Inhibition of Neurite Growth by the NG2 Chondroitin Sulfate Proteoglycan

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The chondroitin sulfate proteoglycans (CSPGs) have been implicated as both positive and negative modulators of axonal growth; however, the functional properties of only a few specific CSPGs have been investigated. Here we demonstrate that NG2, an integral membrane CSPG expressed on the surfaces of glial progenitor cells, inhibits neurite growth from neonatal rat cerebellar granule neurons when presented to the cells as a component of the substrate. Growth inhibition occurred when NG2 was mixed with either laminin or L1, two potent promoters of axonal extension. Moreover, when given a choice between surfaces coated with NG2 and laminin or L1, the axons of the cerebellar neurons extended preferentially on laminin or L1 and avoided areas of the substrate containing NG2. The NG2 proteoglycan inhibited neurite growth after digestion with chondroitinase ABC, demonstrating that the inhibitory activity is a property of the core protein and not the covalently attached chondroitin sulfate glycosaminoglycan chains. NG2 also inhibited neurite growth from embryonic rat dorsal root ganglia neurons on substrates containing laminin. However, when the sensory neurons were plated onto surfaces containing the L1 glycoprotein and NG2, neurite growth was not inhibited. These results demonstrate that the NG2 proteoglycan provides an unfavorable substrate for axonal growth. Cells that express this proteoglycan *in vivo* may participate in axonal guidance by defining areas of the developing CNS that are nonpermissive for axonal extension from specific classes of developing neurons.

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

The proper functioning of the nervous system depends upon the precise and highly stereotyped patterns of neuronal connections that form during development. How growing axons find their appropriate targets remains an essential question in developmental neurobiology. Much attention has been paid to molecules that promote neuronal cell adhesion and neurite outgrowth (for review, see Rutishauser and Jessell, 1988). Cell adhesion molecules such as N-cadherin, L1, and N-CAM are abundant in developing tissues (Rutishauser and Jessell, 1988) as are the

extracellular matrix (ECM) associated molecules, fibronectin and laminin (Sanes, 1989). Although these molecules are capable of supporting neurite outgrowth, most neurons show little ability to discriminate among L1, laminin, and N-cadherin *in vitro* (Lemmon et al., 1992).

Recent work from a number of laboratories has identified molecules that can cause growth cone collapse and inhibit neurite extension *in vitro*. These molecules may provide negative cues or signals important in the formation of axonal connections (for review, see Patterson, 1988; Goodman and Shatz, 1993). For example, Luo and colleagues have identified a 100 kDa membrane glycoprotein, termed collapsin, that induces growth cone collapse of chick dorsal root ganglia (DRG) neurons (Luo et al., 1993). Two glycoproteins of 48 kDa and 55 kDa, isolated from embryonic chick somites, also induce the collapse of chick sensory neuron growth cones (Davies et al., 1990). Lastly, Caroni and Schwab (1988a,b) identified two membrane glycoproteins of 250 and 35 kDa from rat CNS myelin that inhibit the growth of rat sensory neurons. With the exception of the myelin associated proteins, which are expressed by oligodendrocytes, most of these putative growth inhibitory molecules have not been associated with specific cell types within the developing nervous system.

One class of macromolecules capable of providing both positive and negative influences over axonal growth are the proteoglycans. These complex molecules consist of a polypeptide chain with covalently attached glycosaminoglycan (GAG) polymers. When complexed to ECM molecules such as laminin, some proteoglycans increase neuronal cell adhesion and axonal elongation (Lander et al., 1985a; Riopelle and Dow, 1990). However, other proteoglycans are nonpermissive for cell attachment and neurite growth (Cole and McCabe, 1991; Oohira et al., 1991; Grumet et al., 1993). Given the molecular diversity of proteoglycans (Herndon and Lander, 1990) and the cellular specificity of their distribution (Levine and Card, 1987; Zarimba et al., 1989; Maeda et al., 1992), the growth-promoting or inhibitory properties of proteoglycans may best be studied by analyzing the effects of individual species of proteoglycans on defined populations of neurons.

One well-characterized CSPG of the CNS is the NG2 proteoglycan. This molecule, which is found on the surfaces of glial progenitor cells in developing and adult tissues (Stallcup et al., 1983; Levine and Card, 1987; Levine et al., 1993), consists of a single core polypeptide of approximately 300,000 Da and two or three chondroitin sulfate chains (Nishiyama et al., 1991). Since glial cells and their precursors may participate in the guidance of growing axons to their targets, we have analyzed the growth modulating properties of the NG2 proteoglycan. Here, we show that developing cerebellar granule neurons extend neu-

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rites poorly on substrates composed of the NG2 proteoglycan and either laminin or L1. This growth inhibitory activity is a property of the NG2 core protein and not the chondroitin sulfate (CS) GAG chains. These data suggest that the developing glial cells that express the NG2 proteoglycan can play a role in axonal targeting by defining regions of the CNS that are nonpermissive for axonal extension.

Materials and Methods

Reagents. Monoclonal antibody D31.10 against the NG2 proteoglycan core protein and polyclonal rabbit anti-NG2 antibodies were described previously (Stallcup et al., 1983; Levine and Stallcup, 1987). Monoclonal antibody 74-5H7 against L1 was generously provided by Dr. V. Lemmon (Lemmon et al., 1989). Poly-L-lysine (PLL), phenylmethylsulfonyl fluoride (PMSF), *N*-ethyl maleimide (NEM), glucose oxidase, lactoperoxidase, chondroitin disaccharide 4-sulfate, chondroitin disaccharide 6-sulfate, normal rabbit serum, and rabbit anti-mouse IgG antiserum were purchased from Sigma. Pansorbin cells and chondroitin sulfate A were purchased from Calbiochem (San Diego, CA). Protease-free chondroitinase ABC and quick spin protein columns were obtained from Boehringer Mannheim (Indianapolis, IN). Keratanase was from Seikagaku America Inc. (Rockville, MD). Laminin and basic fibroblast growth factor (bFGF) were from Upstate Biotechnology Inc. (Lake Placid, NY). Lithium 3,5-diiodosalicylate (LIS) was obtained from Eastman Kodak Company (Rochester, NY). ¹²⁵I-iodine was purchased from ICN (Irvine, CA). Protein assay kit was from Bio-Rad (Richmond, CA). Nitrocellulose (type BA85) was purchased from Schleicher and Schuell (Keene, NH); 48-well tissue culture plates were from Costar (Cambridge, MA). Sprague-Dawley rats were maintained and bred in the university animal facility.

