

# Interaction of Astrochondrin with Extracellular Matrix Components and Its Involvement in Astrocyte Process Formation and Cerebellar Granule Cell Migration

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**Abstract.** We have recently characterized a chondroitin sulfate proteoglycan from the murine central nervous system which is expressed by astrocytes in vitro and carries the L2/HNK-1 and L5 carbohydrate structures. In the present study, we provide evidence that its three core proteins of different size are similar in their proteolytic peptide maps and thus designate this group of structurally related molecules astrochondrin.

During development, astrochondrin and the L5 carbohydrate were hardly detectable in the brain of 14-d-old mouse embryos by Western blot analysis. Expression of astrochondrin and the L5 epitope was highest at postnatal day 8, the peak of cerebellar granule cell migration and Bergmann glial process formation, and decreased to weakly detectable levels in the adult. Immunocytochemical localization of astrochondrin in the cerebellar cortex of 6-d-old mice showed association of immunoreactivity with the cell surface of astrocytes, including Bergmann glial processes and astrocytes in the internal granular layer or prospective white matter. Endfeet of astrocytes contacting the basal lamina of endothelial and meningeal cells and contact sites between Bergmann glial processes and granule cells also showed detectable levels of astrochondrin. Furthermore, granule cell axons in the molecular layer were astrochondrin immunoreactive. In the adult, astrochondrin immunoreactivity was weakly present in the internal granular layer and white matter.

Both Fab fragments of polyclonal antibodies to astrochondrin and monovalent fragments of the L5 mono-

clonal antibody reduced the formation of processes of mature GFAP-positive astrocytes on laminin and collagen type IV, but not on fibronectin as substrata. Interestingly, the initial attachment of astrocytic cell bodies was not disturbed by these antibodies. Antibodies to astrochondrin also reduced the migration of granule cells in the early postnatal mouse cerebellar cortex.

In a solid phase radioligand binding assay, astrochondrin was shown to bind to the extracellular matrix components laminin and collagen type IV, being enhanced in the presence of  $Ca^{2+}$ , but not to fibronectin, J1/tenascin or other neural recognition molecules. Furthermore, astrochondrin interacted with collagen types III and V, less strongly with collagen types I, II, and IX, but not with collagen type VI. The interaction of astrochondrin with collagen types III and V was saturable and susceptible to increasing ionic strength, and could be competed by chondroitin sulfate, heparin, and dextran sulfate, but not by hyaluronic acid, glucose-6-phosphate, or neuraminic acid.

These observations indicate that astrochondrin may contribute to morphogenetic processes in the developing central nervous system by supporting the interaction of astrocytes with extracellular matrix components, such as laminin and certain collagen types, thus influencing their capacity to extend their processes, possibly by interacting with the basal lamina of meninges and blood vessels. These interactions appear to be, at least in part, dependent on the L5 carbohydrate epitope.

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**A**STROCYTES participate in numerous processes essential for the morphogenesis and functional maintenance of the central nervous system (for reviews see

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Vernadakis, 1988; Jessen and Mirsky, 1990; Hertz et al., 1990). In the developing vertebrate brain, radial glial cells constitute one of the first cell types to form a characteristic cellular element destined to guide migrating neuronal cell bodies to their target positions (Rakic, 1972). These radial glia abut with their endfeet upon the pial surface on one side and the ventricular lumen on the other, thus forming a spatial roster for the assembly of functional neuronal units (Rakic,

1990). Similarly, astrocytes send out star-shaped processes to appose their endfeet onto the abluminal side of capillaries or onto the pia mater, all in close association with the basal lamina of endothelial and meningeal cells. Such contacts appear to be instrumental in the determination of tight junctions between endothelial cells (Janzer and Raff, 1987) and are characterized by intramembranous specializations in the plasma membrane of astrocytes (Landis and Reese, 1982). Astrocytic endfeet also appear in close association with myelinated axons at the node of Ranvier (for review see Black and Waxman, 1988) and thus have been implicated in the development and maintenance of internodal specializations, possibly by topically restricted expression of recognition molecules in the extracellular matrix (French-Constant et al., 1986). A metamorphosis in glial morphology is not only observed during development, when radial glia transform into astrocytes, but also after damage to the adult nervous system. Astrocytic gliosis is accompanied by extensive process outgrowth and changes in the disposition of the cytoskeleton (Bignami and Ralston, 1969; Latov et al., 1979; Janeczko, 1989; Davis et al., 1990).

These observations have elicited an interest in the cellular and molecular determinants underlying the elaboration of astrocytic morphology in the context of their interactions with the surrounding target structures. Growth factors have been found to alter the morphological appearance of astrocytes in vitro (Toru-Debauffe et al., 1990), possibly by influencing intracellular messenger systems including protein kinase C (Harrison and Mobley, 1990) and cAMP (Cavanaugh et al., 1990). Also neurons have been shown to influence the morphology and proliferative behavior of astrocytes in vitro (Hatten, 1985; Nagata and Schachner, 1986; Gasser and Hatten, 1990; Rogister et al., 1990). Furthermore, neurons influence the expression of cell surface components on astrocytes (Nagata and Schachner, 1986) and may thus determine their communication with the cellular and acellular environment. The expression of extracellular matrix components, such as the glia-associated glycoprotein tenascin, appears to correlate with characteristic morphological features of astrocytes (Gierson et al., 1990).

Recently, we have characterized a chondroitin sulfate proteoglycan containing three core protein moieties which is expressed by cultured astrocytes and not by other neural cell types in the murine central nervous system (Streit et al., 1990). This proteoglycan was isolated by virtue of its expression of the novel L5 carbohydrate epitope recognized by the monoclonal L5 antibody. This antibody recognizes an N-linked carbohydrate structure additionally, but much less prominently, present on other molecules in the central nervous system, such as the recognition molecules L1 or Thy-1 (Tiveron et al., 1992). Although the latter molecules carry the L5 carbohydrate, astrochondrin is the only L5-positive molecule expressed by astrocytes (Streit et al., 1990). The L5 epitope itself appears to play an important role during early neurogenesis in chicken (Roberts et al., 1991). Moreover, the L5 expressing chondroitin sulfate proteoglycan was shown to be a new member of the L2/HNK-1 family of recognition molecules (Kruse et al., 1984, 1985; Schachner et al., 1990). These observations encouraged us to investigate the functional properties of this proteoglycan in the interactions between different neural cell types and their surrounding matrix. Here, we show that the three core proteins of the pro-

teoglycan are structurally closely related to each other on the basis of similar proteolytic peptide maps. Furthermore, we provide evidence that the proteoglycan is involved in cerebellar granule cell migration and process formation of astrocytes on specific substrata, such as laminin and collagen type IV. Independently, binding assays demonstrate a specific interaction between the proteoglycan and these extracellular matrix components. Immunoelectron microscopic investigations show a distinct localization of the proteoglycan in close contact with the basal lamina of meningeal and endothelial cells, thus colocalizing with laminin and collagen type IV. Because of its unique association with astrocytes and since it contains chondroitin sulfate as glycosaminoglycan component we have named it astrochondrin.

## Materials and Methods

### Extracellular Matrix Components and Adhesion Molecules

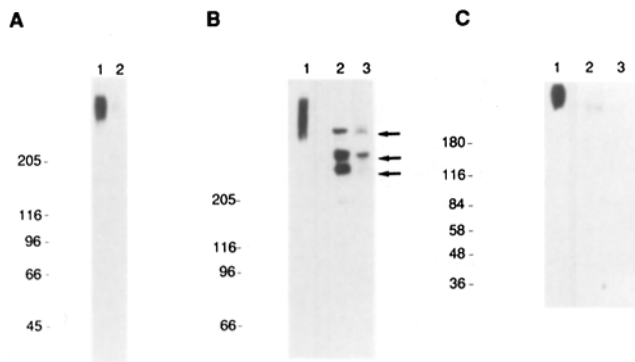
Collagens I, II, III, IV, V, VI, and IX, were purified as described by Fahrig et al. (1987) and kindly provided by Dr. K. Kühn (Max-Planck-Institute for Biochemistry, Martinsried, Germany). Collagen G (Seromed, Heidelberg, Germany) consists of collagen types I (90%) and III (10%). Englebreth-Holm sarcoma laminin and human plasma fibronectin were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Rat chondrosarcoma chondroitin sulfate proteoglycan (ICN, Radiochemicals, Irvine, CA), heparin (No. 375095; Calbiochem-Behring Corp., San Diego, CA), chondroitin sulfate (No. 230687; Calbiochem-Behring Corp.), dextran sulfate (No. D 4911; Sigma Chemical Co.), hyaluronic acid (No. H 4015; Sigma Chemical Co.), glucose-6-phosphate (Sigma Chemical Co.) and neuraminic acid (Sigma Chemical Co.) were obtained commercially. The cell adhesion molecule L1 (Rathjen and Schachner, 1984), the neural cell adhesion molecule (N-CAM)<sup>1</sup> (Hirn et al., 1981), the myelin-associated glycoprotein (MAG) (Fahrig et al., 1987), the adhesion molecule on glia (AMOG) (Antonicek et al., 1987), J1/tenascin (Faissner et al., 1988), and J1-160/180 (Kruse et al., 1985; Pesheva et al., 1989) were purified from mouse brain by immunoaffinity chromatography as described. AMOG was a kind gift from Dr. H. Antonicek, J1/tenascin from Dr. K. Husmann and J1-160/180 from Dr. B. Fuß (Department of Neurobiology, Swiss Federal Institute of Technology, Zürich). Astrochondrin was purified from early postnatal mouse brain by sequential immunoaffinity chromatography on a monoclonal L5 antibody column after removal of L1 (Rathjen and Schachner, 1984) and synneurin (Faissner et al., 1992) followed by anion exchange chromatography (Streit et al., 1990).

### Antibodies

Polyclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from DAKOPATTS (Copenhagen, Denmark). Polyclonal antibodies to astrochondrin were raised in rabbits (Streit et al., 1990) and purified by protein A affinity chromatography using fast protein liquid chromatography (FPLC) (Pharmacia/LKB; Uppsala, Sweden). Fab fragments were generated according to Porter (1959). Fig. 1 shows the specificity of the polyclonal astrochondrin antibodies for the proteoglycan purified by the L5 mAb column (Fig. 1 A); there is no reaction detectable with other, L5 negative proteoglycans. When Western blots of total brain homogenates digested with chondroitinase ABC were probed with the antibodies, only the three core proteins described for astrochondrin (Fig. 1 B, lanes 2 and 3) could be identified again showing their specificity for the proteoglycan. Polyclonal antibodies to astrochondrin only react with cultured astrocytes and not with neurons or oligodendrocytes as shown by immunoprecipitation of radioactively labeled cells (Fig. 1 C).

Monoclonal L5 antibody (Streit et al., 1990) was purified from the ascites fluid of nu/nu mice carrying L5 hybridoma cells or from the superna-

1. *Abbreviations used in this paper:* AMOG, adhesion molecule on glia; BrdU, bromodeoxyuridine; CTB, cytotoxic binding; FPLC, fast protein liquid chromatography; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; MI, migratory index; N-CAM, neural cell adhesion molecule.



