Immunoelectron Microscopic Localization of Hyaluronic Acid-binding Region and Link Protein Epitopes in Brain

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Abstract. The 1C6 monoclonal antibody to the hyaluronic acid-binding region weakly stained a 65-kD component in immunoblots of the chondroitin sulfate proteoglycans of brain, and the 8A4 monoclonal antibody, which recognizes two epitopes in the polypeptide portion of link protein, produced strong staining of a 45-kD component present in the brain proteoglycans. These antibodies were utilized to examine the localization of hyaluronic acid-binding region and link protein epitopes in rat cerebellum. Like the chondroitin sulfate proteoglycans themselves and hyaluronic acid, hyaluronic acid-binding region and link protein immunoreactivity changed from a predominantly extracellular to an intracellular (cytoplasmic and intraaxonal) location during the first postnatal month of brain development. The cell types which showed staining of hyaluronic acid-binding region and link protein, such as granule cells and their axons (the parallel fibers), astrocytes, and certain myelinated fibers, were generally the same as those previously found to contain chondroitin sulfate proteoglycans and hyaluronic acid. Prominent staining of some cell nuclei was also observed. In agreement with earlier conclusions concerning the localization of hyaluronic acid and chondroitin sulfate proteoglycans, there was no intracellular staining of Purkinje cells or nerve endings or staining of certain other structures, such as oligodendroglia and synaptic vesicles. The similar localizations and coordinate developmental changes of chondroitin sulfate proteoglycans, hyaluronic acid, hyaluronic acid-binding region, and link protein add further support to previous evidence for the unusual cytoplasmic localization of these proteoglycans in mature brain. Our results also suggest that much of the chondroitin sulfate proteoglycan of brain may exist in the form of aggregates with hyaluronic acid.

In previous immunocytochemical studies at the light and electron microscopic levels we have demonstrated changes in the localization of chondroitin sulfate proteoglycans in the developing postnatal rat cerebellum (Aquino et al., 1984*a,b*). More recent light and electron microscopic studies on the localization of hyaluronic acid, using a highly sensitive and specific biological probe consisting of the biotinylated hyaluronic acid-binding region prepared from rat chondrosarcoma proteoglycan aggregates (Ripellino et al., 1985), revealed coordinate developmental changes in cerebellar hyaluronic acid and chondroitin sulfate proteoglycans (Ripellino et al., 1988).

In cartilage and certain other tissues, a hyaluronic acid-binding site at the NH_2 terminus of chondroitin sulfate proteoglycan monomers interacts with decasaccharide or larger oligomers of hyaluronic acid to form proteoglycan aggregates. This binding is stabilized by the presence of a third component, link protein, which occurs in two forms having molecular sizes of ~45 and 48 kD (Hassell et al., 1986). Rat brain chondroitin sulfate proteoglycans can be demonstrated to aggregate with hyaluronic acid to the extent of only ~10% when assayed by gel filtration (Kiang et al., 1981), and more recent attempts using other techniques did not demonstrate any greater degree of aggregation (Ripellino et al., 1988). However, considerable aggregation with hyaluronic acid has been reported for chondroitin sulfate proteoglycans from other types of nervous tissue, such as cultured human glial and glioma cells (Norling et al., 1984) and embryonic chick brain (Crawford, 1988). The similar localizations and coordinate developmental changes of hyaluronic acid and chondroitin sulfate proteoglycans in rat cerebellum suggested that a significant proportion of the chondroitin sulfate proteoglycans in rat brain may occur in the form of larger aggregates with hyaluronic acid in situ, even though this is not readily demonstrable by in vitro biochemical assays. We have therefore further studied this question by conducting parallel immunocytochemical studies on the localization of link protein and hyaluronic acid-binding region epitopes using monoclonal antibodies prepared to these components of cartilage proteoglycans (Caterson et al., 1987).

Materials and Methods

Electrophoresis and Immunoblotting

Chondroitin sulfate proteoglycans from 30-d-old rat brain were prepared either as described by Kiang et al. (1981) or together with the heparan sulfate proteoglycans (Klinger et al., 1985), since the products obtained by both procedures have identical properties. In the latter case, a deoxycholate ex-

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tract was chromatographed on DEAE-cellulose, followed by affinity chromatography on lipoprotein lipase-Sepharose (to isolate the heparan sulfate proteoglycans). The unbound fraction from the lipoprotein lipase affinity column, eluted with 0.2 M NaCl, contained almost exclusively chondroitin sulfate proteoglycan and nucleic acid. Residual Tween 80 (from the original DEAE-cellulose chromatography step) was removed by reabsorption and elution of the proteoglycans from a small column of DEAE-cellulose. After dialysis and lyophilization, the proteoglycans were dissolved in 0.2 M sodium acetate buffer, pH 5.6, for gel filtration on Sepharose CL-6B to remove nucleic acids and some smaller proteins (Kiang et al., 1981).

For chondroitinase digestion, the proteoglycans were treated for 1.5 h at 37°C with chondroitinase ABC (obtained from Seikagaku Kogyo Co., Tokyo, Japan through ICN Immunobiologicals, Irvine, CA) in a ratio of 1 mU/μg protein in the presence of protease inhibitors (Oike et al., 1980).

Proteoglycan samples were heated for 5 min at 100°C in sample buffer containing SDS and mercaptoethanol, and electrophoresed on a 6-12% SDS-polyacrylamide slab gel or a 7% "minigel" (7 × 10 cm, Bio-Rad Laboratories, Cambridge, MA) using the discontinuous buffer system of Laemmli (1970).