Immunoaffinity purification of NG2 and L1. The NG2 proteoglycan was immunoaffinity purified from B49 cells, a NG2-rich rat neuroglial line (Schubert et al., 1974), using a solid phase immunoabsorbent technique (MacSween and Eastwood, 1978). Briefly, the B49 cells were lysed in 1% Nonidet P40 (NP40), 50 mM Tris, pH 8.0, 0.15 M NaCl containing 2 mM PMSF. The nuclei were pelleted in a microcentrifuge and discarded. The lysate was cleared by incubation with Pansorbin to which normal rabbit serum had been adsorbed. The cleared lysate was incubated for 1 hr at 4°C with Pansorbin that had been preloaded with D31.10 and rabbit anti-mouse IgG and then fixed in 0.5% paraformaldehyde. Following three washes, the NG2 proteoglycan was eluted from the immunoabsorbent in 0.1 M LIS and the LIS was removed with a quick spin protein column that had been equilibrated in PBS plus 0.05% sodium azide. The NG2 preparation was then incubated with rabbit anti-mouse IgG-coated Pansorbin to remove any contaminating mouse immunoglobulins that may have leached off the first immunoabsorbent. In some cases, the B49 cells were surface labeled with ¹²⁵I-iodine by the glucose oxidase-lactoperoxidase method (Hubbard and Cohn, 1972) and NG2 was purified as described above. Protein concentrations were determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. Figure 1*A* shows the intact and chondroitinase ABC-digested NG2 subjected to SDS-PAGE and visualized by Coomassie blue staining. The NG2 appears as a mixture of a broad high-molecular-weight smear and a single polypeptide band of approximately 300 kDa (lane 2). Digestion with chondroitinase ABC, which removes chondroitin sulfate glycosaminoglycans from CSPGs, converted most the smear into 300 kDa component (lane 3).

The L1 glycoprotein was purified from postnatal day 6 rat brain membranes using monoclonal antibody 74-5H7 and the same immunoabsorbent technique. Brain tissue was homogenized in 0.3 M sucrose, 4 mM HEPES, pH 7.4 in the presence of protease inhibitors and centrifuged at 12,000 × *g* for 30 min. The supernatant was then centrifuged at 100,000 × *g* for 45 min to pellet the membranes. The membranes were extracted in 1% deoxycholate, 50 mM Tris, pH 8.0, 0.15 M NaCl plus 2 mM PMSF and 2 mM NEM. The membrane extract was then cleared with normal rabbit serum-coated Pansorbin and immunoprecipitated with 74-5H7-bound immunoabsorbent. For each preparation, the purified proteins were subjected to SDS-PAGE under reducing conditions to monitor the purity and yield of the L1 glycoprotein. Figure 1*B* shows that the L1 preparation contains equivalent amount of two polypeptides of approximately 180 and 140 kDa.

Enzymatic treatment of the NG2 proteoglycan. The NG2 proteoglycan was digested with protease-free chondroitinase ABC in 40 mM Tris, pH

8.0, 40 mM sodium acetate, 0.1 mg/ml BSA. Typically, 4 μg of pure NG2 was incubated with 0.02 U of chondroitinase ABC at 37°C for 1 hr. The reaction mixture was cooled on ice and 1 mM CaCl₂ was added to inactivate the enzyme. In control experiments, NG2 was incubated with keratanase (0.1 U/μg NG2) 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. For every digestion, an aliquot of reaction mixture was run on a 6% polyacrylamide gel to monitor the completeness of digestion.

Substrate preparation. Forty-eight well tissue culture plates were coated with 25 μg/ml of PLL overnight followed by 125 μl of either L1 or laminin, both at 2 μg/ml, or a mixture of the same amount of L1 or laminin and 0.5–10 μg/ml of the NG2 proteoglycan for 3 hr at 37°C. In some experiments, the PLL wells were first coated with L1 or laminin and then coated with NG2 (10 μg/ml). The protein-coated surfaces were washed with PBS before seeding the neurons. To determine the amount of protein that had bound to the PLL-coated surfaces, the PLL wells were coated with ¹²⁵I-labeled protein mixed with unlabeled protein and the amount bound determined by gamma counting of the substrates and/or the washes. The L1 and laminin were labeled with ¹²⁵I-iodine using chloramine T as described (Klinman and Howard, 1980) and ¹²⁵I-labeled NG2 was purified from iodinated B49 cells as described above. In the antibody-blocking experiments, the PLL wells were coated as described above and then, after washing, treated with sterile rabbit anti-NG2 or normal rabbit IgG (both at 2 mg/ml) for 1 hr before seeding the cells.

The growth-modulating properties of NG2 were also evaluated using nitrocellulose-coated dishes (Lagenaur and Lemmon, 1987). Petri dishes (35 mm) were coated with 250 μl of nitrocellulose dissolved in methanol (5 cm² nitrocellulose in 12 ml of methanol) and air dried in a tissue culture hood. One microliter droplets of different protein solutions (laminin, L1, intact NG2 proteoglycan, or the chondroitinase ABC-digested NG2) were spotted onto nitrocellulose surface for 10 min. After aspiration, the entire dish was washed with medium containing 10% fetal bovine serum and blocked in the same medium for 2 hr at 37°C. To create a boundary between NG2 and laminin or L1, we first spotted a small drop (1 μl) of NG2 (40 μg/ml) onto nitrocellulose, aspirated the drop off after 10 min and then spotted a larger droplet (5 μl) of laminin (10 μg/ml) or L1 (40 μg/ml) over the NG2 spot so that a circular border was formed with NG2 in the inner circle and laminin or L1 in the outer annulus. The dish was washed and blocked in medium with serum prior to adding the cells.

Cell cultures. B49 cells were grown in DMEM containing 10% fetal bovine serum (FBS) and expanded every 3 d.

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/mm²) and onto nitrocellulose-coated dishes at 2 × 10⁶ cells/dish in DMEM containing 10% 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 rat fetuses and digested with 0.25% trypsin for 20 min at 37°C. Following washing with serum-containing medium, the ganglia were mechanically dissociated by passage through flame-narrowed Pasteur pipettes. The cells were washed and preplated onto petri dishes in serum-containing medium for 2 hr at 37°C to allow the non-neuronal cells to attach to the substrate. The DRG neurons were harvested, washed, and seeded at 3000 cells/well in DMEM containing 10% FBS and 60 ng/ml nerve growth factor (a gift from Dr. S. Hälegoua). After 24 hr, the cultures were washed in PBS and fixed in 2% glutaraldehyde in PBS.

