0014-4754/93/050429-18$1.50 + 0.20

Nervous tissue proteoglycans

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Abstract. The structure, biosynthesis, localization, and possible functional roles of nervous tissue glycosaminoglycans and proteoglycans were last reviewed several years ago[70,74]. Since that time, there has been an exponential increase in publications on the neurobiology of proteoglycans. This review will therefore focus on reports which have appeared in the period after 1988, and especially on those concerning the properties of individual characterized nervous tissue proteoglycans. Related areas such as the regulation of glycosaminoglycan biosynthesis and the roles of cell surface proteoglycans in adhesion and growth control are covered in other contributions to this special topic issue.

Key words. Chondroitin sulfate; heparan sulfate; keratan sulfate; cell interactions; cell adhesion molecules; growth factors; cDNA cloning; monoclonal antibodies.

A. Structure and localization of chondroitin sulfate and keratan sulfate proteoglycans

Chondroitinase treatment of purified chondroitin sulfate (or total) soluble proteoglycans of rat brain has indicated the presence of possibly a dozen or more chondroitin sulfate proteoglycan core proteins of different sizes as determined by SDS-PAGE[34,41,88]. However, some of these bands are now known to result from proteolytic processing of a single proteoglycan species (e.g., neurocan, see below), while others may represent the products of mRNA splice variants of a single proteoglycan gene. Biochemical analyses of bulk-isolated neurons and astrocytes demonstrated the presence of chondroitin sulfate and heparan sulfate, which were not detected in oligodendrocytes[73], and the biosynthesis of chondroitin sulfate proteoglycans has been reported for both neurons[43,83] and astrocytes[29,30,50,86]. The demonstrated biochemical heterogeneity of brain chondroitin sulfate proteoglycans is consistent with the finding that although certain of these proteoglycans may be limited to particular types of neurons or glia, studies using monoclonal antibodies to individual chondroitin sulfate preoteoglycan species have revealed both the presence of a particular proteoglycan in multiple cell types and of several different proteoglycans in a single type of cell[98]. Early immunocytochemical studies at the light and electron microscopic levels on the localization of the quantitatively major class of PBS-soluble chondroitin sulfate proteoglycans demonstrated their presence in the extracellular space of early postnatal rat cerebellum, whereas in adult brain they were found predominantly in the cytoplasm or axoplasm, and in the nuclei of some astrocytes and granule cells[3,4]. A similar localization and coordinate developmental changes were found using a specific biotinylated (non-antibody) probe for hyaluronic acid[102], and using monoclonal antibodies for hyaluronic acid binding region and link protein epitopes associated with aggregating species of chondroitin sulfate proteoglycans[103]. In contrast, certain less abundant chondroitin sulfate proteoglycans of brain, such as the NG2 and Cat-301 antigens (see below), appear to be integral membrane components or cell surface associated at all stages of development. Other studies using either partially characterized monoclonal antibodies which are thought to recognize proteoglycan epitopes[28,122] or antibodies to chondroitin sulfate[9,26,28] have also provided data supporting the presence of cell surface or extracellular chondroitin sulfate proteoglycans in mature brain. In a study using monoclonal antibodies specific for unsulfated, 4-sulfated, and 6-sulfated disaccharide 'stubs' which remain attached to the core protein after chondroitinase ABC digestion of chondroitin sulfate proteoglycans, the immunocytochemical localization of chondroitin and the two isomeric chondroitin sulfates was examined in developing rat cerebellum[26]. The possible unsulfated, and mono-, di-, and trisulfated disaccharides were also fractionated and quantitated by HPLC analysis, and localized to specific chondroitin sulfate proteoglycan core proteins present on immunoblots. The different localizations and developmental changes demonstrated for chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate indicate that chondroitin and the two chondroitin sulfates do not result from the random sulfation (or non-sulfation) of a chondroitin precursor polysaccharide, but may reflect specific and biologically significant properties of these different molecular forms in neurobiological processes.

1. Chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of brain

Neurocan (1D1) and the 3F8/3H1 proteoglycans. Most of the recent information on individual species of nervous tissue proteoglycans has concerned molecules initially identified using monoclonal antibodies. Several of these chondroitin sulfate proteoglycans of brain are developmentally regulated with respect to their concentration and/or molecular size, carbohydrate composition, sulfation, and immunocytochemical localization[98]. The biochemical properties of three such proteoglycans isolated by immunoaffinity chromatography with monoclonal antibodies are summarized in the table. Neurocan (formerly designated 1D1) consists of a major component with a molecular size of ~300 kDa in 7-day brain, containing a 245 kDa core glycoprotein and an average of three 22 kDa chondroitin sulfate chains. A ~180 kDa proteoglycan with a core glycoprotein having an apparent molecular size on SDS-PAGE of 150 kDa is also present at 7 days, and by 2–3 weeks postnatal this becomes the major species recognized by the 1D1 monoclonal antibody. Peptide maps indicated that the 150 kDa core protein is part of the larger 245 kDa protein found in early postnatal brain. Neurocan aggregates with hyaluronic acid, and both core proteins are recognized by the 8A4 monoclonal antibody to rat chondrosarcoma link protein. This antibody also reacts with a 45 kDa link protein which copurifies with the proteoglycans isolated from either early postnatal or adult brain.

Neurocan has recently been cloned and its primary structure reported[99]. An open reading frame of 1257 amino acids encodes a protein with a molecular mass of 136 kDa containing 10 peptide sequences present in the adult and/or early postnatal brain proteoglycans. The deduced amino acid sequence revealed a 22 amino acid signal peptide followed by an immunoglobulin domain, tandem repeats characteristic of the hyaluronic acid-binding region of aggregating proteoglycans, and an RGDS sequence. The C-terminal portion (amino acids 951–1215) has ~60% identity to regions in the C-termini of the fibroblast and cartilage proteoglycans, versican and aggrecan, including two epidermal growth factor-like domains, a lectin-like domain, and a complement regulatory protein-like sequence (fig. 1). The central 595 amino acid portion of neurocan has no homology with other reported protein sequences. The proteoglycan contains six potential *N*-glycosylation sites and 25 potential threonine *O*-glycosylation sites. In the adult form of the proteoglycan (which represents the C-terminal half of neurocan) a single 32 kDa chondroitin 4-sulfate chain is linked at serine-944, whereas three additional potential chondroitin sulfate attachment sites (only two of which are utilized) are present in the N-terminal portion of the larger proteoglycan species. A probe corresponding to a region of neurocan having no homology with versican or aggrecan hybridized with a single band at ~7.5 kb on Northern blots of mRNA from both 4-day and adult rat brain (but not with muscle, kidney, liver, or lung mRNA), indicating that the 1D1 proteoglycan of adult brain, containing a 68 kDa core protein, is generated by a developmentally regulated in vivo proteolytic processing of the 136 kDa species which is predominant in early postnatal brain. Another monoclonal antibody has recently been used for the isolation of two proteoglycans derived from the N-terminal half of neurocan but which do not carry the 1D1 epitope (R. K. Margolis and R. U. Margolis, unpublished results).

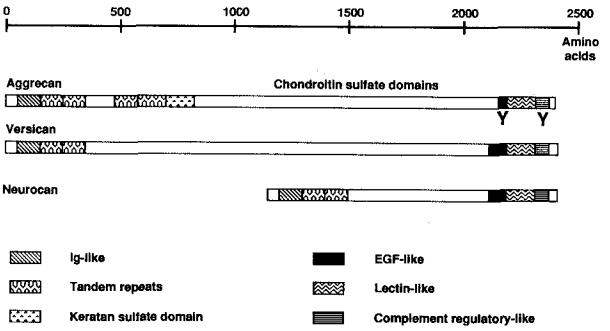

Figure. Summary of the primary structures and homology domains of aggrecan, versican, and neurocan. **Y** indicates two aggrecan domains which may be deleted by alternative splicing.

