Differential effects of glycosaminoglycans on neurite outgrowth from hippocampal and thalamic neurones

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SUMMARY

Chondroitin sulphate proteoglycans are expressed in a temporally restricted pattern from embryonic day 17 to postnatal day 0 in both the thalamus and the cortical subplate, to which thalamic neurones transiently project. To study whether chondroitin sulphate proteoglycans could be specifically involved in the modulation of thalamic axon outgrowth, we compared neurite outgrowth from cultured rat embryonic hippocampal and thalamic neurones, in the presence of chondroitin sulphate type C (isolated from shark cartilage) and chondroitin sulphate type B (dermatan sulphate; isolated from bovine mucosa). When added to the culture medium, both types of glycosaminoglycan lowered the adhesion to laminin and polylysine of both hippocampal and thalamic neurones. However, only chondroitin sulphate specifically modified the pattern of thalamic but not hippocampal neurone

outgrowth, promoting axon growth. The morphological changes induced by chondroitin sulphate were concentration dependent and correlated with the selective binding of chondroitin sulphate to the neuronal plasma membrane and its subsequent internalisation. Chondroitin sulphate loosely bound to the surface of hippocampal neurones, but was not internalised. These results indicate that proteoglycans, and in particular the glycosaminoglycan component of these molecules, can differentially modulate neurite outgrowth, depending on their biochemical composition and on the type of neurones they bind to; this would be a possible mechanism of controlling axon guidance *in vivo*.

Key words: development, neuritogenesis, neuron polarity, proteoglycan, cell adhesion

INTRODUCTION

The mechanisms that control the establishment of neuronal connections are most crucial to neural development. To reach their intended targets, axons depend on a complex pattern of guidance cues present in their local environment (Dodd and Jessell, 1988). Among these cues are: soluble chemoattractants, released by intermediate or final targets; growth promoting molecules, present either on the surface of neuronal and non-neuronal cells or localised in the extracellular matrix; and repulsive molecules, which may block axon extension, probably by contact inhibition of growth cone movements (Grumet, 1991; Tessier-Lavigne and Placzek, 1991; Reichardt and Tomaselli, 1991; Schawb et al., 1993). Recent *in vitro* studies have shown that there also exist molecules that can exert both growth promoting and inhibiting activities, depending on their exact molecular composition and how they are presented to the neurones. An example of such molecules is, for instance, J1/tenascin, which promoted axon extension as a substratum-bound molecule, but inhibited outgrowth as a soluble molecule (Lochter et al., 1991).

Sulphated proteoglycans (PG) are another class of molecules that appear to modulate neurite outgrowth in both a positive and a negative way. Heparan sulphate PGs, associated with laminin, have been reported both to promote neurite outgrowth

and to inhibit it (Lander et al., 1983; Sandrock et al., 1987; Muir et al., 1989). Heparan sulphate (HS) and dermatan sulphate (DS), appear to modulate neuronal polarity *in vitro*, favouring axon and dendrite growth, respectively (Lafont et al., 1992). Similar molecules are present on the surface of astrocytes *in vivo* and chondroitin sulphate proteoglycans (CSPG) are present in axon growth-permissive regions (Ard and Bunge, 1988; Sheppard et al., 1991). However, the same type of molecules, chondroitin (CS) and keratan sulphate (KS) proteoglycans, also inhibit axon extension *in vitro* and have been detected in regions consistently avoided by axons during development and regeneration (Snow et al., 1990a, 1991; Oakely and Tosney, 1990; Fichard et al., 1991; Britts et al., 1992; Cole and McCabe, 1991; Bovolenta et al., 1992, 1993). Many of these studies also suggest that the glycosaminoglycan (GAG) parts of the molecule are responsible for the effect on neurones (Snow et al., 1990b; Cole and McCabe, 1991; Lafont et al., 1992; Bovolenta et al., 1993), while the protein moiety has been shown to participate in the activity only in one case (Oohira et al., 1991).

PGs are extremely heterogeneous; several different core proteins, carrying various amounts of one or more types of GAG attached, contribute to determine the structure of these molecules (Gallagher, 1989; Kjellén and Lindahl, 1991). In addition, each GAG class may vary both in molecular mass

and in the number of sulphate residues per disaccharide unit. This extreme variability of the PG molecules might be an explanation for both the wide variety of PG functions and their apparent conflicting effects on neurite outgrowth (Wight et al., 1992). Even further, this high polymorphism and the presence of different PGs in the developing brain (Margolis and Margolis, 1989; Herndon and Lander, 1990) suggest that these macromolecules could be specific guidance cues for the growth of axon subsets.