**Figure 1.** Characterization of polyclonal antibodies to astrochondrin. (A) Using polyclonal antibodies to astrochondrin in Western blot analysis of the protein fractions binding (lane 1) and not binding (lane 2) to the L5 monoclonal antibody column. Only the proteoglycan binding to the L5 column is reactive. (B) Crude total brain homogenates from 8 (lanes 1 and 2) and 15 (lane 3) d-old mice were used for Western blot analysis before (lane 1) and after treatment with chondroitinase ABC (lanes 2 and 3) and assayed with polyclonal antibodies to astrochondrin. The antibodies only recognize the three core proteins described for astrochondrin (arrows). (C) Immunoprecipitation of [<sup>35</sup>S]methionine labeled astrocytes (lane 1), oligodendrocytes (lane 2), and neurons (lane 3) using polyclonal antibodies to astrochondrin. Astrochondrin is only detectable in astrocyte cultures. The faint band detected in oligodendrocytes is due to the presence of precursor cells and few contaminating astrocytes. Molecular weights of standard protein mixtures are indicated at the left margins.

tant of hybridoma cells cultured in 1% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN) by affinity chromatography on a mouse anti-rat kappa chain column (Bazin, 1982). Monovalent fragments were obtained from the L5 IgM antibodies by tryptic digestion as described by Matthew and Reichardt (1982). The rat mAb 412 to the L2/HNK-1 carbohydrate epitope was obtained by immunization of Lou x Sprague Dawley F1 hybrid female rats with crude membrane fractions of early postnatal mouse cerebellum following the procedures described by Kruse et al. (1984). Polyclonal antibodies to mouse liver membranes (Lindner et al., 1982) and to L1 (Rathjen and Schachner, 1984) were generated in rabbits by Dr. V. Künemund and A. Spitzauer at the Department of Neurobiology (University of Heidelberg).

Alkaline phosphatase-coupled antibodies to rabbit IgG (Promega Biotech, Madison, WI) or mouse IgM (Dianova, Hamburg, Germany) were used as secondary antibodies for Western blot analysis. For indirect immunofluorescence, primary antibodies were visualized by FITC-conjugated swine antibodies to rabbit IgG (Dakopatts) or FITC-conjugated goat antibodies to rat Ig (Cappel Laboratories, Cochranville, PA) or TRITC-conjugated goat antibodies to mouse Ig (Dianova). For immunoelectron microscopy, primary antibodies were visualized by HRP-coupled protein A (Sigma Chemical Co.) at a concentration of 0.75  $\mu$ g/ml.

### Peptide Mapping

Immature cerebellar astrocytes were metabolically labeled with [<sup>35</sup>S]methionine followed by immunoprecipitation with polyclonal antibodies to astrochondrin as described previously (Streit et al., 1990). Collected immunoprecipitates were washed twice with 50 mM Tris-acetate, pH 8.0, containing 1 mM ovomucoid (Sigma Chemical Co.) and 1 mM PMSF and digested with chondroitinase ABC (Boehringer Mannheim Biochemicals) in the same solution for 3 h at 37°C using 10 mU per precipitate. Then, 1 vol of 40 mM sodium phosphate buffer, pH 7.2, containing 1% 2-mercaptoethanol, 100 mM EDTA was added and digestion with peptide N-glycosylase F (25 mU/precipitate; Boehringer Mannheim Biochemicals) was carried out overnight at room temperature. Subsequently, samples were separated by 6% SDS-PAGE and radioactive bands visualized by autoradiography. After exposure, the three core protein bands (380, 360, and 260 kD) were cut out and proteolysis of the gel slices with *Staphylococcus aureus* V8 protease (0.1–2  $\mu$ g; Boehringer Mannheim Biochemicals) was

performed according to Cleveland et al. (1977) using 15% slab gels and SDS-PAGE. The resulting peptides were then visualized by autoradiography.

### Western Blot Analysis

For Western blot analysis, total brains of 14-d-old NMRI mouse embryos (E14), and cerebrum and cerebellum of 1-, 5-, 8-, and 15-d-old (P1, P5, P8, and P15) and adult (older than 6 wk) NMRI mice were used. Tissue extracts were prepared by homogenization in 50 mM Tris-acetate, pH 8.0 (1 ml per gram wet weight of tissue), containing trypsin inhibitors from soybean and turkey egg white (10  $\mu$ g/ml each) and iodoacetamide (0.1 mM). As shown previously (Streit et al., 1990) buffers containing detergents or chaotropic agents were not necessary for optimal recovery of the proteoglycan. Homogenates were shaken at 4°C for 1 h and centrifuged for 10 min at 200 g and 4°C. Protein concentrations in the supernatant were determined according to Bradford (1976). In some cases homogenates were digested with chondroitinase ABC (Boehringer Mannheim Biochemicals; 20 mU per 100  $\mu$ g protein) for 5 h at 37°C. Equal amounts of protein (100  $\mu$ g per lane) were separated by SDS-PAGE on 6% slab gels followed by transfer to nitrocellulose filters according to Kyhse-Andersen (1984) as described previously (Streit et al., 1990). Alkaline phosphatase coupled secondary antibodies (Dianova) or <sup>125</sup>I Protein A (Amersham Corp.) were used for detection.

### Cell Culture

Highly enriched cultures of GFAP-positive, mature astrocytes were obtained from brains of 14- to 16-d-old mouse embryos (Trotter et al., 1989). Cells from 10 to 12 brains were plated into two T75 poly-L-lysine-coated tissue culture flasks in Eagle's Basal Medium containing 10% horse serum (culture medium). After 4 to 5 d in culture, neurons were removed by immunocytolysis with mAb M5 (Keilhauer et al., 1985) and guinea pig complement. After another 5 d, loosely attached macrophages were removed by gently shaking the flasks. Oligodendrocytes and glial precursor cells were then removed by vigorously shaking the flasks for ~15 s. The removal of cells was followed microscopically. The procedure resulted in confluent astrocyte monolayer cultures with a purity of ~95% GFAP-positive cells. Astrocytes were maintained in culture medium for another 2 to 5 d before they were taken as single cell suspensions for the cell-to-substratum adhesion assays. Glial precursor cells appearing during this period were shaken off immediately before the preparation of single cell suspensions. Oligodendrocytes were prepared according to the procedure described above, harvested by shaking and plated on poly-L-lysine-coated Petri dishes. Purification of small cerebellar neurons from 6-d-old mice was carried out as described (Keilhauer et al., 1985). Astrocytes, oligodendrocytes, and neurons were metabolically labeled with [<sup>35</sup>S]methionine and processed for immunoprecipitation with polyclonal antibodies to astrochondrin as described previously (Streit et al., 1990).

Cerebellar microexplant cultures were prepared from 6-d-old mice (Fischer et al., 1986). For antibody perturbation experiments, Fab fragments of polyclonal antibodies to astrochondrin were used at concentrations between 150–500  $\mu$ g/ml and monovalent IgM fragments of monoclonal L5 antibody at 100–300  $\mu$ g/ml. Fab fragments of polyclonal antibodies to mouse liver membranes reacting with the cell surface of all cell types were used as control antibodies at the same concentrations. Antibodies were added to the cultures 4 or 16 h after seeding the microexplants onto coverslips.

Migration of granule cells in cerebellar folium explant cultures was determined as described by Lindner et al. (1983, 1986) with the following modifications: Premigratory proliferating cells were labeled with bromodeoxyuridine (BrdU; Del Rio and Soriano, 1989) instead of labeling with [<sup>3</sup>H]thymidine. Explants of cerebellar cortex folia from 10-d-old C57BL/J6 mice were labeled in vitro with 5 mM BrdU (No. B 5200; Sigma Chemical Co.) for 90 min in serum-free defined culture medium. The explants were then washed thoroughly with defined medium and five to seven folia were maintained for 3 d in 3 ml medium with or without addition of antibodies. Frozen sections (10- $\mu$ m thick) were cut in sagittal orientation and fixed for 30 min at room temperature in PBS, pH 7.3, containing 0.5% paraformaldehyde. The sections were processed at room temperature as follows: after several washes in PBS they were treated for 20 min at room temperature with 4 N HCl in PBS containing 0.5% Tween 20 for denaturation of DNA and permeabilization, washed several times in PBS, followed by a 35-min incubation step with mouse mAb against BrdU (Sigma Chemical Co.) diluted 1:100 in PBS containing 0.5% BSA and 0.5% Tween 20. After washing in PBS, sections were blocked with PBS containing 1% horse serum and 0.1% BSA (blocking buffer) for 10 min, washed in PBS, incubated

with TRITC-coupled goat anti-mouse immunoglobulin antibodies diluted 1:100 in blocking buffer, and finally mounted in PBS containing 90% glycerol and 2% NaI. The spatial distribution of BrdU-labeled cells was determined by automated image analysis using an IBAS system (Kontron; Carl Zeiss, Oberkochen, Germany) (Husmann et al., 1992). Briefly, microscopic images of the immunostained frozen sections of cerebellar folia were recorded with a video camera. For quantitative comparison of the distribution patterns of labeled cells under various experimental conditions, the mean values of the distances of labeled cells ( $\mu\text{m}$  from pial surface) of every histogram were calculated and designated as migratory index (MI). Inhibition of cell migration was calculated as follows:

$$\% \text{ inhibition} = (\text{MI} [\text{control}] - \text{MI} [\text{experimental}]) / (\text{MI} [\text{control}]) \times 100.$$

For each experimental value, three independent experiments were carried out in triplicates.

### Cell-to-Substratum Adhesion Assay

Collagen type IV, fibronectin, and laminin were each coated onto tissue culture Petri dishes as single spots in a 2  $\mu\text{l}$  droplet at a concentration of 20  $\mu\text{g}/\text{ml}$  in HBSS overnight at 37°C in a CO<sub>2</sub> incubator. The droplets were then removed by aspiration. The Petri dish was blocked with 3% heat-inactivated BSA in HBSS for 1 h at 37°C and afterwards washed three times with HBSS. Astrocytes were harvested from cell monolayers after mild trypsin treatment (20  $\mu\text{g}/\text{ml}$  trypsin, 0.2% EDTA in HBSS for 5 min at room temperature), suspended in modified Sato medium (Trotter et al., 1989) in the presence or absence of antibodies and added to the test plates at a density of  $2.5 \times 10^5$  cells/ml. For antibody perturbation experiments, Fab fragments of polyclonal antibodies to astrochondrin were used at concentrations between 125–500  $\mu\text{g}/\text{ml}$ . Fab fragments of polyclonal antibodies to mouse liver membranes reacting with the surface of astrocytes were used as control antibodies at the same concentrations. 30 min after plating, the non-attached cells were removed by washing twice with HBSS. The remaining cells were again incubated in Sato medium in the presence or absence of antibodies. The number of attached cells was evaluated by phase contrast microscopy after 5 h. Cells were then further maintained in culture and fixed after 24 h in PBS containing 4% paraformaldehyde for 10 min at room temperature and stained with 0.2% toluidine blue in 2.5% Na<sub>2</sub>CO<sub>3</sub>. Cell process formation was evaluated microscopically: cells with processes longer than two thirds of the cell diameter were counted as process bearing cells. Cells with round, flattened morphology (cf. Fig. 9f) and with triangular morphology (cf. Fig. 9h) were counted as non-process bearing cells. The percentage of process bearing cells (between 50 and 80% of all adherent cells) in the absence of antibodies or in the presence of Fab fragments of antibodies to mouse liver membranes was set to 100% and inhibition of process formation in the presence of antibodies to astrochondrin was calculated in relation to this value.