Immunostaining of protein-coated nitrocellulose substrate. Some cultures were immunofluorescently stained to visualize the protein spots on the nitrocellulose-coated surfaces. After 24 or 48 hr growth, the cultures were washed in PBS and fixed in 3.7% formaldehyde in PBS. Following extensive PBS washes, the dishes were incubated with rabbit anti-NG2 (1:200) or rabbit anti-laminin antibodies (produced in the laboratory, 1:500) for 30 min at room temperature (RT). After 3 washes, the dishes were incubated with FITC labeled goat anti-rabbit IgG (Tago; 1:50) for 30 min at RT. The dishes were then washed in PBS and examined with a Leitz fluorescence microscope to reveal the substrate identities.

Quantitation of neurite length and cell attachment. Cultures were examined with an inverted phase contrast microscope and images were taken with a video camera (MTI 65) and stored in an imaging computer.

Figure 1. Purification of the NG2 proteoglycan and the L1 glycoprotein. Protein samples were electrophoresed on 6% SDS-polyacrylamide gels and visualized by Coomassie blue staining. *A*, Purification of NG2. *Lane 1*, NP40 lysate of B49 cells. *Lane 2*, Immunoaffinity-purified NG2 (2 µg). NG2 appears as a mixture of a high-molecular-weight smear and a single polypeptide band of approximately 300 kDa. *Lane 3*, NG2 digested with chondroitinase ABC (0.02 U, 37°C, 1 hr). Chondroitinase digestion causes the high-molecular-weight smear to disappear and increases the amount of 300 kDa component. *Lane 4*, Chondroitinase ABC and digestion buffer alone. Arrows to the left indicate the mobility of molecular weight standards; from top to bottom, they are 200,000, 116,000, 97,400, and 66,200 Da. *B*, Purification of the L1 glycoprotein. *Lane 1*, Postnatal day 6 rat brain membranes extracted in deoxycholate as described in Materials and Methods. *Lane 2*, Immunoaffinity-purified L1 (2 µg). L1 appears as two polypeptides of approximately 180 and 140 kDa. The mobility of the same molecular weight markers is indicated by the arrows to the left. The low-molecular-weight material in *lane 2* of *A* and *B* is likely to be immunoglobulin light chain that leached off the immunoabsorbents.

For each substrate condition, the number of cells attached and the number of cells with neurites per unit area were determined. The length of a minimum of 50 neurites from duplicate wells for each substrate condition were measured using software from Optimas and Infrascan Inc. Following the suggestion of Lagenaur and Lemmon (1987), a neurite was defined as a process extending from neuronal cell body by more than a cell diameter. Length was measured from the center of a cell body to the tip of the neurite. For cells that had more than one neurites, only the longest neurite was measured. A cumulative neurite length histogram was constructed for each experiment and data from separate experiments were pooled and analyzed. For the tables, percentage 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

NG2 does not support neuronal cell attachment and neurite elongation

To examine the effects of NG2 on cell adhesion and neurite outgrowth, we adsorbed NG2 (40 µg/ml) onto nitrocellulose-coated surfaces (Lagenaur and Lemmon, 1987). Partially purified cerebellar granule neurons were seeded onto these substrates and cell attachment and neurite growth was assessed after 24 hr. As shown in Figure 2*A*, there is almost no cell attachment

or neurite growth on the NG2-coated nitrocellulose. In control experiments, granule neurons were seeded onto nitrocellulose that had been coated with either laminin (10 µg/ml) or L1 (40 µg/ml). As shown in Figure 2*B*, laminin-coated nitrocellulose promoted extensive cell attachment and neurite outgrowth. Although cell attachment to L1-coated nitrocellulose was reduced relative to laminin, neurite outgrowth on the L1-coated nitrocellulose was robust (Fig. 2*C*). The cerebellar granule neurons did not attach to nitrocellulose alone (data not shown). Thus, the NG2 proteoglycan does not support neuronal cell attachment or neurite outgrowth.

NG2 inhibits neurite growth on laminin-coated surfaces

To assay the effects of the NG2 proteoglycan on neurite elongation in the presence of the growth-promoting molecule laminin, we compared the growth of postnatal day 5 or 6 cerebellar granule neurons on surfaces coated with laminin to growth on surfaces coated with laminin and NG2. To determine the concentration of laminin that promotes optimal neurite growth from cerebellar granule neurons, the PLL-coated surfaces were coated with 0.5, 2, 10, 25, or 50 µg/ml of laminin and purified cerebellar granule neurons were seeded onto these substrates.

Figure 2. NG2 does not support cell adhesion and neurite outgrowth. Partially purified neonatal cerebellar granule neurons were seeded onto nitrocellulose-coated 35 mm dishes spotted with various proteins as described in Materials and Methods and were allowed to grow for 24 hr. *A*, Cells on the NG2 (40 µg/ml)-coated nitrocellulose surface. *B*,

Neurite length was determined after 24 hr. On surfaces coated with 0.5 µg/ml of laminin, the mean neurite length was 60 µm. The mean neurite length increased with increasing laminin concentrations and reached a plateau at 10 µg/ml of laminin with a mean length of 95 µm. Surfaces coated with 2 µg/ml of laminin resulted in 90% of the maximal stimulation of neurite growth (mean length = 85 µm). Therefore, in the experiments described below, we used laminin at a concentration of 2 µg/ml. We determined the amount of the input laminin that was bound to the substrate using ¹²⁵I-laminin as described in Materials and Methods. At 2 µg/ml, approximately 65% of the input laminin or 0.2 µg/cm² of laminin bound to the PLL-coated surfaces. When surfaces were coated with the same concentration of laminin mixed with the NG2 proteoglycan at a concentration of 10 µg/ml, 55% of the input laminin and 65% of the input NG2 bound to the PLL-coated surfaces. Thus, the amounts of laminin bound were sufficient to promote neurite outgrowth from cerebellar granule neurons under all conditions tested.