We began testing this hypothesis by studying the distribution in the developing rat brain of CSPGs, using monoclonal antibodies against chondroitin 4-sulphate (C4S) and chondroitin 6-sulphate (C6S). Because C6S, but not C4S, showed a restricted spatiotemporal distribution both in the thalamus and in the cortical subplate, to which thalamic neurones transiently project, we questioned whether C6S could specifically modulate outgrowth of thalamic axons. To this end, we compared the *in vitro* effect on neurite outgrowth from thalamic versus hippocampal neurones, of chondroitin sulphate C and chondroitin sulphate type B (dermatan sulphate), two closely related GAGs. Both GAGs are composed of disaccharide repeating units of galactosamine and glucuronic acid with the difference that, in some cases, glucuronic acid epimerized to iduronic acid in DS. Because of the similarity in their biochemical composition, DS appeared to be a good control to use in the study of the specificity of CS effects. Furthermore, because chondroitin sulphate C contains the sulphate predominantly in the 6 position and dermatan sulphate only in the 4 position (Margolis and Margolis, 1989), these molecules may also reflect the distribution of C6S and C4S epitopes in the brain. We found that high concentrations of both DS and CS lower cell adhesion and inhibit neurite extension of both types of neurones. However, lower concentrations of CS but not DS, promoted axon extension from thalamic neurones only, an effect that correlated with binding and internalisation of CS.

MATERIALS AND METHODS

Cell culture

Neuronal cultures were set up on 10 mm glass coverslips, placed into 4-well Petri dishes (Greiner). The coverslips were precoated with polylysine (10 µg/ml) for 30–45 minutes at room temperature, and washed three times in distilled water. One half of the coverslips was incubated further with laminin (2–10 µg/ml, Gibco) at 37°C, for 90 minutes. Before cell plating, coverslips were rinsed in Hanks' balanced salt solution (Gibco) at 37°C, for 10–15 minutes.

Thalamic and hippocampal cultures were prepared from E15 and E17.5–18 Wistar rat embryos, respectively. After dissection, the tissue was digested in a mixture of trypsin (0.7 mg/ml), bovine serum albumin (3 mg/ml) and DNase (100 mg/ml), in Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution, for 15 minutes, at 37°C. After the addition of soybean trypsin inhibitor (500 µg/ml, Sigma Chemical Co., St Louis, MO), the tissue was mechanically dissociated in the same digestion mixture. Cells were recovered by centrifugation at 200 g and resuspended in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and F12 medium (DMEM/F12, Gibco), containing 10% foetal calf serum (FCS, Gibco). Hippocampal and thalamic neurones were plated at the density of 7,500 and 10,000 cells per well, respectively, in a volume of 100 µl of DMEM/F12 with 10% serum, and placed in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. One or two hours after plating, 2 ml of serum-free DMEM/F12

containing N2 additives (Bottstein and Sato, 1979) and either bovine mucosa chondroitin sulphate type B (dermatan sulphate, DS) or shark cartilage chondroitin sulphate C (CS) (Sigma) were added and the plates replaced in the incubator.

Sixteen to 20 hours after the medium was added to the plates, the cells were fixed in a mixture of 100 mM cacodylate (Sigma), 100 mM sucrose and 0.25% glutaraldehyde (Merck) and rinsed three times in phosphate buffered saline solution, pH 7.2 (PBS). The glass coverslips were mounted over a slide with glycerol/gelatine, and analysed as indicated below.

Quantification of cell adhesion and neurite outgrowth

The effect of the two different GAGs was quantified in at least four independent experiments performed in duplicate. For the conditions tested in each experiment, the following parameters were measured in five separate fields (48.5×10⁴ µm²) using an Argus-10 Image Processor (Hamamatsu) coupled to an inverted microscope (Nikon, Diaphot): (1) total number of cells, *N*; (2) percentage of cells exhibiting at least one process over the total number of cells in the field, % *P*; (3) percentage of the total number of cells exhibiting only one process, either short or long, % *P*₁; (4) percentage of cells with two or three processes, % *P*₂₋₃; (5) percentage of cells with more than three processes, % *P*_{>3}; (6) percentage of cells with processes shorter than twice the body diameter, with respect to the cells with processes, % *SP*; and (7) percentage of cells with at least one process longer than twice the body diameter, with respect to the cells with processes, % *LP*. Neurones were distributed in morphological categories, according to these parameters. The effect of CS and DS on neuritic length was also quantified in the same preparation as follow. The length of all the neurites of each neurone present in five separate fields (48.5×10⁴ µm²) was measured with the Argus-10 Image Processor. The neuritic lengths were classified in four different classes (<21 µm; 21–50 µm; 50–100 µm; >100 µm). The results were represented as the percentage of neurites falling in a particular class over the total number of neurones with processes (neurite length frequency).

Immunohistochemistry and culture immunocytochemistry

Rats of ages ranging from embryonic day 15 (E15) to postnatal day 0 (P0, the day of birth) were decapitated and their heads fixed by immersion in 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.2 for 3 hours, followed by 30% sucrose, 24 hours. Cryostat sections (sagittal and coronal), 12–14 µm thick, were cut and collected on gelatine-coated slides for immunohistochemistry.

Sections were first digested with chondroitinase ABC (0.5 unit/ml, 37°C, 3 hours) and then incubated overnight at 4°C with monoclonal antibodies against the 4-sulphate (clone 2B6) and the 6-sulphate (clone 3B3) stubs of dermatan and chondroitin sulphate proteoglycans, respectively (ICN Biomedicals). FITC (fluorescein isothiocyanate)-conjugated secondary antibodies (Jackson ImmunoResearch) were used to localise the primary antibodies. Sections were mounted with glycerol/gelatine (Sigma) for examination.