A chondroitin/keratan sulfate proteoglycan (designated 3H1) with a size of ~500 kDa was isolated from rat brain using monoclonal antibodies to the keratan sulfate chains. The size of the core glycoprotein decreases and the concentration of the 3H1 proteoglycan increases during development (see table), and there is a developmental decrease in the branching and/or sulfation of the keratan sulfate chains. The 3F8 monoclonal antibody was used to isolate a chondroitin sulfate proteoglycan of similar size comprising a 400 kDa core glycoprotein and an average of four 28 kDa chondroitin sulfate chains. Recent data obtained from amino acid sequencing and cDNA cloning studies have demonstrated that the 3F8 and 3H1 proteoglycans contain very similar or identical core proteins, and that the 3H1 chondroitin/keratan sulfate proteoglycan is a glycosylation variant of the 3F8 proteoglycan (P. Maurel, U. Rauch, R. K. Margolis, and R. U. Margolis, unpublished results). The 1D1, 3H1, and 3F8 proteoglycans all contain 3-sulfated HNK-1 carbohydrate epitopes (which are present in a number of neural cell adhesion molecules) as well as the novel *O*-glycosidic mannose-linked oligosaccharides which have previously been characterized in the chondroitin sulfate proteoglycans of brain[58, 59], and these glycans show different developmental patterns in the three proteoglycans. (In the table, the relative proportions of mannose-linked oligosaccharides in the different proteoglycans at differ-

Table. Properties of neurocan (1D1), and the 3H1 and 3F8 proteoglycans

	Neurocan		3H1 Proteoglycan[b]		3F8 proteoglycan	
	7-Day brain	Adult brain	7-Day brain	Adult brain	7-Day brain	Adult brain
Average molecular size	300, (180)[a] kDa	180 kDa	~ 500 kDa	~500 kDa	~500 kDa	~500 kDa
Core protein size (SDS-PAGE)	245, (150) kDa	150 kDa	~360 kDa	~280 kDa	400 kDa	400 kDa
Glycosaminoglycan chain size						
Chondroitin sulfate	22,000	32,000	25,000	25,000	28,000	28,000
Keratan sulfate[c]	-	-	8,400	10,000	-	-
% of total CSPG protein	18–21%	5–7%	3%	11%	12%	9%
Carbohydrate (% by weight)						
Chondroitin sulfate	22%	22%	15–23%	12–19%	23%	20%
4-sulfate	80[d]	97	>99	>99	67	96
6-sulfate	20	<3	<1	<1	33	<4
Keratan sulfate	-	-	5–11%	17–19%	-	-
Glycoprotein oligosaccharides	20%	17%	25–31%	20–22%	35%	30%
HNK-1 epitopes	+	+	+	+	+	-
Yield of mannitol after $NaOH/NaBH_4$[e]	<1	15	26–29	28–52	31	42

[a]Values in parentheses are for the less abundant component. [b]Data for proteoglycan, core glycoprotein, and glycosaminoglycan chain sizes apply to both 0.5 M NaCl and pH 11.5 eluates, % of total chondroitin sulfate proteoglycan protein represents the sum of the two fractions, and carbohydrate concentrations are ranges of the values given in ref. 98. [c]Also contains some short keratan sulfate chains with a size of 3,000–4,500. [d]As percent of total chondroitin sulfate. [e]As percent of total mannose.

ent ages is indicated by the yield of mannitol derived from *O*-glycosidically linked mannose by alkaline borohydride treatment.)

Immunocytochemical studies demonstrated that like the total population of soluble chondroitin sulfate proteoglycans of brain whose localization was previously examined using polyclonal antibodies, staining with monoclonal antibodies is generally most intense in the prospective white matter and absent from the external granule cell layer of early postnatal cerebellum, while in adult brain staining is strongest in the molecular layer and also present in the granule cell layer. However, 3F8 proteoglycan staining is most intense in the molecular layer of 7-day cerebellum. Two other monoclonal antibodies to chondroitin sulfate proteoglycans of brain, 5F3 and 3H7, which react on immunoblots of chondroitinase-treated proteoglycans with core glycoproteins in the 350–450 and 61–105 kDa range, respectively, show a significantly different staining pattern insofar as they also stain Purkinje cells, and strongly stain astrocytes in the granule cell layer[98]. In situ hybridization studies have demonstrated that neurocan is synthesized by neurons (cerebellar granule cells and cerebral cortical neurons), whereas in cerebellum the 3F8/3H1 proteoglycans are synthesized by Golgi epithelial cells, which are the cell bodies of the Bergmann glia fibers (M. Engel, U. Rauch, R. U. Margolis and R. K. Margolis, unpublished results).

The NG2 proteoglycan. The antigen recognized by the NG2 monoclonal antibody is a membrane-associated chondroitin sulfate proteoglycan of ~500 kDa containing a 300 kDa core protein. It was originally identified on rat neural cell lines with characteristics of both neuronal and glial cells, and is found in rat optic nerve and cerebellum on O2A glial progenitor cells which have the potential to differentiate in vitro into oligodendrocytes or type 2 astrocytes[62, 115]. In vivo NG2 has been found on stellate, process-bearing cells in the rat cerebellum, but not on cells with morphological characteristics of mature oligodendrocytes[61], suggesting that its expression is turned off before the terminal differentiation of the progenitors into oligodendrocytes. Immunocytochemical studies of developing rat embryos have also shown a widespread distribution in non-neural tissues, especially in the developing mesenchyme, and the expression of NG2 appears to be developmentally regulated insofar as the level of expression is highest on immature proliferating cells and decreases as these cells differentiate[84]. Antibodies to NG2 coimmunoprecipitate type VI collagen from some cells, and the binding between the two molecules appears to be mediated by protein-protein interactions[116]. NG2 and type VI collagen are also colocalized in frozen sections of certain rat tissues such as the intervertebral discs and spinal arteries, as well as on the surface of several cell lines. Treatment of these cells resulting in a change in the distribution of NG2 on the cell surface also causes a parallel change in the distribution of type VI collagen, suggesting that NG2 may mediate cell-matrix interactions by binding to type VI collagen[116].

The primary structure of the NG2 proteoglycan core protein has recently been reported[85]. cDNA clones coding for the proteoglycan hybridize with a single 8.9 kb band in mRNA from several NG2-positive rat neural cell lines. An 8.1 kb composite sequence of overlapping cDNA clones contains an open reading frame for 2,325 amino acids which encodes a protein with a molecular mass of 252 kDa. The predicted protein is an integral membrane protein with a large extracellular domain (2,224 amino acids), a single transmembrane domain (25 amino acids), and a short cytoplasmic tail (76 amino acids). Based on the deduced amino acid sequence and immunochemical analysis of proteolytic fragments of NG2, the extracellular region can be divided into two cysteine-containing domains which are separated by a 950-amino acid region containing nine serine-glycine pairs, at least two of which would appear to be potential chondroitin sulfate attachment sites. The extracellular domain also contains 14 potential *N*-glycosylation sites and four internal repeats, each consisting of 200 amino acids and containing a short sequence that resembles the putative calcium-binding region of the cadherins. However, the major portion of the sequence has no significant similarity to other known proteins, indicating that NG2 is a novel species of integral membrane proteoglycan.

Versican. There has been a rather large number of reports describing the properties and localization of various small molecular size (usually 60–70 kDa) hyaluronic acid-binding proteins detected in brain and other tissues. The early literature in this area was reviewed by Toole[119], and the further development of these studies can be followed in reports which have appeared since that time[6, 8, 23, 77, 90, 91]. Two of these hyaluronic acid binding proteins have also been used for the histochemical detection of hyaluronic acid in brain[10, 32], in a manner similar to that earlier employed for the light and electron microscopic localization of hyaluronic acid in developing brain using the biotinylated hyaluronic acid binding domain isolated after clostripain or trypsin treatment of rat chondrosarcoma proteoglycan[101, 102].

Because hyaluronectin, the first of these proteins to be described, and the closely related or identical 'glial hyaluronic acid binding protein' (GHAP), are both isolated from brain homogenates in the absence of protease inhibitors and under acidic conditions that would favor lysosomal enzyme activity, it was suggested that they may be proteolytic degradation products of larger proteoglycan core proteins[69]. This probable

origin was also noted by Zimmermann and Ruoslahti[128] in their report on the cloning of versican, an aggregating chondroitin sulfate proteoglycan[60] closely related to cartilage aggrecan (fig. 1) and first identified in human fibroblasts, in view of the fact that peptide sequences present in the 60 kDa glial hyaluronic acid-binding protein of brain[90] are virtually identical with sequences within the amino-terminal hyaluronic acid-binding domain of versican.