After blocking with 1% fat-free dry milk in TBS, pH 7.5, nitrocellulose immunoblots were incubated with a 1:100 dilution of rabbit antisera to the proteoglycans (Aquino et al., 1984*a*) in TBS containing 0.05% Tween-20 and 1% milk followed by peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories; diluted 1:2,000 in Tween-TBS containing 5% BSA). The 12/21/1C6 and 9/30/8A4 monoclonal antibodies (isotypes IgG$_1$ and IgG$_{2b}$, respectively; generously provided by Dr. Bruce Caterson, West Virginia University Health Science Center) were diluted 1:30 in Tween-TBS containing 1% milk followed by peroxidase-conjugated rabbit anti-mouse IgG (ICN Immunobiologicals; diluted 1:250 in Tween-TBS containing 5% BSA). Color development was with hydrogen peroxide and 3,3'-diaminobenzidine.

Immunocytochemistry

Rats were perfused with 4% formaldehyde/0.1% glutaraldehyde, and Vibratome sections of the cerebellum were prepared as described previously (Aquino et al., 1984*a*). For staining with the 1C6 and 8A4 monoclonal antibodies, sections were incubated for 30 min with 5% BSA and then overnight at 4°C with 1C6 or 8A4 ascites fluid diluted (1:100 and 1:50, respectively) in 0.1 M PBS, pH 7.4. Control sections were incubated in PBS without primary antibody. Sections were then washed three times for 15 min with 50 mM TBS, pH 7.4, incubated for 30 min with a 1:30 dilution of rabbit anti-mouse IgG (CooperBiomedical Inc., Malvern, PA) in TBS containing 1% normal rabbit serum, washed with TBS, incubated for 30 min with peroxidase-antiperoxidase complex (monoclonal mouse origin; Sternberger-Meyer Immunocytochemicals, Jarrettsville, MD; 1:100 in TBS containing 1% normal rabbit serum), and washed again with TBS. Color was developed by incubation for 10 min in 0.05% 3,3'-diaminobenzidine/0.01% hydrogen peroxide in TBS, pH 7.6, followed by washing with TBS as above. Monoclonal antibodies (both IgG$_1$ isotype) to glial fibrillary acidic protein (clone G-A-5) and to α-tubulin (clone DM-1A) were obtained from ICN Immunobiologicals and used as described above for 1C6 and 8A4. Staining of chondroitin sulfate proteoglycans was performed by the peroxidase-antiperoxidase procedure using F(ab')$_2$ fragments prepared from a rabbit antiserum to the proteoglycans (Aquino et al., 1984*a*). Sections were processed for electron microscopy as described previously (Ripellino et al., 1988).

Results

Immunostaining of Proteoglycan Components Fractionated by SDS-PAGE

Immunoblots of the chondroitin sulfate proteoglycans of rat brain after SDS-PAGE and stained with polyclonal antisera

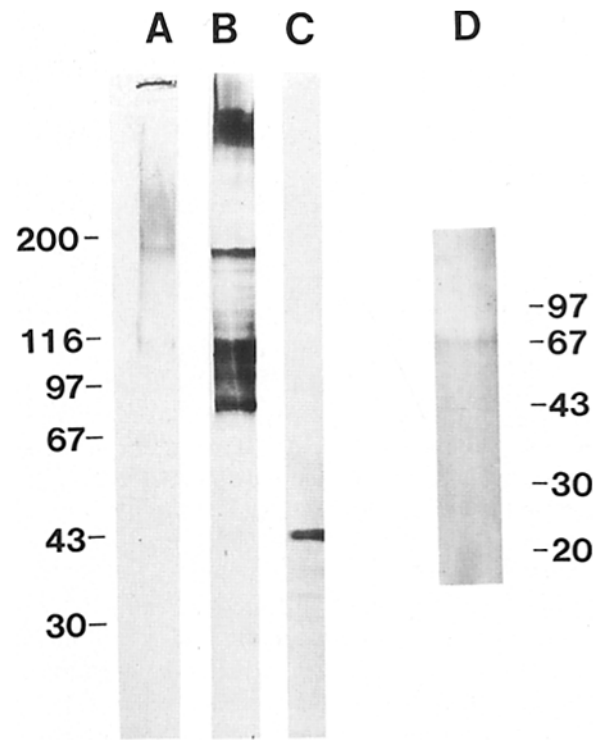
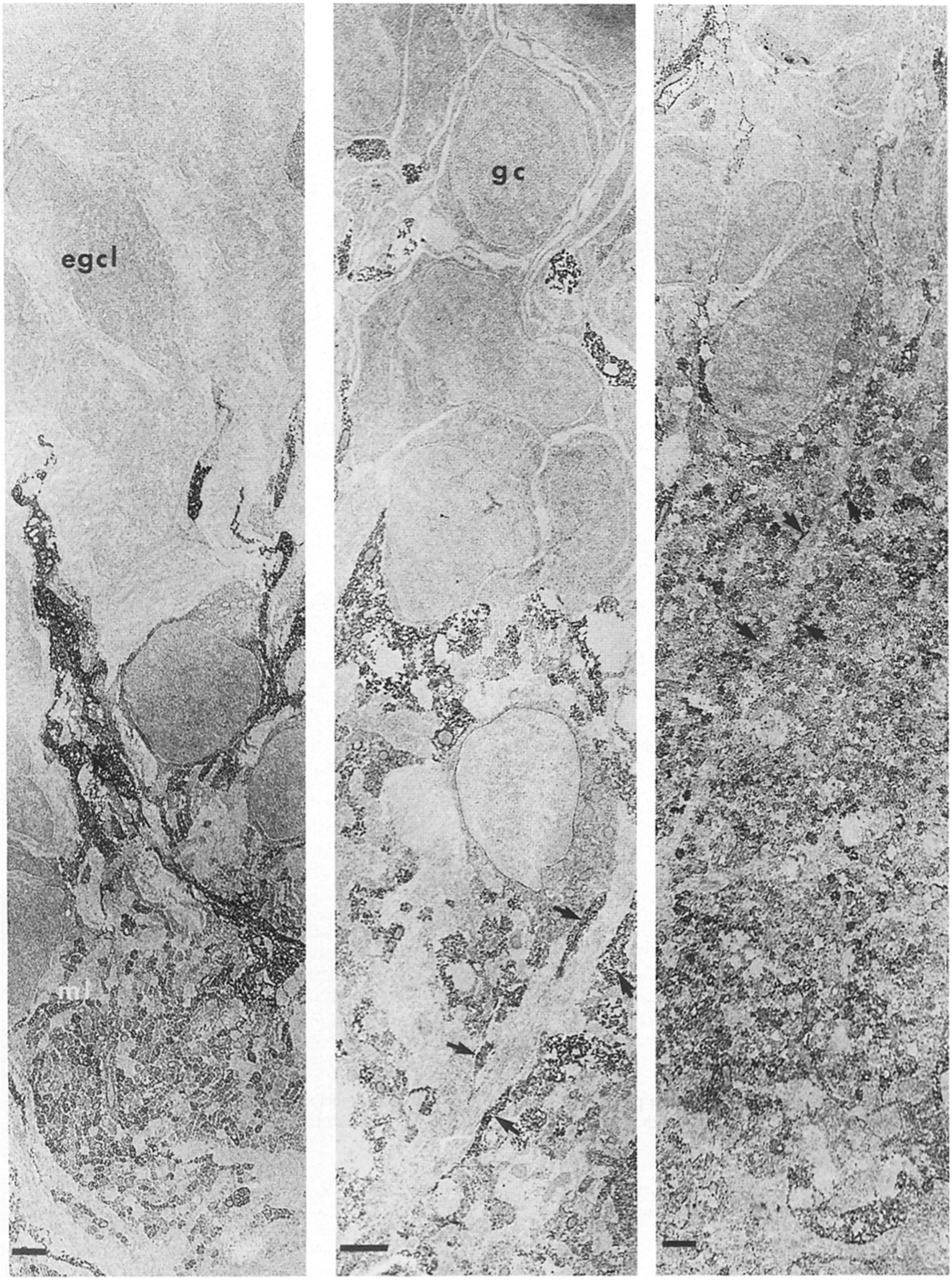