### Immunocytochemistry

**Light Microscopy.** Animals were sacrificed by cervical dislocation. Cerebella were quickly frozen in isopentane cooled by liquid nitrogen. Cryosections, 10- $\mu\text{m}$  thick, were mounted on poly-L-lysine-coated glass coverslips and allowed to dry for at least 1 h at room temperature. Indirect immunofluorescence was carried out as described previously (Goridis et al., 1983). For immunocytochemistry of cultured cerebellar microexplants, cultures were fixed and permeabilized in ice-cold 96% ethanol, and indirect immunofluorescence was carried out as described (Schnitzer and Schachner, 1981). Specificity of the immunoreaction was verified by incubation of sections or cultures with 1% BSA containing 0.1% horse serum instead of primary antibodies.

**EM.** Animals were deeply anaesthetized by intraperitoneal injection of 3% chloral hydrate (aqueous solution, 0.01 mg/g body weight) and perfused intracardially with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M Palay buffer (Palay and Chan-Palay, 1974). Cerebella were post-fixed in fixation solution without glutaraldehyde for  $\sim 2$  h at 4°C and embedded in 5% Agar-Agar (Difco Laboratories, Detroit, MI) diluted in PBS, pH 7.3. Sections, 50–70- $\mu\text{m}$  thick, were cut on a vibratome and rinsed for up to 2 h in PBS containing 0.1% BSA. Pre-embedding staining, using primary polyclonal antibodies and peroxidase-coupled protein A, was performed as described (Martini and Schachner, 1986; Nolte et al., 1989). In brief, vibratome sections were incubated first in 0.1 M NaIO<sub>4</sub> (10 min) and then in 1% NaBH<sub>4</sub> (15 min), and finally in 5% DMSO (30 min) at room temperature. Sections were incubated with polyclonal antibodies to as-

trochondrin overnight at 4°C, washed, incubated with peroxidase-coupled protein A for at least 2 h at room temperature, and washed again. After fixation with 0.1% glutaraldehyde for 15 min, sections were immersed in 0.02% 3,3'-diaminobenzidine-4HCl (Sigma Chemical Co.) in 0.04 M Tris buffer, pH 7.6, for 30 min, then in the diaminobenzidine solution containing 0.01% H<sub>2</sub>O<sub>2</sub>, also for 30 min, all at room temperature. Sections were post-fixed in 2% OsO<sub>4</sub>, dehydrated in acetone, and embedded in Spurr (Plano, Marburg, Germany). Ultrathin sections were analyzed by EM (EM 10C; Carl Zeiss).

### Solid Phase Radioligand Binding Assay

Iodination of purified astrochondrin was performed according to the method of Salacinski et al. (1981) as described (Streit et al., 1990). Radioligand binding assays were also carried out as described (Fahrig et al., 1987). Briefly, individual wells of flexible microtiter plates (No. 3911 Falcon, Cockeysville, MD) were incubated overnight at 4°C with different proteins at concentrations of 20  $\mu\text{g}/\text{ml}$  in 0.1 M NaHCO<sub>3</sub>, pH 8.2 (coating buffer). Plates were then treated with coating buffer containing 1% BSA for 1 h at room temperature and afterwards washed three times with hypotonic (10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 15 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4) or isotonic incubation buffer (10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4). For experiments carried out in the presence of Ca<sup>2+</sup>, 1 mM CaCl<sub>2</sub> was added to the incubation buffers that contained no EDTA. Wells were incubated with <sup>125</sup>I-labeled astrochondrin (usually 50,000–70,000 cpm) diluted in the appropriate incubation buffer for 4 to 5 h at room temperature and washed five times in incubation buffer. Bound radioactivity was counted immediately after cutting the bottom of the plastic wells. Coating efficiencies of the different proteins were determined as described (Fahrig et al., 1987) and found to be similar ( $\sim 10\%$  of the total input) for all collagen types and recognition molecules. Values of saturation curves in the binding assay were plotted according to Scatchard (1949) and could be approximated with one linear function by linear regression. Binding of <sup>125</sup>I-labeled astrochondrin to BSA (unspecific background) was subtracted from experimental values unless indicated.

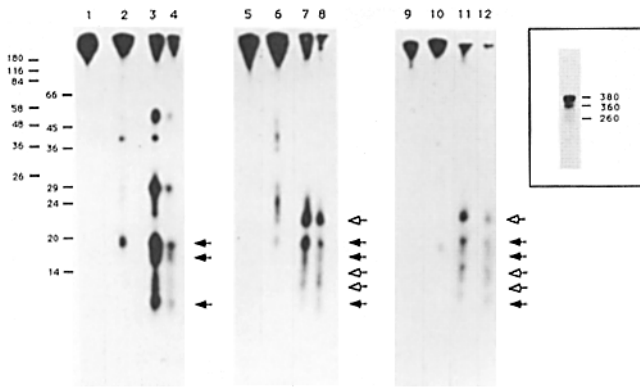
When binding assays were carried out in the presence of glycosaminoglycans or monosaccharides, these substances were mixed with <sup>125</sup>I-labeled astrochondrin immediately before the assay at concentrations indicated in the figure legends.

The percentage of maximal binding was determined as follows: maximal binding in each experiment was set to 100% and the other values calculated in relation to this value. Mean values of different experiments carried out in triplicates were calculated and are shown  $\pm$ SD.

## Results

### Relationship between the Three Core Proteins of the Chondroitin Sulfate Proteoglycan

We previously demonstrated that digestion with chondroitinase ABC of the L5 carbohydrate expressing proteoglycan isolated from mouse brain generated three core proteins of different molecular size (Streit et al., 1990). To distinguish whether these core proteins are structurally related to each other, proteolytic peptide maps were generated from biosynthetically labeled proteoglycan derived from astrocyte cell cultures (Fig. 2). After treatment with chondroitinase ABC and N-glycohydrolase F and digestion with V8 protease, the three core proteins yielded similar peptide maps. Whereas the peptide patterns of 260- and 360-kD core proteins were very similar, the 380-kD core protein not only yielded corresponding, but also different peptide bands. Three peptides with apparent molecular weights of  $\sim 19$ , 17, and 7.5 kD were found to be identical in all the core proteins (Fig. 2, filled arrows). The 380-kD protein contained additionally one major peptide of  $\sim 30$  kD (Fig. 2, lanes 3 and 4), also occurring as an intermediate product in digestion of the 360-kD molecule (Fig. 2, lane 6). Moreover, the similarity between the different protein bands is underscored by the fact that 40.5- and 24-kD fragments appear during digestion of both the

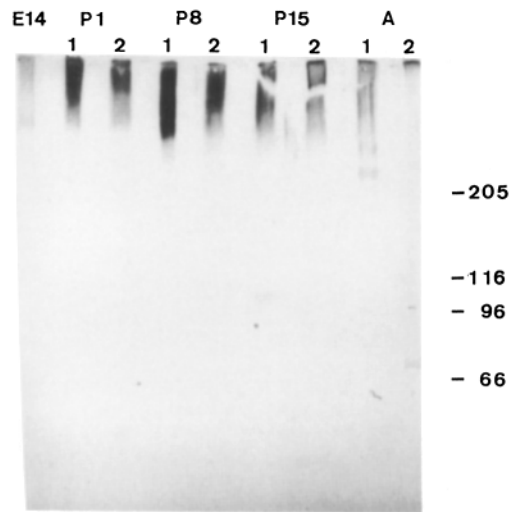


**Figure 2.** Peptide mapping of the core proteins of astrochondrin. Immunoprecipitates of astrochondrin with polyclonal antibodies from  $^{35}\text{S}$ -labeled cultures of immature astrocytes were digested with chondroitinase ABC and peptide *N*-glycohydrolase F. The inset shows the pattern of the deglycosylated core proteins in 6% SDS-PAGE. Numbers refer to the molecular weights of protein bands after chondroitinase treatment only as originally described in Streit et al. (1990). Proteolytic digestion of the deglycosylated 380 kD (lanes 1–4), 360 kD (lanes 5–8) and 260 kD (lanes 9–12) components was carried out with 0.1  $\mu\text{g}$  (lanes 2, 6, and 10), 1  $\mu\text{g}$  (lanes 3, 7, and 11) and 2  $\mu\text{g}$  (lanes 4, 8, and 12) V8 protease. Lanes 1, 5, and 9 show the core proteins before treatment with V8 protease. Filled arrows mark peptides identical in all three core proteins, open arrows those which are similar in the 360- and 260-kD proteins only. Molecular weights of two different standard protein mixtures are indicated at the left margin.

380- and the 360-kD core proteins (Fig. 2, lanes 3 and 6). The peptides with the apparent molecular weights of  $\sim 10$ , 12, and 22.5 kD (Fig. 2, *open arrows*) derived from the 360- and 260-kD components were not present in the 380-kD component. Thus, the three proteins show considerable similarities in their proteolytic peptides and appear to be comprised of closely related molecules for which we propose the name of astrochondrin, reflecting its unique localization on astrocyte cell surfaces and the presence of chondroitin sulfate as glycosaminoglycan component. It is worth emphasizing here that in accordance with our previous observations (Streit et al., 1990) immunofluorescence labeling of cerebellar cell cultures (not shown) and immunoprecipitation of biosynthetically labeled cultures of purified small cerebellar neurons, astrocytes, and oligodendrocytes using polyclonal antibodies to astrochondrin demonstrated that the proteoglycan is expressed by astrocytes only (Fig. 1 C).

### Immunochemical and Immunocytochemical Detection of Astrochondrin during Development

To gain first insights into the role of astrochondrin in the central nervous system, its expression at different developmental stages was investigated immunochemically and immunocytochemically. Western blot analysis of crude homogenates of murine cerebellum and cerebrum of different developmental stages (from embryonic day 14 until adult; Fig. 3) using polyclonal antibodies to astrochondrin showed that at embryonic day 14, astrochondrin was hardly detectable. Astrochondrin expression increased during the first postnatal week, with a peak at approximately day 8, and then decreased to low levels in the adult. At all developmental stages, expression of astrochondrin was more prominent in