This proposed origin of hyaluronectin/GHAP has recently been confirmed by the isolation from human brain, using a pH 7.4 buffer containing protease inhibitors, of a 365 kDa chondroitin sulfate proteoglycan with a 345 kDa core protein which binds to hyaluronic acid and contains N-terminal amino acid sequences previously reported for versican and GHAP[92]. Certain inconsistencies in the immunocytochemical localization of hyaluronectin, GHAP, and, more recently, the 365 kDa proteoglycan have led to some confusion in this area and were originally used to support the contention that GHAP and hyaluronectin were not derived from a larger proteoglycan core protein. Although GHAP/hyaluronectin may be present in brain together with versican, either as a result of in vivo proteolytic processing or conceivably as a small alternatively spliced product of the versican gene, any conclusions regarding this question must await further studies demonstrating the isolation of the 60–70 kDa N-terminal domain of versican under conditions which would preclude artifactual proteolysis during purification, or Northern blot analyses compatible with a small splice variant of versican. However, it should be emphasized in this connection that other small hyaluronic acid-binding proteins unrelated to versican may also be present in brain, such as three proteins from embryonic chick brain (ranging in size from 69–93 kDa) which were identified with a monoclonal antibody[8]. It was proposed that one or more of these proteins may represent a hyaluronic acid receptor involved in early stages of central nervous tissue histogenesis.

The Cat-301 proteoglycan. The Cat-301 monoclonal antibody was originally shown to recognize a surface-associated antigen on subsets of mammalian central nervous system neurons. The antigen was later demonstrated to be a large (~680 kDa) chondroitin sulfate proteoglycan which is apparently a peripheral membrane or extracellular matrix protein and does not require detergents for extraction from brain[127]. Studies of the effects of early visual deprivation on Cat-301 immunoreactivity in the cat lateral geniculate nucleus and visual cortex indicate that neuronal activity during the critical period of the development of central visual pathways regulates the expression of the Cat-301 proteoglycan[38], and that Cat-301 specifically recognizes Y-cells in the cat lateral geniculate nucleus[42]. Cat-301 and another monoclonal antibody to this proteoglycan (Cat-304) were also found to recognize Lugaro cells in cat cerebellar cortex, demonstrating that this neuronal cell type is both molecularly and morphologically distinct from Purkinje and Golgi cells, which do not bind the Cat-301/304 monoclonal antibodies[106]. Other studies demonstrated that the expression of the Cat-301 proteoglycan on the surface of hamster motor neurons requires input relayed in the early postnatal period by large diameter primary afferents[51], and that during this period its expression on motor neurons could be specifically inhibited by blockade of the *N*-methyl-D-aspartate receptor at the spinal segmental level[52].

It has recently been reported that the Cat-301 proteoglycan, which was detected in a number of non-neural tissues, shares immunochemical determinants with cartilage aggrecan (but has a lower buoyant density), and is capable of binding to hyaluronic acid[27]. The size of the Cat-301 core protein (~400–580 kDa; refs 27, 127), its ability to bind to hyaluronic acid, and its immunochemical cross-reactivity with aggrecan all suggest that Cat-301 may in fact be versican. Although differences in the staining of brain sections with the Cat-301/304 monoclonal antibodies as compared to antibodies to glial hyaluronic acid-binding protein or versican would appear to indicate that these are different antigens[27], until Cat-301 is more completely characterized it remains an open question whether this brain proteoglycan is actually versican or a fourth member of the aggrecan/versican/neurocan family.

The L5 proteoglycans. The L5 monoclonal antibody recognizes *N*-linked carbohydrate epitopes which are present on a number of mouse brain glycoproteins including the L1 neural cell adhesion molecule (which is the mouse equivalent of the rat and human NILE glycoprotein and related to chicken Ng-CAM), and are also present on several chondroitin sulfate proteoglycans. This antibody has been used to isolate from PBS extracts of early postnatal to adult mouse brain, or from cultured astrocytes, a mixture of proteoglycans with an average molecular size of ~500 kDa and which yield core glycoproteins having apparent molecular sizes of 260, 360, and 380 kDa after chondroitinase treatment[117]. Immunoblots of the mixture of native proteoglycans (which remained at the top of the gel) stained with the L2 monoclonal antibody to HNK-1 epitopes, indicating that these sulfated oligosaccharides are present on one or more of the component proteoglycans. Although these proteoglycans share carbohydrate epitopes with a number of brain glycoproteins, their structural relationship to one another and to other characterized chondroitin sulfate proteoglycans of brain remains to be determined.

The T1 proteoglycan. A chondroitin sulfate proteoglycan of ~450 kDa with a major 300 kDa core protein was

extracted from rat brain using 6 M guanidine HCl and partially purified by CsCl density gradient centrifugation and ion exchange chromatography[48]. The brain proteoglycan is recognized by the T1 monoclonal antibody generated to a large basement membrane-associated chondroitin/keratan sulfate proteoglycan (designated PG-1000) from elasmobranch electric organ[47], and the T1 monoclonal antibody shows a diffuse staining of both grey and white matter in sections of adult rat brain, consistent with a general extracellular localization of the proteoglycan. The nature of the T1 epitope is unknown, but it is destroyed by treatment of PG-1000 or the T1 proteoglycan of brain with either pronase or chondroitinase ABC. Only 10–20% of the T1 antigen is extracted from adult brain with 0.15 M NaCl in the presence or absence of various detergents, as compared to extraction with 6 M guanidine HCl/2% CHAPS, and under nonreducing conditions the T1 proteoglycan copurified with two other proteins of 53 and 119 kDa. The proteoglycan binds to hyaluronic acid[49], but appears to be distinct from aggrecan or versican. It was proposed[48] that the T1 proteoglycan may be a component of the adult brain extracellular matrix.

The 6B4 proteoglycan. A chondroitin sulfate proteoglycan with a 250 kDa core protein was identified in embryonic through adult rat brain using the 6B4 monoclonal antibody[68]. This proteoglycan, which also contains HNK-1 epitopes, was found in immunocytochemical studies to be expressed on the surface of a subset of neurons including cerebellar Purkinje cells and Golgi cells, and in certain nuclei which are connected to the cerebellum through the mossy fiber system. In view of significant developmental changes in its expression and localization, it was suggested that the 6B4 proteoglycan is closely involved in the formation of the cerebellar mossy fiber system.

The S103L proteoglycan. Krueger et al.[57] have used the S103L monoclonal antibody to a protein epitope in the chondroitin sulfate attachment domain of chick cartilage proteoglycan (aggrecan) to immunoprecipitate a structurally related chondroitin sulfate proteoglycan from a PBS extract of embryonic chick brain. It has a core glycoprotein with an apparent molecular size of 370 kDa on SDS-PAGE, and is a quantitatively minor component, accounting for only 4–5% of the [^{35}S]sulfate-labeled brain proteins (in which most of the labeling is present in proteoglycans). Based on the results of enzyme digestions, S103L appears to contain both *O*-glycosidic and a small amount of *N*-glycosidically linked oligosaccharides, but lacks both HNK-1 epitopes and keratan sulfate chains. It resembles the cartilage proteoglycan insofar as both have 370 kDa core glycoproteins, and an RNA probe based on the cartilage proteoglycan cDNA sequence hybridizes with a single 7.5 kb message in both embryonic chick cartilage and brain mRNA preparations. However, the S103L proteoglycan of brain differs from chick aggrecan insofar as it is freely soluble in PBS rather than being matrix bound, it has a considerably smaller molecular size (K_{av} on Sepharose CL-2B of $\sim$0.31 as compared to $\sim$0.14 for the cartilage proteoglycan), and it lacks keratan sulfate chains. A better understanding of the relationships between neurocan, Cat-301 and S103L, all of which have primary structural and/or immunochemical similarities to aggrecan and versican, will have to await cloning of the Cat-301 and S103L proteoglycans. Although the authors attached special significance to the disappearance of the S103L proteoglycan during chick embryonic development (as compared to rat proteoglycans which remain present in early postnatal brain), it should also be noted in this connection that many of the developmental processes which take place in embryonic chick brain occur during the early postnatal period in rat brain and in other species, and that this is the same period during which major changes have been found in the concentration, core protein size, carbohydrate composition, sulfation, and immunocytochemical localization of rat neurocan[98].