Figure 1. Nitrocellulose immunoblot of the chondroitin sulfate proteoglycans of brain before (lane *A*) and after (lane *B*) chondroitinase treatment, stained with a rabbit antiserum to the native proteoglycans. Untreated proteoglycans were stained with the 8A4 monoclonal antibody to link protein epitopes (lane *C*) and with the 1C6 antibody to the hyaluronic acid-binding region (lane *D*). Lanes *A*-*C* were electrophoresed on a 6-12% gradient gel; lane *D* on a 7% minigel (see Materials and Methods for details).

to the proteoglycans (before and after chondroitinase treatment) revealed a polydisperse and heterogeneous mixture of molecular species (Fig. 1, lanes *A* and *B*). In vitro translation studies and Coomassie blue staining of chondroitinase-treated proteoglycans fractionated by SDS-PAGE support the conclusion that the chondroitin sulfate proteoglycans of brain contain multiple core proteins (Gowda et al., 1989), some of which may be specific to particular cell types. When immunoblots were stained with the 8A4 monoclonal antibody, which recognizes two epitopes in the polypeptide portion of rat chondrosarcoma link protein (Caterson et al., 1985), strong staining was seen at 45 kD (Fig. 1, lane *C*). The stained component of the brain proteoglycan preparation presumably represents one of the two forms (45 and 48 kD) of link protein present in cartilage and other connective tissues.

The 1C6 monoclonal antibody to the hyaluronic acid-binding region of cartilage, muscle, aorta, and other proteogly-

Figure 2. Similarities in staining of 7-d-old rat cerebellum using antibodies to the chondroitin sulfate proteoglycans (*left*), hyaluronic acid-binding region (*center*), and link protein (*right*). The external granule cell layer (*egcl*) shows only extracellular staining surrounding the unstained granule cells (*gc*), whereas in the developing molecular layer (*ml*) there is axonal staining in addition to extracellular staining (*arrows*) surrounding the processes of Golgi epithelial cells (the Bergmann glial fibers) which ascend in bundles from the Purkinje cell layer. Bars, 1 μm.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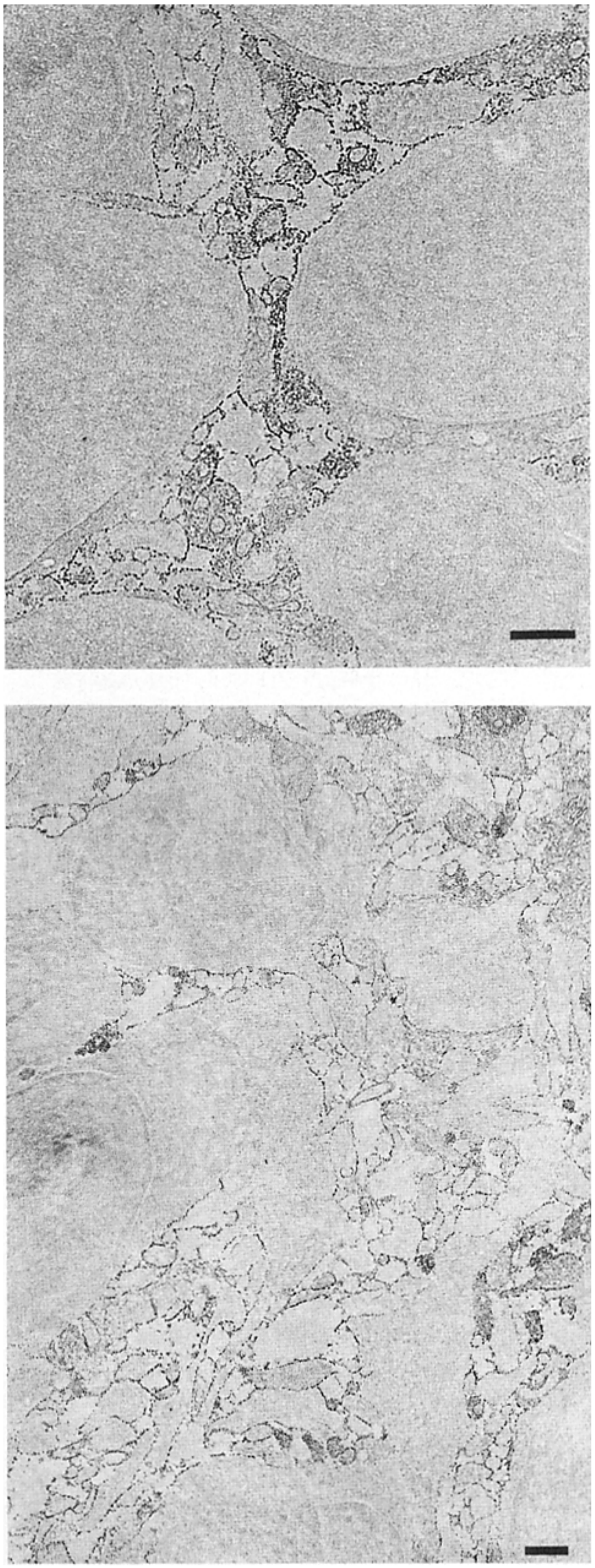