The cerebellar granule cells attached equally well to the laminin-coated surfaces and surfaces coated with a mixture of laminin and the NG2 proteoglycan (Table 1). However, whereas 52% of the attached cells extended neurites on laminin surfaces, only 27% of the cells did so on surfaces composed of laminin and the NG2 proteoglycan (Table 1). On the laminin and NG2 surfaces, the neurites were significantly shorter than on laminin alone. The results from 12 independent experiments are summarized in Figure 3*A*. Fifty percent of all neurites measured were greater than 80 µm on the laminin substrates whereas on surfaces coated with laminin plus NG2 only 12% were longer than 80 µm. Despite this difference in neurite length, there were no obvious morphological differences between the cells grown on laminin alone and those grown on laminin mixed with the NG2 proteoglycan (compare Fig. 4*A,B*). When surfaces were coated first with laminin and then with NG2 rather than coated with a mixture of these two molecules, both the percentage of cells that extended neurites (34%) and the mean neurite length (54 µm) were decreased compared to cells growing on laminin alone (Table 1, Fig. 3*A*). This control experiment demonstrates that the NG2 proteoglycan need not interact with laminin in solution in order to neutralize the growth-promoting effects of laminin.

To demonstrate that the growth inhibitory effects observed are due to the NG2 proteoglycan, we treated the NG2/laminin-coated surfaces with rabbit anti-NG2 antibodies and compared the growth of granule neurons on these substrates with growth on control surfaces that had been coated with laminin and treated with the same antibodies. As shown in Table 1, the mean neurite length on antibody-treated NG2/laminin surfaces (69 µm) was not statistically different from the mean neurite length on the antibody-treated laminin control surfaces (72 µm). When nonimmune rabbit IgG was used in place of the anti-NG2 IgG, neurite growth was inhibited to approximately the same extent as in cultures grown without any antibodies present (Table 1). These results indicate that the rabbit anti-NG2 antibodies can

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Cells on laminin (10 µg/ml)-coated substrate. *C*, Cells on L1 (40 µg/ml)-coated surfaces. Whereas cell attachment and neurite growth was extensive on laminin-coated nitrocellulose, there is almost no adhesion on the NG2-coated surfaces. Neurite outgrowth was robust on L1-coated surfaces although cell attachment was reduced relative to laminin-coated nitrocellulose. Scale bar, 50 µm.

Figure 3. Quantitative analysis of neurite growth. *A*, A cumulative neurite length histogram showing the distribution of neurite lengths of cerebellar neurons grown on laminin alone, laminin mixed with NG2, laminin mixed with NG2 that had been digested with chondroitinase ABC, and laminin followed by NG2. 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). *B*, Distribution of neurite lengths of cells grown on L1, L1 mixed with NG2, L1 mixed

abolish the inhibitory activity of the NG2 proteoglycan and provide evidence that the NG2 proteoglycan is responsible for the inhibition of neurite outgrowth on laminin-coated surfaces.

NG2 inhibits neurite extension on L1 substrates

The data presented above demonstrate that the NG2 proteoglycan can inhibit the potent growth-promoting effects of laminin. However, little laminin has been detected in the developing CNS (Rogers et al., 1986; Letourneau et al., 1988). Rather, many CNS neurons extend their axons in areas rich in the L1 glycoprotein (Faissner et al., 1984; Stallcup et al., 1985). Therefore, we evaluated the ability of the NG2 proteoglycan to influence axonal elongation on L1-coated surfaces.

We quantitatively analyzed the neurite growth from cerebellar granule neurons on surfaces coated with 0.5, 1, 2, 10, or 25 $\mu\text{g/ml}$ of immunoaffinity-purified L1. In agreement with previous studies using chick and mouse L1 (Lagenaur and Lemmon, 1987; Lemmon et al., 1989), the L1 glycoprotein isolated from rat brain membranes (see Fig. 1*B*) promoted extensive neurite outgrowth. Maximal neurite growth was obtained with 2 $\mu\text{g/ml}$ of L1 (mean = 95 μm). At this concentration approximately 80% of the input L1 bound to the substrate. Higher concentrations of L1 resulted in a slight inhibition of neurite growth (data not shown). This inhibition may be due to contaminating polypeptides in the L1 preparation or to the small amounts of anti-L1 antibody that may leach off the immunoaffinity resins. Accordingly, we carried out all the following experiments using L1 at 2 $\mu\text{g/ml}$.

As was the case with laminin-coated surfaces, the granule neurons attached equally well to the L1 or the NG2/L1-coated substrates (Table 1, Fig. 4*D,E*). On the L1-coated surfaces, 47% of the cells established neurites whereas on surfaces coated with L1 and the NG2 proteoglycan, only 20% of the attached cells extended neurites. As shown in Figure 3*B* and Table 1, neurite growth on the L1/NG2-coated surfaces was reduced significantly relative to the growth on surfaces coated with L1 alone. The inhibition of neurite growth by NG2 occurred in a dose-dependent manner (Fig. 3*C*). Little or no inhibition was seen when the NG2 was used at 0.5 $\mu\text{g/ml}$. The percentage of inhibition increased with increasing concentration of NG2, reaching a maximum of 45–50% inhibition when 10 $\mu\text{g/ml}$ of NG2 was mixed with 2 $\mu\text{g/ml}$ of L1. To determine whether NG2 needs to interact with L1 in solution in order to inhibit neurite growth, we employed the sequential coating procedure described above. As shown in Table 1 and Figure 3*B*, this sequential coating procedure resulted in the same degree of inhibition of neurite extension as did coating surfaces with a mixture of L1 and NG2. Treatment with rabbit anti-NG2 antibodies abolished the inhibitory effects of the L1/NG2-coated substrates (Table 1). Taken together, these experiments demonstrate that the NG2 proteoglycan is a potent inhibitor of neurite outgrowth from cerebellar granule neurons on L1-coated surfaces, a molecule which is abundant within the developing cerebellar molecular layer (Faissner et al., 1984; Stallcup et al., 1985).

with NG2 that had been digested with chondroitinase ABC, and L1 followed by NG2. The error bars in *A* and *B* are SDs. Laminin and L1 were used at 2 $\mu\text{g/ml}$ and NG2 used at 10 $\mu\text{g/ml}$ in both *A* and *B*. *C*, Dose-response curve of the NG2 proteoglycan in the presence of L1. Percentage of inhibition by NG2 on L1-coated surfaces (*y*-axis) was plotted against concentration of NG2 (*x*-axis). L1 was used at 2 $\mu\text{g/ml}$ throughout.