Chondroitin sulfate proteoglycans of embryonic chick brain which contain the HNK-1 epitope. It was originally reported[43] that embryonic chick brain contains a chondroitin sulfate proteoglycan with a 280 kDa core protein and HNK-1 epitopes which binds to and is copurified with cytotactin (tenascin), a glycoprotein involved in neuron-glia adhesion. During early development of the chicken embryo, cytotactin and the proteoglycan become differentially distributed in the rostral and caudal halves of the developing sclerotome[118]. Their differential expression is crest cell-independent, although they inhibit crest cell migration, and it was proposed that cytotactin and the chondroitin sulfate proteoglycan may contribute to the localization of neural crest cells in the rostral half of the sclerotome. In later studies using antibodies to cytotactin and the cytotactin-binding proteoglycan, it was found that during neural development both the levels and molecular forms of each molecule varied, following different time courses[44]. A 250 kDa form of cytotactin that contained chondroitin sulfate was also reported, and using a monoclonal antibody raised to rat brain chondroitin sulfate proteoglycans we have found that a small portion of rat brain tenascin occurs in the form of a chondroitin sulfate proteoglycan containing HNK-1 epitopes (M. Engel, M. Flad, R. U. Margolis and R. K. Margolis, unpublished results). Unlike the molecules from neural tissue, cytotactin and the cytotactin-binding proteoglycan from non-neural tissues such as fibroblasts lacked the HNK-1 epitope, which, like the chondroitin sulfate glycosaminoglycan chains,

does not appear to be directly involved in binding. Cell culture experiments indicate that cytotactin is specifically synthesized by glia whereas the cytotactin-binding proteoglycan appears to be a product of neurons. Both molecules were found either associated with the cell surface or in an intracellular, perinuclear pattern. This proteoglycan may be the chick homolog of rat neurocan (see above), although a neurocan probe based on the rat cDNA sequence does not hybridize at high stringency with chick brain mRNA (M. Flad and R. K. Margolis, unpublished results).

Krueger et al.[57] have also described a major chondroitin/keratan sulfate proteoglycan of embryonic chick brain which was immunoprecipitated with the HNK-1 monoclonal antibody and accounted for 30–40% of the incorporated [^{35}S]sulfate radioactivity. This proteoglycan, which in distinction to S103L was present at all stages of embryonic development, has a larger core glycoprotein with an apparent molecular size of 340 kDa on SDS-PAGE, but a relatively small molecular size for the native proteoglycan ($K_{av} = 0.56$ on Sepharose CL-2B) as compared to S103L. Although this proteoglycan is reported to contain keratan sulfate and to have a larger core protein than the cytotactin-binding chondroitin sulfate proteoglycan of chick brain described above, it is possible that these two proteoglycans are closely related or identical molecules.

A second HNK-positive proteoglycan was also detected in these studies. This ~175 kDa proteoglycan was converted to a ~155 kDa core protein after keratanase treatment, and did not appear to contain chondroitin sulfate chains.

Synaptic vesicle proteoglycans. Classical neurotransmitters, such as acetylcholine, are stored in small clear synaptic vesicles and secreted from the nerve terminal by exocytosis. Synaptic vesicle glycoproteins and proteoglycans were recently reviewed[18]. A proteoglycan which is a specific component of cholinergic synaptic vesicles from electric organ has been identified by two monoclonal antibodies (Tor 70 and anti-SV1) which bind to the same (SV1) epitope. This proteoglycan behaves like an integral membrane protein with the SV1 epitope present on the luminal surface. The SV1 epitope is also present on the external nerve terminal surface of the electric organ synapse, where it is restricted to the junctional region. It was originally proposed to be either a heparan sulfate or a keratan sulfate proteoglycan. A 100 kDa transmembrane glycoprotein has also been identified as a specific component of purified electric organ synaptic vesicles using the SV2 monoclonal antibody to a cytoplasmic epitope. The SV2 antigen is present in vesicles of neurons and endocrine cells, but is not detected in exocrine cells.

It has recently been found that two forms of the SV2 antigen are present in electric organ synaptic vesicles: the previously described 100 kDa protein (now designated the L form), and a more heavily glycosylated H form of ~250 kDa[108]. The H form is the previously identified synaptic vesicle proteoglycan containing the SV1 epitope. Based on their reactivity with various monoclonal antibodies, it was concluded that both forms are keratan sulfate proteoglycans (collectively designated synaptoglycan), although only the L form shows a mobility shift after keratanase treatment, and the SV2 antigen appears to account for most of the synaptic vesicle keratan sulfate. The L and H forms of synaptoglycan were also detected in electric fish brain and rat brain.

The presence of keratan sulfate containing the SV1 antigenic marker specific for one group of neurons in electric fish makes synaptoglycan unique among synaptic vesicle proteins. SV1 is removed from electric organ synaptoglycan by keratanase treatment but is not present in bovine corneal or cartilage keratan sulfate. It is found in highest concentration on the H form of synaptoglycan from electromotor neurons but is present in only very low amounts on the H form elsewhere in the fish brain. Previous studies have indicated that this epitope is synthesized in the nerve terminal and also occurs on the nerve terminal surface. The possible biological significance of the specific synaptic localization of synaptoglycan remains to be determined.

In a recent study of *Torpedo* electric organ synaptic vesicles[7], it was found that a proteoglycan is strongly linked to the acetylcholine transporter and the receptor for vesamicol (a drug which inhibits acetylcholine storage). Of the glycosaminoglycan lyases tested on permeabilized vesicles, only keratanase and testicular hyaluronidase (in the absence of detectable protease activity) inactivated vesamicol binding and destroyed the presumed glycosaminoglycan epitope for the SV1 monoclonal antibody. The effects of these two glycosaminoglycan lyases on vesamicol binding are difficult to reconcile with their known effects on previously characterized glycosaminoglycans. However, it appears that a synaptic vesicle glycosaminoglycan may control the conformation of the vesamicol receptor, and the unexpected linkage to a proteoglycan suggests that the acetylcholine transporter/vesamicol receptor in intact nerve terminals might communicate with the extracellular matrix, and thereby participate in the stabilization and operation of this cholinergic synapse.

Chromogranin A. Chromogranin A, the major matrix protein of chromaffin granules (the catecholamine and peptide storage organelles of adrenal medulla) occurs to the extent of ~1–2% in the form of a 110 kDa chondroitin sulfate proteoglycan which yields a 95 kDa core glycoprotein after chondroitinase treatment[35, 104, 123]. A similar but slightly larger chromogranin A proteoglycan was also detected in PC12 pheochromocytoma cells[33, 35].

In terms of size, carbohydrate composition, and immunochemical reactivity the minor 'full-length' chromogranin A proteoglycan appeared to be quite distinct from two major chondroitin/dermatan sulfate proteoglycans present in the bovine chromaffin granule matrix, with average molecular sizes of 35–40 kDa and which both yield a 14 kDa core glycoprotein after chondroitinase treatment[35]. However, recent N-terminal microsequencing of the core glycoprotein of one of these proteoglycans revealed an 18-amino acid sequence which was identical to the sequence of bovine chromogranin A beginning at residue 383 (A. Flaccus, R. K. Margolis, and R. U. Margolis, unpublished results). These results indicate that the C-terminal portion of chromogranin A, containing one chondroitin sulfate chain and 15% of the amino acids, is removed by proteolytic processing to generate a considerably smaller proteoglycan fragment of the 431 amino acid mature chromogranin A protein. In view of these results and a comparison of immunoblots of chromogranin A and the two chromaffin granule proteoglycans (before and after chondroitinase treatment) stained with antibodies to specific chromogranin A peptide sequences[35], it appears that a large portion of the chromogranin A molecules may have originally carried a chondroitin sulfate chain, and that the 66 C-terminal amino acids may not be present in much of the chromogranin A in chromaffin granules.

