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Proteoglycans in brain development

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Proteoglycans, as part of the extracellular or cell-surface milieu of most tissues and organ systems, play important roles in morphogenesis by modulating cell-matrix or cell-cell interactions, cell adhesiveness, or by binding and presenting growth and differentiation factors. Chondroitin sulfate proteoglycans which constitute the major population of proteoglycans in the central nervous system may influence formation of neuronal nuclei, establishment of boundaries for axonal growth and act as modulators of neuronal outgrowth during brain development, as well as during regeneration after injury. There is a paucity of information on the role of chondroitin sulfate proteoglycans in central nervous system organogenesis. In the chick embryo, aggrecan has a regionally specific and developmentally regulated expression profile during brain development. By Northern and Western blot analysis, aggrecan expression is first detected in chick brain on embryonic day 7 (E7), increases from E7 to E13, declines markedly after E16, and is not evident in hatchling brains. The time course and pattern of aggrecan expression observed in ventricular zone cells suggested that it might play a role in gliogenesis. We have analyzed the role of aggrecan during brain development using a aggrecan-deficient model, nanomelia. In nanomelic chicks, expression and levels of neurocan and brevican is not affected, indicating a non-redundant role for these members of the aggrecan gene family. Our analysis of the aggrecan-deficient model found a severely altered phenotype which affects cell behavior in a neuronal culture paradigm and expression of astrocytic markers *in vivo*. Taken together our results suggest a function for aggrecan in the specification of a sub-set of glia precursors that might give rise to astrocytes *in vivo*. *Published in 2004.*

Keywords: **aggrecan, nanomelia, chondroitin sulfate proteoglycan, hyalectins, glycosaminoglycan**

Introduction

As components of the extracellular matrix and cell surface, proteoglycans (PGs) are critical environmental modulators, playing important roles in cell differentiation, tissue morphogenesis and phenotypic stabilization of all tissue and organ systems via cell: matrix adhesiveness, cell: cell interactions and/or binding to growth regulators and differentiation factors [1–5]. This is particularly evident during central nervous system (CNS) development where proteoglycans may promote neurite outgrowth [6,7], inhibit neurite outgrowth [8,9] or stabilize new synapse formation [10]. Proteoglycans are up-regulated in CNS lesions [11,12] and proteoglycan degradation enhances axon regeneration [13–15], suggesting a role in recovery from injury as well. The functional roles of neural proteoglycans during development and regeneration have only been partially elucidated; this knowledge is essential to fully appreciate the contributions of proteoglycans in establishment and maintenance of the nervous system.

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Proteoglycans

Proteoglycans are a large and diverse group of molecules defined both by their protein and carbohydrate constituents. They are composed of repeated disaccharide glycosaminoglycan (GAG) chains, consisting of a sulfated hexosamine and uronic acid, covalently linked to a central protein core (Figure 1). Type, size and composition of GAG chains, primary sequence and domain arrangement of the protein core, and degree of substitution and distribution of the GAG chains along the protein core all vary, leading to proteoglycan structures that are complex and diverse. Hybrid molecules with additional structural diversity may arise by substitution with N- and O-linked glycoprotein-type oligosaccharides or by having more than one type of GAG chain attached to the same core protein (Figure 1). Although all proteoglycans have these features in common, six distinct classes of GAG chains are recognized based on differences in monosaccharide composition, sulfation, and epimerization of the uronic acid. Four GAG classes, chondroitin sulfate