Neuronal cultures, established as described above, were grown for 1–5 days in the continuous presence of CS and DS. After fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, the cells were rinsed in PBS and incubated for 30 minutes at room temperature in 0.2 M glycine in PBS containing 5% normal goat serum. Neurones were further incubated for 1 hour, at 37°C, with a solution in PBS, containing 0.05% Triton X-100, of a Pan axonal marker directed against the highly phosphorylated isoform of neurofilament H (Affiniti Research Products Limited, UK). After 3 rinses in PBS, the cells were incubated for 45 minutes at 37°C with a fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). After several rinses in PBS, plates were further incubated for 1 hour, at 37°C, with a solution in PBS of the 3A10 monoclonal antibody that selectively recognises neurones, to visualise the cell body and all the neurites (Furley et al., 1990). The presence of this monoclonal antibody was revealed with a secondary antibody conjugated with

Texas Red (Jackson ImmunoResearch). Cells were rinsed, mounted on a slide with glycerol/glycine solution and the preparations viewed and photographed with a Labophot microscope, equipped for epifluorescence (Nikon).

CS and DS biotinylation

GAGs were biotinylated with biotin hydrazide (Pierce, Rockford, IL), following the manufacturer's procedure. Free biotin hydrazide was removed with several washes and subsequent centrifugations at 1500 *g* for 20 minutes, using a Centricon-30 Microconcentrator (Amicon Corporation, Danvers, MA). Labelling of both CS and DS was checked by blotting the biotinylated GAGs. The blots were incubated with peroxidase-conjugated streptavidin and revealed with DAB (0.5 mg/ml) and H₂O₂ (0.003%).

Biotinylated CS and DS were used to determine their prospective binding sites on hippocampal and thalamic neurones. Cultures were established as described above and biotinylated GAGs were added to the culture medium at a concentration of 10 µg/ml. Thirty minutes to 20 hours later, the cultures were fixed in 2% paraformaldehyde solution in 0.1 M phosphate buffer containing 0.5% *N*-cetylpyridinium (Merck) for 10 minutes, rinsed with PBS, incubated with peroxidase-conjugated streptavidin and developed with DAB (0.5%) and H₂O₂ (0.003%). Photographs were taken with an Axiophot (Zeiss), equipped for Nomarski optics.

Preparations for confocal scanning laser microscopy (Laser Scan Microscope, Zeiss) were incubated with fluorescein/streptavidin, washed in PBS and mounted in glycerol/gelatine. Preparations were excited with an Argon laser at 488 nm with optical sections of 1 µm.

Binding of GAG to cells and to substratum

Bovine mucosa dermatan sulphate and shark cartilage chondroitin sulphate C were not totally devoid of core protein, allowing the iodination of the molecules with chloramine-T (Hunter, 1978). Labelled GAGs were separated from free iodine by gel permeation chromatography on a G-25-desalting column (Pharmacia). The GAG concentration was adjusted to 100 µg/ml and the resulting specific activity was 8.75×10⁵ cpm/mg for CS and 1.5×10⁶ cpm/mg for DS.

Glass coverslips, prepared as described above, were incubated with ¹²⁵I-labelled CS or DS in PBS (50 µl, 2–10 µg/ml) for 30, 90 or 180 minutes. After several washes with PBS, the coverslips were placed in separate tubes and counted (1282 Compugamma Counter, LKB, Sweden). The amount of bound GAGs was expressed as ng/mm².

Acutely dissociated thalamic (1×10⁶) and hippocampal (5×10⁵) cells were incubated in 3 ml of DMEM/F12 medium containing ¹²⁵I-labelled CS or DS (10 µg/ml). After 5, 15, 60 and 120 minutes of incubation at 4°C, cells were collected, washed by centrifugation at 200 *g* for 6 minutes in PBS, and counted in the γ-counter. The amount of bound GAGs was calculated as ng per 10⁶ cells.

Subcellular fractionation of hippocampal and thalamic cultures

Hippocampal and thalamic neurones, prepared as described above, were plated on 60 mm PLL-coated Petri dishes at a density of 5×10⁶ cells/dish. Biotinylated CS and DS were added to the chemically defined culture medium 2 hours later and cultures were grown for 24 hours. Cells were collected then with a cell scraper, recovered by centrifugation at 200 *g* and the supernatant was immediately discarded. Cells were lysed in 10 mM Tris, 0.25 mM sucrose, containing DNase (1 µg/ml), aprotinin (1 µg/ml), PMSF (1 mM) and leupeptin (10 µg/ml), using two 30 second bursts of an Ultra-Turrax T 25 homogeniser (Janke and Kunkel Co., Germany). Centrifugation at 1000 *g* for 15 minutes separated a nuclear pellet and a supernatant containing the rest of the membranes. Endoplasmic reticulum, lysosomes, Golgi and plasma membranes were separated on a 20% Percoll gradient in 10 mM Tris, containing 0.25 mM sucrose, as described by Morand and Kent (1986). Sixteen fractions of 625 µl

were collected, starting from the bottom of the centrifuge tube, and tested for biotinylated CS or DS by 'dot-blot', revealed with avidin-peroxidase (BioMakor, Rehovot, Israel).

