Sulfated proteoglycans as modulators of neuronal migration and axonal decussation in the developing midbrain

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Abstract

Proteoglycans are abundant in the developing brain and there is much circumstantial evidence for their roles in directional neuronal movements such as cell body migration and axonal growth. We have developed an in vitro model of astrocyte cultures of the lateral and medial sectors of the embryonic mouse midbrain, that differ in their ability to support neuritic growth of young midbrain neurons, and we have searched for the role of interactive proteins and proteoglycans in this model. Neurite production in co-cultures reveals that, irrespective of the previous location of neurons in the midbrain, medial astrocytes exert an inhibitory or nonpermissive effect on neuritic growth that is correlated to a higher content of both heparan and chondroitin sulfates (HS and CS). Treatment of astrocytes with chondroitinase ABC revealed a growth-promoting effect of CS on lateral glia but treatment with exogenous CS-4 indicated a U-shaped dose-response curve for CS. In contrast, the growth-inhibitory action of medial astrocytes was reversed by exogenous CS-4. Treatment of astrocytes with heparitinase indicated that the growth-inhibitory action of medial astrocytes may depend heavily on HS by an as yet unknown mechanism. The results are discussed in terms of available knowledge on the binding of HS proteoglycans to interactive proteins, with emphasis on the importance of unraveling the physiological functions of glial glycoconjugates for a better understanding of neuron-glial interactions.

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• Astrocytes
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Introduction

Proteoglycans are polyanionic molecules consisting of core proteins and unbranched sugar polymers, glycosaminoglycans (GAGs), which are covalently attached to specific serine residues of the protein core side chains. GAGs are linear polysaccharides of 20-200 sugars in length, built by sequential addition of identical disaccharide units onto a characteristic linkage region (1). Three types of disaccharide may be used, giving rise to
three families of GAGs: the heparin/heparan family, the chondroitin/dermatan family, and the keratan family. The sugars of most GAGs are further modified, e.g., by O- or N-sulfation, and GAGs are subsequently referred to as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin, and heparan sulfate (HS). However, core proteins are not just scaffolds for GAGs but contain domains that have specific biological activities (2).

HS side chains are covalently attached to a protein core in HS proteoglycans (HSPGs) which may belong to one of three broad classes: i) those associated with the cell membrane which may have a glycosylphosphatidylinositol anchor such as the glypicans; ii) the transmembrane forms such as the syndecans; iii) those HSPGs that are secreted by cells and located in the extracellular matrix such as perlecan (2). HS chains can vary in length, epimerization of glucuronic acid to iduronic acid, overall sulfation of the chains, and position of sulfation of the monosaccharides.

Proteoglycans were originally detected in vertebrate tissues but GAGs have been identified in a wide variety of invertebrate species (for a review, see Ref. 3). The phylogenetic conservation of the physiological functions of proteoglycans has been underscored by recent work with mutants of the fruit fly Drosophila that established that proteoglycans, their associated GAGs and sulfation of heparan have a role in Wnt signaling and are required for normal development (4). However, since HS interacts with various molecules that are essential for early morphogenesis, Drosophila mutants (and transgenic mice) that lack proper HS biosynthesis show multiple developmental defects (5). Thus, it has been difficult to study the role of HS in late developmental processes such as the main directional movements of neurons, i.e., migration of the cell body and axonal elongation.

The midbrain is a structure characterized by a remarkable dependence on the Wnt-1 (int-1) proto-oncogene, being selectively ablated in mice homozygous for null alleles of Wnt-1 (6). Closure of the neural tube at the midbrain and forebrain level is prevented by heparitinase treatment at doses that do not impair closure at more caudal levels (7). The proteoglycans and/or their glycidic moieties - GAGs - involved in neuronal migration and in axon guidance or growth have not been well characterized in vivo although they are expected to be important for several reasons including the in vitro binding to GAG chains of proteins involved in these phenomena (8).