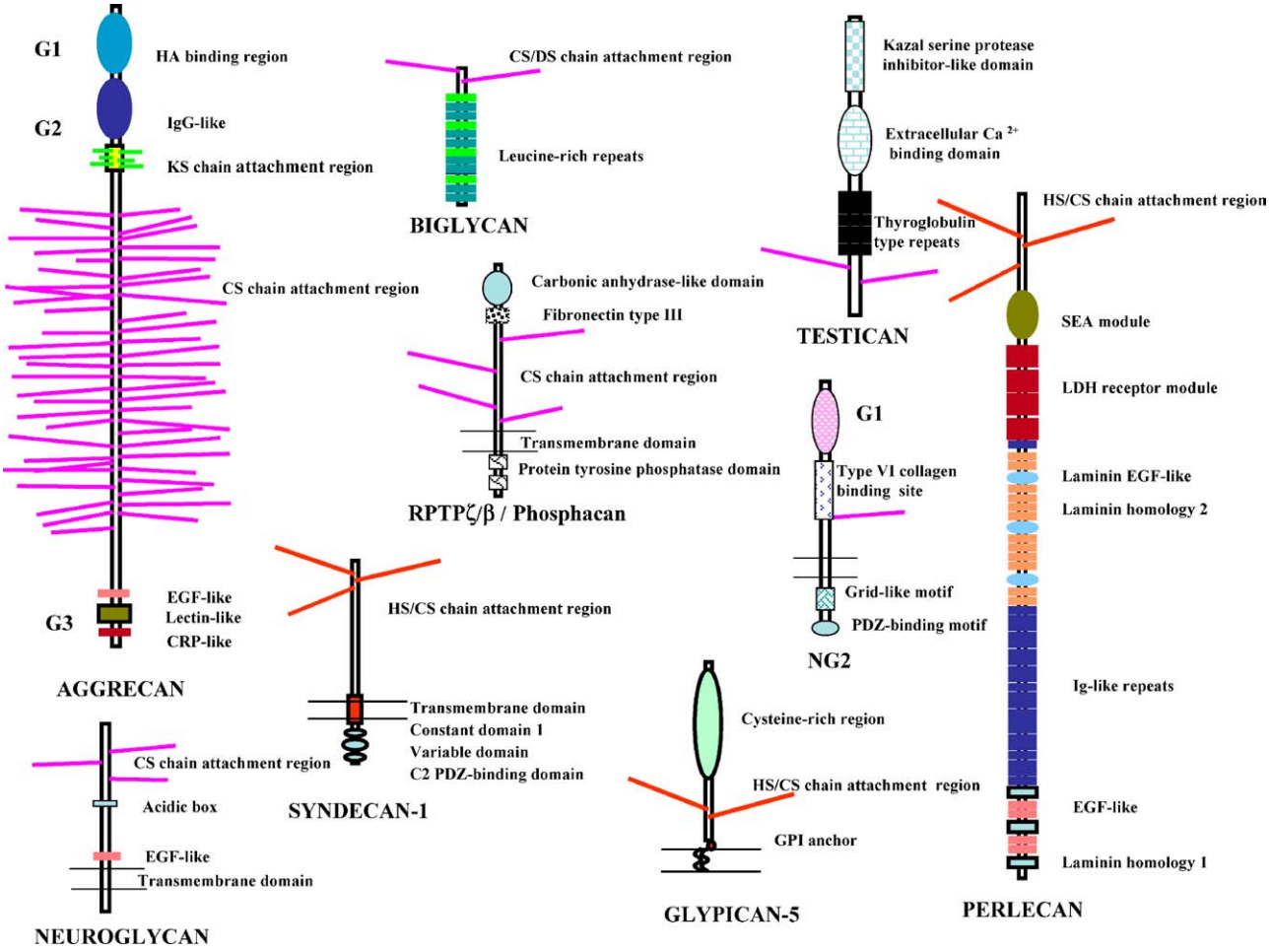

Figure 1. Modular structures of representative members of several proteoglycan families expressed during brain development.

(CS), dermatan sulfate (DS), heparin and heparan sulfate (HS) are linked to serines of the protein core via a common tetrasaccharide (xylose-galactose-galactose-glucuronic acid) [16–23], while keratan sulfate (KS) is attached to the core protein through an *N*-acetylglucosamine by N-linkage to asparagine or O-linkage to serine. Hyaluronate, a simple polymer of *N*-acetylglucosamine and glucuronic acid disaccharides, is not linked to protein.

The diversity of proteoglycan structure and function is reflected by the cloning of more than forty full-length cDNAs encoding proteoglycan core proteins and the accompanying development of a system for classifying proteoglycans into gene families [22,24–26]. From analysis of deduced primary structures, the concept of modular proteoglycans with discrete structural and functional domains, consisting of both carbohydrate-attachment and carbohydrate-free regions, has evolved (Figure 1). The major chondroitin sulfate proteoglycan (CSPG) gene family, comprising those most abundant in nature, consists of four members, aggrecan, versican, neurocan and brevican. The family members have a structural organization consisting of one or two N-terminal globular domains and a C-terminal multifunctional binding domain (G3), separated by a variable-length carbohydrate-rich domain, and are designed hyalectins because of their hyaluronan- and lectin-interacting domains (Figure 1).

Other proteoglycan families have been described (Figure 1): the cell-associated proteoglycans comprising the membrane-bound syndecan family, which have a short C-terminal cytoplasmic domain and a large extracellular domain substituted with HS and CS chains; the large basement membrane proteoglycan, perlecan, which has a complex modular structure consisting of five major domains with multiple functions and a single N-terminal heparan sulfate attachment domain; the small leucine-rich proteoglycans which are characterized by the dermatan/chondroitin sulfate-substituted decorin and biglycan; and the keratan sulfate-substituted fibromodulin and lumican. Variation within and among these distinct gene families is based on modular core protein organization and diversity in GAG type and provides a vast combinatorial potential for functional specificity that has been exploited by nature [20,22,25,26].

Proteoglycans fulfill a variety of biological functions, such as molecular concentration, growth modulation, ionic filtration, and biomechanical lubrication [1,27–29]. Spatial

immobilization of growth factors and cytokines may be one of the most important functions of proteoglycans. For instance, cell-surface heparan sulfate proteoglycans (HSPGs) bind growth modulators such as fibroblast growth factor, which serves to protect the growth factors from degradation in the extracellular milieu and sequester a surface reservoir of growth factor, which is released only by degradation of the proteoglycan, or act as co-receptors to alter the conformation of growth factors thereby facilitating binding to their receptors and triggering signal transduction pathways [30]. Proteoglycans may also act as molecular organizers of the extracellular matrix (ECM) and promoters of cell adhesion [30]. The functional interactions that lead to the large electron-dense multimolecular aggregates characteristic of cartilage extracellular matrix [31] involve the unique terminal domains of the aggrecan core protein. These interact noncovalently with other matrix constituents *e.g.*, hyaluronan and type II collagen, thereby interconnecting the ECM constituents. Members of the low molecular weight leucine-rich proteoglycan family (*i.e.* decorin and fibromodulin) also participate in organizing the extracellular matrix by binding types I and II collagen [32,33].

All proteoglycans, with the exception of hyaluronan, follow the same general pattern of synthesis. CS, DS and HS chains are initiated by addition of xylose to the hydroxyl of a serine residue embedded in a specific peptide sequence [34,35] in a reaction catalyzed by the chain-initiating xylosyltransferase [36]. Elongation of the tetrasaccharide linkage region is catalyzed by distinct glycosyltransferases, each specific with respect to acceptor, donor and linkage formed. The repeating polymer is synthesized by the concerted action of *N*-acetylgalactosaminyltransferase and glucuronosyltransferase activites, concomitant with sulfation of the GAG chains at either the 4 or 6 position of the hexosamine [21,36,37]. In contrast, heparin and HS syntheses require the concerted action of several additional modifying enzymes, including *N*-deacetylase/*N*-sulfotransferase, glucuronic acid C-5 epimerase, iduronic acid 2-*O*-sulfotransferase, and glucosamine 6-*O*- and 3-*O*-sulfotransferases [16]. Most likely, coordination between chain elongation and modification leads to the observed diversity of the HS synthesized by different cells and tissues [38].

