS4, CS6, and KS to inhibit neurite growth at low concentrations suggests that these effects are mediated by mechanisms other than those dependent on charge density alone.

Throughout the experiments reported here, we used laminin at a concentration of 2 μg/ml. When used at this concentration, the mean length of neurites was 90% of the maximum achieved on substrates coated with higher concentrations of laminin (Dou and Levine, 1994). As the concentration of laminin used to coat surfaces is decreased, neurite length also decreases. However, a reduction in the mean neurite length of 40% (a value comparable to the degree of inhibition caused by CS4, CS6, and KS) occurred only when laminin was used at a concentration of 0.2 μg/ml (Dou and Levine, unpublished observations). As the data in Figure *1B* precludes the possibility that the GAGs are reducing the amount of laminin bound to the substrate, the growth inhibition reported here must be due to some specific action of the GAGs per se rather than a reduction in substrate-bound laminin.

In the case of the L1 glycoprotein, CS4, CS6, DS, and HS all reduced the amount of substrate bound L1 in a dose-dependent manner. When the GAGs were used at a concentration of 1 mg/ml, this reduction was as high as 50–60%. Although this data suggests that the growth inhibition caused by DS and HS may be due to a reduction in the amount of L1 bound to the PLL-coated surfaces, the following observations rule out this possibility. First, although CS4, CS6, DS, and HS were equally effective in reducing the amount of L1 bound to the substrates, HS and DS inhibited neurite extension from cerebellar neurons whereas CS4 and CS6 did not. The data in Figure 2 also eliminate the possibility that CS4 and CS6 were not incorporated into the substrate. Second, when cerebellar neurons were plated onto surfaces coated with L1 at a concentration of 1 μg/ml, a value comparable to the amount of substrate-bound L1 detected after coating surfaces with mixtures of L1 and GAGs at a concentration of 1 mg/ml, the mean neurite length was approximately 85 μm, a value that is only 11% reduced from control values. Thus, the ability of HS and DS, at moderate concentra-

tions, to inhibit the growth of cerebellar neurons on L1 surfaces is not due to a reduction in substrate-bound L1, rather, it is likely due to a specific interaction between the GAGs and L1 or between the substrate bound GAGs and the neurons. If the interactions are between the GAGs and the neurons, there must be a specificity to the manner in which different types of neurons respond to substrate bound GAGs as the growth of DRG neurons was not inhibited under conditions that inhibit neurite growth from cerebellar neurons.

We can only speculate regarding the mechanisms by which GAGs exert their growth inhibitory effects. One possibility is that the inhibitory GAGs interact with laminin/L1 in a manner such that the neurite promoting domains of the molecules are inaccessible to the neurons. Detailed analyses of laminin using either proteolytic fragments or synthetic peptides have identified several different structural domains that promote cell adhesion and neurite outgrowth *in vitro* (Edgar et al., 1984; Aumailley et al., 1987; Goodman et al., 1987; Skubitz et al., 1991). Some of these fragments and peptides bind heparin with high affinity (Edgar et al., 1984; Skubitz et al., 1991). The binding of GAGs other than heparin to either intact laminin, proteolytic fragments of laminin or synthetic laminin-derived peptides has not been demonstrated. Thus, it is unlikely that those GAGs with growth inhibitory activity (i.e., CS4, CS6, and KS) are exerting their inhibitory actions through interactions with specific domains of laminin. The lack of any significant effect of heparan sulfate on neurite growth from either cerebellar or DRG neurons on laminin surfaces suggests that either heparan sulfate is not functionally equivalent to heparin or that heparan sulfate binding to laminin does not sterically hinder cellular integrins' access to those domains of laminin that promote neurite extension.

It is not yet known whether there are specific molecular interactions between GAGs and the L1 glycoprotein such as those occurring between some proteoglycan core proteins and L1 (Grumet et al., 1993; Friedlander et al., 1994). The ability of HS and DS to inhibit neurite extension from cerebellar neurons on L1-coated surfaces suggests that such interactions may indeed be taking place although we cannot rule out the possibility that these GAGs are altering the neuronal surface membrane in a manner that prevents L1 from interacting with either its homophilic or heterophilic binding partners whether in *cis* (Kadmon et al., 1990a,b) or in *trans* (Kuhn et al., 1990; Felsenfield et al., 1994).