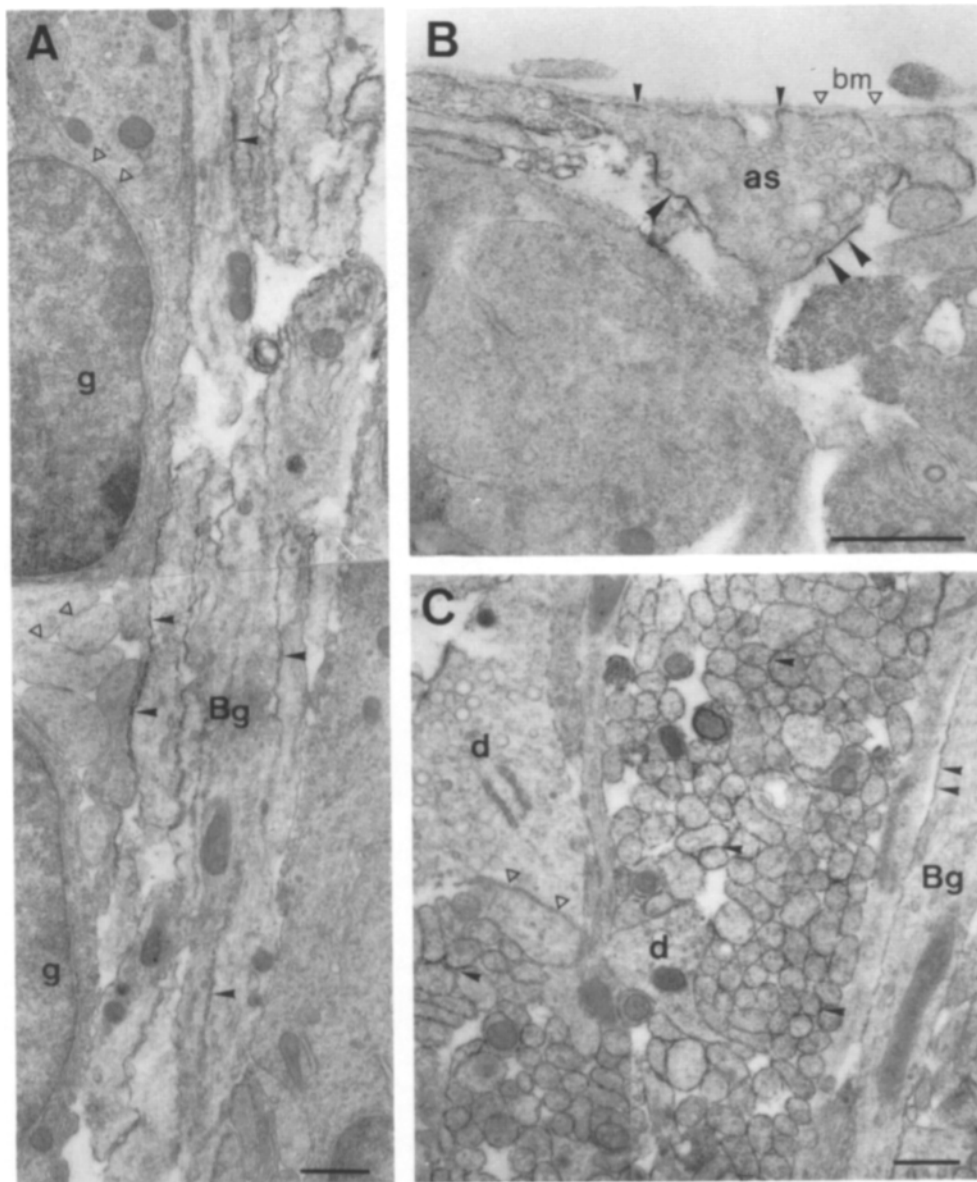
**Figure 3.** Western blot analysis of astrochondrin expression during development in crude homogenates of cerebellum (1), cerebrum (2), or total embryonic brain (E14). Homogenates were prepared from 14-d-old mouse embryos (E14), 1 (P1), 5 (P5), 8 (P8), and 15 (P15)-d-old and adult (A) mice and assayed with polyclonal antibodies to astrochondrin. Molecular weight markers are indicated at the right margin.

the cerebellum than in the cerebrum. Identical results were obtained with monoclonal L5 antibody (not shown).

Immunoprecipitations with polyclonal antibodies to astrochondrin from detergent lysates of biosynthetically labeled immature and mature astrocytes (not shown) demonstrated that all three core proteins are present in both cell populations suggesting that no major changes in the expression of the core proteins occur during development.

To investigate the cellular and subcellular localization of astrochondrin in situ, immunocytochemical studies were carried out in histological sections of postnatal mouse cerebellum at the light and electron microscopic level. Indirect immunofluorescence was performed with monoclonal L5 antibody and polyclonal antibodies to astrochondrin on fresh frozen sections of 6- and 14-d-old adult mice (not shown). Both antibodies showed almost identical staining patterns at these stages. In agreement with the immunochemical data, the most distinct and highest immunoreactivity was observed at postnatal day 6. At this stage, the antibodies reacted most prominently with the developing molecular layer in a homogeneous distribution. Immunoreactivities for both antibodies were also detectable in the external and internal granular layers, with the latter being more intensely stained by the antibodies than the external granular layer. The internal granular layer showed labeling around granule cell bodies. The staining pattern was often punctate in the outer margin of the external granular layer. At day 14, immunoreactivity became very weak in the molecular layer when compared to earlier stages and was hardly or not at all detectable in the adult cerebellum. In the adult, only the internal granular layer and the white matter were very weakly labeled with both antibodies.

Immunoelectron microscopic analysis using polyclonal antibodies to astrochondrin in the cerebellum of 6-d-old mice showed immunoreactivity at the cell surface of astrocytes including those of the radial Bergmann glial processes in the external granular and molecular layers (Fig. 4, A and



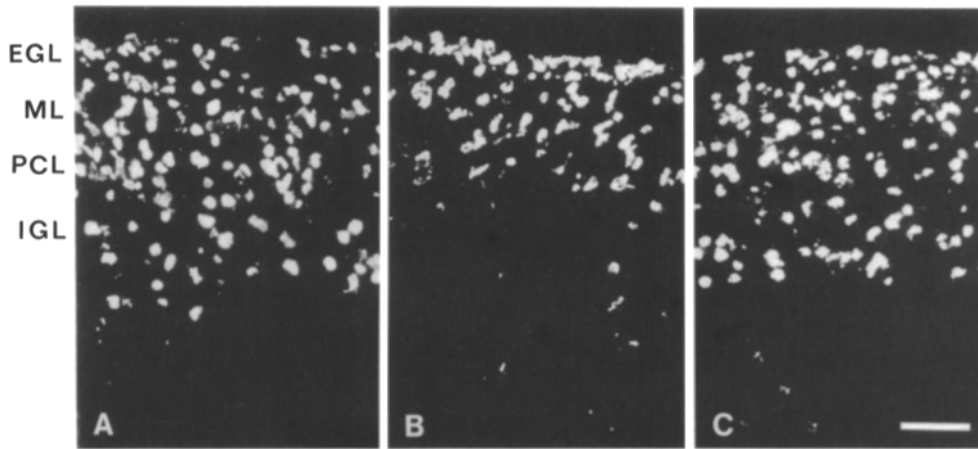
**Figure 4.** Immunoelectron microscopic localization of astrochondrin in the cerebellar cortex of 6-d-old mice using polyclonal antibodies. (A) External granular layer. Bergmann glial fibers (Bg) are positive for astrochondrin at contact sites with each other and with granule cells (g), (solid arrowheads). Contact sites between granule cell bodies and their axonal processes do not show detectable levels of immunoreactivity (empty arrowheads). (B) External granular layer. Astrocytic endfeet (as) abutting onto the basement membrane (bm) at the pial surface are weakly antigen-positive (solid arrowheads). The basement membrane itself does not show detectable levels of immunoreactivity (empty arrowheads). (C) Molecular layer. The cell surfaces of parallel fibers are immunoreactive (solid arrowheads), whereas contact sites between dendrites (d) appear negative (empty arrowheads). Bergmann glia fibers (Bg) express the proteoglycan at contact sites (solid arrowheads). Bars, 0.2  $\mu\text{m}$ .

C). Endfeet of astrocytes contacting blood vessels or endfeet of Bergmann glia abutting onto the pia mater were astrochondrin positive (Fig. 4 B). The basal lamina juxtaposed between glial endfeet and pia did not show detectable immunoreactivity (Fig. 4 B). Within the external granular layer, astrochondrin was not detectable on somata or processes of granule cells where then contacted each other (Fig. 4 A, empty arrowheads). Contact sites between granule cells and Bergmann glial processes, however, were astrochondrin positive (Fig. 4 A, solid arrowheads). In agreement with the observations at the light microscopic level strong immunoreactivity in the molecular layer was seen at the cell surface of parallel fibers (Fig. 4 C). In the molecular and internal granular layers, astrochondrin was detectable at the surface of neurons only where they were contacted by an astrocytic process or by the astrochondrin-positive parallel fibers (Fig. 4, A and C). In the adult cerebellar cortex, hardly any immunoreactivity was observed by immuno EM neither in association with the surface nor intracellularly.

### ***Astrochondrin Is Involved in Cerebellar Granule Cell Migration***

To gain insights into the functional roles of astrochondrin in the early postnatal morphogenesis of the cerebellar cortex, antibody perturbation experiments were performed in different cell culture systems. Since astrochondrin was most prominently expressed in the cerebellum and its expression peaked at the beginning of the second postnatal week, the developmental stage at which cerebellar granule cell migration along astrochondrin-positive Bergmann glial fibers takes place, we first tested whether astrochondrin may be involved in this migration process. In tissue pieces of 10-d-old mouse cerebellum maintained in vitro for 3 d, the dislocation of bromodeoxyuridine-labeled neurons from the external to the internal granular layer can be determined quantitatively. In this assay system, the IgG fraction of polyclonal antibodies to astrochondrin inhibited granule cell migration by  $28 \pm 6.7\%$  (Figs. 5 and 6). This value has previously been ob-





**Figure 5.** Effects of polyclonal antibodies to astrochondrin on the migration of cerebellar granule cells. Sagittal sections of cerebellar folium explants were immunocytochemically stained with antibodies to bromodeoxyuridine used to pulse-label dividing cells. (A) Explant maintained in the absence of antibodies. (B) Explant maintained in the presence of polyclonal antibodies to astrochondrin (IgG fraction, 500  $\mu\text{g/ml}$ ). (C) Explant maintained in the presence of monoclonal antibody 412 to the L2 carbohydrate epitope (IgG fraction, 500  $\mu\text{g/ml}$ ). Bar, 50  $\mu\text{m}$ .

served with polyclonal antibodies to L1 (both IgG fraction and Fab fragments). No inhibition was observed when explants were maintained in the presence of monoclonal antibody 412 to the L2/HNK-1 carbohydrate structure (IgG fraction).

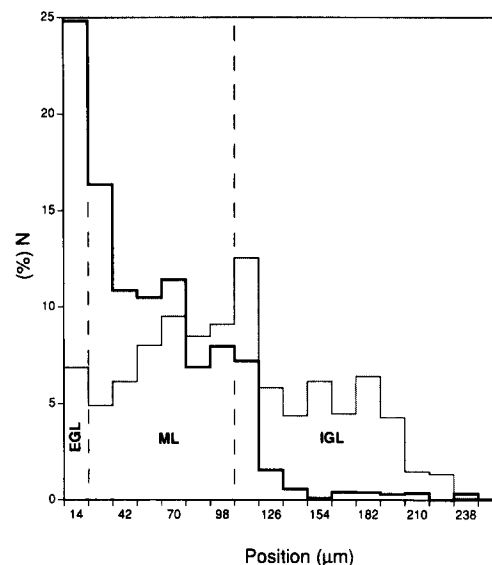
#### ***Astrochondrin Is Involved in Astrocytic Process Formation***

***Astrocyte Outgrowth in Cerebellar Microexplant Cultures.*** Since Bergmann glial cells continue to divide and elaborate radially oriented processes abutting with their endfeet onto the meninges early postnatally, the question arose whether astrochondrin is involved in astrocyte-mediated cell interactions other than cerebellar granule cell migration. Thus, the cellular outgrowth pattern of cultured microexplants from early postnatal mouse cerebellum was investigated in the absence and presence of polyclonal antibodies to astrochondrin and monoclonal L5 antibodies. These microexplant cultures provide an *in vitro* assay system to monitor migration of cell bodies and process outgrowth of neurons and astrocytes.