Figure 4. Appearance of neonatal cerebellar cells. Cells were plated onto PLL-coated tissue culture wells containing various substrates and grown for 24 hr as described in Materials and Methods. The substrates are as follows: *A*, laminin; *B*, laminin mixed with the intact NG2 proteoglycan; *C*, laminin mixed with chondroitinase ABC-digested NG2; *D*, L1; *E*, L1 mixed with intact NG2; *F*, L1 mixed with chondroitinase ABC-digested NG2. Laminin and L1 were used at 2 $\mu\text{g}/\text{ml}$ and NG2 at 10 $\mu\text{g}/\text{ml}$ throughout. Note that the neurites are significantly shorter on surfaces containing either intact or chondroitinase ABC-digested NG2 than on laminin or L1 alone. Scale bar, 25 μm .

Inhibitory activity of the NG2 core protein

The GAG moieties of proteoglycans have been shown to exert both negative and positive influences over axonal growth *in vitro* (Snow et al., 1990; Cole and McCabe, 1991; Lafont et al., 1992). To determine whether the CS GAG chains are required for the growth inhibitory activity of the NG2 proteoglycan, we digested immunoaffinity-purified NG2 with either chondroitinase ABC or keratanase and assayed the ability of the digestion products to modulate the growth of cerebellar granule neurons. Figure 1*A* shows that digestion with chondroitinase ABC causes the high-molecular-weight smear component of NG2 to disappear and increases the relative amount of the 300 kDa component. As described previously (Stallcup et al., 1983; Nishiyama et al., 1991), the high-molecular-weight smear represents the intact proteoglycan and the 300 kDa component is the glycosylated core protein. Digestion of NG2 with keratanase had no effect on the electrophoretic mobility of the high-molecular-weight

smear (not shown), indicating a lack of keratan sulfate chains in the molecule.

When cerebellar granule neurons were plated onto surfaces coated with laminin mixed with chondroitinase ABC-digested NG2, cell attachment did not differ significantly from attachment to surfaces coated either with laminin alone or with laminin and undigested NG2 (Table 1). Neurite outgrowth was reduced by 46% in agreement with the studies described above using the intact NG2 proteoglycan (Fig. 3*A*, Table 1). As shown in Figure 4*C*, the morphology of cells grown on laminin mixed with the NG2 core protein was identical to that of cells grown on laminin mixed with the intact proteoglycan. Thus, the NG2 core protein is sufficient to inhibit neurite growth on laminin-coated surfaces.

The experiments described above suggest that the CS chains of the NG2 proteoglycan are not required for its growth inhibitory activity when mixed with laminin. To determine whether growth inhibition in the presence of the L1 glycoprotein is also

Table 1. Inhibition of neurite growth from cerebellar neurons by the NG2 proteoglycan

Substrate	Cells/mm ²	Cell with neurites/mm ²	Neurite length (μ m)	% Inhibition
Laminin (2 μ g/ml)	95 $\pm$ 24	49 $\pm$ 15	85 $\pm$ 12	
Laminin + NG2 (10 μ g/ml)	86 $\pm$ 21	23 $\pm$ 10 ^b	48 $\pm$ 11 ^b	44
Laminin + NG2 (C'ase digested)	88 $\pm$ 22	26 $\pm$ 11 ^b	46 $\pm$ 5 ^b	46
Laminin then NG2 ^a	81 $\pm$ 22	28 $\pm$ 13 ^b	54 $\pm$ 7 ^b	36
Laminin + RantiNG2	94 $\pm$ 16	45 $\pm$ 13	72 ^c	
Laminin + NG2 + RantiNG2	103 $\pm$ 29	46 $\pm$ 15	69 ^c	4
Laminin + NG2 + NRS	ND	ND	49 ^{b,c}	32
L1 (2 μ g/ml)	102 $\pm$ 21	48 $\pm$ 11	95 $\pm$ 6	
L1 + NG2 (10 μ g/ml)	100 $\pm$ 38	20 $\pm$ 9 ^b	50 $\pm$ 5 ^b	47
L1 + NG2 (C'ase digested)	107 $\pm$ 42	23 $\pm$ 11 ^b	54 $\pm$ 7 ^b	43
L1 then NG2 ^a	113 $\pm$ 11	23 $\pm$ 4 ^b	57 ^{b,c}	40
L1 + RantiNG2	84 $\pm$ 14	33 $\pm$ 6	78 $\pm$ 3	
L1 + NG2 + RantiNG2	80 $\pm$ 9	27 $\pm$ 9	74 $\pm$ 2	5
L1 + NG2 + NRS	ND	ND	46 ^{b,c}	41

Substrates were prepared and neurite outgrowth determined as described in Materials and Methods. Laminin and L1 were used at 2 μ g/ml and NG2 was used at 10 μ g/ml throughout. RantiNG2, rabbit IgG directed against NG2 core protein; NRS, normal rabbit serum. Both were used at 2 mg/ml. Data shown are the means $\pm$ SD from 3–12 separate experiments for each condition. ND, not determined; "C'ase digested" indicates digestion with chondroitinase ABC.

^a Sequential coating as described in Materials and Methods.

^b $p < 0.001$ (Student's t test); where not indicated, the numbers are not statistically different from the appropriate controls.

^c Mean from duplicate wells of a single experiment.

independent of the GAG chains, we tested the ability of both chondroitinase ABC- and keratanase-digested NG2 to alter neurite outgrowth on surfaces coated with PLL and L1. As shown in Figure 3*B* and Table 1, the inhibitory activity of the chondroitinase ABC-digested NG2 was quantitatively identical to that of the intact proteoglycan (also compare Fig. 4*E,F*). Digestion of NG2 with keratanase had no effect on the ability of the NG2 proteoglycan to inhibit neurite outgrowth on L1-coated surfaces (data not shown). To rule out any nonspecific effects of the GAG lyases on L1-coated surfaces, we treated L1-coated substrates with calcium inactivated chondroitinase ABC and then plated cerebellar granule neurons onto these surfaces. In those experiments, 41% of attached cells extended neurites that had a mean length of 90 μ m, a number that is not statistically different from that on control, L1-coated surfaces. Since digestion of NG2 with chondroitinase ABC may leave short stubs of CS attached to the core protein, we also tested the ability of chondroitin disaccharide sulfates to inhibit neurite growth on laminin and L1. Neither substrate-bound chondroitin disaccharide 4-sulfate nor chondroitin disaccharide 6-sulfate at 10–100 μ g/ml inhibited the growth of cerebellar granule neurons (data not shown). Taken together, these experiments suggest that the ability of the NG2 proteoglycan to inhibit the growth of cerebellar granule neurons under a variety of substrate conditions is a specific property of the core protein and is not necessarily associated with the covalently attached GAG chains.