Figure 3. Extracellular staining of hyaluronic acid–binding region (*top*) and link protein (*bottom*) in the internal granule cell layer of 7-d-old cerebellum. Bars, 1 μm.

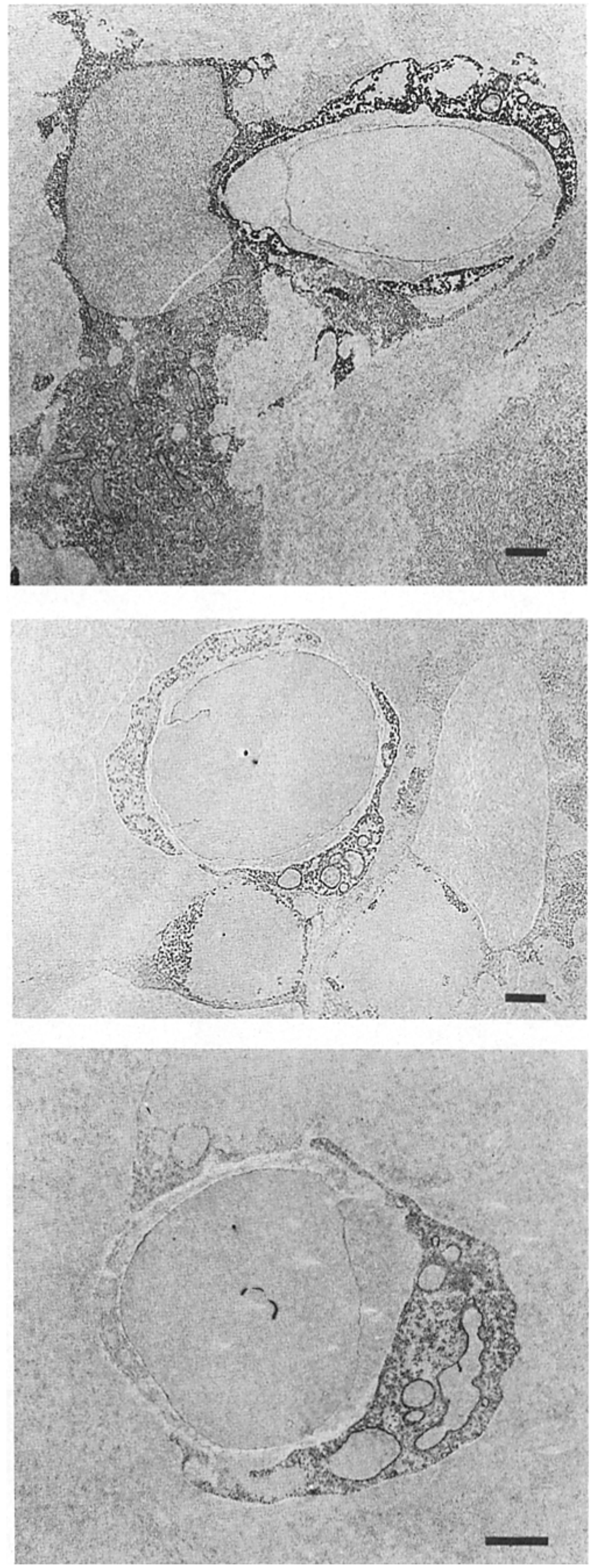
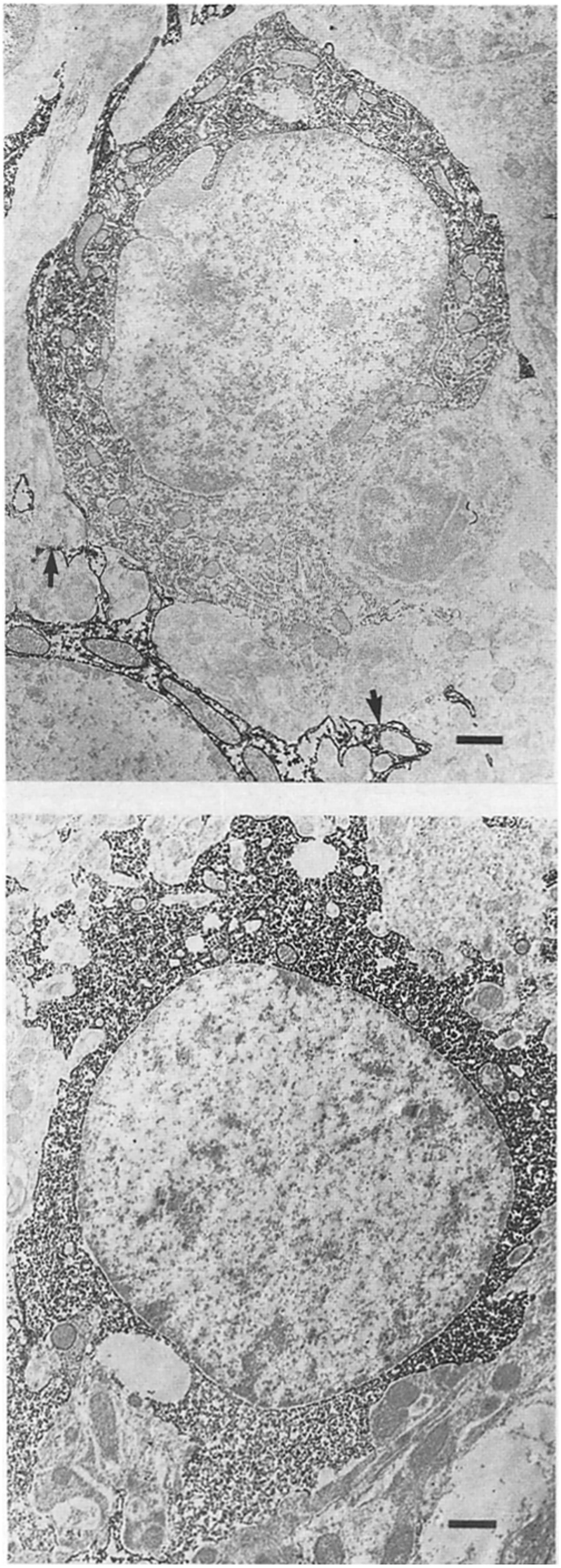