We review here some recent studies on the effects of HS of axonal and glial proteoglycans on the specific events of neuronal migration in the midbrain and neuritic growth and guidance at choice points such as the midbrain midline and analogous sites of the central nervous system (CNS).

Nervous tissue proteoglycans

Within vertebrate tissues, there are wide variations in the extracellular matrix, with that of the mature neural tissue being considered unique by the abundance of CS proteoglycans (CSPGs) and hyaluronan (9). Most proteoglycans that are constituents of the extracellular matrix in the mature brain are hyalectans (hyaluronic and lectin-binding proteoglycans) or lecticans and carry mainly CS side chains (CSPGs) (for a review, see Ref. 10). There are also some sequence similarities in the genes encoding hyalectans and CD44, a transmembrane glycoprotein that is the main cell surface receptor for hyaluronic acid and that carries a variable number of CS side chains. CD44 has 10 or more different isoforms, with variable abilities to bind hyaluronic acid or HS (11). Other secreted proteoglycans are the keratan-containing and keratan-devoid variants of the soluble form of RPTPδ phosphatase or phosphacan and its mouse homologue DSD-1 (12). In the developing mammalian brain, aggrecan fam-
ily proteoglycans, phosphacan/RPTPβ/ζ, and
neuroglycan C are the major classes of CSPGs
(13).

In recent years, a considerable advance
has been made with respect to HSPGs repre-
sented in the neural tissue and it is known
that several members of the syndecan and
glypican families of HSPGs appear in neu-
rons and glia (10,14,15).

Proteoglycans as part of scaffolds
in the developing brain

Cell migration is an important determi-
nant of brain structure. In vertebrates with
complex brains such as mammals, most, if
not all, neurons have to migrate from the
sites where they are born to places where
they terminally differentiate and integrate
into the brain circuitry. Radial migration of
young neurons from germinal regions lining
lateral ventricles to more superficial layers
has been recognized as a prerequisite for
proper morphogenesis and function of the
cerebral cortex (16) and could be the basis
for the inside-out sequence of formation of
the cortex and certain cortical-like structures
such as the midbrain superior colliculus (17).
Tangential migration of late-born neurons
has also been characterized in the cortex but
it is the prototypical tangential migration
that provides interneurons for the olfactory
bulb (18).

A rather complex pattern of migration
has been known to occur for the midbrain
dopaminergic neurons destined to form the
reticular formation, the substantia nigra pars
compacta and the midbrain ventral tegmen-
tal area (19). Such neurons migrate in two
phases, first following a ventral direction
from the ventricular surface along radial pro-
cesses of neuroepithelial cells and then later-
ally along tangentially arranged nerve fibers
(19,20). It is not yet clear whether migration
for the midbrain tectum follows rules similar
to those described for the midbrain tegmen-
tum although the morphology of a special-
ized set of radial glia, the median ventricular
formation (21), suggests that selected tectal
populations may follow a sequence of radial
and tangential migration.

Recent work has suggested that the HSPG
N-syndecan is involved in the tangential mi-
gration of luteinizing hormone-releasing hor-
monc neurons from the olfactory placode to
the hypothalamus (22). To our knowledge,
there is no indication of a role of HSPGs in
the complex pattern of neuronal migration in
the midbrain tegmentum. However, there is
circumstantial evidence for a role of a CSPG.

Kawano and co-workers (20) first showed
that the extracellular matrix protein tenascin
is transiently expressed on radial processes
of neuroepithelial cells coincident with the
radial migration of dopaminergic neurons
and that the adhesion molecule L1 was tran-
siently expressed on tangentially arranged
axons in the ventral midbrain coincident with
the lateral migration of dopaminergic neu-
rons. Interestingly, immunoreactivity for the
6B4 proteoglycan (phosphacan), but not
neurocan, was present on the cell surfaces of
migratory dopaminergic neurons from the
time when they were ready to attach to the
L1-positive tangentially oriented fibers.
These results led Kawano and collaborators
(20) to suggest that heterophilic interactions
between phosphacan on dopaminergic neu-
rons and L1 (23) on tangentially arranged
fibers mediate the lateral migration of dopa-
minergic neurons. However, there was no
indication whether the GAG moiety or a
peptide domain of the core protein is in-
volved in these interactions and the direc-
tional movement they promote.