The amyloid β protein precursor. Amyloid fibrils in Alzheimer's disease accumulate in the neuritic plaque cores and in cerebral blood vessels. The major component of the amyloid depositions is the 4 kDa amyloid β protein (ABP) which forms part of at least three larger integral membrane proteins termed amyloid β precursor proteins (ABPP). Based on studies of PC12 pheochromocytoma cells, it was earlier suggested that the ABPP is a 65 kDa heparan sulfate proteoglycan core protein[107]. However, the actual size of the ABPP was considered to be approximately 110–135 kDa, and investigations of PC12 cell proteoglycans[33] indicated that the identification of the ABPP as a heparan sulfate proteoglycan was a result of its copurification with previously characterized PC12 cell heparan sulfate proteoglycans having average molecular sizes of 110–130 kDa[76].

Recent studies on the possible relationship of the ABPP and proteoglycans have demonstrated that a portion of the ABPP secreted by C6 glioma cells (as well as that present in rat and human brain; V. Vassilakopoulou, J. Ripellino, R. Margolis, and N. Robakis, unpublished results) occurs in the form of a 140–250 kDa chondroitin sulfate proteoglycan, which is converted by chondroitinase treatment to a 120 kDa core glycoprotein but is unaffected by heparitinase treatment[110]. As in the case of chromogranin A and tenascin, the ABPP therefore also occurs as a 'part-time' proteoglycan, with the important quantitative difference that ~90% of the ABPP secreted by C6 glioma cells is found in a proteoglycan form. Two of the ABPP isoforms contain a 56 amino acid extracellular region homologous to the Kunitz-type serine protease inhibitors (KPI). Following glycosylation, ABPPs are cleaved by a secretase at the extracellular region close to the transmembrane sequence, and secreted. Insofar as nexin II, the secreted ABPP form derived from the KPI-containing precursors, migrates on SDS-PAGE as a 120 kDa protein which is recognized by antisera specific to the KPI domain, the reported size of the chondroitin sulfate proteoglycan core protein is in excellent agreement with the expected molecular size of these major ABPP segments. The proteoglycan nature of the ABPP may have important implications for the production of ABP, which is thought to be the result of aberrant ABPP processing. Two of the three potential chondroitin sulfate attachment sites in ABPP are at serine-637 and serine-660, and therefore closely flank Asp-653 which represents the N-terminus of the ABP. It is therefore possible that the presence or absence of chondroitin sulfate chains at these potential attachment sites may modulate the proteolytic processing of the ABPP and therefore affect the deposition of ABP in Alzheimer's amyloid fibrils, although some type of 'autocatalytic' noncovalent modification of the ABPP conformation appears to be equally plausible.

2. Keratan sulfate proteoglycans of brain

Cole and McCabe[19] have described a keratan sulfate proteoglycan which is primarily expressed in embryonic chick brain. It migrates on SDS-PAGE as a compact band at 320 kDa, and after keratanase treatment yields a major band at 105 kDa above a large diffuse region of antibody staining. This proteoglycan, which was identified and purified using monoclonal antibodies and is not detectable in adult chicken brain, appears to contain only keratan sulfate glycosaminoglycan chains since its molecular size was unaffected by treatment with chondroitinase. It was also unaffected by treatment with peptide N-glycosidase and does not contain HNK-1 epitopes, but the proteoglycan transferred to nitrocellulose binds peanut lectin. It is synthesized by glial cells and secreted into the medium of glial cultures. Immunocytochemical studies showed that this protein (which has been named claustrin) is present in putative midline barrier structures in the developing chick central nervous system. When added to laminin or neural cell adhesion molecule (N-CAM) adsorbed onto nitrocellulose-coated dishes, claustrin abolished cell attachment and neurite outgrowth on these adhesive substrata, and these effects could be prevented by keratanase treatment or incubation with a monoclonal antibody whose epitope is removed from claustrin by

keratanase. It therefore appears that as has been proposed for other proteoglycans (see section C.1. below), claustrin may be involved in modulating cell interactions in developing brain. Further studies of chicken claustin have demonstrated immunochemical cross-reactivity and considerable amino acid sequence identity with the rat and mouse microtubule associated protein MAP1B (which has the same apparent molecular size as claustrin on SDS-PAGE), and it was therefore suggested that MAP1B may be a keratan sulfate proteoglycan[12]. However, a number of observations such as the absence of polydispersity usually characteristic of native proteoglycans and the very low labeling with [^{35}S]sulfate (in spite of the fact that keratan sulfate is reported to account for ~70% of the apparent molecular size of claustrin), as well as the possibility that the effects of keratanase treatment might be due to contaminating proteases, all indicate the need for further characterization, including a direct biochemical demonstration of the presence of keratan sulfate chains.

B. Structure and localization of heparan sulfate proteoglycans

1. Heparan sulfates of nervous tissue

There is known to be a wide range of variability in the fine structure of both heparan sulfate, which is a component of proteoglycans present in many cell types, and of the closely related glycosaminoglycan, heparin, which occurs in a proteoglycan produced specifically by mast cells. These glycosaminoglycans are composed of disaccharide units containing a uronic acid (D-glucuronic acid or L-iduronic acid) and a glucosamine residue which may be either *N*-acetylated or *N*-sulfated, and sulfate may also be present at the 2- and 6-positions on the uronic acid and glucosamine residues, respectively. The epimerization of D-glucuronic acid to L-iduronic acid and the deacetylation and sulfation reactions, all of which occur at the polymer level, are effected by a series of enzymatic reactions which must proceed in a defined sequence[107b].

Heparan sulfates from brain and other tissues have recently been characterized after depolymerization with a mixture of three heparin and heparan sulfate lyases from *Flavobacterium heparinum* and separation of the resulting disaccharides by HPLC (H. Tekotte, R. K. Margolis, and R. U. Margolis, unpublished results). In rat, rabbit, and bovine brain, 46–69% of the heparan sulfate disaccharides are *N*-acetylated and unsulfated, and an additional 17–21% contain a single sulfate residue in the form of a sulfoamino group. In rabbit, bovine, and 1-day postnatal rat brain, disaccharides containing both a sulfated uronic acid and *N*-sulfate account for an additional 10–14%, together with smaller and approximately equal proportions (5–9%) of mono-, di- and trisulfated disaccharides having sulfate at the 6-position of the glucosamine residue. Based on nitrous acid degradation studies, it was previously reported that there is relatively little change in the *N*-sulfation of rat brain heparan sulfate during the first month of postnatal development, and that there are no large *N*-acetylated segments since predominantly di- and tetrasaccharide nitrous acid degradation products were obtained[71]. The finding that unsulfated disaccharides containing GlcNAc represent the major component in both 1-day postnatal and adult rat brain is consistent with these earlier conclusions, and indicates that these must alternate with disaccharides containing *N*-sulfated glucosamine residues to yield the relatively small nitrous acid degradation products previously observed.

Comparison of the effects of heparinase and heparitinase treatment of a heparan sulfate proteoglycan of brain (see below) revealed that this glycosylphosphatidylinositol-anchored proteoglycan contains a significant proportion of relatively short *N*-sulfoglucosaminyl 6-*O*-sulfate(α1–4)iduronosyl 2-*O*-sulfate(α1–4) repeating units, and that the portions of the heparan sulfate chains in the vicinity of the carbohydrate-protein linkage region are characterized by the presence of D-glucuronic acid rather than L-iduronic acid[100].

It has been established that the anticoagulant activity of heparin depends on its specific binding to antithrombin, and that the antithrombin binding site is a pentasaccharide containing a unique 3-*O*-sulfated glucosamine *N*-sulfate residue. A survey of various rat tissues showed that brain contained the highest proportion of heparan sulfate chains with a high affinity for antithrombin (21.5% of the total heparan sulfate), and that there is a sparse but nonrandom distribution of 3-*O*-sulfated glucosamine *N*-sulfate residues in the heparan sulfate chains[45].