It is well recognized that the GAG chains are assembled while the proteoglycan core protein substrate is traversing the intracellular secretory pathway. An outline of the dynamic and topological aspects of GAG synthesis has emerged from studies on the aggrecan system [21,36,39]. While N-linked oligosaccharides are added co-translationally to the nascent core protein in the endoplasmic reticulum (ER), CS chains are initiated by xylose addition after extrusion of the core protein into the lumen of the ER is completed. The xylosylated precursor core protein is then translocated to early compartments of the Golgi for further modification, subsequently moves through the secretory pathway, and is secreted as a fully glycosylated and sulfated aggrecan molecule [40,41]. Other members of the aggrecan gene family and other types of proteoglycans exhibit aspects of GAG synthesis and assembly onto the various core proteins similar to those elucidated for aggrecan [37].

Brain proteoglycans

Since the early studies of [42], evidence has accumulated that proteoglycans are located on the surface and in the extracellular space of neural cells and that they play important roles in cell-cell and cell-matrix interactions during neural development. Because of the cellular complexity and numerous developmental stages involved in migration and maturation of the various neural elements, it could be expected that multiple proteoglycans are involved during this developmental cell- and stage-specific program. Because of the enormous structural diversity arising from the type and content of carbohydrates attached to numerous core proteins, the isolation, identification, characterization, and elucidation of biological functions of the potentially large number of proteoglycans that may be elaborated during neural development has been a challenge. Studies using immunological, biochemical, or molecular analyses confirmed the presence of multiple proteoglycan core proteins in the brain and the rich diversity and abundance of these extracellular macromolecules in neural tissue [43–48].

CNS proteoglycans have been found to be diverse with regard to size and structure of their core proteins, as well as the nature and number of attached glycosaminoglycan chains and other carbohydrate substituents, *i.e.*, HS, KS, CS, and DS [49–52]. CSPGs in the CNS have been partially characterized from chick brain [53–55], adult rat brain [48,56], cultured glial cells [57–59], and postnatal cat brain [60]. Immunocytochemical studies using monoclonal antibodies to a generic CS epitope have shown several types of neurons to be surrounded by CSPGs [49,61,62]. More specific antibodies have localized other CSPGs such as: cytotactin binding proteoglycan in Bergmann glial fibers and along parallel fibers of adult cerebellum [63]; two large CSPG core proteins in granule cells and parallel fibers of adult rat brain [48]; Cat-301 CSPG [60]; somatoglycan-S [64]; 6B4 CSPG expressed in a subset of neurons in adult mammalian brain and spinocerebellar system [65]; and the cell-associated NG2, expressed by a class of protoplasmic astrocytes in rat cerebellum [66] and by oligodendrocyte precursors [67]. This diversity of location suggests that several different CSPGs are associated with distinct cellular components and functions of the CNS.

All members of the aggrecan gene family are expressed in neural tissue. Versican is expressed postnatally in rat brain and spinal cord [68,69]. Neurocan is found in early postnatal rat brain as a large CSPG with a 245 kDa core protein and in adult brain as a smaller CSPG with a 150 kDa core protein, and is predominantly a neuronal product [48,70]. Brevican has N- and C-terminal domains similar to those of other CSPGs, however, the central carbohydrate-rich region is much shorter than, and shows little homology to, those of the other CSPGs [71]. A significant amount of unglycosylated brevican can be

demonstrated in brain; thus this proteoglycan is considered a part-time proteoglycan, analogous to several other proteoglycan core proteins that exist in forms with or without GAG chains [72]. Brevican is expressed predominantly by astrocytes, and not by neurons, in the rat [73,74]. Among the CSPGs studied in mammalian brain, Cat-301, a neuronal cell surface CSPG identified with a specific monoclonal antibody, has been shown to be identical to aggrecan and to be expressed in adult mouse brain [60,75,76].

Recently, other CSPG families have been characterized in brain. The testican family of secreted CSPG comprises three members that have been found to be abundant in brain during neurogenesis [77–79] (Figure 1). Their function is still unclear, but it has been suggested that they work in concert to modulate the activity of metaloproteases by interacting with MT-MMPs [80,81]. Neuroglycan C is a part-time trans-membrane CSPG, the core protein of which exist in three different forms resulting from alternative mRNA splicing [111] (Figure 1). It is expressed in developing brain and in developing retina, and is immunolocalized primarily to Purkinge cells and their dendritic trees in the cerebellum [82,83]. Its distribution suggests a function in adhesion and synaptogenesis [82]. Phosphacan/DSD-1, an mRNA splicing variant form, lacks the cytoplasmic and transmembrane domains of the receptor-type protein tyrosine phosphatase (RPTPζ/β) [84,85]. This proteoglycan contains CS and KS chains and the different isoforms are developmentally regulated. Phosphacan is expressed in astrocytes and their precursors [84,86–88]. NG2 is a transmembrane CSPG expressed by oligodendrocyte precursors that may be involved in regulating the stability and function of synapsis [89–91]. Other glycoproteins in brain have been described as part-time CSPGs, such as CD44 [92–94] and APP (amyloid precursor protein) [95–97]. Furthermore, three classes of HSPGs that bear CS chains as well have been described in brain during development: syndecan-1; glypican-5 and perlecan (Figure 1).