RESULTS

Transient expression of CSPGs in embryonic rat brain

Monoclonal antibodies against C6S or C4S showed that PG of these types were present in several defined regions of the developing rat brain embryo. The pattern detected by anti-C6S was strikingly spatially and temporally restricted to two regions of the brain, the thalamus and the cortex. In the thalamus, C6S was first expressed at E16 and reached its maximal expression by E17 in the dorsolateral aspect of this region (Fig. 1A). In the cortex, low C6S levels were first detected around E14 in both the ventricular zone and the pial surface. By E17, C6S immunoreactivity was strongly localised in the subplate region (Fig. 1B), as previously described in mice (Sheppard et al., 1991). At birth (P0), C6S was no longer detectable in either the subplate or the thalamus, where labelling was confined to the blood vessels (Fig. 1C,D). This pattern of staining was not observed with the antibody against C4S, which instead stained other brain regions (unpublished observations). The specific and transient expression of C6S epitopes correlates with the ingrowth of thalamic axons through the subplate, before entering their final targets, which are mainly in layer 4 of the cortex (Ghosh and Shatz, 1992, 1993). These observations raised the possibility that CSPGs, particularly their GAG portion, could specifically modulate outgrowth of thalamic processes. To test this hypothesis, we compared the effect of chondroitin sulphate type C (CS) and chondroitin sulphate type B (dermatan sulphate, DS), two closely related GAGs, on the cell adhesion and neurite outgrowth of thalamic neurones. These effects were further compared with those on hippocampal neurones, used as control. Because CS contains the sulphate in the 6 position and DS in the 4 position, these molecules appeared to be good candidates to reflect the distribution of C6S and C4S epitopes in the brain.

Proteoglycan effect on cell adhesion

Bovine mucosa DS and shark cartilage CS were added to the culture medium of hippocampal or thalamic neurones 1 hour after they were seeded on either polylysine or laminin, and their effect was examined 2, 6 and 20 hours after addition of GAGs. Both CS and DS generally lowered adhesion of hippocampal and thalamic neurones to both culture substrata, independently of the time of culture. About 35% fewer neurones remained attached to the substratum in the presence of GAGs, independently of the concentration of these molecules in the culture medium, at least in the range tested (10 ng/ml to 100 µg/ml; Table 1). These GAGs appeared to have a general effect on the adhesion of cells of neuronal lineage, since they also lowered the adhesion to laminin of the pheocromocytoma PC12 or the neuroblastoma Neuro2A (Table 1). In this latter case, however, the effect was more pronounced, since only 50% of the cells attached to laminin in the presence of CS or DS, in comparison with untreated cultures.

Fig. 1. Immunohistochemical detection of C6S. Coronal sections of E17 (A,B) and P0 (C,D) rat cortex (B,D) and thalamus (A,C), stained with antibody 3B3 against C6S. (A) CS staining in embryonic thalamus was punctate and diffuse in the ventrolateral (VL) portion, but very intense in the dorsolateral (DL) aspect. (B) Staining in the embryonic cortex was intense in the subplate (SP), and very diffuse in the cortical plate (CP) and intermediate cortical layer (ICL). (C) Immunoreactivity was lost in P0, where only blood vessels stained. (D) Staining was no longer detectable in P0. Bar, 50 µm.

CS enhanced axon outgrowth from thalamic neurones

Neurite outgrowth from thalamic neurones could be specifically enhanced by CS, as shown by comparing the neurite outgrowth effects of CS and DS on cultured thalamic and hippocampal neurones (Fig. 2). Neuronal cultures were estab-

lished on polylysine alone or on polylysine/laminin, in the presence of chemically defined culture medium. Under these conditions, more than half of both thalamic and hippocampal neurones extended neurites ($63.7\pm2.5\%$ and $67.7\pm3.2\%$, respectively). In general, both types of neurones developed two or three processes per cell, independently of whether the culture substratum was polylysine or laminin. However, the overall morphology of both neuronal populations suggested intrinsic differences. Thalamic neurones, when cultured on polylysine, generally had small soma (average diameter, 11 µm) and short processes, usually tipped by large, lamellipodial growth cones (Fig. 3D). By comparison, hippocampal neurones had larger cell bodies (16 µm in diameter), with longer and thinner processes, tipped by smaller, filopodial growth cones (Fig. 3A).

GAGs were added to the cultures 3 hours after seeding, at concentrations ranging from 0.01 to 100 µg/ml, and their effect was examined 20 hours later. In the presence of 50 µg/ml or greater of either CS or DS, the number of both hippocampal and thalamic neurones that extended at least one process dramatically dropped, reaching 20% of control cultures. The majority of neurones attached to the culture dish, but remained round and did not extend neurites (Fig. 3G,H). However, lower

Table 1. Effect of GAGs on cell adhesion over laminin-coated substrata

	Control	CS		DS	
		1 µg	10 µg	1 µg	10 µg
Thalamus	89±10	66±21.6	57±14.8	66±22	66±16.8
Hippocampus	82±10.5	76±13	63±12.5	46±11.8	51±14.5
PC12	242±25.6	156±9	103±12.4	137±14.8	108±21.4
N2A	157±34.9	122±18.9	77±19.9	120±11.5	69±24.4

Acutely dissociated neurones or neuronal cell lines were incubated on polylysine-laminin-coated coverslips in chemically defined medium or in the same medium containing different concentration of GAGs. After 2 hours of incubation, coverslips were washed with Hanks' balanced salt solution, fixed in paraformaldehyde and the number of cells bound in each field ($48.5\times10^4\text{ }\mu\text{m}^2$) was counted. In each experiment 5 separate fields were counted. The results are the average of three experiments performed in duplicate.