The findings described above that implicate the NG2 core protein and not the GAG chains as an inhibitor of neurite outgrowth are in conflict with previous studies which suggested that the GAG chains are required for growth inhibition by proteoglycans (Snow et al., 1990; Cole and McCabe, 1991). These discrepancies could be due to differences between either the type of substrates or the type of neurons used in our experiments and in previous studies. We therefore tested the effects of purified GAGs on neurite outgrowth from neonatal cerebellar gran-

ule neurons. As shown in Table 2, chondroitin sulfate, over a wide range of concentrations, inhibited the growth of cerebellar granule neurons on laminin-coated surfaces. Digestion of CS GAG with chondroitinase ABC completely reversed this inhibition of neurite outgrowth. However, CS GAG was much less effective as a growth inhibitor when tested on the L1-coated surfaces. For example, whereas CS GAG at 10 μ g/ml caused a 32% reduction in the mean neurite length on laminin substrates, a 10-fold higher concentration of chondroitin sulfate did not have a significant effect on neurite outgrowth on the L1-coated surfaces (Table 2). When CS GAG was used at 1 mg/ml, however, neurite growth on L1-coated surfaces was inhibited. These results are consistent with the notion that the CS GAG chains of the NG2 proteoglycan are not required for an inhibition of neurite growth on L1-coated surfaces. It appears that both the NG2 core protein and the CS GAGs can each independently inhibit neurite outgrowth on laminin-coated surfaces.

Neurites avoid the NG2 substrate

In vitro, elongating growth cones will avoid substrates composed of inhibitory or repellent molecules (Walter et al., 1987; Caroni and Schwab, 1988a; Fawcett et al., 1989; Pesheva et al., 1989, 1993; Faissner and Kruse, 1990; Fichard et al., 1991). To determine whether elongating axons avoid surfaces coated with the NG2 proteoglycan, we examined the patterns of neurite growth from cerebellar granule neurons at borders of NG2 and laminin or L1. These borders were created by spotting a larger aliquot of either laminin or L1 over a smaller NG2 spot on nitrocellulose as described in Materials and Methods. Figure 5, *A* and *C*, demonstrates that, in a choice situation, cerebellar cells attach to the laminin- or L1-coated substrates and very few cells adhere to those areas where the NG2 proteoglycan was spotted. Neurites growing on these substrates extended over the laminin- or L1-containing annular surrounds and did not grow over the border between the laminin- or L1-containing annulus

Table 2. Chondroitin sulfate inhibits neurite growth on laminin but not on L1-coated surfaces

Substrate	Cells/mm ²	Cells with neurites/mm ²	Neurite length (μm)	% Inhibition
Laminin (2 μg/ml)	95 ± 24	49 ± 15	85 ± 12	
Laminin + CS (10 μg/ml)	80 ± 17	27 ± 9 ^a	58 ± 7 ^a	32
Laminin + CS (10 μg/ml, digested) ^b	89 ± 15	43 ± 12	78	8
Laminin + CS (100 μg/ml)	88 ± 19	16 ± 10 ^a	49 ± 5 ^a	42
Laminin + CS (1 mg/ml)	74 ± 24	9 ± 6 ^a	48 ± 11 ^a	44
L1 (2 μg/ml)	102 ± 21	48 ± 11	95 ± 6	
L1 + CS (10 μg/ml)	98 ± 9	59 ± 13	94 ± 9	1
L1 + CS (100 μg/ml)	92 ± 14	57 ± 10	97 ± 11	0
L1 + CS (100 μg/ml, digested) ^b	102 ± 14	37 ± 5	97	0
L1 + CS (1 mg/ml) ^b	92 ± 17	16 ± 4 ^a	47 ^a	50

Cerebellar granule neurons were purified as described in Materials and Methods and plated into 48-well plates containing the indicated substrates. After 24 hr, cell attachment and neurite length were measured as described in Materials and Methods. Data shown are the mean and SD from at least three separate experiments. The data for growth on laminin and L1 alone are from Table 1. CS, chondroitin sulfate (a mixture containing approximately 90% chondroitin 4-sulfate and 10% chondroitin 6-sulfate). "Digested" indicates treatment of chondroitin sulfate with chondroitinase ABC.

^a $p < 0.001$ (Student's t test); where not indicated, values are not statistically different from the appropriate controls.

^b Data from duplicate wells of a single experiment.

and the NG2-containing center. This avoidance of the NG2-coated substrate was maintained for at least 48 hr in culture at which time long neurites extended over those parts of the dish coated with the L1 glycoprotein (Fig. 5D). Immunofluorescence staining with rabbit anti-NG2 antibodies confirmed that the NG2 proteoglycan was present in the central area but not in the surrounding annulus (data not shown). NG2 that had been digested with chondroitinase or keratanase exhibited a similar inhibitory activity (data not shown). When nitrocellulose surfaces were spotted first with BSA at 40 μg/ml and then with laminin, no border effects were observed. Cells attached uniformly to the entire area and neurites grew in both the central area and in the surrounding annulus (not shown). These results demonstrate that in a choice situation, cerebellar granule neurons and their processes avoid areas containing either the intact NG2 proteoglycan or its core protein.

NG2 effects on dorsal root ganglia neurons

To determine whether the NG2 proteoglycan can inhibit the growth of other types of neurons, we tested the ability of the

NG2 proteoglycan to modulate neurite elongation from embryonic DRG neurons. As shown in Figure 6A and Table 3, extensive neurite growth occurred on surfaces coated with 2 μg/ml of laminin. However, on surfaces that had been coated with a mixture of laminin and the NG2 proteoglycan (10 μg/ml), neurite length was reduced significantly (Table 3). The morphology of DRG neurons growing on the NG2-containing surfaces differed from that on the control surfaces. On laminin, most of the DRG neurons extended one or two neurites with 0–3 branches from their cell bodies. On surfaces coated with laminin and the NG2 proteoglycan, the neurons gave rise to short neurites with many filopodia extending from both the growth cones and neurite shafts (compare Fig. 6A,B). Surprisingly, the NG2 proteoglycan had no significant effects on the growth of DRG neurons on L1-coated surfaces. Neurons extended neurites equally well on surfaces coated either with L1 alone or with L1 and NG2 (Table 3) and the cells growing on both surfaces were morphologically indistinguishable (compare Fig. 6C,D). These results suggest that the NG2 proteoglycan specifically inhibits the growth of cerebellar neurons but not DRG neurons on the