Heparan sulfate accounts for ~11% of the chromaffin granule membrane glycosaminoglycans and 4–5% of those in the granule contents[31,55]. It is interesting that bovine chromaffin granule membranes contain heparan sulfate with a low degree of sulfation similar to that of adult rat brain, insofar as almost all of the disaccharides are either unsulfated (71%) or monosulfated (18%). In sympathetic nerves, norepinephrine is stored in large dense-cored vesicles which in biochemical composition and properties closely resemble adrenal chromaffin granules. The concentration of glycosaminoglycans in dense-cored vesicles is 15% of that present in chromaffin granules, but heparan sulfate accounts for ~75% of the total, as compared to the much smaller proportion (<10%) present in chromaffin granules[75]. The heparan sulfate in large dense-cored vesicles differs considerably from that in chromaffin granules and more closely resembles heparin, insofar as it contains only 21% unsulfated disaccharides, 10% of mono- and disulfated disaccharides, and 69% trisulfated disaccharides (H. Tekotte,

R. K. Margolis and R.U. Margolis, unpublished results). The significant differences between heparan sulfates from different sources (including nervous tissue, kidney and lung) support the possibility that structural variations in heparan sulfate may be related to specific biological functions.

2. Heparan sulfate proteoglycans

Glypican. A membrane-associated heparan sulfate proteoglycan isolated and characterized from rat brain was found to be very similar in many of its properties (e.g., size, charge, density, etc.) to the population of more abundant chondroitin sulfate proteoglycans, from which it was separated by affinity chromatography on lipoprotein lipase-Sepharose[56]. This ~220 kDa proteoglycan contains a 55 kDa core glycoprotein, 15 kDa heparan sulfate chains, and predominantly tri- and tetraantennary *N*-glycosidic as well as *O*-glycosidic oligosaccharides[56, 100]. N-terminal amino acid sequences of the core glycoprotein and of tryptic peptides derived from it demonstrated a high degree of identity with sequences originally reported for a glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycan (glypican) of human lung fibroblasts[21], and which was shown by Northern blot analysis to be also present in a large number of human cell lines including glioma and neuroblastoma cells[66].

Cloning of the heparan sulfate proteoglycan of brain revealed an open reading frame of 558 amino acids which encodes a protein with a molecular mass of 62 kDa containing three peptide sequences found in the isolated proteoglycan[53]. The deduced amino acid sequence and the 3'- and 5'-untranslated sequences have 89% and 66–80% identity, respectively, with those of human glypican. There is no obvious transmembrane domain, and it is therefore likely that this heparan sulfate proteoglycan is also GPI-anchored at the cell surface. Herndon and Lander[41] have previously reported data indicating that two heparan sulfate proteoglycans of rat brain, with core proteins of 50 and 59 kDa, are linked to the membrane by GPI anchors.

Although the GPI anchor of glypican could provide a means for its selective release from the cell surface in brain and other tissues by stimulation of a specific phospholipase under appropriate physiological conditions, as has been demonstrated for other GPI-anchored proteins[65, 67], there is as yet no definitive evidence that such a process actually occurs for heparan sulfate proteoglycans. In the case of rat ovarian granulosa cells none of the GPI-anchored heparan sulfate proteoglycans are shed into the medium, but it appears that the GPI-anchored and membrane-intercalated heparan sulfate proteoglycans in these cells have distinct secretory, endocytic, and intracellular degradation pathways which may be due to differences in their anchor structures[125]. The 89% identity of amino acid sequences in the rat and human proteoglycans and 66–80% identity over ~2 kb in the nucleotide sequences of the untranslated regions indicate that the glypican gene has been highly conserved, and the apparently wide cell and tissue distribution of the proteoglycan suggests that it serves a fundamental biological function.

N-Syndecan. A cDNA clone coding for a portion of the core protein of a membrane-intercalated heparan sulfate proteoglycan (designated *N*-syndecan) was isolated from a neonatal Schwann cell cDNA library by screening with an oligonucleotide complementary to a conserved 33-base sequence in the cytoplasmic domains of murine epithelial (syndecan) and human lung fibroblast transmembrane heparan sulfate proteoglycan core protein sequences[17]. The partial amino acid sequence deduced for the C-terminal portion of the protein predicted a 353 amino acid polypeptide with a single membrane-spanning domain and a 34-amino acid C-terminal cytoplasmic domain, both of which have ~50–70% identity to similar regions in syndecan and the human lung fibroblast heparan sulfate proteoglycan. However, there was only a 17–25% identity between the extracellular domains of these proteoglycans and the partial N-terminal sequence of *N*-syndecan. The putative extracellular domain contains three potential glycosaminoglycan attachment sites, and antibodies to a fusion protein containing a 16 kDa portion of the *N*-syndecan core protein reacted on immunoblots of neonatal rat Schwann cell and brain proteins with a ~120 kDa band which was apparent only after heparitinase treatment. These antibodies stained peripheral nerve, neonatal rat brain, heart, aorta, and other neonatal tissues, but not embryonic or adult brain. Northern blots revealed a 5.6 kb band in mRNA from rat Schwann cells, neonatal brain, and smooth muscle, while a ribonuclease protection assay revealed high levels of message in neonatal rat brain, heart, and Schwann cells, but the mRNA was barely detectable in neonatal or adult liver, or adult brain. Its expression during a relatively restricted period suggests that it may be involved in one or more specific developmental processes, and since the message size is over twice that of the reported mRNA (which includes ~ 1.6 kb of 3'-untranslated sequence), interesting features of the major N-terminal extracellular domain of this proteoglycan may also emerge when its complete amino acid sequence is available.

Phosphatidylinositol-anchored Schwann cell proteoglycans. Schwann cells synthesize both hydrophobic, membrane-anchored heparan sulfate proteoglycans and peripheral cell surface heparan sulfate proteoglycans which can be released by treatment with polyanions such as heparin or phytic acid (inositol hexaphosphate).

Analysis of the kinetics of labeling of Schwann cell heparan sulfate proteoglycans suggested that the peripheral proteoglycans are derived from the membrane-anchored forms[14]. Heparitinase treatment of heparan sulfate proteoglycans purified from cultured neonatal rat sciatic nerve Schwann cells revealed the presence of at least six core proteins ranging in size from 57 to 185 kDa[15]. Antibodies raised to this mixture of proteoglycans immunoprecipitated a subpopulation of [^{35}S]sulfate-labeled heparan sulfate proteoglycans that was released from Schwann cells by treatment with a phosphatidylinositol-specific phospholipase C, as well as smaller amounts of peripheral proteoglycans present in a phytic acid extract. In both cases, a single proteoglycan with a 67 kDa core protein was present in immunoprecipitates from extracts of [^{35}S]methionine-labeled Schwann cells. These antibodies also stained the surface of cultured Schwann cells and the outer ring of Schwann cell membrane in sections of adult rat sciatic nerve, and treatment of cultured Schwann cells with phosphatidylinositol-specific phospholipase C (but not phytic acid) significantly reduced the cell surface staining. Other experiments demonstrated a reversible binding of the purified GPI-anchored heparan sulfate proteoglycan to laminin and fibronectin[16]. Since the myelination and ensheathment of axons by Schwann cells in tissue culture is dependent on Schwann cell contact with basement membrane, it would appear that this heparan sulfate proteoglycan may be involved in binding the Schwann cell plasma membrane to the adjacent basement membrane surrounding the individual axon-Schwann cell units, and that its interactions with laminin may play a role in laminin-dependent Schwann cell spreading.

Other heparan sulfate proteoglycans of nervous tissue. Haugen et al.[40] have recently described a 160–175 kDa heparan sulfate proteoglycan isolated from B104 neuroblastoma cells, containing a 78 kDa core protein and 25–35 kDa heparan sulfate chains. This proteoglycan may be related to a heparan sulfate proteoglycan of similar molecular size containing a 65 kDa core protein and which represents the major heparan sulfate proteoglycan of PC12 pheochromocytoma cells[33]. Heparitinase treatment of neuroblastoma cells and antibodies generated to a mouse melanoma heparan sulfate proteoglycan core protein both inhibited neuroblastoma cell adhesion and spreading on a defined peptide sequence from the C-terminal heparin binding domain of fibronectin. These antibodies, which recognized the 78 kDa neuroblastoma heparan sulfate proteoglycan core protein on immunoblots, also stained the neuroblastoma cell surface and neurons and glia in primary cultures of nervous tissue. This proteoglycan may therefore be involved in neuronal cell binding to fibronectin or related ligands.