Fig. 2. Quantification of GAG effects on neuronal morphology and neurite length. Hippocampal (HC) and thalamic (Th) neurones were grown over polylysine (PLL) or laminin (LN), in chemically defined medium, in the presence of increasing concentrations (μg/ml) of CS (A) or DS (B). After 24 hours, the cultures were fixed and morphometric analysis of the neurones was performed. The effect on neuronal morphology of increasing GAG concentrations added to the medium, is represented by: (A,B) the ratio of the percentage of neurones with only one process, P1, to the percentage of those with two or three processes, P2-3; (C,D) neurite length frequency for thalamic (C) and hippocampal (D) neurones (μ, μm). In the absence of GAG, the total number of neurites was much higher than in its presence, and the neurites were shorter. In the presence of CS, cells shifted from multipolar towards unipolar. The effect of CS and DS on neurite length was also quantified in the same preparation as follows. The length of all the neurites of each neurone present in 5 separate fields ($48.5 \times 10^4 \mu\text{m}^2$) was measured with the Argus-10 Image Processor. The different neuritic lengths were classified in 4 different classes. The results are represented as the percentage of neurites falling in a particular class over the total number of neurones with processes (neurite length frequency).

concentrations of both CS and DS did not significantly reduce the total number of neurones extending at least one neurite with respect to control culture. Independently of the culture substratum and the type of GAG, the number of neurones extending at least one process averaged $65.3 \pm 4.5\%$ for thalamic neurones and $64.7 \pm 8\%$ for hippocampal neurones. Surprisingly, however, CS specifically modified the polarity of thalamic, but not of hippocampal, neurones (Figs 2 and 3B,E), when compared with their respective controls (Figs 2 and 3A,D). DS, in contrast, did not influence the neuritic growth from either hippocampal or thalamic neurones (Figs 2B and 3C,F). In the presence of CS, thalamic but not hippocampal neurones tended to develop one single process, whereas neurones in control thalamic cultures generally had at least two processes. Particularly when cultured on polylysine, the proportion of thalamic neurones with only one process exceeded three times that of cells with two processes or more (Fig. 2A). This specific effect of CS was concentration dependent. DS had no significant influence on the number of processes per cell from either hippocampal or thalamic neurones (Fig. 2B). In the presence of CS, thalamic neurones not only became predominantly unipolar, but the length of their processes increased significantly with respect to controls (Fig. 2C). The CS-induced dose-dependent increase in the number of cells with processes longer than two somas was matched by a corresponding decrease in the number of cells with processes shorter than twice the body diameter (Fig. 2C). By comparison, process length of cultured hippocampal neurones was not affected by various concentrations of CS (Fig. 2D).

These results suggested that CS influenced the polarity of thalamic neurones, biasing the cells toward the *in vitro* development of a single neurite, at the expense of neurones with

several processes. To test whether CS induced the formation of axons, thalamic neurones were cultured for five days, with and without daily addition of CS to the culture medium (final concn, 10 μg/ml). Longer periods in culture were necessary to achieve a complete segregation of neuronal polarity. Cultures were doubly stained with a general neuronal marker (the 3A10 monoclonal antibody that stains the cell body and processes) and a Pan axonal marker, directed to the highly phosphorylated isoform of neurofilament H (NF-H), specifically present in axons. After five days, neurones in control cultures had developed several processes per cell (Fig. 4B), one of which was in some cases longer than all the others (Fig. 4A). These processes, however, did not express (Fig. 4D), or only at low level (Fig. 4C), the highly phosphorylated isoform of neurofilament H. Thalamic neurones grown in the presence of CS grew fewer processes per cell (Fig. 4E,F). In all cases, the neurones developed one long and sometime branched process, which was immunolabelled with antibodies against NF-H (Fig. 4G,H). These results strongly suggest that CS biases neurite differentiation towards axon-like processes.