Aggrecan in developing brain

Fewer studies have attempted to characterize CSPGs expressed in the embryonic nervous system. Using antibody and molecular probes developed for our cartilage proteoglycan studies, we have explored the hypothesis that particular genes may play multiple roles in development, by being modified, used in different combinations, or expressed at different times, in order to carry out unique functions in various tissue or organ systems. We identified three distinct developmentally regulated CSPGs in embryonic chick notochord, brain, and cartilage, all of which share common reactivity with S103L, the monoclonal antibody that reacts with a species-specific epitope in the CS-rich region of CSPG core proteins [55,98,99]. We then demonstrated that the cartilage, notochord, and brain CSPGs are all products of the same aggrecan gene; however, in each tissue the CSPG product exhibits significantly different chemical, structural, and immunological properties, indicating that although each is initiated on the identical core protein, they are processed differently, yielding distinct proteoglycans. The brain S103L CSPG has a core protein of similar size to the cross-reacting cartilage species (about 370 kDa), but a much smaller hydrodynamic size (K_{av} about 0.31), is substituted with fewer CS chains and has virtually no KS chains. Interestingly, the S103L-reactive CSPG has CS chains that are mainly either substituted with 6-sulfate groups or remain nonsulfated. This is in contrast to the sulfation pattern of cartilage aggrecan, which exhibits the typical ΔDi-4S: ΔDi-6S ratio of 70:30, and contains a substantial amount of KS as well [100] (Table 1). Immunohistochemical methods have detected expression of another predominant CSPG as early as stage 16, long before chondrogenesis occurs; expression then continues during the time of active neural crest migration through the onset of sclerotomal differentiation. Although this notochordal CSPG shares S103L cross-activity with the cartilage species and is a product of the same aggrecan gene, striking differences distinguish these two CSPGs at the post-translational level. The notochord CSPG is devoid of KS (the common biochemical hallmark of cartilage CSPG) and is much smaller, indicating less CS chain substitution. Most notably, mature cartilage and brain CSPGs lack the HNK-1 epitope, while the notochord and early cartilage CSPGs have a high content of HNK-1 [98,101]. A predominant and ubiquitously expressed CSPG that does contain the HNK-1 epitope has also been isolated and cloned from brain. This HNK-1 CSPG has a smaller core protein (340 kDa), an even smaller hydrodynamic size (K_{av} about 0.56) and is substituted with both, but presumably fewer, CS and KS chains [54,55,99] (Table 1).

Table 1. Biochemical properties of the cartilage, brain and notocord aggrecans

	Notochord	*Brain*	*Cartilage*
Solubility	Soluble	Soluble	Matrix Bound
Expression	E2-E4	E6-E18	E6-adult
S103L reactivity	+	+	+
HNK-1 reactivity	+	−	+/−
KS	−	−	+
ΔDi6S/ΔDi4S	n.d.	1:0	3:7

n.d.: not determined.

Together, these results suggest that variation in sulfate content and localization on the same GAG chains on the same core protein is under tissue- or cell-specific regulation. It is possible that the expression of specific GAG biosynthetic and modifying enzymes by individual cell types dictates the type of carbohydrate modification that the core proteins expressed in each cell type will incorporate, as has been previously suggested [102]. Alternatively, specific modifications could be dictated by the type of core protein to accommodate specific functions. A better understanding of the expression of biosynthetic and modifying enzymes in relationship to the core protein distribution would shed light into these possibilities.

Although significant progress has been made in identifying the major CSPGs synthesized by embryonic brain, it is not clear which CSPGs are contributed to the ECM milieu by different types of CNS cells. Therefore, a study was undertaken to determine the contribution of specific CNS cell types to the ECM of the developing brain with respect to the distribution of the two well-characterized CSPGs, S103L and HNK-1, as well as to identify and characterize biochemically and developmentally other distinct CSPGs of the different cell types. Specifically, addressing questions concerning the expression of CSPGs within specific cell lineages from the same or different germinal layers, we analyzed the high density CSPGs from a single species (chick) during the course of CNS development using whole brain preparations and specific-cell-enriched culture systems. The two large brain CSPGs were found to differ in their temporal expression patterns during embryogenesis. The HNK-1 CSPG is expressed constitutively from day 4 through hatching, while S103L-CSPG is developmentally regulated (detected from embryonic day 6 (E6 to E18) in all major areas of CNS, *i.e.*, cerebral hemispheres, optic lobes and spinal cord [103]. The peak expression at E11-E13 coincides with the organization of neuronal nuclei [104]. Conspicuously absent at the protein level of analysis are higher MW CSPGs of the versican type. This finding is commensurate with results which indicate very low expression of versican mRNA by neurons [99]. In addition to the large HNK-1 and S103L-CSPGs, CNS cells synthesize and secrete into the ECM several other CSPGs of lower molecular weight (~220, 145, and 120 kDa) which were monitored using the ΔDi-4S, ΔDi-6S, ΔDi-0S or anti-KS antibodies and developmentally regulated, but with different temporal expression patterns from each other [103].

To determine which cell types synthesize the various CSPGs that constitute the cell surface/ECM milieu of the developing brain, CSPG profiles of major neuronal subtypes derived from different brain regions, *i.e.*, cerebral hemisphere, spinal and retinal neurons, and astrocytes, all of neuroectodermal derivation, were identified and compared to whole brain and to meningeal cells, which are of mesenchymal origin, using cell type- and CNS region-specific culture systems. CSPG profiles obtained from the major neuronal type cultures exhibited robust S103L CSPG production [54,103,105]. In contrast with the pattern observed in neuronal cultures, astrocytic cultures are characterized by the presence of predominantly low molecular weight CSPGs, although some very large proteoglycan core proteins (>500) were also distinguished, most likely indicating the presence of versican-like proteoglycans in this cell culture system. Thus it appears that versican in developing brain is contributed predominantly by astrocytes. Astrocytes also synthesize the HNK-1 CSPG, which is expressed constitutively over the course of embryonic development [104]. In mammalian systems, astrocyte-specific proteoglycans that are HNK-1 positive have also been described; these are recognized by the L5 antibody [106]. The expression of HNK-1 CSPGs throughout gliogenesis and by differentiated astrocytes [7,54,105,106] suggests a role in general cell homeostasis and possibly in specific astrocytic functions.

Of the other types of non-neuronal cells that can be established in culture as highly enriched cell systems, meningeal cells also express the HNK-1 CSPG but not the S103L-CSPG. Meningeal cells are of mesenchymal origin, as are the chondrocytes that form the cranial membraneous bone. The differential temporal expression of S103L-CSPG as the cranial, meningeal and neural layers are formed cannot be explained on the basis of cell lineage, since the two predominant cell types of mesenchymal origin (chondrocytes and meninges) do not share common expression of the S103L-CSPG gene. Overall, these results suggest that the specific temporal pattern of expression of the S103L-CSPG may contribute to conditions that induce or stabilize specific cell phenotypes during CNS development. In contrast, the other major HNK-1-positive CSPG in the CNS, is synthesized by all cell types of different lineages over the entire embryonic period, suggesting a more global cell-maintenance function for this CSPG.