Figure 4. Staining of chondroitin sulfate proteoglycans (*top*), hyaluronic acid–binding region (*center*), and link protein (*bottom*) in perivascular astrocytes of 7-d-old cerebellum. Cytoplasmic staining of chondroitin sulfate proteoglycans is also seen in an adjacent cell. Bars, 1 μm.



cans (Caterson et al., 1987) weakly stained a 65-kD component of the proteoglycans in the more compact bands seen after SDS-PAGE on a minigel (Fig. 1, lane *D*), although this was usually not apparent on regular size gels and the staining intensity was not affected by chondroitinase treatment of the proteoglycans. The hyaluronic acid–binding region epitope may also be present on larger molecular size core proteins, but in a conformation which is not recognized on immunoblots by the 1C6 monoclonal antibody. It is likely that the 65-kD band represents a proteolytic product of the chondroitin sulfate proteoglycan core protein, since it is known that the hyaluronic acid–binding region occurs as a 65–67-kD fragment after trypsin digestion of most cartilage proteoglycans (Faltz et al., 1979) and since the protein stained by the 1C6 monoclonal antibody also corresponds in molecular size to hyaluronic acid–binding proteins which have been isolated from brain and other tissues (Delpech et al., 1986, 1987). No other bands were stained with either monoclonal antibody when tested with total brain proteins rather than a purified proteoglycan preparation.

The cross-reactivity between these components in connective and nervous tissues is not surprising in view of our previous finding (based on ELISA and immunocytochemical studies) of a significant degree of immunochemical cross-reactivity between brain and cartilage proteoglycans (Aquino et al., 1984*a*). This can also be demonstrated in immunoblots, where it is seen that a rabbit antiserum to the brain proteoglycans recognizes a number of components in chondroitinase-treated cartilage proteoglycans, and that antibodies raised to a high molecular size core protein purified from chondroitinase-treated brain proteoglycans cross-react equally well with most of the other brain proteoglycan core proteins and with those of cartilage proteoglycans (unpublished results).

Immunocytochemical Localization

Preliminary light microscopic studies revealed that developmental changes in the localization of hyaluronic acid–binding region and link protein epitopes in rat cerebellum were very similar to those previously found for chondroitin sulfate proteoglycans and hyaluronic acid (Ripellino et al., 1988). These sudies were therefore extended to the electron microscopic level to permit us to determine whether these similarities also applied to other features, such as their extracellular, cytoplasmic, and nuclear localizations.

Antibodies to the chondroitin sulfate proteoglycans, the hyaluronic acid–binding region, and link protein produced a similar staining pattern in 7-d-old rat cerebellum–predominantly extracellular staining in the internal and external granule cell layers and the developing molecular layer (Figs. 2 and 3). However, there was also intraaxonal staining in the molecular layer (Fig. 2), and some cytoplasmic (Fig. 4) and nuclear staining of hyaluronic acid–binding region, link protein, and chondroitin sulfate proteoglycans was seen in 7-d-

Figure 5. Cytoplasmic staining of hyaluronic acid–binding region (*top*) and link protein (*bottom*) in Golgi epithelial cells (protoplasmic astrocytes) of adult cerebellum. Staining of hyaluronic acid–binding region in an adjacent cell also extends into fine processes (*arrows*). Bars, 1 μm.