As there is little direct experimental support for a steric hindrance model of GAG action, we suggest that the inhibitory actions of CS4, CS6, and KS on neurite growth on laminin are due to effects of the GAGs on the neurons themselves. In such a model of GAG action, substrate bound GAGs interact with specific GAG binding proteins that act as receptors for these extracellular ligands. These putative receptors might be linked to cellular second messenger systems such that one consequence of this receptor-ligand interaction would be an interference or dampening of the integrin-dependent activation of C kinase (Bixby, 1989). This alteration in the activation state of C kinase or other second messenger systems would change the neuronal physiology such that neurite elongation is inhibited or proceeds at a slower rate. Such a receptor-ligand model of GAG action could also account for the lack of any significant effect of CS6 and KS on neurite growth from both cerebellar and DRG neurons on L1-coated surfaces. L1-mediated axon growth is likely to involve different intracellular second messenger systems than does growth on laminin substrates (Bixby and Jhabvala, 1992; Ignelzi et al., 1994). Glycosaminoglycan binding to cell surface receptor sites may not interfere with the activation of G proteins or the changes in intracellular calcium levels or pH that follow from L1 activation. In this manner, an array of cellular GAG receptors with different binding specificities and patterns of cellular expression each linked differentially to intracellular signaling mechanisms would provide for the specificity of GAG action reported here and the ability of some GAGs to inhibit laminin-mediated growth without inhibiting L1-mediated growth while other GAGs inhibit L1-mediated neurite growth without effecting laminin-mediated growth.

There is increasing evidence for cell surface binding sites for extracellular GAGs, in particular heparin and HS. First, macrophages appear to have cell surface receptors for heparin whose activation increases the secretion of urokinase-type plasminogen activator (Falcone, 1989). Second, when either GAGs or synthetic GAG-like carbohydrates are added to the medium in which mesencephalic neurons are growing, the GAGs bind to the cell surface in a specific and saturable manner and are subsequently internalized (Lafont et al., 1992, 1994). In this system, soluble GAGs, particularly CS4 and CS6, promote axonal and dendritic growth (Lafont et al., 1992, 1994). Third, heparin and HS bind with high affinity to a site close to the amino terminal of the neuronal cell adhesion molecule NCAM (Cole et al., 1986). Thus it is possible that some of the growth inhibitory actions of GAGs reported here are due to a direct interaction between the GAGs and the target neurons.

In vivo, elongating axons reproducibly avoid certain cells and tissues. For example, in the chick, developing sensory and motor axons avoid the posterior somite, perinotochordal mesenchyme and the precursor of the pelvic girdle (Tosney and Oakley, 1990). In the developing CNS, spinal axons do not cross the dorsal midline and fibers of the optic nerve do not cross the midline of the optic tectum (Snow et al., 1990b). It has been suggested that these specialized areas can be considered barriers to axonal growth (for review, see Steindler, 1993). Peanut lectin binding glycoproteins and several GAGs, particularly CS6 and KS, are molecular markers for these barrier tissues (Steindler and Cooper, 1987; Snow et al., 1990b; Steindler et al., 1990; Cole and McCabe, 1991; Oakley and Tosney, 1991) and CS and KS have been shown to inhibit axon growth *in vitro* (Carbonetto et al., 1983; Snow et al., 1990a; Cole and McCabe, 1991). The data presented here demonstrating a cellular and a substrate specificity to the growth inhibitory properties of isolated GAGs suggests that caution must be used in extrapolating the results of *in vitro* studies to the behavior of growing axons *in vivo*. In cases where an axon is elongating on surfaces containing the L1 glycoprotein, GAGs may have only minor inhibitory effects on axon growth and could even enhance the rate of elongation. On the other hand, the elongation of axons growing in an environment rich in laminin may be quite sensitive to moderate levels of GAGs in their pathway.

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