Rat brain mRNA has also been shown to hybridize with a probe for a recently cloned major cell surface heparan sulfate proteoglycan of rat liver[96], whose deduced amino acid sequence (which codes for a 23 kDa protein) has a high degree of identity with the predicted partial sequence of the human lung fibroblast heparan sulfate proteoglycan, fibroglycan[78].

Finally, it should be noted that plasma membrane and cell surface associated heparan sulfate proteoglycans, which presumably account for most of those present in central nervous tissue parenchyma, differ considerably in structure and immunochemical reactivity from basement membrane heparan sulfate proteoglycans, such as are found in peripheral nerve and other tissues[54,64].

C. Functional roles of proteoglycans in nervous tissue

1. Chondroitin sulfate proteoglycans and cell interactions

Effects on neurite outgrowth and cell migration. There is increasing evidence that glycosaminoglycans and proteoglycans are involved in modulating cell interactions in developing nervous tissue. In some of the initial studies in this area it was found that soluble glycosaminoglycans or glycosaminoglycan-coated substrata modify the adhesion and neurite extension of cultured PC12 cells[1,20] and dorsal root ganglia[13,120]. More recent studies indicate that chondroitin sulfate and keratan sulfate proteoglycans may be components of barriers for axonal migration[11,19,79,87,112,114], and that chondroitin/keratan sulfate proteoglycans can inhibit neural crest cell migration[94]. Studies of spatial and temporal changes in the distribution of proteoglycans during avian neural crest development showed that in general, chondroitin/keratan sulfate proteoglycans are abundant in regions where neural crest cells are absent, and that their segmental distribution inversely correlates with migration pathways of neural crest cells[95]. It has also been reported that neurite outgrowth from cultured embryonic chicken dorsal root ganglion neurons, chicken retinal ganglion neurons, and rat forebrain neurons was progressively inhibited by increasing concentrations of bovine nasal cartilage chondroitin sulfate proteoglycan presented as a step gradient on a laminin substratum[111]. In contrast, the heparan sulfate chains of the Engelbreth-Holm-Swarm mouse sarcoma basement membrane proteoglycan, as well as free chondroitin sulfate and heparan sulfate, were found to significantly enhance neurite outgrowth from embryonic rat brain neurons, and to also affect neuronal polarity[59a].

Distinct alterations in cell adhesion and morphology have been demonstrated following inhibition of chondroitin sulfate proteoglycan biosynthesis by β-xyloside treatment of PC12 pheochromocytoma cells and primary cultures of rat cerebellum[72]. β-Xyloside inhibition of chondroitin sulfate proteoglycan biosynthesis or antibodies to chondroitin sulfate have also been shown to

abolish the avoidance of chick epidermis by dorsal root ganglia fibers[25], suggesting that an epidermal chondroitin sulfate proteoglycan is involved in the neurite avoidance reaction.

Although there is in certain cases a degree of chemical specificity for the effects observed in vitro, it is not clear to what extent they are due to factors such as modification of the surface charge of the substrate, alteration of the binding of growth-promoting or extracellular adhesive molecules produced by the cultures, or a reduction in binding mediated by cell adhesion molecules in the plasma membrane. In tissues, sulfated glycosaminoglycans occur as covalent complexes with a core protein in the form of proteoglycans, and both the glycosaminoglycan and protein components have been suggested to affect cell behavior. Although a number of previous studies indicate the involvement of the glycosaminoglycan portion of these molecules[11, 19, 94, 112, 114], it appears that the glycosaminoglycan chains do not contribute significantly to the cytotactin/tenascin-binding properties of a chondroitin sulfate proteoglycan of embryonic chicken brain[44] and are not required for the stimulatory[46] or inhibitory[89] effects of chondroitin sulfate proteoglycans of brain on neurite outgrowth, or for their interactions with neural cell adhesion molecules[37]. It may be significant that all of these effects of core proteins have been obtained using chondroitin sulfate proteoglycans purified from brain. In contrast, studies utilizing cartilage or basement membrane proteoglycans, in which only the native proteoglycan (containing glycosaminoglycan chains) but not the core protein was found to have effects on neurite outgrowth or other aspects of cell behavior are particularly difficult to evaluate with respect to their relevance to nervous tissue proteoglycans having quite different biochemical properties, and with regard to the extent to which such effects might be due merely to a high negative charge density. We have found, for example, that adhesion of neurons to cell adhesion molecules was inhibited to a relatively small degree (20–35%) by native (but not chondroitinase-treated) aggrecan[37], but these effects may have little or no biological significance in view of the fact that chondrosarcoma aggrecan contains ~100 chondroitin sulfate chains as compared to the 3–4 chains in brain proteoglycans such as neurocan and the 3F8 proteoglycan. These brain proteoglycans also differ significantly from aggrecan in a number of properties including their size, primary structure, degree and type of substitution with glycosaminoglycans and *N*- and *O*-glycosidic oligosaccharides, and sulfation.

In studies on the localization of chondroitin sulfate proteoglycans[4], hyaluronic acid[102], and hyaluronic acid binding region and link protein epitopes[103] in developing rat cerebellum, it was found that these proteoglycans and associated macromolecules were absent from the external granule cell layer during the early postnatal period when active migration of granule cells is taking place, and similar results were obtained using monoclonal antibodies to a number of individual proteoglycans[98]. It is therefore possible that, at least in the case of granule cells, chondroitin sulfate proteoglycans represent a *non*permissive environment for cell migration. Such a hypothesis is supported by recent studies indicating that chondroitin sulfate or chondroitin/keratan sulfate proteoglycans may be components of astroglial axon barriers studied in vivo and in vitro. For example, the glial roof plate, a putative axon barrier located along the dorsal midline of the developing spinal cord, was found to stain with a monoclonal antibody for keratan sulfate when axons grow close to but do not cross the midline[113]. In a related study, neurite outgrowth from isolated E9 chick dorsal root ganglia was evaluated in nitrocellulose-coated petri dishes onto which stripes of various purified macromolecules were attached. It was found that whereas laminin promoted neurite outgrowth, the chondroitin sulfate and keratan sulfate glycosaminoglycan chains of bovine or chick cartilage chondroitin/keratan sulfate proteoglycan or rat chondrosarcoma chondroitin sulfate proteoglycan formed a barrier to axon growth[112]. Although this latter report confirms and extends the results of previous studies which demonstrated that glycosaminoglycans are capable of inhibiting neurite outgrowth from dorsal root ganglia and rat PC12 pheochromocytoma cells, the widespread occurrence of extracellular chondroitin sulfate proteoglycans and hyaluronic acid in most areas of developing cerebellum (with the notable exception of the external granule cell layer) indicates that these macromolecules do not constitute a general barrier to neurite extension and cell migration. It is therefore likely that the processes by which extracellular proteoglycans can affect cell interactions in developing nervous tissue will ultimately be found to involve the concerted actions of several molecules, possibly acting at different sites or time periods.

Interactions with neurons and neural cell adhesion molecules. Other cell surface molecules that have been implicated in adhesion during neural development include various cell adhesion molecules (CAMs) that are members of the Ig superfamily (reviewed in ref. 36). N-CAM and Ng-CAM are two of the CAMs that are expressed on neurons and have been implicated in various aspects of cell adhesion, cell migration and neurite fasciculation that correlate with their general patterns of expression during development. Each CAM can mediate homophilic binding and has at least one heterophilic ligand. Although these neural CAMs are functionally and structurally distinct, they share features including certain carbohydrate structures, and both contain related Ig-like and fibronectin-like domains. In view of evidence that extracellular proteoglycans are involved in

cell interactions, we have begun to explore the question of whether purified brain proteoglycans can affect the behavior of brain cells and, in particular, interfere with mechanisms of cell adhesion[37].