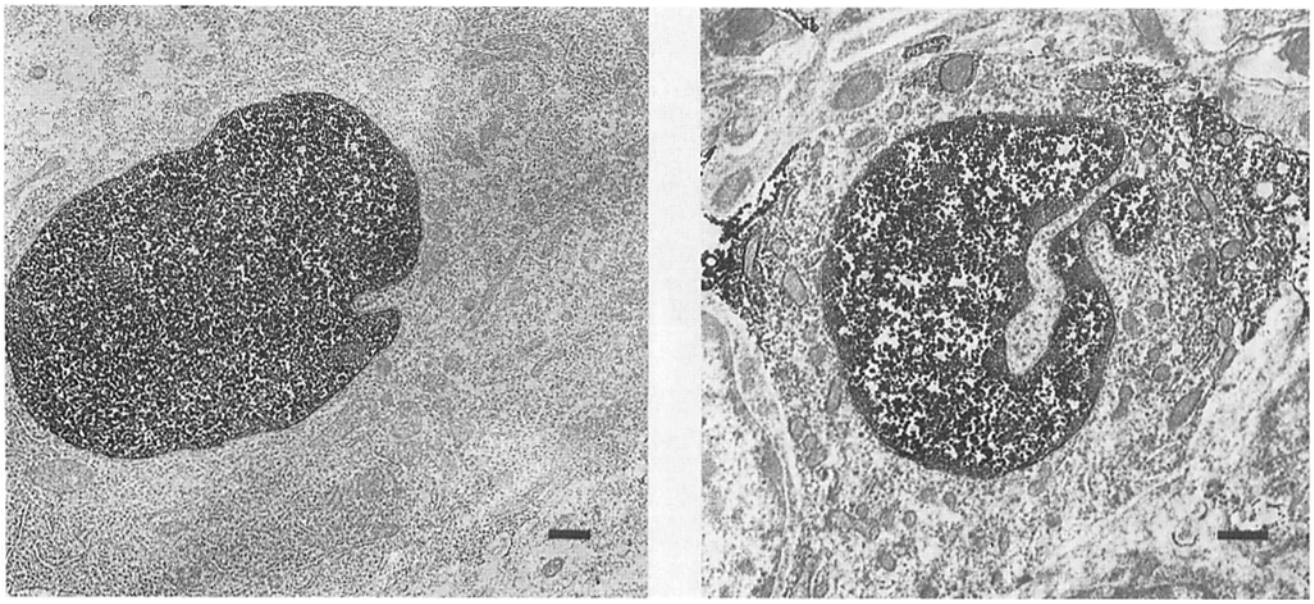


Figure 6. Nuclear staining of hyaluronic acid–binding region (*left*) and link protein (*right*) in large Golgi neurons of adult cerebellum. Bars, 1 μm.

old brain. In adult brain, staining of all three components appeared to be exclusively intracellular (cytoplasmic, nuclear, and intraaxonal), as seen in Figs. 5–9. In certain cases there was also staining in the vicinity of plasma membranes, as was previously found for hyaluronic acid and the chondroitin sulfate proteoglycans (Aquino et al., 1984*a*; Ripellino et al., 1988). Whether or not this indicates some persistence of extracellular staining in adult brain cannot be definitely determined because of the size of the diaminobenzidine reaction product, which does not permit clear resolution of the plasma membrane from the 20–40-nm intercellular space of adult brain.

Discussion

We found that staining of brain sections with the 1C6 and 8A4 antibodies did not require reduction and alkylation, which is known to be necessary to expose sequestered epitopes recognized by these antibodies in cartilage proteoglycans (Caterson et al., 1987). Because the relatively prolonged reduction and alkylation procedure is highly deleterious to good morphological preservation at the electron microscopic level, it was omitted in all studies of this type. Since the present report is, to our knowledge, the first application of these antibodies to the study of noncartilagenous tissues, it is possible that other ultrastructural investigations of chondroitin sulfate proteoglycans in such tissues might be similarly facilitated by the existence of different types or conformations of the hyaluronic acid–binding region and link protein which do not require treatment of tissue sections to obtain antibody reactivity.

The specificity of the staining obtained in our studies in the absence of reduction and alkylation is supported by several types of evidence. These include the facts that (*a*) the 1C6 and 8A4 antibodies do not stain other components present in immunoblots of brain proteins; (*b*) irrelevant monoclonal antibodies (e.g., to α-tubulin and glial fibrillary acidic protein) used under identical conditions do not produce a similar staining pattern; and (*c*) this unusual distribution of staining is identical to that previously found for hyaluronic acid and the chondroitin sulfate proteoglycans themselves, using independent methods (i.e., affinity-purified F[ab']$_2$ prepared from a polyclonal antiserum to the protein moiety of the proteoglycans, and a biotinylated nonantibody probe for hyaluronic acid).

The nuclear staining seen with the 1C6 and 8A4 antibodies is similar to that previously found for chondroitin sulfate proteoglycans and hyaluronic acid (Aquino et al., 1984*a*,*b*; Ripellino et al., 1988). Nuclear staining was present in all of the cell types which showed intracellular staining and, although we did not specifically study this aspect, it would appear that only a small proportion (~20–30%) of the nuclei were stained. There have been numerous previous reports, mostly based on biochemical analyses, demonstrating the presence of hyaluronic acid, chondroitin sulfate, and heparan sulfate in highly purified nuclei, including those of rat brain (Bhavanandan and Davidson, 1975; Stein et al., 1975, 1981; Margolis et al., 1976; Fromme et al., 1976; Furukawa and Terayama, 1977, 1979; Fedarko and Conrad, 1986; Ishihara et al., 1986). Although cultures of a rat hepatocyte cell line accumulate a nuclear pool of free heparan sulfate chains (Fedarko and Conrad, 1986; Ishihara et al., 1986), our immunocytochemical studies using antibodies to protein epitopes indicated that the chondroitin sulfate present in brain nuclei occurs in the form of proteoglycans. Later morphological evidence that these nuclei also contain hyaluronic acid, as well as hyaluronic acid–binding region and link protein epitopes, strongly suggests that at least a portion of the nuclear chondroitin sulfate proteoglycans may occur in the form of aggregates with hyaluronic acid.

Our studies indicate that the chondroitin sulfate proteoglycans of brain contain a link protein and a hyaluronic acid–

binding region which, in terms of molecular size and immunochemical reactivity, correspond to the smaller (45-kD) species of cartilage proteoglycan link protein and to the hyaluronic acid–binding region fragment obtained by trypsin treatment of cartilage proteoglycans, respectively. Using monoclonal antibodies to these two components of chondroitin sulfate proteoglycan aggregates to study their localization in rat cerebellum, it was found that, like the chondroitin sulfate proteoglycans themselves (Aquino et al., 1984*a*,*b*) and hyaluronic acid (Ripellino et al., 1988), their localization changed from a predominantly extracellular to an intracellular (cytoplasmic and intraaxonal) location during the first postnatal month of brain development. The cell types which showed staining of hyaluronic acid–binding region and link protein, such as granule cells and their axons (the parallel fibers), astrocytes, and certain myelinated fibers, were generally the same as those previously found to contain chondroitin sulfate proteoglycans and hyaluronic acid. However, in agreement with earlier conclusions concerning the localization of hyaluronic acid and chondroitin sulfate proteoglycans (Aquino et al., 1984*a*,*b*; Ripellino et al., 1988) there was no intracellular staining of Purkinje cells or nerve endings, and other structures, such as oligodendroglia and synaptic vesicles, also remained unstained.