CS effects seem mediated by specific interactions with thalamic neurones

The observed effects of CS on thalamic neurones could be the consequence of a direct specific interaction between CS and the neurones, or may be mediated by CS binding to the culture substrata. To distinguish between the two possibilities, CS and DS were labelled with ^{125}I and coverslips coated with polylysine or polylysine/laminin were incubated at 37°C with the labelled GAGs for 30, 90 or 180 minutes. After extensive washing, the amount of ^{125}I -labelled GAGs attached to the coverslip was measured. Both CS and DS bound maximally to

Fig. 3. Effect of CS and DS on the morphology of cultured hippocampal and thalamic neurones. Hippocampal (A-C) and thalamic (D-H) neurones were grown over polylysine in chemically defined culture medium alone (A,D); with the addition of DS, 10 $\mu\text{g/ml}$ (C,F) and 50 $\mu\text{g/ml}$ (H), or with the addition of CS, 10 $\mu\text{g/ml}$ (B,E) and 50 $\mu\text{g/ml}$ (G). In control cultures hippocampal (A) and thalamic (D) neurones showed intrinsic morphological differences. Thalamic neurones generally had small soma and short processes, with lamellipodial growth cones. Hippocampal neurones had larger cell bodies and longer processes, with small filopodial growth cones. DS did not alter this morphology in either hippocampal (C) or thalamic neurones (F). Thalamic neurones exposed to CS developed one single and long process (curved arrow, E), but hippocampal neurone morphology did not change in the presence of CS (B). Most thalamic neurones did not extend processes in the presence of high concentrations of either CS (G) or DS (H). Bar, 20 μm .

Fig. 4. CS effect on neurite polarity of cultured thalamic neurones. Thalamic neurones were cultured for 5 days on polylysine-coated glass coverslips, in chemically defined culture medium alone (A,B,C,D) or with CS added at a concentration of 10 $\mu\text{g/ml}$ (E,F,G,H). Cultures were double stained with the monoclonal antibody 3A10 (A,B,E,F), and a pan axonal marker directed against the highly phosphorylated isoform of neurofilament H (C,D,G,H). (A,B) Neurones in control cultures develop several processes per cell (small arrowheads), all visualised by the neurone-specific marker 3A10. (C,D) These processes are not stained by the pan axonal marker. (E,F) Thalamic neurones grown in the presence of CS always develop one long and sometime branched process (arrows). (G,H) These processes were immunolabelled with antibodies against NF-H. Large arrowheads indicate the position of neuronal cell bodies in C, D, G, H. Bar, 35 μm .

the laminin coat within the first 30 minutes; the amount bound decreasing slowly thereafter (Fig. 5A). Larger amounts of DS and lesser of CS bound to the polylysine-coated coverslips within the first 30 minutes of incubation; the amount bound rapidly decreasing with time. For example, about 10 ng/cm^2 of CS bound to polylysine-coated coverslips in the first 30 minutes, but less than 5% of it remained attached after 3 hours

(Fig. 5A). In the second experiment, suspensions of acutely dissociated hippocampal or thalamic neurones, were incubated at 4°C for 5, 15, 60 and 120 minutes with ^{125}I -labelled DS and CS. Relatively low concentrations of CS and slightly higher of DS bound to hippocampal neurones, reaching their maximum after 15 minutes of incubation and slowly decreasing thereafter (Fig. 5B). Relatively higher amounts of both DS and CS bound

Fig. 5. Binding of iodinated CS and DS to substrata and neurones. (A) Polylysine- (PLL), or polylysine/laminin- (LN) coated substrata, were incubated with 100 ng of ^{125}I -labelled CS or DS (●, CS-PLL; *, DS-PLL; □, CS-LN; ▲, DS-LN) in PBS for various periods of time, before removal of unbound GAGs. After several washes with PBS, the coverslips were placed in separate tubes and counted. The amount of bound GAGs is expressed in ng/cm^2 . (B) Acutely dissociated thalamic (T) and hippocampal (H) neurones were incubated at 4°C in medium containing ^{125}I -labelled CS or DS (10 $\mu\text{g/ml}$)

to thalamic neurones. The amount of DS bound to thalamic neurones increased with time, reaching a plateau after 1 hour. In contrast, the amount of CS bound to thalamic neurones progressively increased over the 2 hours of incubation (Fig. 5B). These experiments suggested that whereas the transient

binding of CS to culture substrata (especially to polylysine, where the major morphological differences were observed) was unlikely to mediate outgrowth of thalamic axons, this was probably caused by specific interactions of the GAG with thalamic neurones.

Fig. 6. Time course of CS binding to hippocampal and thalamic neurones. Thalamic (A,B,D,E,G,H) and hippocampal (C,F,I) neurones were seeded on polylysine-covered glass coverslips and cultured in chemically defined culture medium alone (A), or with the addition of biotinylated CS (B-I). Cultures were fixed 30 minutes (A-C), 3 hours (E, F), and 20 hours (D, G-I) after GAG addition. (B) Thalamic neurones, still round, readily accumulate CS on their plasma membranes (arrowheads). (E) After 3 hours, CS appears bound to the plasma membranes (arrowheads) and the growth cones (curved arrow); some labelling was also observed within the neuronal cytoplasm. (D, G-H) With time, CS accumulated inside both the cell bodies (arrows) and the growth cones (curved arrows). Glial cells, present in the cultures (stars) did not bind CS. (C,F,I) Hippocampal neurones bound CS on the cell body surface (arrowheads) but not on their processes (curved arrows) or growth cones. (A) No staining was observed in control cultures, grown in defined medium alone, but processed as those treated with labelled CS. Bars: C,H, 7 μ m; all others, 15 μ m.