The expression pattern of aggrecan in neuronal cultures derived from embryonic day 6 chick telencephalon (E6) is analogous to that in embryonic brain tissue, providing an in-culture model for investigating the regulation of aggrecan expression and its role in telencephalon development [54,104]. Specifically, the expression profile of S103L-CSPGs in the E6 cultures, analyzed both by Western blot and immunocytochemical staining, showed aggrecan-positive cells as early as one day after plating. As neuronal aggregates formed and neurons extended processes over the course of the next five days, both the number of S103L-reactive cells and the intensity of staining increased significantly. Maximal reactivity occurred at culture day 5, when most neurons, especially those in aggregates, were positive. Reflecting the course of *in vivo* events, staining of neurons in aggregates began to decline by culture day 7, and by day 15 no S103L-positive cells were detectable. Cultured neurons remained metabolically active and expressed other developmentally regulated neuron-specific markers [54,104] (Figure 3). The staining pattern of aggrecan expression was mirrored by quantitative immunoprecipitation and Western blot analysis [54,55,104], and on Northern blots [99].

In order to demonstrate that aggrecan may participate in the adhesion and migration processes required for establishment of neuronal aggregates in culture, a model for CNS nuclei formation [54,107,108], we analyzed the involvement of different components of the aggrecan molecule in this process. Normal neuronal cultures start out as single dissociated cells which migrate on the polylysine substrate to form neuronal aggregates that increase in size over time in culture and which are connected by long thick bundles of neurites (Figure 3). Immunocytochemistry using the S103L antibody against aggrecan [55] was performed on neuronal cell cultures derived from day-8 telencephali and maintained in culture for 6 days, and showed robust aggrecan accumulated in the aggregates where it localized extracellularly, as had been previously determined biochemically

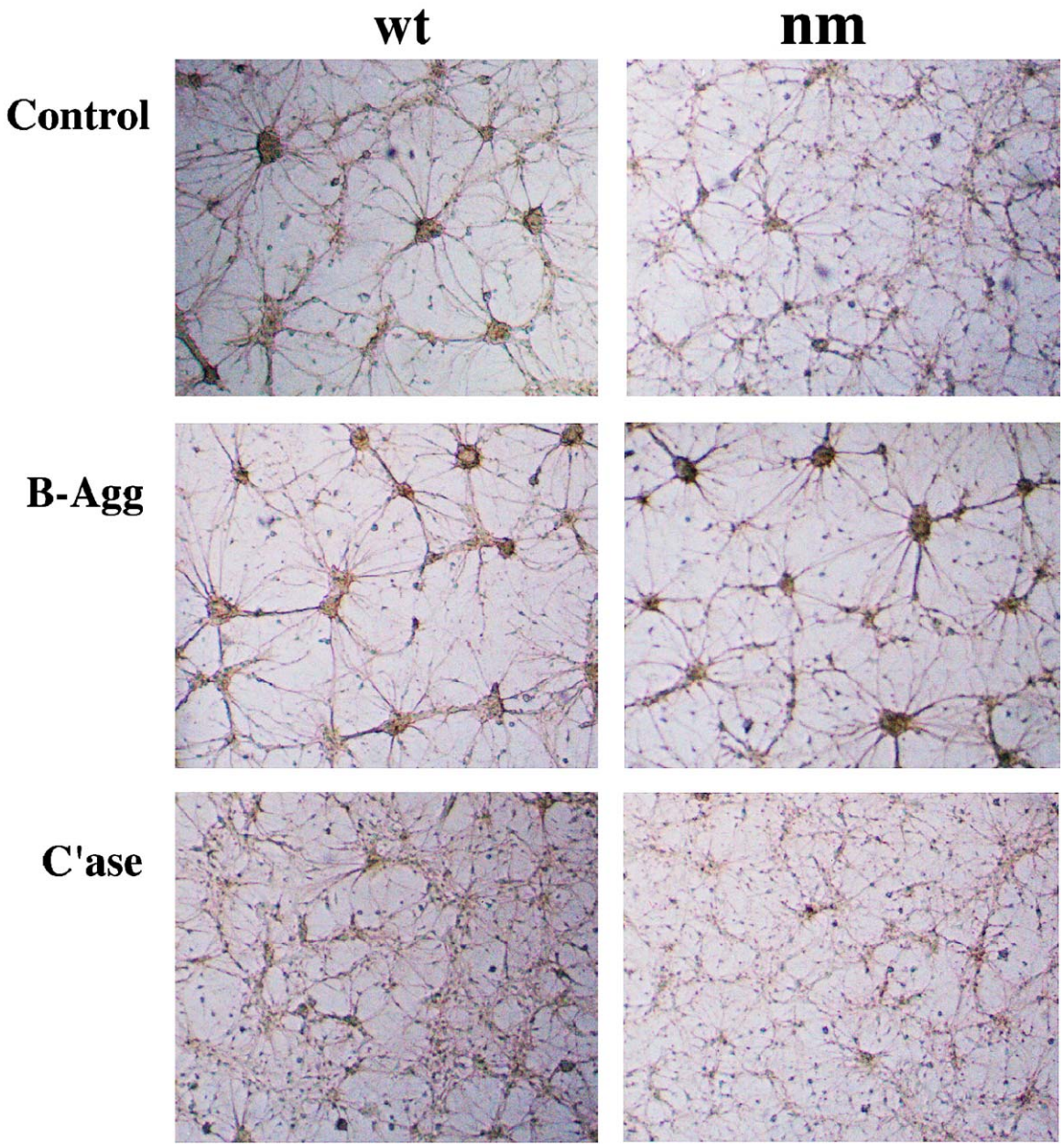

Figure 2. Aggrecan rescue of the nanomelic neuronal phenotype. Immunocytochemistry using anti-neurofilament-200 on telencephalic neurons in culture for 6-days. The cells were obtained from wild type (wt) and nanomelic (nm) E8 chicks embryos. One day after plating, the cultures were not treated (control), treated with purified brain aggrecan (5 μg/ml) (B-Agg) or with chondroitinase ABC (80 mU/ml) (C'ase) [110].