Table 3. NG2 inhibits the growth of dorsal root ganglia neurons on laminin but not on L1-coated surfaces

	Neurons/mm ²	Cells with neurites/mm ²	Neurite length (μm)	% Inhibition
Laminin (2 μg/ml)	10.2 ± 2.1	7.5 ± 1.7	189 ± 32	
Laminin + NG2 (10 μg/ml)	8.2 ± 2.3	3.5 ± 0.7 ^a	87 ± 2 ^a	54
Laminin + NG2 (C'ase digested)	12.2 ± 2.8	4.3 ± 0.9 ^a	78 ^{a,b}	59
L1 (2 μg/ml)	8.9 ± 1.8	5.8 ± 0.9	110 ± 9	
L1 + NG2 (10 μg/ml)	9.1 ± 1.5	5.4 ± 0.9	119 ± 15	0

Dorsal root ganglia neurons were removed from ED15 rat embryos and seeded into 48-well plates at a density of 3000 neurons per well as described in Materials and Methods. After 24 hr, cell attachment and neurite length were measured. Data shown are the mean and SD from at least three separate experiments. "C'ase digested" indicates treatment with chondroitinase ABC.

^a $p < 0.001$ (Student's t test); the mean neurite length on L1 + NG2 is not statistically different from that on L1 alone.

^b Mean length from duplicate wells of a single experiment.

Figure 5. Neurites avoid NG2-containing substrates in a choice situation. Neonatal cerebellar granule neurons were seeded onto nitrocellulose-coated dishes containing NG2 in the center (3-mm-diameter circle) and laminin or L1 in a surrounding annulus as described in Materials and Methods. The cultures were photographed after 24 and 48 hr growth. *A*, A low-magnification photograph showing the top half of such an annular arrangement 24 hr after plating the cells. Few cells adhered to the NG2-containing central area and cells attached extensively to the laminin-containing annulus. *B*, An enlargement of the area indicated by the arrow in *A*. Note that neurites do not grow into the NG2-containing central area. *C*, A small section of an annular arrangement containing L1 to the left and NG2 in the center (shown on the right). This culture was photographed after 24 hr growth, at which time neurites extended on the L1-containing surround but not on the NG2-containing center. *D*, After 48 hr growth, neurites ramify extensively on the L1-containing surround (*bottom half of D*) and do not cross into the NG2-containing central area (shown to the top). Scale bars: *A*, 250 μ m; *B–D*, 50 μ m.

L1-coated surfaces. On the other hand, the NG2 proteoglycan can inhibit the growth of both cerebellar and sensory neurons on laminin-coated surfaces.

Discussion

The proteoglycans of the nervous system comprise a highly diverse group of multifunctional macromolecules (Herndon and Lander, 1990). Although the chondroitin sulfate and keratan sulfate proteoglycans have attracted much attention recently as possible endogenous inhibitors of axonal growth (Snow et al., 1990; Cole and McCabe, 1991), the observations that proteoglycans can have both positive and negative effects on neurite growth (Iijima et al., 1991; Oohira et al., 1991) suggest that the biological properties of proteoglycans and their covalently attached GAG chains are complex. Here we have shown that NG2, a membrane-bound proteoglycan associated with the surfaces of O2A^{neonatal} and O2A^{adult} progenitor cells (Levine et al., 1993), inhibits the growth of neonatal cerebellar granule neurons on substrates composed of either laminin or L1. When granule neurons were given a choice between NG2-coated surfaces and laminin- or L1-coated surfaces, the neurites extended prefer-

entially on laminin/L1 and appeared to avoid areas of the substrate containing NG2. The growth inhibitory activity was associated with the NG2 core protein and not the covalently attached CS GAG chains. The growth of DRG neurons was also inhibited by the NG2 proteoglycan; however, in this case, axonal growth was inhibited in the presence of laminin but not in the presence of the L1 glycoprotein. Thus, the developing glial precursor cells that express the NG2 proteoglycan *in vivo* are capable of defining areas of the developing CNS that are nonpermissive for axonal growth from selective populations of neurons.

The NG2 proteoglycan is a membrane spanning CSPG that was first identified with antibodies raised against the B49 cell line, a rat cell line with properties of both neurons and glial cells (Schubert et al., 1974; Wilson et al., 1981). There is no evidence for either dermatan sulfate (Stallcup et al., 1990) or keratan sulfate chains (Stallcup et al., 1983) in the NG2 proteoglycan. The primary sequence of the NG2 core protein suggests that it is a unique molecule and is not related to other families of either membrane spanning or extracellular proteoglycans (Nishiyama et al., 1991). Little is known about the functional properties of the NG2 proteoglycan. In previous studies, NG2 has been shown

Figure 6. NG2 inhibits the growth of DRG neurons on laminin but not on L1-coated surfaces. Embryonic rat DRG neurons were seeded onto various substrates and allowed to grow for 24 hr as described in Materials and Methods. *A*, Laminin substrate. Most neurons extended one or two long neurites. Notice the small growth cones with long filopodia (arrowhead). *B*, Laminin mixed with NG2. The neurites are significantly shorter than those on laminin alone. Numerous filopodia extend from flattened growth cones (arrowhead). *C*, L1. The DRG neurons extended one or two neurites with large growth cones characterized by lamellipodia. *D*, L1 mixed with NG2. The neurons extended long neurites that were indistinguishable from cells grown on L1 alone. Laminin and L1 were used at 2 $\mu\text{g}/\text{ml}$ and NG2 used at 10 $\mu\text{g}/\text{ml}$ throughout. Scale bar, 50 μm .

to associate with type VI collagen, although the significance of this association for developing neurons and glial cells is unclear since type VI collagen has not been detected in brain (Stallcup et al., 1990).

The data presented here demonstrate that NG2 can inhibit neurite growth *in vitro*. Because surfaces coated with NG2 alone are nonadhesive for dissociated cerebellar cells, we used an experimental design in which the effects of NG2 on neurite extension were studied independent from any possible effects on cell adhesion. Under these conditions, the inhibition of axon growth from cerebellar neurons on L1-coated surfaces was con-

centration-dependent with half maximal inhibition occurring when 3–5 $\mu\text{g}/\text{ml}$ of NG2 was mixed with 2 $\mu\text{g}/\text{ml}$ of L1. Thus, NG2 is active at concentrations (5–8 nM) comparable to those used in other studies of the growth inhibitory properties of brain proteoglycans (Cole and McCabe, 1991; Oohira et al., 1991) and between 200 and 300 times lower than that used in studies of a cartilage proteoglycan (Snow et al., 1990). The NG2 proteoglycan was an effective inhibitor of neurite growth on laminin at the same low concentrations.