Intracellular (cytoplasmic and nuclear) staining of link protein and hyaluronic acid–binding region epitopes in early postnatal brain was more apparent than that of chondroitin sulfate proteoglycans and hyaluronic acid seen in our previous studies, although some cytoplasmic staining of chondroitin sulfate proteoglycans was also seen in 7-d-old cerebellum. While no attempt was made to quantitate the proportion of intracellular, as compared with extracellular, staining of these four components in early postnatal brain, any differences in their relative intracellular staining may reflect differences in the developmental time course and sites of occurrence of chondroitin sulfate proteoglycans in the form of aggregates with hyaluronic acid, as compared with free proteoglycan monomers.

The rat brain chondroitin sulfate proteoglycans which we have studied are probably closely related to the chicken brain proteoglycans recently described by Hoffman et al. (1988) insofar as they both contain HNK-1 (glucuronic acid 3-sulfate) epitopes (Margolis et al., 1987; Hoffman and Edelman, 1987; Gowda et al., 1989), and, in the adult, the partially glycosylated core proteins obtained after chondroitinase treatment of the native proteoglycans have a similar range of molecular sizes. However, our inability to stain immunoblots of the brain proteoglycans with a rabbit antiserum to tenascin (cytotactin) indicates that this protein does not copurify with the rat brain proteoglycans prepared under our conditions (unpublished results). It is more difficult to draw conclusions concerning the possible relationship of these proteoglycans with the NG2 antigen, a cell surface protein which is reported to be a chondroitin sulfate proteoglycan and is associated with protoplasmic astrocytes (Levine and Card,

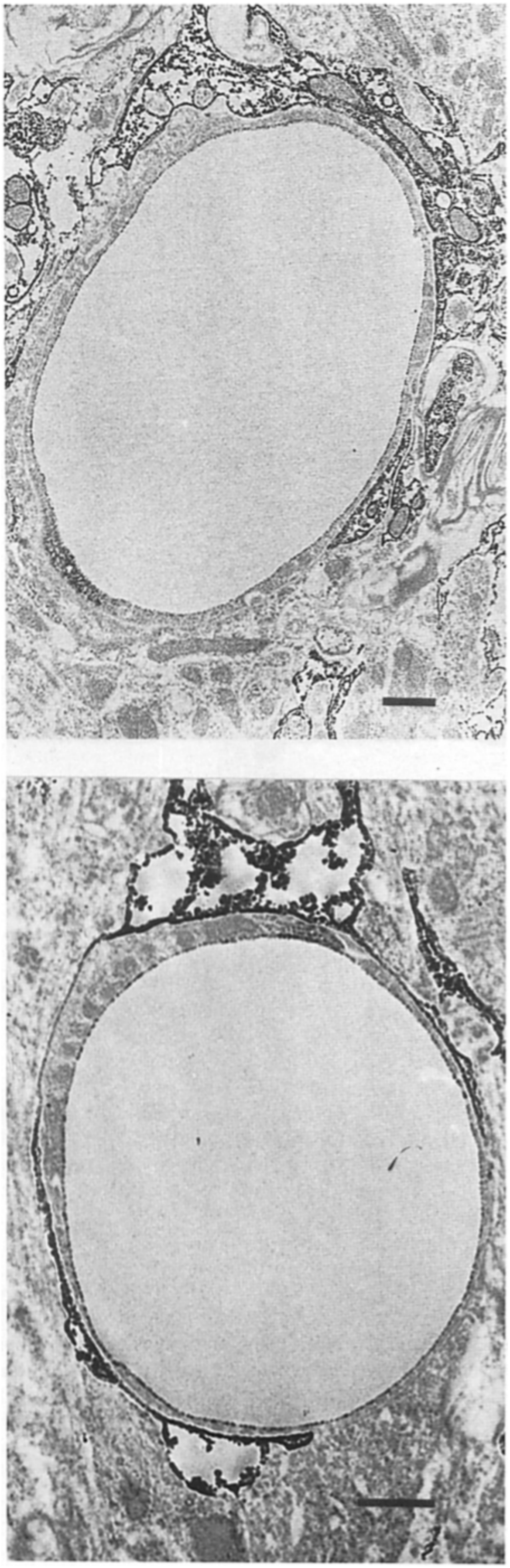


Figure 7. Cytoplasmic staining of hyaluronic acid–binding region (*top*) and link protein (*bottom*) in perivascular astrocytes of adult cerebellum. Bars, 1 μm.