[54]. To determine whether the neuronal-aggregation-promoting activity of aggrecan resides in the GAG side-chains or in the core protein, experiments were designed to test each component separately. Based on our previous inhibition studies with β-xyloside [54], the importance of GAG chains in this experimental paradigm was analyzed. Chondroitinase ABC treatment (80 mU/ml) of E6C1 neuronal cultures caused fewer and smaller aggregates (20%, $p < 0.005$) after 5 days in culture (E6C6) (Figure 2). As well, a large number of single cells between aggregates were observed, mimicking the distribution observed in the β-xyloside-treated neuronal cultures [54] and suggesting the effect was not due to loss of cell attachment to the dishes. In contrast to the results with chondroitinase, other glycanases (*i.e.*, heparatinase and hyaluronidase) did not produce similar reductions in aggregate size or number, although cells tended to associate into aggregates more rapidly after heparatinase treatment. Keratanase also induced smaller aggregate size but not number, possibly by acting on the KS proteoglycan

phosphacan [109] expressed by neurons in these cultures. Treatment with glycosaminoglycans, *e.g.* chondroitin 4-sulfate, chondroitin 6-sulfate or hyaluronan had no effect on normal cultures, while somewhat larger and less-numerous cellular aggregates (but with fewer well-defined neuritic processes) were observed in DS and HS-treated cultures, indicating that the effects of these molecules, or lack thereof, cannot be simply explained by charge-mediated competition with the substrate.

It is well documented that the E6 cerebral hemisphere neuronal cultures produce copious amounts of aggrecan, as demonstrated by S103L antibody staining, suggesting that the origin of the aggrecan around the cell aggregates is from the more abundant neurons. However, since aggrecan is a secreted extracellular component that may associate with a cell other than the one which produces it, we identified the cell-type responsible for aggrecan production in these cultures by *in situ* hybridization (Figure 3). Unexpectedly, the fully-differentiated neurons do not express aggrecan, rather its expression is confined to a small population of cells usually localized to the center of the neuronal aggregates (Figure 3). Also, single cells not in aggregates do not express aggrecan. To determine whether there were other sources of CS chains in these cultures, the sources of two other members of the gene family, brevican and neurocan, were analyzed [110]. Expression of mRNA for brevican (Figure 3) is similar to that of aggrecan mRNA, as it was found to be expressed by only a small population of cells in the center of the aggregates. In contrast, aggrecan expression is clearly distinct from that of neurocan (Figure 3) and class III-β-tubulin, a known marker of neurons, which are both strongly expressed by all cells in aggregates, as well as by single cells. Thus the presence of other CSPGs in these cultures may contribute to the pronounced phenotype observed in chondroitinase-treated cultures [110].

Aggrecan-deficient mutants

The studies discussed thus far, suggest a possible function for aggrecan as a diffusible signal in CNS histomorphogenesis. To further explore this possibility we employed the nanomelic chick mutant. Chick embryos homozygous for the autosomal recessive gene *nanomelia* (nm) exhibit an extreme form of micromelia [111], with reduced trunk and head sizes and gross skeletal abnormalities including extremely shortened, broad and malformed limbs. This lethal chondrodystrophy results from a nonsense mutation in the aggrecan gene leading to the absence of expression of glycosylated aggrecan in the brain and cartilage extracellular milieu [112,113]. The nanomelic chick phenotype is consistent with the characteristics of the murine cartilage matrix deficiency (cmd) phenotype for which two allelic deletion mutations in the mouse aggrecan gene have been identified [114,115]. Thus far, there are no reports of any mutations in the human aggrecan gene that yield identifiable phenotypes. We are using the nanomelic model system to investigate the role of aggrecan in CNS development. When neurons from normal and nm chick embryo telencephalon were cultured, scored for aggregate formation and analyzed for distribution of aggrecan protein and expression of aggrecan mRNA, distinctly different pattern formation, with respect to aggregate size (smaller) and number (fewer) was observed in poly-L-lysine plated nm neuronal cultures (Figure 2).

To quantitate the effect of aggrecan on neuronal aggregation, we stained plate cultures with neurofilament-200 monoclonal antibody, counted the number and measured the areas of cell aggregates in randomly selected fields, and performed statistical analysis. Our results indicate that nm neurons formed significantly smaller and fewer aggregates compared to the normal controls (Figure 2). Counting neuronal nuclei in 6-day wt and nm cultures derived from E8 embryos (E8C6) revealed no significant difference in the total number of cells associated with the polylysine substratum, indicating that the observed differences were not attributable to differential cell adhesion to the substrate. Moreover, aggregate size increased progressively between days 4 and 8 in wt cultures, while in nm cultures no increase in aggregate size was observed over the same time period.