The homophilic binding of cell adhesion molecules can be demonstrated by the ability of CAM-coated fluorescent beads (Covaspheres) to self-aggregate. We have found that the extent of aggregation of Covaspheres coated with either Ng-CAM or N-CAM was strongly inhibited by low concentrations (2–10 μg/ml) of the 1D1 (neurocan) and 3F8 chondroitin sulfate proteoglycans of rat brain (see section A.1. above), and by the core glycoproteins resulting from chondroitinase treatment of the proteoglycans. Neurocan and the 3F8 proteoglycan also inhibited binding of neurons to Ng-CAM when mixtures of these proteins were adsorbed to polystyrene dishes. In both assays, much higher concentrations of rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) had no significant effect. These findings support other evidence indicating that extracellular chondroitin sulfate proteoglycans may act as repulsive molecules which modulate cell-cell and cell-matrix interactions by providing a mechanism for diminishing adhesive forces, thereby permitting cell rounding, division, differentiation, and cell movement in developing brain. Direct binding of neurons to the proteoglycan core glycoproteins from brain but not from chondrosarcoma was also demonstrated using an assay in which cell-substrate contact was initiated by centrifugation, and neuronal binding to neurocan was specifically inhibited by the 1D1 monoclonal antibody. These results therefore indicate that certain proteoglycans can bind to neurons, and that Ng-CAM and N-CAM may be heterophilic ligands for neurocan and the 3F8 proteoglycan. Recent studies have also demonstrated direct binding of radiolabeled neurocan and 3F8 proteoglycan to Ng-CAM and N-CAM, with dissociation constants in the range of 10^{-10} to 10^{-11} M (P. Milev, D. Friedlander, M. Grumet and R. Margolis, unpublished results). Secondary structuré predictions based on the N-terminal primary sequences of aggrecan and cartilage link protein, and comparison with the known crystal structures of immunoglobulin constant and variable region domains, have identified a pattern of β-sheet structure found in immunoglobulin folds[93]. The Ig-like domain of neurocan (and the copurified link protein) can therefore also be considered to place this brain proteoglycan in the Ig superfamily, almost all of whose members are cell surface molecules involved in cell recognition, cell adhesion, and immune function. Although these properties may contribute to the somewhat greater potency of the early postnatal 'full-length' form of the 1D1 proteoglycan in producing the biological effects described above, they are evidently not a major factor insofar as the adult form of the proteoglycan which contains only the C-terminal half of neurocan has very similar effects. The ability of these brain proteoglycans to inhibit cell adhesion to CAMs may be one mechanism to modulate cell adhesion and migration in the nervous system. It will obviously be important to determine the nature of the binding sites that may mediate interactions between proteoglycans and cell adhesion molecules, and cDNA constructs have now been prepared to express specific domains of neurocan and Ng-CAM to explore potential interactions.

Immunocytochemical studies using the 1D1 and 3F8 monoclonal antibodies[82] have shown that neurocan and the 3F8 proteoglycan are present in the subplate region of the developing mouse cerebral cortex in regions that were previously found to contain chondroitin sulfate and fibronectin[109]. Based on correlations between the patterns of protein expression and axonal migration during development, it has been suggested that these molecules may play a role in defining a destination for migrating axons that form the cortical plate, and in delineating pathways for early axonal extension[109].

2. Effects of heparan sulfate proteoglycans on neurite outgrowth and cell interactions

Several reports indicate that heparan sulfate glycosaminoglycan chains are capable of promoting[20, 24, 59a, 120] or inhibiting[1] neurite outgrowth, but as in the case of chondroitin sulfate proteoglycans discussed above, the biological relevance of studies employing free glycosaminoglycan chains (as compared to proteoglycans) may be difficult to evaluate. In addition to the presumably direct effects of proteoglycans on neurite outgrowth, numerous observations indicate a general role for proteoglycans and glycosaminoglycans (especially heparan sulfate) in the control of cell proliferation via interactions with growth factors. For example, it appears that in dorsal root ganglion neurons and brain membranes, cell surface heparan sulfate proteoglycans serve as an anchor for growth factors having Schwann cell mitogenic activity[22, 97] and for neurite outgrowth-promoting factors in brain[63, 80, 81]. It is also possible that quantitatively minor cell surface chondroitin sulfate proteoglycans of brain, such as the NG2 and Cat-301 antigens described above, may function in the binding, storage, and release of locally high concentrations of growth factors, as may the chondroitin/heparan sulfate proteoglycan (betaglycan) formerly referred to as the type III transforming growth factor-β (TGF-β) 'receptor'[2]. Expression of high levels of a small chondroitin/dermatan sulfate proteoglycan (decorin) in Chinese hamster ovary cells has a dramatic effect on their morphology and growth properties which is probably due at least in part to its ability to bind TGF-β, and there is evidence suggesting that this proteoglycan may be a component of a feedback system regulating cell growth[124].

Other possible functions of proteoglycans in relation to growth factors are indicated by the considerable work

on the role of heparan sulfate proteoglycans as modulators of fibroblast growth factor (FGF) action. It has been known that the binding of FGFs to heparan sulfate (or heparin) chains of proteoglycans appears to protect the growth factors from degradation, and may be important in providing a matrix-bound or cell surface-bound reservoir of FGF from which active FGF-glycosaminoglycan complex can be generated by proteolysis of the proteoglycan core proteins or partial degradation of the heparan sulfate chains (for a review, see ref. 105). More recently, it has been shown that the binding of basic FGF to its receptor requires prior binding either to the heparan sulfate chains of a membrane proteoglycan or to free heparan sulfate chains[126]. It would appear that the glycosaminoglycan may change the conformation of basic FGF so that it acquires an ability to bind to the receptor, or binding to the glycosaminoglycan might produce oligomerization of bFGF in a mode compatible with receptor dimerization. Although these reports do not pertain directly to nervous tissue, a recent study suggests that a chondroitin sulfate proteoglycan in the interphotoreceptor matrix is involved in the binding of basic FGF in primate retina[39]. It has also been reported that a 480 kDa chondroitin sulfate proteoglycan isolated from neonatal rat superior colliculus, and which yields 300 and 370 kDa core glycoproteins after chondroitinase treatment, acts as a neurotrophic factor for neonatal rat retinal ganglion cells[107a].

There have up to now been relatively few reports concerning the potential role of heparan sulfate proteoglycans in cell interactions in the central nervous system. Cultured astrocytes secrete an extracellular matrix containing both heparan sulfate proteoglycan(s) and laminin. Although expression of these molecules appears to be regulated together with morphological differentiation (as determined by the culture conditions) and by contact with neurites, the capacity of astrocytes to interact with growing neurites is not dependent on heparan sulfate proteoglycan or laminin expression[5]. Evidence has also been presented indicating that heparan sulfate proteoglycans are involved in the guidance of a subset of pioneer axons in cultured cockroach embryos[121]. Since studies of this type involve evaluation of the effects of heparitinase treatment, their conclusions once again pertain only to the potential biological roles of the heparan sulfate chains (rather than those of the core protein), and also implicitly rely upon the specificity of the enzyme preparations used.

D. Future prospects

During the past several years, the isolation and biochemical characterization of individual proteoglycan species, followed by the cloning of some of these proteoglycans, has provided an increasingly firm foundation for elucidating their neurobiological functions. Data on the primary structure of their core proteins have revealed often unexpected relationships to other proteins with specific biological functions, and has allowed a more rational design of experiments directed towards exploring their interactions with cell surface and extracellular matrix proteins, including the use of recombinant proteins to define the roles of specific protein domains. Immunocytochemical and in situ hybridization studies are also beginning to reveal the cellular sites of synthesis of characterized proteoglycans and their association with specific neuronal pathways and developmental processes. The use of homologous recombination and other strategies for the knockout of particular proteoglycan genes offers further prospects for more clearly defining their functional roles. The ability to express cloned membrane proteoglycans in cells in which they are not usually present, and to use antisense strategies to inhibit the expression of membrane-associated and secreted proteoglycans by other cell types, should aid in evaluating their involvement in cell interactions and other processes. Much work also remains to be accomplished in defining the structures and functions of their glycoprotein-type oligosaccharides, and the neurobiological significance of fine structural variations in the glycosaminoglycan chains. It is however clearly apparent that we are now in a very advantageous position to make rapid progress in understanding the roles of proteoglycans in a tissue as cellularly and functionally complex as the central nervous system, and that one can expect to see a number of exciting developments in the next several years.

Acknowledgements. Research cited in this review which was performed in the authors' laboratories was supported by grants from the National Institutes of Health (NS-09348, NS-13876, and MH-00129).

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