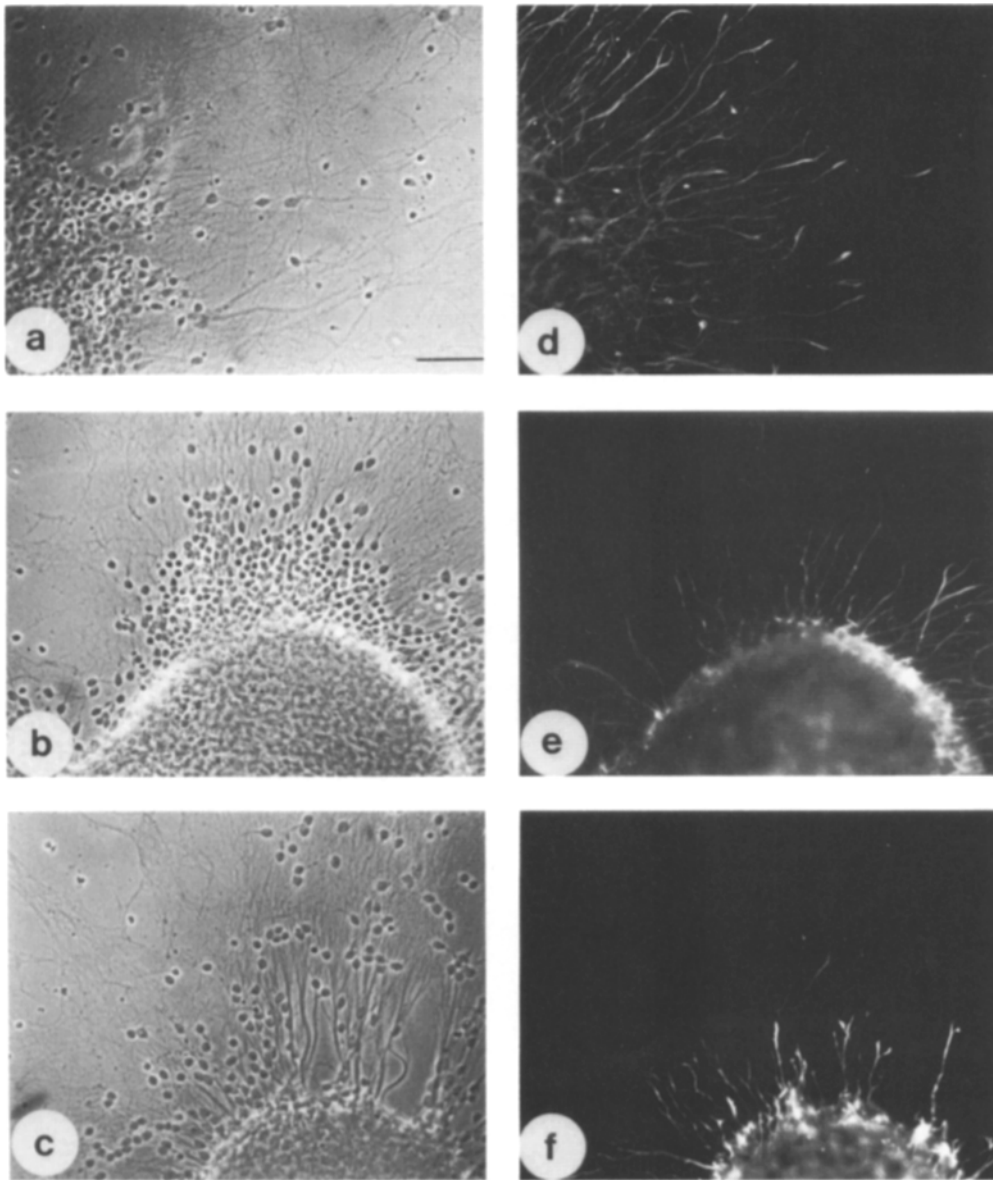
When microexplants were plated onto glass coverslips coated with poly-D-lysine, the characteristic outgrowth pattern described by Fischer et al. (1986) could be observed. Microexplants attached to the substratum within the first 2 h. Neurites started to leave the explant after 24 h. Neuronal cell bodies migrated out of the explant core and neurites began to fasciculate in the periphery after 48–72 h. Astrocytes sent out processes more slowly than neurons and rarely left the explant core. When Fab fragments of polyclonal antibodies to astrochondrin (250  $\mu\text{g/ml}$ ) or monovalent fragments of polyclonal antibodies to astrochondrin (250  $\mu\text{g/ml}$ ) or monovalent fragments of the monoclonal L5 antibody (300  $\mu\text{g/ml}$ ) were added to the cultures 16 h after plating, the outgrowth of astrocytic processes was found to be modified (Fig. 7), as visualized by immunocytochemical staining with antibodies to GFAP, a marker for mature astrocytes. Formation of processes was markedly reduced (Fig. 7, *b*, *e*, *c*, and *f*) when compared to the controls, in which either no antibodies or Fab fragments of polyclonal antibodies to mouse liver membranes (250  $\mu\text{g/ml}$ ) had been added (Fig. 7, *a* and *d*). Furthermore,  $\sim 20\%$  of all explants maintained in the presence of antibodies to astrochondrin or monoclonal L5

antibody showed strong fasciculation of neurites in the vicinity of the explant core (Fig. 7 *c*).

When microexplants were plated on coverslips coated with laminin, the outgrowth of neurites and astrocytes as well as their migration out of the explant core were enhanced in time and space when compared to poly-D-lysine as substratum. Therefore, Fab fragments of polyclonal antibodies to astrochondrin or mouse liver membranes and monovalent fragments of monoclonal L5 antibody were added to the cultures



**Figure 6.** Relative distribution of bromodeoxyuridine pulse-labeled cells with respect to their distance from the pial surface. Cerebellar folium explants pulse labeled with bromodeoxyuridine were maintained for 3 d *in vitro* in the absence (*weak line*) or presence (*solid line*) of polyclonal antibodies to astrochondrin. The position of the bromodeoxyuridine-labeled cells in sagittal sections is plotted as a function of distance from the pial surface (in  $\mu\text{m}$ ). In the presence of antibodies to astrochondrin (IgG fraction, 500  $\mu\text{g/ml}$ ), migration of pulse-labeled cells was inhibited by  $28 \pm 6.7\%$  when compared with controls. The histogram shows mean values of three independent experiments carried out in triplicate. EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; N, number of labeled cells expressed in percentage.



**Figure 7.** Effect of monoclonal L5 antibody and polyclonal antibodies to astrochondrin on the outgrowth pattern of astrocytes in cultures of cerebellar microexplants on poly-D-lysine. Microexplants from cerebella of 6-d-old mice were plated on poly-D-lysine-coated coverslips. Fab fragments of polyclonal antibodies to mouse liver membranes (*a* and *d*; 500  $\mu\text{g}/\text{ml}$ ) or to astrochondrin (*b* and *e*; 250  $\mu\text{g}/\text{ml}$ ) or monovalent IgM fragments of monoclonal L5 antibody (*c* and *f*; 300  $\mu\text{g}/\text{ml}$ ) were added 16 h later. After 72 h in vitro, explants were stained by indirect immunofluorescence with antibodies to GFAP to visualize mature astrocytes. *a-c* show corresponding phase contrast micrographs to fluorescence images *d-f*, respectively. Bar, 50  $\mu\text{m}$ .

already 4 h after plating. After 48 h of culture, GFAP-positive astrocytes were monitored in their outgrowth pattern (Fig. 8). As on poly-D-lysine, monoclonal L5 antibody and polyclonal antibodies to astrochondrin reduced the formation of astrocytic processes, whereas they did not interfere with neurite outgrowth or migration of neuronal cell bodies.

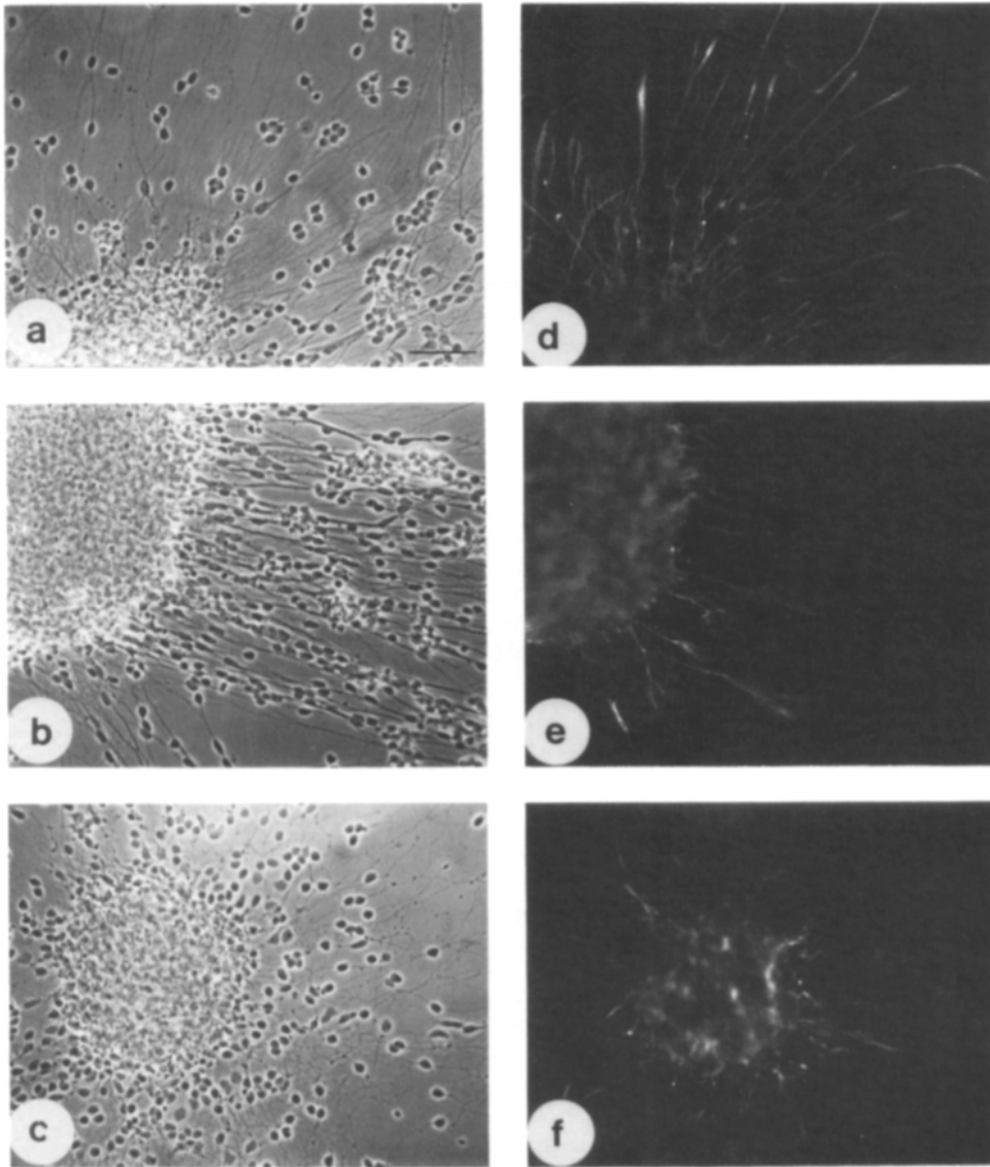
The outgrowth pattern of microexplants plated on monolayers of mature astrocytes as substratum closely resembled that observed on laminin. Addition of monoclonal L5 antibody and polyclonal antibodies to astrochondrin 4 h after plating did not reveal an altered pattern of neurite outgrowth after 48 h as visualized immunocytochemically by antibodies to the neural recognition molecule L1 (not shown). The outgrowth pattern of astrocytes could not be monitored in these cultures, since explant-derived astrocytes could not be distinguished immunocytochemically from the astrocyte monolayer underneath.