The growth inhibition described here is dependent upon NG2 core protein and not the covalently attached CS GAG chains.

Digestion of the NG2 proteoglycan with either chondroitinase ABC or keratanase did not alter the ability of this molecule to inhibit neurite growth. In the case of laminin, CS GAG chains and the NG2 core protein each independently inhibited neurite growth. Laminin is a multifunctional molecule and contains an integrin-binding domain and several heparin-binding domains, all of which are capable of supporting cell attachment and neurite growth (Ignatius and Reichardt, 1988; Skubitz et al., 1988, 1991; Hall et al., 1990). Cerebellar neurons are thought to attach to and extend neurites on laminin via both integrin receptors and cell surface heparin sulfate proteoglycans (Werz and Schachner, 1988). The ability of some species of proteoglycans to interact with and modulate the functional properties of laminin is well established (Lander et al., 1985b; Riopelle and Dow, 1990). Therefore, blockage of any of the neurite-promoting domains in laminin by the NG2 core protein could result in a partial inhibition of neurite extension such as reported here. This blockage could result from either a direct molecular interaction between the NG2 core protein and the neurite-promoting domains on laminin or from NG2 binding elsewhere on laminin but sterically hindering granule neurons' access to the neurite-promoting domains.

In addition to inhibiting neurite growth on laminin, the NG2 core protein inhibited neurite growth on L1, a homophilic cell-cell adhesion molecule that, unlike laminin, is abundant within the developing CNS (Faissner et al., 1984; Stallcup et al., 1985; Letourneau et al., 1988). The observation that CS and other GAGs are extremely weak inhibitors of neurite growth on L1 (Dou and Levine, 1993) suggests that the inhibition of growth on L1 surfaces may be the result of an interaction between L1 and the NG2 core protein. The L1 glycoprotein has been shown recently to have several extracellular domains that can independently promote neurite outgrowth from cerebellar neurons (Appel et al., 1993). As was the case with laminin, an interaction between the NG2 core protein and any of these neurite-promoting domains could disrupt the homophilic interactions between cell surface and substrate-bound L1 and consequently inhibit neurite outgrowth. Interestingly, the core protein of neurocan, a soluble CSPG of the developing brain that is inhibitory to neuronal cell adhesion, also interacts with L1 (Grumet et al., 1993). The primary sequence of neurocan, however, shows no homologies with the primary sequence of NG2 (Nishiyama et al., 1991; Rauch et al., 1992).

The hypothesis that the NG2 proteoglycan can disrupt L1-L1 interactions provides a simple molecular mechanism to explain our results with cerebellar neurons but fails to explain why NG2 is not an effective inhibitor of the growth of embryonic DRG neurons on L1-coated surfaces. Although substrate-bound L1 is a ligand for cell surface L1 (Lemmon et al., 1989), other CAMs such as axonin-1, TAG-1, and F3/11 can also serve as ligands for L1 (Kuhn et al., 1991; Pesheva et al., 1993; Felsenfeld et al., 1994). If embryonic rat DRG neurons used axonin-1 to interact with L1, as has been shown for chick sensory neurons, and if the domains of L1 to which axonin-1 binds were different than the domains needed for L1-L1 interactions, this would explain the failure of the NG2 proteoglycan to inhibit axon extension from this particular neuronal type.

An alternate explanation for the growth inhibition reported here is that, rather than preventing cellular access to neurite-promoting domains of laminin and L1, substrate-bound NG2 interacts with neuronal cell surface molecules that act as receptors for NG2. The consequence of this putative receptor-ligand

interaction may be a quenching of the actions of intracellular second messenger systems that are activated by cellular binding to laminin and L1 (Bixby, 1989; Schuch et al., 1989). Although the ability of our rabbit anti-NG2 antibodies to neutralize the growth inhibitory effects of NG2 would seem to favor such a ligand-receptor-second messenger model of NG2 action, these same antibodies could induce a conformational change in NG2 allowing neurons access to domains of laminin or L1 that mediate neurite growth. Experiments are currently under way to determine the mechanisms by which NG2 causes the growth inhibition reported here.

The finding that the NG2 core protein is functionally active as an inhibitor of neurite growth is consistent with previous studies demonstrating that the core proteins of rat brain CSPGs can inhibit neurite growth and cell attachment (Oohira et al., 1991; Grumet et al., 1993) but is in conflict with other studies demonstrating an inhibitory function for the GAG moieties of proteoglycans not the core proteins (Snow et al., 1990; Cole and McCabe, 1991). Differences in experimental design and reagents may explain this discrepancy. For example, Snow et al. (1990) used relatively high concentrations of a cartilage proteoglycan to inhibit the growth of embryonic chick DRG neurons. The CS and KS GAG chains make up a larger proportion (90%) of the total mass of the cartilage proteoglycan than do the CS GAGs of NG2 (Hascall et al., 1991; Nishiyama et al., 1991) so that surfaces coated with the cartilage proteoglycan would contain a higher density of negative charges when compared to surfaces coated with NG2. These negative charges could discourage cell attachment and neurite extension.

The distribution of the NG2 proteoglycan during neuronal development has been studied extensively in the rat cerebellar cortex (Levine and Card, 1987; Levine et al., 1993). There, NG2-positive glial precursor cells are initially found within the internal granule layer and at the level of the Purkinje cell bodies. The superficial aspects of the developing molecular layer are relatively free of NG2-expressing cells. Thus, as the newly generated granule cells extend their axons through the molecular layer (Altman, 1972), they do so above a layer of NG2-positive cells (Bicknese and Levine, unpublished observations). Such a topographical arrangement is consistent with both the growth inhibitory properties of the NG2 proteoglycan and the observation that neurites appear to avoid substrates containing the NG2 proteoglycan. This suggests that NG2-positive glial cells may contribute to cerebellar morphogenesis by helping to keep growing parallel fibers stacked within the molecular layer (Altman, 1972). Expression of the NG2 proteoglycan is also transiently increased after acute brain injury in adult animals (Levine, 1994). Thus, in addition to playing a growth modulatory role during development, the NG2 proteoglycan may also contribute to the failure of damaged CNS neurons to regenerate successfully.

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