Binding and internalisation of CS occurs only in thalamic neurones

To study further the specificity of CS binding to the surface of thalamic neurones, CS and DS were biotinylated and added to the culture media of hippocampal and thalamic neurones, two hours after seeding. Thirty minutes, 3 hours and 20 hours after the addition of biotinylated CS, the cultures were fixed, and the subcellular distribution of the CS followed using peroxidase-conjugated streptavidin. In other experiments, the presence of biotinylated CS was revealed with fluorescein-conjugated streptavidin and observed by confocal microscopy. Thirty minutes after the addition of CS, thalamic neurones were still round cells that had not yet started to extend processes (Fig. 6A,B). However, their plasma membranes were already strongly labelled (Fig. 6B). After 3 hours, biotinylated CS was bound not only to the cell body surface, but also to the processes that had begun to extend and some labelling was also observed within the neuronal cytoplasm (Fig. 6E). Twenty hours after the addition of biotinylated CS, the label had also

strongly accumulated inside both the cell bodies and the growth cones of thalamic neurones (Figs 6D,G,H and 7A,B). This labelling appeared to be neurone-specific, since the few glial cells that occasionally grew in the culture were not labelled (Fig. 6D,G). In parallel experiments, DS was never observed inside thalamic neurone cell body or growth cone. Thirty minutes after the addition of CS, hippocampal neurones bound biotinylated CS on the surface of the cell body but not on their processes, which had already begun to form (Fig. 6C). Labelling of hippocampal neurones did not change over time, but remained confined to the cell body surface (Fig. 7C), and was never observed on the growth cones (Fig. 6F,I).

These results suggested that thalamic neurones rapidly internalised and accumulated CS within the cell. To determine the subcellular localisation of CS within thalamic neurones and the specificity of the binding, hippocampal and thalamic neurones were grown in the presence of either CS or DS conjugated with biotin. After 24 hours, cells were detached from the culture dish with a cell scraper and rapidly subfractionated on a Percoll gradient. Fractions corresponding to endoplasmic reticulum and Golgi apparatus (both of which are contaminated by the lysosomal fraction), plasma membranes and cytosol, were collected as described by Morand and Kent (1986). The presence in each fraction of bound biotinylated GAGs, was determined by dot blot, revealed with peroxidase-conjugated streptavidin (Fig. 8). In thalamic neurones, biotinylated CS, but not DS, was mainly recovered in the fractions corresponding to endoplasmic reticulum and Golgi apparatus and, therefore, lysosomes. Little or no staining was observed in the plasma membrane and cytosolic fractions (Fig. 8). In hippocampal neurones, diffuse labelling was observed in all fractions, including the soluble fractions, with both CS and DS (Fig. 8).

DISCUSSION

We undertook this study to examine whether specific gly-

Fig. 7. CS distribution in thalamic and hippocampal neurones, observed by confocal microscopy. Biotinylated CS was added to the culture medium of thalamic (A, B), or hippocampal neurones (C). The cells were fixed, after 20 hours in culture and reacted with fluorescein-conjugated streptavidin. (A) Confocal section of the thalamic neurones close to the culture dish. Note the labelling inside the cell bodies and the growth cone (arrowhead). (B) Confocal section of the same thalamic neurones through the mid-height of the cells. (C) Confocal section through the mid-height of a hippocampal neurone. Labelling was confined to the cell body surface (small arrowheads). Bars: A,B, 10 μ m; C, 7 μ m.

Fig. 8. Subcellular distribution of biotinylated CS and DS in thalamic and hippocampal neurones. Dot blot analysis of the binding of biotinylated CS and DS to the peak fractions of each subcellular component. Equal amounts of the fractions were dotted on nitrocellulose and the presence of biotinylated CS and DS was revealed with peroxidase-conjugated streptavidin. Dots 1,2 and 3,4 correspond to the peak recovery of endoplasmic reticulum and Golgi apparatus, respectively. Dots 5,6 and 7,8 correspond to the peak recovery of plasma membrane and cytosolic components. (A) Hippocampal neurones, incubated with DS. (B) Hippocampal neurones, incubated with CS. (C) Thalamic neurones, incubated with DS. (D) Thalamic neurones, incubated with CS. a,b,c,d correspond to dot blots of total unfractionated membranes of A,B,C,D, respectively.

cosaminoglycans could modulate the outgrowth of defined neuronal cell types. The results reported here indicate that chondroitin sulphate type C (CS), but not type B (DS), specifically modifies the neurite extension pattern of cultured thalamic neurones, but not hippocampal neurones. The tendency of thalamic neurones to develop one axon-like process correlates with the binding and internalisation of this GAG, which does not happen in hippocampal neurones. These results, together with the transient expression of C6SPG during development in both the thalamus and the cortical subplate, towards which thalamic neurones transiently project, raise the possibility that in vivo CS could modulate thalamic axon outgrowth through the subplate. PGs appear to be involved in many morphogenetic events of the nervous system such as cell adhesion, cell migration and neurite outgrowth (Wight et al., 1992). Our results give further information on the effect of CS on neurite outgrowth, and support the idea that GAGs can modulate neuronal morphology, at least in vitro (Lafont et al., 1992).