These observations indicate that both monoclonal L5 antibody and polyclonal antibodies to astrochondrin interfere

with the outgrowth pattern of astrocytes in cerebellar microexplant cultures suggesting that astrochondrin and the L5 carbohydrate domain may be involved in formation of astrocytic processes. On astrocyte surfaces astrochondrin represents the only L5-expressing molecule so the target of both polyclonal and monoclonal antibodies is the proteoglycan only. Since neurons achieve L5-positivity only after several days in culture and do not express astrochondrin in vitro, modification in neurite outgrowth pattern in the presence of the antibodies may thus be secondary to the inhibition of astrocyte outgrowth.

**Astrocyte Adhesion and Process Formation.** We next investigated the influence of polyclonal antibodies to astrochondrin on astrocyte adhesion and process outgrowth in cell-to-substratum adhesion assays. As substrata, fibronectin, laminin, collagen type IV, and astrochondrin were chosen and coated on tissue culture Petri dishes as individual spots. Mildly trypsinized single cell suspensions of mature astrocytes expressing astrochondrin at the cell surface were plated onto these substrates in the absence or presence of Fab





**Figure 8.** Effect of monoclonal L5 antibody and polyclonal antibodies to astrochondrin on the outgrowth pattern of astrocytes in cultures of cerebellar microexplants on laminin. Microexplants from cerebella of 6-d-old mice were plated on coverslips coated with 20  $\mu\text{g}/\text{ml}$  laminin. Fab fragments of polyclonal antibodies to mouse liver membranes (a and d; 500  $\mu\text{g}/\text{ml}$ ) or to astrochondrin (b and e; 250  $\mu\text{g}/\text{ml}$ ) or monovalent IgM fragments of monoclonal L5 antibody (c and f; 300  $\mu\text{g}/\text{ml}$ ) were added 4 h later. After 48 h in vitro, explants were stained by indirect immunofluorescence with antibodies to GFAP to visualize mature astrocytes. a-c show corresponding phase contrast micrographs to fluorescence images d-f, respectively. Bar, 50  $\mu\text{m}$ .

fragments (250  $\mu\text{g}/\text{ml}$ ) of polyclonal antibodies to astrochondrin or mouse liver membranes. Cell adhesion and process formation were evaluated after 5 and 24 h. Astrocytes attached well to laminin, fibronectin and collagen type IV within 30 to 45 min, whereas astrochondrin itself proved to be a very poor substratum and almost no cells adhered. Antibodies to astrochondrin did not interfere with the initial adhesion of astrocytes to any of these substrata.

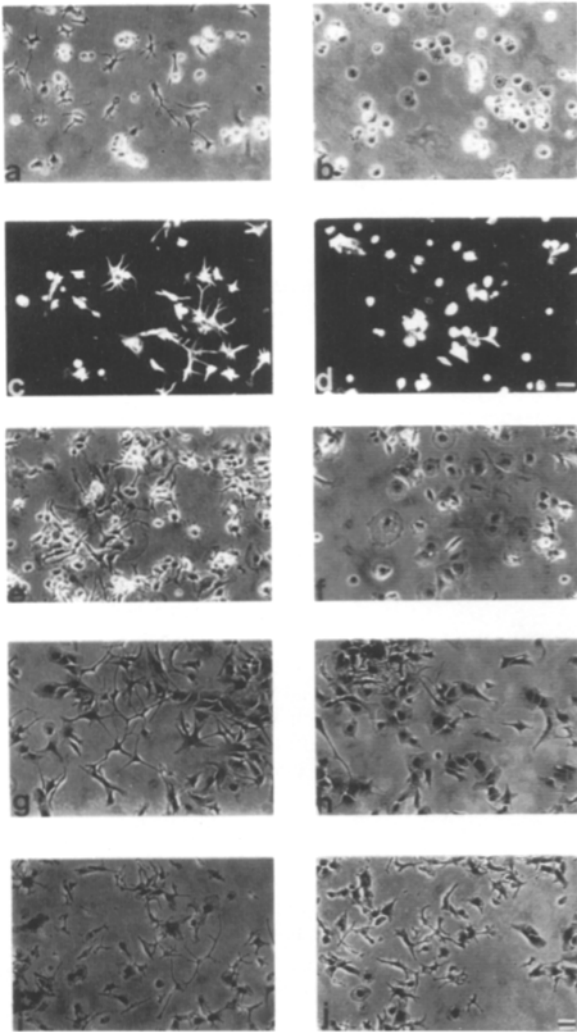
After 5 h in culture, astrocytes had begun to extend processes almost equally well on the three substrata in the absence or presence of antibodies to mouse liver membranes (Fig. 9, a and c, for collagen type IV) and formed a network of processes after 24 hours (Fig. 9, e, g, and i, for collagen type IV, laminin and fibronectin, respectively). In the presence of polyclonal antibodies to astrochondrin, astrocytes remained without processes 5 h after plating (Fig. 9, b and d, for collagen type IV) and, after 24 h, hardly formed any processes but showed a round, somewhat flattened morphology on collagen type IV (Fig. 9f) and a triangular morphology on laminin (Fig. 9 h). Formation of cell processes was

markedly reduced on both substrata in the presence of antibodies to astrochondrin, with the inhibition being more prominent on collagen type IV ( $\sim 80\%$ ) than on laminin ( $\sim 40\%$ ) (Fig. 10 A). The extent of inhibition of process formation depended on the antibody concentration (Fig. 10 B). When fibronectin was used as substratum, no differences in morphology could be observed between astrocytes cultured in the presence or absence of polyclonal antibodies to liver membranes or to astrochondrin (Figs. 8, i and j and 10 A).

These observations indicate that astrochondrin may be involved in the formation of astrocytic processes by interaction with the extracellular matrix components laminin and collagen, but not fibronectin.

#### **Binding of Astrochondrin to Extracellular Matrix Components**

Since these cell biological studies suggested that astrochondrin interacts with particular extracellular matrix components we addressed the question, whether the purified mole-



**Figure 9.** Effect of polyclonal antibodies to astrochondrin on process formation of mature astrocytes on the substrata laminin, collagen type IV, and fibronectin. Collagen type IV (*a-f*), laminin (*g* and *h*), and fibronectin (*i* and *j*) were coated onto Petri dishes as individual spots and single cell suspensions of mature astrocytes were added in the presence of Fab fragments of polyclonal antibodies to mouse liver membranes (*a, c, e, g, and i*) or to astrochondrin (*b, d, f, h, and j*). Astrocyte morphology was evaluated either 5 h (*a-d*) or 24 h (*e-j*) after plating the cells. Cells were either immunocytochemically labeled with antibodies to GFAP (*c* and *d*) or stained with toluidine blue (*a, b, and e-j*). Bars, 50  $\mu$ m.

cule could directly bind to these components in a solid phase radioligand binding assay. Under hypotonic conditions (10 mM phosphate buffer, 15 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4) astrochondrin could be shown to interact with collagen types I, II, III, IV, V, IX, and G, and laminin (Fig. 11 *A*) with different efficiencies, but not with collagen type VI and BSA. Interaction was most pronounced with collagen types III and V, with up to  $40 \pm 5\%$  of the added astrochondrin being bound. Astrochondrin bound 30- to 40-fold more efficiently to the collagens type III and type V than to BSA. When the proteoglycan concentration was reduced (from 70,000 to  $\sim 10,000$  cpm) binding of up to 80% of the total input and a 50- to 60-fold better binding to collagens type III and V than to BSA was found, suggesting that not only a subset of molecules, but all components of astrochondrin

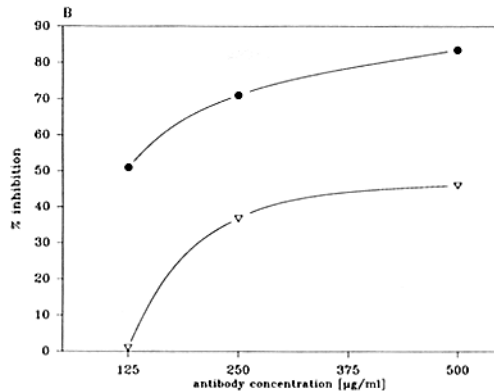
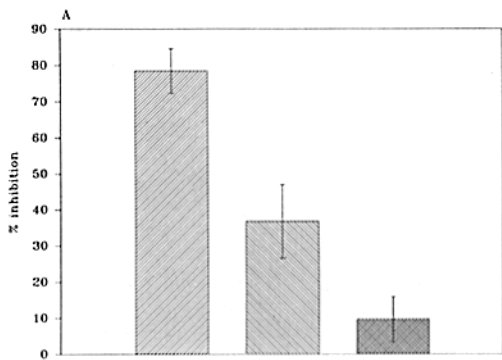
are able to interact with these collagens. Binding of astrochondrin to collagen types IV or IX and laminin was stronger in the presence of 1 mM  $\text{Ca}^{2+}$  (10 mM phosphate buffer, 15 mM NaCl, 1 mM  $\text{CaCl}_2$ , 0.1% BSA, pH 7.4) than in its absence. Under these conditions, astrochondrin bound up to sevenfold better to collagen types IV and IX ( $\sim 30\%$  of the total input) and 3.5-fold better to laminin ( $\sim 15\%$  of the total input) than to BSA. These results indicate that divalent cations are promoting the interactions between astrochondrin and collagen types IV and IX or laminin. Binding of astrochondrin to the other collagens was not markedly different in the presence or absence of  $\text{Ca}^{2+}$  (Fig. 11 *B*).

Under isotonic conditions (10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4) binding of astrochondrin to extracellular matrix components was generally reduced, remaining pronounced for collagen types III and V with 7-12% of the input being bound ( $\sim 10$ -fold above binding to BSA; Fig. 11 *C*). Considerable binding of astrochondrin to laminin and collagen type IV under isotonic conditions could only be observed in the presence of 1 mM  $\text{Ca}^{2+}$  (10 mM phosphate buffer, 150 mM NaCl, 1 mM  $\text{Ca}^{2+}$ , 0.1% BSA, pH 7.4; Fig. 11 *D*) with values being approximately five- to sevenfold higher than binding to BSA (3.5-5% of the total input). Under none of the conditions tested, astrochondrin was found to bind to the adhesion molecules L1, N-CAM, MAG and AMOG and to the extracellular matrix components J1/tenascin, J1-160/180, and fibronectin (Fig. 11, *A-D*).

To analyze the specificity of the interaction between astrochondrin and collagen types III and V, increasing amounts of astrochondrin were allowed to bind to hypotonic (Fig. 12) and isotonic (not shown) buffers. Under both conditions, saturation could be achieved, suggesting a specific and finite number of proteoglycan binding sites. Scatchard plot analysis (Scatchard, 1949) of these data (Fig. 12, *A* and *B*, insets) yielded linear plots for collagen types III ( $r = 0.94$ ) and V ( $r = 0.93$ ) indicating that these collagens contain a single class of binding sites for astrochondrin.