CSPG and HSPG modulation
of neurite growth

The notion that proteoglycans or, more
precisely, CSPGs, may contribute to a bar-
rier function for axons crossing the CNS
midline can be dated to the seminal work of
Snow et al. (24), detailing the developmental
cytological changes in the cord roof plate in rat embryos, a site avoided by dorsal root ganglion axons (25), and reporting on the presence of a KS-like immunoreactivity in the roof plate. The same epitope disappears from the dorsal midline of the spinal cord at the time of formation of the dorsal commissure (24,25) and is detected in the dorsal midline of the optic tectum in neonate hamsters during the growth of retinal axons (26) that must be prevented from crossing the midline. In a subsequent analysis employing nitrocellulose-coated culture dishes as a substrate for the attachment of laminin (or NCAM) and proteoglycans of interest, the behavior of the main target population - dorsal root ganglion neurons - in explant cultures was evaluated and showed a general inhibitory action of KS/CSPGs, DSPG and, in a much smaller measure, of a chondrosarcoma tumor cell CSPG devoid of KS and predominantly 4-sulfated (27). It is interesting to note that Dou and Levine (28) subsequently showed that chondroitin 4-sulfate (CS-4), CS-6 and KS inhibit neurite outgrowth from both cerebellar and dorsal root ganglion neurons on laminin-coated surfaces, whereas DS and HS had no effect on these neurons under similar conditions.

Additional work by Snow and co-workers (29) showed that soluble CSPGs (versican-like, aggregan-like, etc.) at different concentrations inhibited neurite outgrowth from dorsal root ganglion neurons and decreased the rate of neurite elongation on a fibronectin substrate but had little or no effect on neurite initiation or outgrowth on a laminin substrate. However, not all neurite inhibitory effects of CSPGs should be attributed to CS. For instance, CS chains of CSPG-enriched fractions such as brevican and versican V2-enriched fractions from bovine myelin contribute only to a minor extent to the inhibition of neurite growth of cerebellar neurons and the major activity seems to reside in the protein cores (30).

Unilateral application of a mixture of soluble CS-4 and CS-6 to the exposed brain of stage 28 *Xenopus* embryos caused navigation errors of a subpopulation of forebrain axons within the tract of the postoptic commissure (TPOC) (31). Instead of crossing the ventral midline in the preexistent ventral commissure to join the contralateral TPOC, this axonal subpopulation either continued growing longitudinally into the ventral longitudinal tract or, less often, prematurely exited the ipsilateral TPOC and extended dorsally into the diencephalon or midbrain. It was not determined whether navigational errors were restricted to a subpopulation expressing a novel glycoform of the neural cell adhesion molecule, but it was clear that axon elongation was neither inhibited nor promoted. Furthermore, immunostaining revealed that native proteoglycans containing CS-4 or CS-6 were widely expressed in the *Xenopus* brain and neither delineated channels, pathways, or specific choice points. For this and other reasons, these investigators proposed that exogenous CS affected axon guidance by competing for a bound molecule, possibly netrin 1, from native CSPGs.

It should also be mentioned, in passing, that axonal response to CS-4 and CS-6 might differ in some commissural systems (32). Within the developing rat anterior commissure, CS-4 was localized on the pathway of growing, neurofilament-reactive axons while the course of tightly fasciculated axons was totally devoid of CS-6 which was, however, present in the surrounding regions.