In order to determine whether these differences in the behavior of nm neurons in culture were due directly to the absence of aggrecan from the extracellular milieu or the indirect consequence of the presence of the intracellular, truncated aggrecan precursor, we tested whether adding an enriched preparation of brain aggrecan (B-aggrecan) [54,55] to the nm neuronal cultures could rescue the ability to form normal size aggregates. Indeed, the normal radial pattern of migration and aggregation was restored in nm cultures after addition of exogenous B-aggrecan (Figure 2). Quantitative analysis further established that treatment with B-aggrecan rescued the ability of the nm cultures to form aggregates both of normal size and number. Similar treatment of wt cultures slightly increased the number of aggregates but had little effect on aggregate size. Thus, modulation of neuronal aggregate formation mimicked that previously observed after chondroitinase ABC treatment, which removes CS chains, and after treatment with ß-xylosides, which prevent GAG-chain addition, confirming that the aggregate-promoting activity resides predominantly in aggrecan rather than any of the other CSPGs produced by CNS derivatives. Furthermore, the effect on neuronal aggregation appears to be specific to chondroitin-6-sulfate, as addition of free chondroitin-6-sulfate GAG chains restores the normal pattern of aggregation in nanomelic neuronal cultures. This result is particularly interesting in light of the fact that the main modification of the CS chains of brain aggrecan is 6-sulfation, and that in purified preparations of brain CSPGs, aggrecan is the main source of chondroitin-6-sulfate [103].

Interestingly, these findings are in contrast to those from the other brain CSPG knockouts, where ablation of expression of certain CSPGs, which presumably exhibit the earlier-mentioned inhibitory or regenerative properties, does not elicit corresponding developmental deficient phenotypes. Specifically,

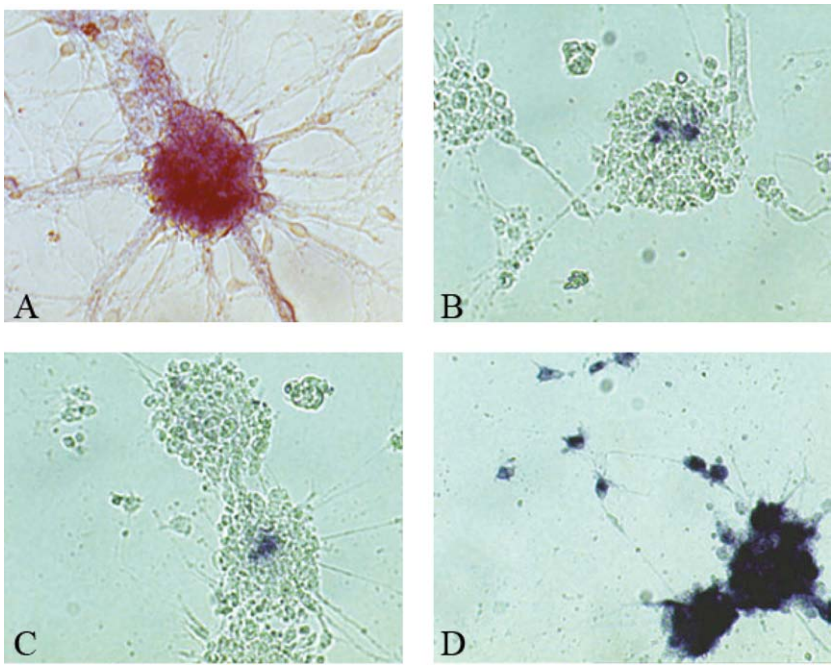

Figure 3. Detection of aggrecan in neuronal cultures. E8 wild type telencephalon neuronal cultures were probed by immunocytochemistry with the aggrecan-specific antibody S103L (A) or by mRNA *in situ* hybridization for expression of aggrecan (B), brevican (C) and neurocan (D) gene transcripts in comparable cultures [110].

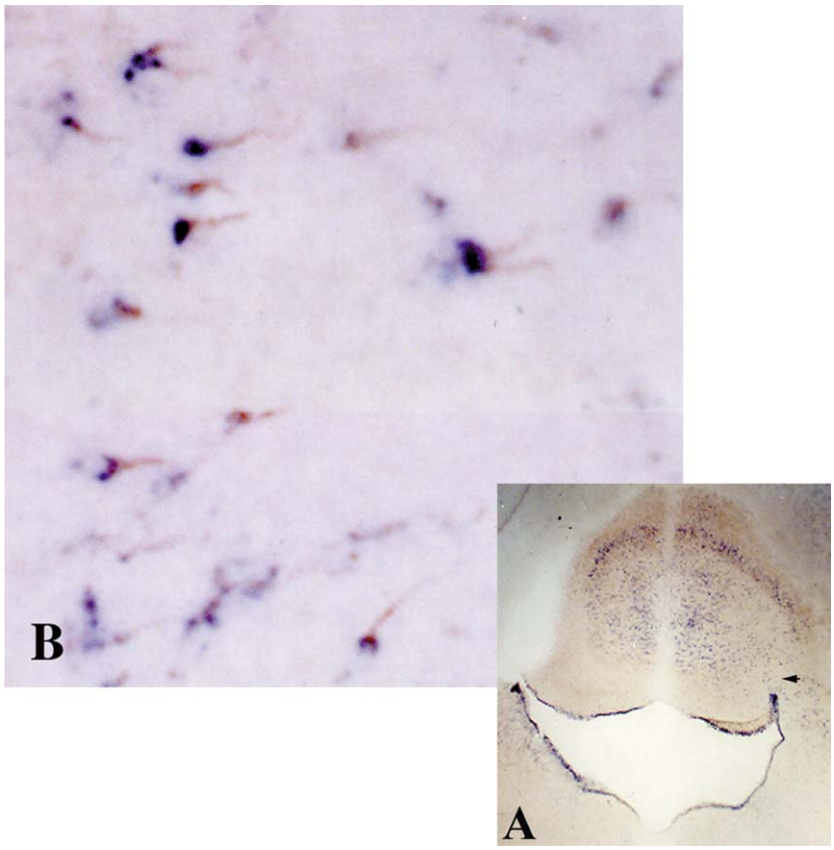

Figure 4. mRNA *in situ* hybridization of aggrecan (blue stain) and immuno-localization of aggrecan with S103L Mab (brown stain). Transverse section of E12 brainstem (A); an enlarged view of the area in A indicated by an arrow, showing expression in individual cells, is depicted in B.

knockouts of neurocan, predominantly a neuronal product, and brevican, mostly a glial product, suggest they have non-essential roles in development [116–118].