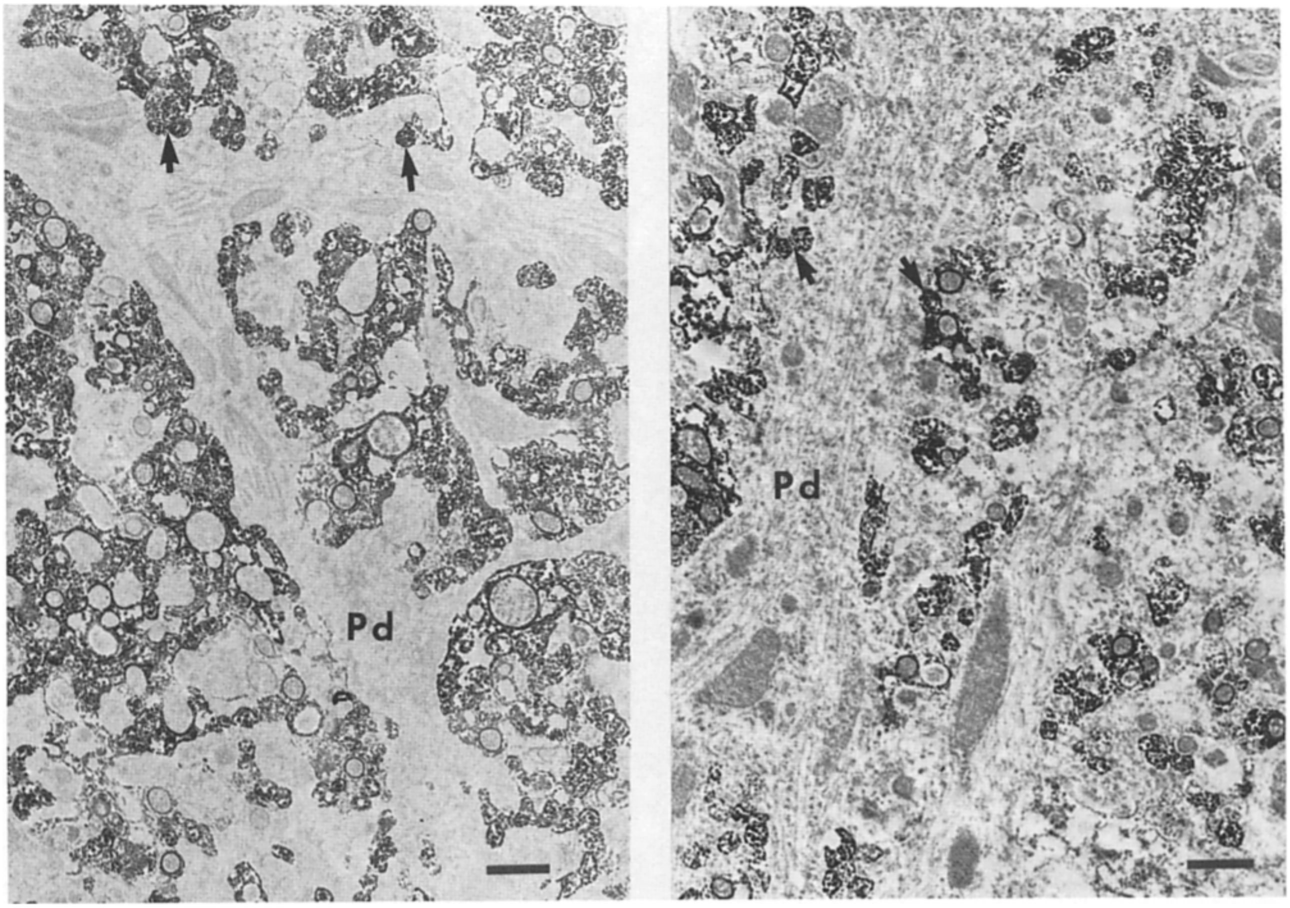


Figure 8. Intraaxonal staining of hyaluronic acid–binding region (*left*) and link protein (*right*) in parallel fibers of adult cerebellum (*arrows*), surrounding unstained Purkinje cell dendrites (*Pd*). Bars, 1 μm.

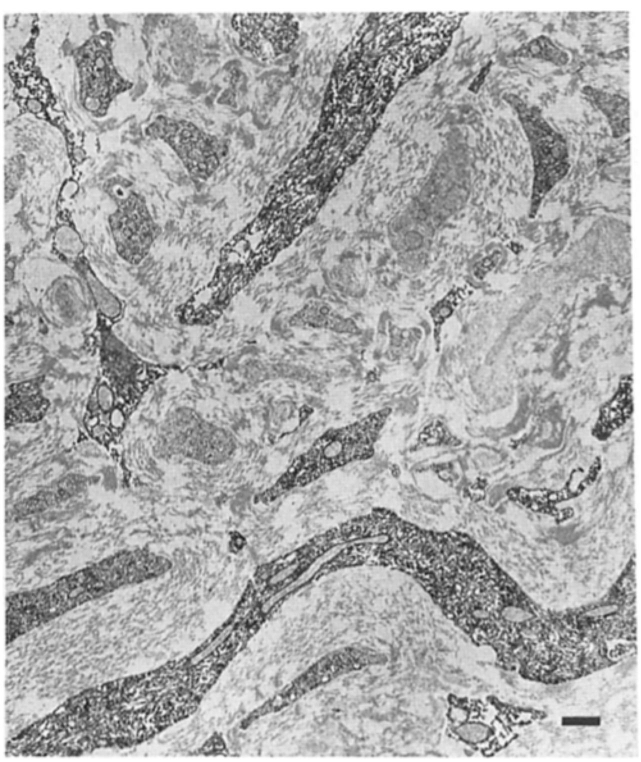


Figure 9. Intraaxonal staining of hyaluronic acid–binding region in myelinated axons of adult cerebellar white matter. Bar, 1 μm.

1987), since the biochemical properties of this antigen have not been characterized in detail.

While it is not yet clear what might be the biological significance of the developmental change in localization of chondroitin sulfate proteoglycans in the brain (from being predominantly extracellular in the early postnatal period to almost exclusively intracellular in mature brain), there is no evidence that this represents an actual movement of proteoglycan from an extracellular to an intracellular compartment. The most likely explanation for our observation may be that as the extracellular space decreases dramatically during brain development together with the concentrations of hyaluronic acid and chondroitin sulfate proteoglycans (Margolis et al., 1975) and intracellular staining becomes more pronounced, the net effect appears as a change in localization, although the differences are more quantitative than qualitative. The relatively large amounts of hyaluronic acid and chondroitin sulfate proteoglycans present in early postnatal brain could serve as a space-filling matrix through which neuronal migration and differentiation can take place during early brain development and may also partially account for the higher water content of brain at these ages. Their subsequent removal from the extracellular space may then allow various adhesive processes to begin. Aside from these considerations, the biological roles of chondroitin sulfate proteoglycans in the cytoplasm, axoplasm, and nuclei of mature brain remain to be elucidated.

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