An analysis of axon routing in the embryonic mouse optic chiasm after enzymatic removal of CS from living slices showed several age-related errors. At an early stage there was crossing of the midline at aberrant positions including formation of a retinoretinal pathway, at an intermediate stage there was abolition of the transient ipsilateral projection from the central retina, and at a late stage there was marked reduction of the ipsilateral projection from the temporal retina.
Sulfated proteoglycans in axonal growth in the midbrain

At any stage, there were increases in the area and complexity of growth cones both at premidline and postmidline sites. It remains to be determined how the CS moieties of proteoglycans modulate axon routing in the optic chiasm.

In view of the experimental evidence for a midline barrier to developing retinal axons at the midbrain tectum of mammals (21,34), a simplified system was developed by our groups (35,36) to test the behavior of embryonic midbrain neurons on astroglial substrates derived from medial and lateral sectors of the embryonic (E-14) midbrain. Furthermore, correlations were sought between the outgrowth-inhibitory (medial astrocytes) and outgrowth-promoter (lateral astrocytes) activities of these relatively immature glial cells and their content of sulfated GAGs (37) and revealed that midbrain medial astrocytes synthesize about 1.65 times more (extracellular + pericellular) CS per unit time than lateral astrocytes. A similar ratio (medial/lateral) was also found for the entire complement of sulfated GAGs in comparisons of the whole tectal midbrain tissue of neonate hamsters (38). These investigators also demonstrated that proteoglycan cores are expressed at similar relative levels in the midline and lateral tectum including those with molecular masses similar to those of neurocan, considered to be responsible for the inhibitory activities of reactive astrocytes (39).

Attempts to determine whether the higher content of CS in midbrain medial astrocytes was responsible for their outgrowth-inhibitory properties did not confirm this hypothesis (40). Neurons growing on medial glia previously treated with chondroitinase ABC underwent only a very minor increase in the proportion of cells bearing neurites and in the median total length of these processes, although there was a significant increase in the length of their largest set of neurites (95th percentile). Interestingly, the effect of chondroitinase ABC on lateral glia was the opposite of that on medial glia, causing a non-negligible decrease in the total length of neurites grown on the treated lateral substrate (Figure 1). It should be noted that addition of chondroitinase ABC had no effect on the length of neurites growing on a noncellular laminin substrate, thus reinforcing the notion that effects are exerted through glial proteoglycans.

More recent work aiming to clarify the issue of the effect of CS/CSPGs on neurite length have revealed a rather complex picture. Treatment of glial cultures with xyloside, that impairs the synthesis of CSPGs and causes pronounced release of soluble CS, revealed an enormous capacity of medial glia to secrete CS (41). Both treatment with xyloside and addition of exogenous CS-4, the major chondroitin form in midbrain glia (41), showed concentration-dependent, regionally specific effects (42). Thus, treatment of lateral astrocytes with xylosides resulted in a minor increase in the length of neurites while addition to the same glial type of CS-4 resulted in a U-shaped curve with a significant decrease of neurite length at intermediate concentrations (about 2.5 nM; Figure 2) (42).

Figure 1. Effect of glycosaminoglycan lyase treatment on total length of neurites cultured on lateral (L) or medial (M) astrocytes (see also Figure 3). A, Control co-culture (L) and co-cultures treated with chondroitinase ABC (L + chase ABC) or heparitinase 1 (L + hept). B, Control co-culture (M) and co-cultures treated with chondroitinase ABC (M + chase ABC) or heparitinase 1 (M + hept). Box-and-whisker plots show the total length of neurites. The boxes enclose the 25th and 75th percentiles and are bisected by the median; whiskers indicate the 5th and 95th percentiles, i.e., conservative estimates for the shortest and the longest set of neurites. L + chase ABC and M + hept are significantly different from the respective controls L (P < 0.05) and M (P < 0.0001). Modified from Ref. 40, courtesy of Wiley-Liss.
It is possible that both the enzymatic treatment and the addition of excess exogenous soluble CS disrupt CS-mediated binding of molecules that need not themselves be proteoglycans, with regionally specific effects as observed in the developing cortex (43). At present, there are no clues about the identity of such presumptive molecules which should be different in lateral and medial cultures. Attempts to determine differences in the complement of proteins considered to be responsible for growth-inhibitory activities of certain astrocytes such as tenascin-C (44) revealed no excess of this protein in medial astrocytes with respect to the outgrowth-promoting lateral astrocytes (40).