In sum, our studies have verified that deficiency of aggrecan results in aberrant neuronal aggregation in a culture system and that this abnormal behavior can be rescued by addition of exogenous B-aggrecan. While differential adhesive properties which influence morphogenetic events in the developing CNS have been shown previously, this is the first such role demonstrated for aggrecan. Moreover, since aggrecan is secreted it is highly likely its action may not be restricted to the cells which express it, but rather may be extended to surrounding cells, effecting either short-range cues in the ECM or long-range signalling in a more diffuse manner. Our studies indeed indicate both potent production of aggrecan by only a few cells (by *in situ* hybridization) and broad distribution of soluble aggrecan amongst all cells in the culture (by immunohistochemistry). Localization of aggrecan-expressing cells to the centers of aggregates suggests either an attractive role in gathering neurons into aggregates or an inhibitory role whereby it signals cells to stop migrating, fasciculate and send out processes. By either mechanism, aggrecan participates in induction of the structural and functional connectivity characteristic of vertebrate nervous system development.

Aggrecan expression *in vivo*

To explore the role aggrecan may be playing during normal CNS histogenesis, we have examined aggrecan expression *in vivo* and assessed the impact of lack of aggrecan during brain development using the aggrecan-deficient mutant models. As we previously showed by Northern and Western blot analyses, aggrecan expression is first detected in chick brain on embryonic day 7 (E7), increases from E8 to E13, then declines markedly after E16, and is not evident in hatchling brains [54,55,104]. The time course of aggrecan expression suggested that it might play a role in later development, possibly including gliogenesis (Figure 4). We therefore compared aggrecan gene expression with that of other extracellular matrix markers (tenascin-C, neurocan, brevican), glial markers (glutamine synthetase, GFAP, PLP) and neuronal markers (class III β-tubulin). Whole mount *in situ* hybridization detected aggrecan mRNA by E6 in the ventricular zone (VZ) of the ventral midbrain; by E7, hindbrain and forebrain were labeled and the location of aggrecan message in the ventricular layer was highly patterned; by E8 in the hindbrain, aggrecan expression progressed rostral to caudal through the VZ of the optic tectum and was observed in the VZ of the telencephalon and hindbrain in stripes of aggrecan-expressing cells extending deep into the pons; by E12, the whole VZ exhibited expression of aggrecan and streams of labeled cells were seen extending into the mantle layer of the tectum [119]. Indeed, aggrecan expression always preceded astrocyte differentiation as determined by GFAP expression, suggesting that aggrecan-positive cells migrate from the ventricle to the deepest layers of the tectum and then differentiate into GFAP-positive cells. Aggrecan was co-expressed with glutamine synthase mRNA during the period that coincides with the generation of glioblasts in the VZ, raising the possibility that aggrecan may be a product of early glial precursors *in vivo* [120] (Figure 4).

To determine whether other members of the aggrecan family can replace the function of aggrecan in the aggrecan-deficient nanomelic chick, studies on the expression patterns and message levels of brevican, neurocan and versican were carried out. Brevican is seen to be expressed in a pattern similar to that of aggrecan in normal E12 brains, in contrast to neurocan which was expressed in a pattern similar to β-tubulin, in agreement with earlier studies indicating neurocan is a neuronal product. In nanomelic chicks, the patterns of expression determined by *in situ* hybridization and the levels of expression determined by Northern analysis of neurocan, brevican, and versican mRNAs were not affected, indicating non-redundant roles for these members of the family [120].

Hints regarding the functional role of aggrecan are also forthcoming from its dynamic temporal/developmental regulation. Because the pattern of aggrecan mRNA expression *in vivo* was associated with putative glial precursors, we analyzed the expression of several glial precursor and mature glial cell marker mRNAs in the nanomelic brain at E12: proteolipid protein (PLP), myelin basic protein (MBP), glutamine synthase (GS), GLAST (glutamate transporter) and glial fibrillary acidic protein (GFAP). There was no detectable difference in the level or pattern of expression of any of these transcripts in the nanomelic mutant versus wild type at this stage. In contrast, when the expression analysis was continued in normal and nanomelic brain sections up to E18-E20, increased levels of expression of GS, GFAP and GLAST in nanomelic specimens were observed by *in situ* hybridization, while levels of expression of PLP and class III-β-tubulin remained unchanged. Furthermore, careful examination of the location of PLP-expressing cells in nanomelic brain sections showed a more restricted distribution of these cells in the laminated wall of the optic lobe and in the cerebellar folia compared to that in wild type sections [121]. In contrast, GFAP-expressing cells occurred with a broader distribution pattern in the cerebellar folia and an increased number of astrocytes was associated with the Purkinje cell layer in the mutant. Taken together, our observations *in vivo* suggest a function for aggrecan during gliogenesis that may be associated with the generation of glial precursors and/or their migration behavior.

Additionally, our results *in vivo* suggest that a radical change in the ECM of the VZ in particular is occurring during the period of gliogenesis and that such remodeling could be establishing a new environment which influences the fate and/or migration pattern of the cells being generated in these areas. Our results in the aggrecan-deficient mutant suggest a disruption in production of the normal cellular profile which leads to a phenotype that does not become apparent until later stages of development. Although it is clear that the timing of gliogenesis onset depends on the specific location of cells within the CNS and that the local

environment may influence glial precursor proliferation and/or migration, the mechanisms which underlie temporal and spatial regulation of gliogenesis are not defined. We hypothesize that the switch in cell type is associated with differential expression of specific adhesion or ECM molecules, most likely including aggrecan, which in turn is controlled by expression of transcription factors. Furthermore, our findings reveal complex regulation of gene expression in the ventricular layer during the period of gliogenesis and suggest that aggrecan may play an important role in late embryonic brain patterning events. Although how differential expression of aggrecan is regulated, either in a cell-specific or temporal manner, is not yet defined, it appears that both the temporally and spatially restricted aggrecan expression pattern and the glial lineage differentiation process are tightly and coordinately regulated.

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