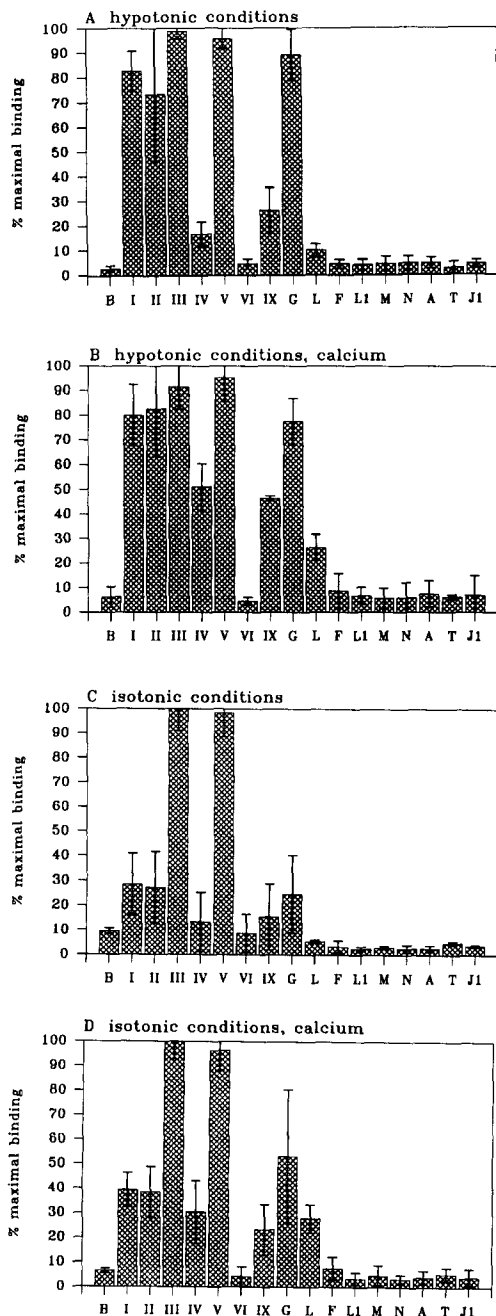
To investigate whether the binding of astrochondrin to collagen types III and V is dependent on ionic interactions, the influence of different concentrations of NaCl on binding efficiencies was determined (Fig. 13). Binding was highest at 15 mM NaCl in 10 mM phosphate buffer, 1 mM EDTA, 0.1% BSA, pH 7.4 (30% of the total input of astrochondrin), decreasing to a constant level of 5 to 7% of the total input at 75 mM NaCl in the same buffer.

To further characterize the properties of the collagen binding site, we studied the effect of polyanionic carbohydrates and charged monosaccharides on the binding of astrochondrin to collagen (Fig. 14). Collagen types III and V were incubated with  $^{125}\text{I}$ -labeled astrochondrin in the presence of increasing concentrations of glucose-6-phosphate ( $10^{-7}$ - $10^{-3}$  M), neuraminic acid ( $10^{-7}$ - $10^{-2}$  M), hyaluronic acid ( $1$ - $3 \times 10^9$  ng/ml), dextran sulfate ( $10^{-10}$ - $10^{-5}$  M), heparin ( $10^{-10}$ - $10^{-3}$  M), and chondroitin sulfate ( $10^{-10}$ - $10^{-3}$  M) (10 mM phosphate buffer, 15 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4). Glucose-6-phosphate, neuraminic acid, and hyaluronic acid did not alter the binding of astrochondrin to all collagen types tested (not shown), whereas dextran sulfate, heparin and chondroitin sulfate were potent inhibitors (Fig. 14, *A* and *B*). At half maximal binding, the astrochondrin-



**Figure 10.** (A) Quantitative evaluation of the effects of Fab fragments of polyclonal antibodies to astrochondrin (250 µg/ml) on process formation by astrocytes on collagen IV, laminin and fibronectin as shown in Fig. 9. The percentage of process bearing cells in control experiments was set to 100%. Bars represent mean values of three independent experiments carried out in duplicates  $\pm$  SD. (B) Inhibition of astrocytic process formation as a function of concentration of Fab fragments of polyclonal antibodies to astrochondrin. Mean values from one representative experiment carried out in duplicates are given. In three independent experiments, SDs were  $<5\%$ . (▨) Collagen IV; (▩) laminin; (■) fibronectin; (●) collagen IV; (▽) laminin.

tion as a function of concentration of Fab fragments of polyclonal antibodies to astrochondrin. Mean values from one representative experiment carried out in duplicates are given. In three independent experiments, SDs were  $<5\%$ . (▨) Collagen IV; (▩) laminin; (■) fibronectin; (●) collagen IV; (▽) laminin.



collagen type III interaction was inhibited  $\sim 30$  times better by dextran sulfate than by chondroitin sulfate and heparin (Fig. 14 A). In contrast, when binding of astrochondrin to collagen type V was measured, concentrations of all three polyanions were similar at half maximal inhibition values with chondroitin sulfate and heparin being two- to fivefold more potent inhibitors than dextran sulfate.

### Discussion

In this study we present evidence that the differently sized core proteins of the astrocyte-derived chondroitin sulfate proteoglycan (Streit et al., 1990) are structurally related to each other. At the moment we cannot distinguish whether the three components are generated by posttranslational modifications or whether they are transcribed from different messenger RNAs. Furthermore, pulse chase experiments ruled out the possibility that the two lower molecular weight proteins are derived by proteolytic cleavage of the 380-kD component (Streit, A., and M. Schachner, unpublished observations). Since the peptide maps were generated from deglycosylated astrochondrin, it does not seem likely that the core proteins represent differentially *N*-glycosylated forms of a single protein backbone (see also Streit et al., 1990). Because of the characteristic expression of the proteoglycan by astrocytes and the structural similarities between the individual core components, we propose to designate this

**Figure 11.** Binding of astrochondrin to extracellular matrix components and neural recognition molecules as determined by the solid phase radioligand binding assay. The interaction of  $^{125}\text{I}$ -labeled astrochondrin with collagen types I (I), II (II), III (III), IV (IV), V (V), VI (VI), IX (IX), and G (G), the extracellular matrix components laminin (L), fibronectin (F), J1/tenascin (T), and J1-160 (J1) and the neural recognition molecules L1 (LI), MAG (M), N-CAM (N), and AMOG (A) was investigated in hypotonic (15 mM NaCl; A and B) and isotonic (150 mM NaCl; C and D) 10 mM phosphate buffer, pH 7.4, in the presence (1 mM  $\text{CaCl}_2$ ; B and D) or absence (1 mM EDTA; A and C) of  $\text{Ca}^{2+}$ . Mean values are from three (C and D) and four (A and B) independent experiments carried out in triplicates  $\pm$  SD. In each experiment maximal binding was set to 100%. Binding of  $^{125}\text{I}$ -labeled astrochondrin to BSA (B) was not subtracted.

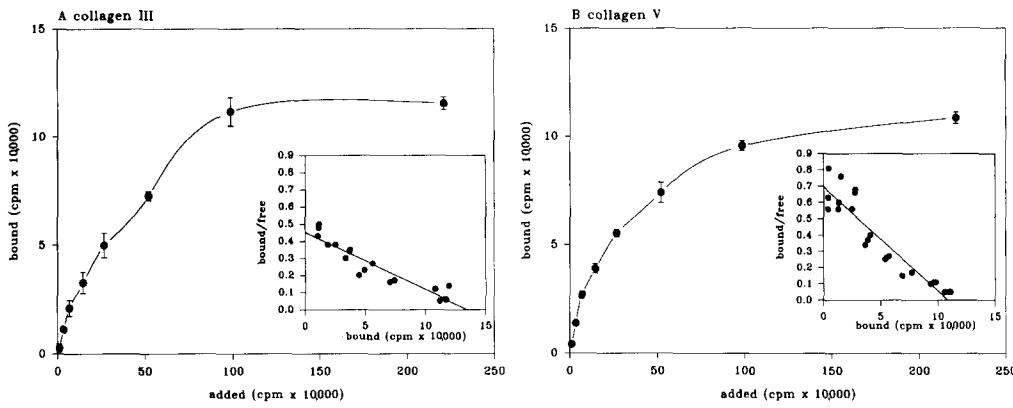


Figure 12. Binding of astrochondrin to collagen types III (A) and V (B) as a function of astrochondrin concentration under hypotonic conditions (see legend to Fig. 9) as determined by the solid phase radioligand binding assay. Mean values from one representative experiment carried out in triplicates  $\pm$  SD are given. Ordinate: Amount of  $^{125}\text{I}$ -labeled astrochondrin (cpm) bound. Abscissa: input of  $^{125}\text{I}$ -labeled astrochondrin (cpm). (Insets) Corresponding Scatchard plots.

group of chondroitin sulfate carrying molecules astrochondrin.

Astrochondrin, expressed and released by astrocytes in vitro and associated with their cell surface (Streit et al., 1990), is involved in granule cell migration and the interaction of astrocytes with certain extracellular matrix components. In particular, process formation on the extracellular matrix substrata laminin and collagen type IV, but not fibronectin, is influenced by antibodies to astrochondrin in vitro. In cerebellar microexplant cultures, antibodies to astrochondrin interfered with astrocytic process formation, whereas they did not affect neurite outgrowth patterns on different substrata. In a cell-to-substratum adhesion assay antibodies to astrochondrin inhibited process formation of astrocytes.

To our knowledge, astrochondrin is the first nervous system-derived chondroitin sulfate proteoglycan shown to be involved in astrocyte process formation. It is noteworthy that astrochondrin does not mediate the initial attachment of astrocytes to laminin or collagen type IV. Thus, adhesion and process outgrowth can be separated into two distinct events suggesting that distinct molecular mechanisms are involved. It is interesting in this context that a fibroblast derived heparan sulfate proteoglycan was shown to be essential to induce

formation of focal contact points, but not to initiate cell attachment in vitro (Woods et al., 1986). At the moment how astrochondrin affects astrocyte process outgrowth is still unclear. Located on their cell surface it could help to stabilize cell-substrate contact by interacting with the respective extracellular matrix components. Also, astrochondrin might interact with cell surface molecules, such as receptors for extracellular matrix constituents, thereby modulating their functional activities. On collagen type IV, astrochondrin appears to be the predominant mediator of astrocyte process outgrowth, since outgrowth is almost completely inhibited by astrochondrin antibodies. The fact that process formation is only partly inhibited by astrochondrin antibodies when cells are maintained on laminin might reflect the fact that astrocytes use more than one mechanism for process outgrowth on this substrate, only one of which is dependent on astrochondrin.

In agreement with our observations on the functional properties of astrochondrin, binding of astrochondrin to collagen type IV and laminin, but not to the extracellular matrix component fibronectin could be observed under hypotonic and isotonic conditions. This interaction was promoted in the presence of  $\text{Ca}^{2+}$ . Since the interaction of astrochondrin with collagen types III and V was least susceptible to the different binding conditions, this interaction was studied in more detail and shown to be specific: both collagens show a single, saturable class of binding sites for astrochondrin which can be competed by excess of astrochondrin (A. Streit, unpublished observations). Binding of astrochondrin to collagen types III and V was diminished by increasing ionic strength suggesting that it partly depends on ionic interactions. Such interactions were shown to be crucial for binding of glycosaminoglycans to collagens (Lindahl and Höök, 1978; Höök et al., 1984; Ruoslahti, 1988). It appears that the extent and pattern of sulfation of the glycosaminoglycan moiety does not play an important role in astrochondrin-collagen interactions as has been suggested for other proteoglycan-collagen interactions (e.g., Koda et al., 1984; for review see Ruoslahti, 1988). In our study, chondroitin sulfate and heparin are approximately equally potent competitors for the interaction of astrochondrin with both collagens, although heparin contains clusters of highly sulfated regions, which potentially occur also in dextran sulfate (see Koda et al., 1984), while sulfate groups are more uniformly distributed along chondroitin sulfate (Lindahl and Höök,