It has been occasionally argued that the true effects of astrocytic CSPGs could not possibly be ascertained in conventional cell cultures in which the extracellular CSPGs would not be retained on the cell surface and would be greatly diluted by the medium (39). The interpretation is that in three-dimensional cultures, as well as in the diminutive extracellular space of the mature brain, CSPGs would be trapped between the cells, where they would be in a position to interfere with access to neurite outgrowth-promoting molecules in astrocytes.

The argument of CSPG trapping as the true environment may be applicable to the mature brain but not necessarily to the large extracellular spaces of the developing CNS tissue. Furthermore, the different effects of chondroitinase ABC on the growth-modulating activities of lateral and medial glia indicate a heterogeneous distribution of CSPGs themselves in our system and/or of attractive or repelling proteins to which they attach. Again, it is interesting to point out that the DSD-1 proteoglycan (the mouse homologue of phosphacan) displays opposite effects on neurite outgrowth dependent on neuronal lineage, showing a CS/DS-dependent growth-promoting effect on neurites of E-14 midbrain and E-18 hippocampal neurons and a growth-inhibitory effect on embryonic dorsal root ganglion neurons (12).

In contrast to CSPGs, little information is available in the older literature about a role of HSPGs in axon growth and guidance at the midline of the CNS. Both the older and the current literature have emphasized the idea that HSPGs play an invariant supportive role in axon growth (for a review, see Ref. 45), in spite of conflicting evidence about some HSPGs as substrates in vitro (46). This uniform viewpoint is surprising if compared to the conflicting concepts of the role played by HS in other events such as tumor growth and metastasis (47). As already emphasized by Turnbull and co-workers (48), there has been an increasing realization that specific sequences in the HS chains are designed for selective interactions with many proteins and that HS functions as a new class of multifunctional cell regulator.

Although most of the early in vitro studies reported an outgrowth-promoting activity of HSPGs (44), there were examples in which HS-binding proteins were viewed as antagonistic to neurite extension by Tobey et
al. in 1985 (49). Ten years after that study, this dependence on the substrate was persuasively demonstrated by Dou and Levine (28) who showed that the percentage of cerebellar neurons with neurites and the length of these neurites on a substratum of L1 + HS (10 µg/ml) was half of the respective values for the same neurons on an L1 (only) substratum. For comparison, similar values for CS-6 were obtained only after adding this GAG at 100-fold concentration (L1 + CS-6, 1 mg/ml).

The relevance of HS to the modulation of axon growth and guidance at the midline may be much higher than usually believed. Heparan is expressed by SSEA-1 hypothalamic neurons in the mouse and may be involved in changes in age-related fiber order in the optic chiasm and chiasm/optic tract transition (50). Furthermore, very consistent findings on the relations of HSPGs to midline crossing have been found in the exposed live brain of Xenopus embryos. Administration of exogenous heparin or perlecan-FGF-2 to the optic tract of stage 37-40 embryos caused advanced optic axons to cross the dorsal midline via the posterior commissure (51) in addition to other navigational errors of bypassing of the optic tectum, with fibers either growing around the anterior or anterior/ventral tectal margins. Heparitinase treatment and removal of axonal HS at these late stages also caused errors in axon guidance including erroneous crossing of the midline. Actual reduction of axonal growth was observed only in early embryos (stages 32-37) in which heparitinase caused a “shortening” of the optic tract that could be reversed by the addition of FGF-2 but without correction of the navigational errors (51).

In our work on the compartmental distributions of GAGs in medial and lateral midbrain glia, we observed, but did not emphasize, that sulfated HS (or, at least, its extent of sulfation) was about 1.5 higher than sulfated CS in both cultures and that sulfated HS in medial astrocytes was 3.25-fold higher than in lateral astrocytes (37). Interestingly, the percentage of the lane’s radioactivity of radiiodinated core proteins was about 4-fold higher for CSPGs than HSPGs in neonatal hamster optic tectum, with the latter being apparently due to syndecan-3 and glypican-1 and to very low levels of a soluble HSPG (38).

Treatment of medial glial cultures with heparitinase before and during co-culturing caused changes in the cell surface (52) and a dramatic reversal of medial glia outgrowth-inhibitory capacity (40). The proportion of midbrain neurons cultured onto heparitinase-treated medial astrocytes that produced two or more neurites rose from about 40% to more than 80% and the median value of their total length increased more than 1.5-fold. A dramatic change also occurred in their maximal total length that became more than twice

Figure 3. Photomicrographs showing predominant morphologies of MAP2-stained E-14 midbrain neurons after culturing for 24 h onto lateral (A, B) and medial (C, D) astrocytes. A and C, Neurons grown onto a substrate of lateral (A) or medial astrocytes (C) without enzymatic treatment; B and D, Neurons in co-cultures treated with heparitinase 1 (hept). Notice that hept treatment reverses the inhibitory properties of medial astrocytes (D) as also shown by β3 tubulin staining (cf. Figure 5 in Ref. 40). Scale bar: 50 µm. L = lateral; M = medial. Reproduced from Ref. 36, courtesy of Academia Brasileira de Ciências.
that of control neurons (Figures 1 and 3) grown on untreated medial cultures. There were also changes in co-cultures including lateral glia but they appeared to be minor in comparison to those occurring in neurons cultured onto medial glia. It is interesting to note that more recent work has shown that heparitinase treatment of striatal live sections causes them to lose their permissivity for attachment of midbrain explants in culture, whereas treatment of cortical sections attenuates their repulsive properties (53).

The main questions arising from the dramatic changes occurring in medial glia co-cultures is which glial HSPG candidates are and which hypothetical mechanisms could be involved in causing inhibition of neurite growth.

At the present time, it is known that astrocytes express glypican-1 (14), which is shed from the cell surface (15) and, particularly, syndecan-2 and -4 (14), which may also be shed (54). A possible explanation for the nonpermissiveness of medial glia for neurite growth is that this cell type is the source (a) of a soluble protein with axon-repulsive activity, which is able to bind a nervous tissue HSPG (b) of (preferentially) soluble or shed HSPG. A possible candidate for the repulsive protein is the slit protein, which is expressed by midline glial specializations such as the roof and floor plates, and the chiasmatic glial palisade (55,56) and, possibly, by the presumptive derivatives of the midbrain primitive midline glia (21). Slit protein is able to bind glypican-1 (8) and shows HS dependence on its collapsing activity for olfactory axons in explant cultures (57). An analogous mechanism may underlie the properties of medial astrocytes but this putative mechanism remains to be tested.

Significant new work has been done in the identification of the structural characteristics of HS that induce aberrant axon targeting such as the erroneous crossing in the Xenopus dorsal diencephalon. This bypass-inducing activity has been correlated to distinct structures, particularly those containing a combination of 2-0- and 6-0-sulfate groups (58) and seems to be unrelated to N-sulfation, which is essential for FGF-2 signaling (59). Thus, it has been hypothesized that 6-0-sulfated HS in the dorsal diencephalon provides a crucial component of a repulsive signal that is essential for correct axonal guidance in Xenopus. It remains to be demonstrated whether a homologous component is responsible for the contribution of HS to the nonpermissive role of the midline glia in the growth of midbrain neurites in a mammal.

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