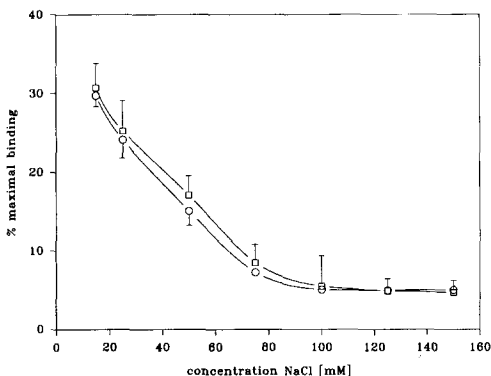
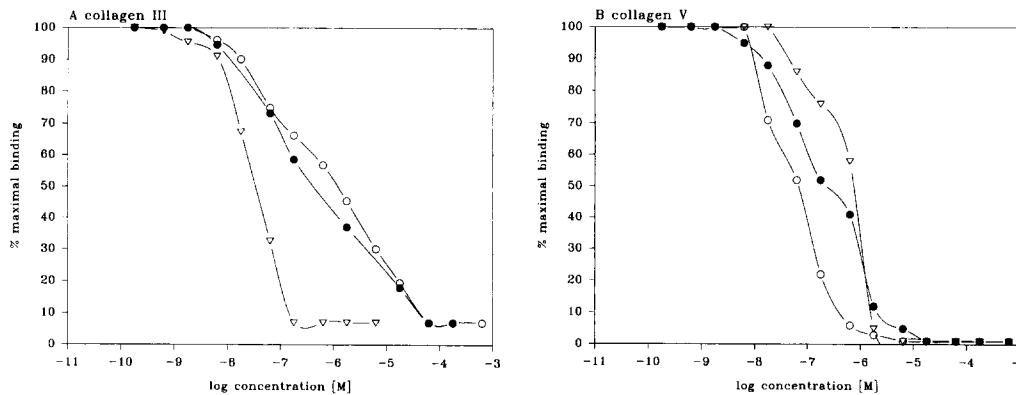


Figure 13. Binding of astrochondrin to collagen types III and V as a function of NaCl concentration in 10 mM phosphate buffer, 1 mM EDTA, 0.1% BSA, pH 7.4, as measured by the solid phase radioligand binding assay. Mean values of two independent experiments carried out in triplicates  $\pm$  SD are given. ( $\square$ ) Collagen III; ( $\circ$ ) collagen V.



**Figure 14.** Binding of astrochondrin to collagen types III (A) and V (B) in the presence of heparin, chondroitin sulfate, and dextran sulfate as determined by the solid phase radioligand binding assay in 10 mM phosphate buffer, pH 7.4, containing 15 mM NaCl and 1 mM EDTA. Mean values from triplicate measurements of one representative experiment are given; maximal binding was set to 100% and other binding efficiencies calculated

in relation to this value. Standard deviation for each value was less than 3% of maximal binding. Ordinate: % maximal binding of the  $^{125}\text{I}$ -labeled astrochondrin. Abscissa: concentration of polysaccharides incubated with astrochondrin before addition to the substrata. (○) Heparin; (●) chondroitin sulfate; (▽) dextran sulfate.

1987). Collagens have been reported to contain specific binding sites which are selective for polysaccharides with regions of extensive sulfation interrupted by non- or low-sulfated regions (Lindahl and Höök, 1978). Chondroitin sulfate chains apparently use different binding sites on collagens (Höök et al., 1984; Lindahl and Höök, 1978). It is therefore possible that inhibition of astrochondrin-collagen interactions by heparin and dextran sulfate is due to steric hindrance rather than to competition for specific binding sites. The nature of the interaction of astrochondrin with laminin is not yet known. Several heparin binding sites have been described for laminin (for review see Martin and Timpl, 1987), but it is not clear whether also chondroitin sulfate or chondroitin sulfate proteoglycans use these or other sites.

The relation of astrochondrin to other chondroitin sulfate proteoglycans in the nervous system (Hoffman and Edelman, 1987; Stallcup and Beasley, 1987; Faissner et al., 1991; Zaremba et al., 1989; Margolis and Margolis, 1989; Oohira et al., 1989; Herndon and Lander, 1990) is not known. It is possible that astrochondrin constitutes a subpopulation of proteoglycans from rat brain (Margolis et al., 1976; Gowda et al., 1989), since both carry the L2/HNK-1 and L5 carbohydrate epitopes (Margolis, R. U., A. Streit, and M. Schachner, unpublished observations; Streit et al., 1990). Furthermore, subpopulations of the glia-derived chondroitin/dermatan sulfate proteoglycan syneurin (Faissner, A., A. Lochter, A. Streit, and M. Schachner, manuscript submitted for publication) and astrochondrin share the L5 carbohydrate and the dermatan sulfate epitope DS-1 (Faissner, A., A. Streit, and M. Schachner, unpublished observations). Whereas astrochondrin is expressed by cultured astrocytes, the cytotactin binding (CTB) proteoglycan (Hoffman and Edelman, 1987; Hoffman et al., 1988) is expressed by neurons. Since astrochondrin could not be found to bind J1/tenascin—the mouse homolog of cytotactin (Grumet et al., 1985; Faissner et al., 1988)—CTB proteoglycan appears to show different binding properties from astrochondrin (Hoffman et al., 1988). The core proteins of CTB proteoglycan, astrochondrin and syneurin, however, share immunogenic determinants (Hoffman, S., G. M. Edelman, A. Streit, A. Faissner, and M. Schachner, unpublished observations). No immunochemical relationships between astrochondrin and the chondroitin sulfate proteoglycans CAT 301 (Zaremba et al., 1989) and NG2 (Stallcup and Beasley, 1987) were observed (Hockfield, S.,

W. B. Stallcup, A. Streit, and M. Schachner, unpublished observations). Furthermore, NG2 interacts specifically only with collagen type VI but not with other types of collagen (Stallcup et al., 1990), whereas astrochondrin binds to different collagen types but never to collagen type VI.

Studies on the role of proteoglycans in the nervous system mainly focused on outgrowth and guidance of axons and proposed different modes of their action. However, less attention has been paid to their role in glial development. While astrochondrin takes part in promoting process extension of astrocytes, several other chondroitin and keratan sulfate proteoglycans have been shown to be inhibitory for either neurite outgrowth (Verna et al., 1989; Muir et al., 1989; Snow et al., 1990; Fichard et al., 1991; Cole and McCabe, 1991; Oohira et al., 1991) or neuronal cell migration (Tan et al., 1987; Perris and Johansson, 1987, 1990; Perris and Bronner-Fraser, 1989). Furthermore, also chondroitin sulfate proteoglycans from non-neural tissue, e.g., decorin (Schmidt et al., 1987), appear to decrease cell adhesion (Lark et al., 1985; Culp et al., 1978), and several models for the interaction between these various molecules, cell surface receptors, such as integrins, and the extracellular matrix have been proposed (for review see Ruoslahti, 1988, 1989). Additionally, these molecules modify cell motility and invasive behaviour of metastatic cells (Schrappe et al., 1991; Faassen et al., 1992), processes which seem to be proportional to the expression of chondroitin sulfate proteoglycans (Iozzo, 1985). On the other hand, chondroitin sulfate proteoglycans (Iijima et al., 1991) and different heparan sulfate proteoglycans have been implicated in neurite outgrowth promotion (Lander et al., 1983; Dow et al., 1988; Riopelle and Dow, 1990). Moreover, there is evidence that proteoglycans, such as syndecan as a cell surface receptor for extracellular matrix components (for review see Jalkanen et al., 1991), CD44 as a ligand for extracellular matrix and cell surface molecules (for review see Jalkanen et al., 1991) or heparan sulfate proteoglycans as components of adhesions (Schubert and LaCorbiere, 1985; Cole et al., 1985; Schubert et al., 1983), play a role in mediating cell adhesion (see also Couchman and Höök, 1989; Wight, 1989) and some of them are probably also involved in signal transduction (see Jalkanen et al., 1991). Recently, fibronectin type III and EGF-like repeats or immunoglobulin-like domains, known to be important in other groups of molecules involved

in cell-cell and cell-matrix interactions have been described for several proteoglycans, e.g., syndecan (Saunders et al., 1989), the large fibroblast-derived proteoglycan versican (Zimmermann and Ruoslahti, 1989), decorin (Krusius and Ruoslahti, 1986), the aggregating cartilage proteoglycan (Doege et al., 1987), and the basement membrane heparan sulfate proteoglycan (Noonan et al., 1988; Kallunki and Tryggvason, 1992). It will be interesting to elucidate the contribution of these domains to the various functional properties of proteoglycans.

The biological significance of the functional properties of astrochondrin *in vitro* is underscored by its increased expression in the developing cerebellar cortex during the first two postnatal weeks. In this period, important morphogenetic events, such as cerebellar granule cell migration and outgrowth of newly generated radial Bergmann glial processes, take place. Immunohistochemical data show that astrochondrin is localized at the cell surface of endfeet of astrocytes and Bergmann glia in apposition to the basal lamina of blood vessels and meninges. This spatially and temporally specific expression of astrochondrin in close association with its cellular and acellular binding partners is likely the basis for these interactions. Thus, astrochondrin may be responsible for the successful interaction of astroglia with endothelial cells, thereby constituting a prerequisite for the induction of the blood-brain barrier by astrocytes (Janzer and Raff, 1987). Furthermore, astrochondrin expressed by Bergmann glia is involved in granule cell migration, perhaps in combination with laminin, which has been reported to be transiently expressed by astrocytes and Bergmann glia *in vitro* and *in vivo* (Liesi et al., 1983; Liesi 1985a, b; Selak et al., 1985). It is not known how astrochondrin may mediate granule cell migration, since it is not detectably involved in neurite extension or dislocation of neuronal cell bodies in microexplant cultures. Nevertheless, astrochondrin may be important for the extension of Bergmann glial processes towards the pial surface—a prerequisite for the migration of granule cells. The biological significance of the transient expression of astrochondrin on the cell surface of parallel fibers during the period of active axon outgrowth is presently unknown, since astrochondrin is not expressed by neurons *in vitro* (Streit et al., 1990). It is possible that neurons have lost the ability to synthesize astrochondrin *in vitro* or that astrochondrin is released by astroglia and absorbed to the axonal cell surface by its receptor(s) *in vivo*. It is noteworthy in this context that also the astrocyte-associated extracellular matrix molecule J1/tenascin (Kruse et al., 1985; Faissner et al., 1988) is detectable on axons in the developing optic nerve and cerebellum (Bartsch et al., 1992a, b). It is therefore tempting to speculate that astrochondrin may subserve a functional role in the formation of axonal processes, which could, however, not be observed in the *in vitro* assay systems.

Finally, our results suggest that not only astrochondrin, but the L5 carbohydrate epitope expressed by it, may mediate and/or stabilize process outgrowth by astrocytes. It is important to mention that astrochondrin is the only molecule on astrocytes and by far the predominant molecule in brain carrying the L5 epitope. Interestingly, another carbohydrate structure, the L2/HNK-1 epitope, which is also expressed by astrochondrin (Streit et al., 1990) and several neural recognition molecules (Kruse et al., 1984, 1985; Schachner et al., 1990) plays an important role as a ligand in astrocyte-lami-

nin interactions (Künemund et al., 1988). However, in contrast to the L5 carbohydrate, the L2/HNK-1 carbohydrate appears to be directly involved in the initial attachment of astrocytes to laminin (Liu, L., H. Hall, M. Schachner, B. Schmitz, submitted for publication). Thus, carbohydrate structures as posttranslational modifications may be instrumental in influencing and modifying cellular behaviour by their specific temporal expression patterns possibly on even one and the same protein backbone (Schachner et al., 1990). Further investigations will elucidate the functional roles of carbohydrates in cell interactions and the structural basis underlying these interactions.

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