Proteoglycans have different effects on the regulation of cell adhesion. For instance, molecules like syndecan or CD44 are clearly involved in mediating lymphocyte adhesion to bone marrow stroma or endothelial cells, but are not expressed on circulating lymphocytes, being, therefore, required to mediate cell to cell adhesion. On the other hand, PGs can interact with extracellular matrix molecules, like fibronectin, thrombospondin or tenascin, thus destabilising cell contact points (reviewed by Wight et al., 1992). Soluble GAGs or GAG-coated substrata, modify the adhesion of PC12 cells and dorsal root ganglia to fibronectin or collagen, and brain purified keratan sulphate (KS) and CS PGs inhibited the adhesion of neurones to laminin or cell adhesion molecules, like NCAM or NgCAM (Carbonetto et al., 1983; Akerson and Warren, 1986;

Verna et al., 1989; Cole and McCabe, 1991; Grumet et al., 1993). Thus, it is not surprising that GAGs lowered the adhesion of thalamic and hippocampal neurones, as well as of neuronal lines PC12 and N2A, to either polylysine or laminin. In our brief analysis of this effect, however, we did not observe a concentration dependence, at least in the ranges tested. Furthermore, both DS and CS gave similar results, suggesting that the structural composition of the galactosaminoglycan chain was not critical. Although these results support the idea that GAGs can destabilise neuronal cell adhesion, it may simply be that the GAGs bound to the two substrata through charge interaction, sterically interfering in the interaction between the substrata and their cell surface receptors. A mechanism of this type has been proposed, for instance, to explain the interference of two different small DSPGs, biglycan and decorin, with the binding of CHO cells to fibronectin (Bidanset et al., 1992). Indeed, in our case, analysis of CS and DS binding to the substrata suggested an unstable interaction. In vivo, direct and stable modulation of cell adhesion processes by PGs may definitively require the cooperation of protein cores, as recently proposed (Grumet et al., 1993).

The effect of different PGs on neurite outgrowth can be quite distinct, just as for cell adhesion. PGs and in particular their GAG components have been reported, in many instances, to inhibit neurite outgrowth. KS, DS and CS, extracted from bovine cartilage or purified from brain tissue, inhibited nerve fibre growth of dorsal root ganglia and retinal ganglion cells in vitro (Carbonetto et al., 1983; Cole and McCabe, 1991; Fichard et al., 1991; Snow et al., 1990b, 1991). The expression of CSPG in the developing mammalian retina precedes the differentiation and growth cone extension of the ganglion cells; the removal of CS from these regions results in ectopic ganglion cell differentiation and disorganisation of axon outgrowth (Brittis et al., 1992). Different types of proteoglycans are also associated with the gliotic tissue that develops after brain injury, and they are likely to be involved in the failure of axon regeneration in the mammalian CNS (McKeon et al., 1991; Bovolenta et al., 1993).

Somehow, in contrast to these reports, we have observed that low concentrations of CS specifically modify the pattern of thalamic, but not hippocampal, neurite growth, favouring the extension of only one long process, with the characteristics of an axon, per cell. Only relatively high concentrations of both CS and DS, non-specifically inhibited neurite extension of both hippocampal and thalamic neurones. The major difference between our study and many of those previously reported was the form in which the GAG was presented to the neurones. GAGs have been used as substratum-bound molecules, in conjunction with growth-promoting molecules like laminin or NCAM (Verna et al., 1989; Fichard et al., 1991; Snow et al., 1990, 1991; Snow and Letourneau, 1992). Since both laminin and NCAM contain GAG-binding sites (Cole et al., 1986), the neurite inhibitory effects could be, at least in part, due to the hindering of neurite promoting sites present on these molecules. In addition, the inhibitory effects may be only detectable when using relatively high concentrations of GAG, as we observed, since only a small but constant proportion of the starting solution binds to the substrata (Snow et al., 1991). In support of this idea, neurones can extend processes on CS/laminin substrata, at low GAG concentrations, when the GAG is presented in the form of a step gradient (Snow and

Letourneau, 1992). Furthermore, different types of neurones respond differently to the substratum-bound, step gradient of the CS (Snow and Letourneau, 1992). This indicates that neurones show intrinsically different responses, even when the GAGs are bound to the substratum.

As our binding studies indicated, soluble CS binds more stably to thalamic neurones than, for instance, to the polylysine substratum, over which the greatest morphological differences were observed. Therefore, when added in solution to the culture medium, GAGs could bind to a neuronal surface receptor involved in the interaction with the substratum or bind to a membrane component located in the vicinity of this receptor, offering steric hindrance. When laminin was used as substratum, the effect of CS on thalamic neurones was less pronounced. Because binding of GAGs to laminin appeared somewhat more stable than on polylysine, the morphological effects seen in this case may be the result of CS binding to both cells and substratum. In all cases, the result will be an imbalance of the adhesion of the growing neurites to the substratum, which is different, however, from that obtained when the GAGs are bound directly only to the substratum. If dendrite growth requires higher adhesion, as proposed (Rousselet et al., 1990; Lafont et al., 1993), CS might simply destabilise adhesion of thalamic neurones, inhibiting dendrite formation but still allowing axon growth, when the concentration of the GAG is low. At higher concentration, GAG could destabilize adhesion in such a way that not even axon-like processes could grow, as suggested by our results. Indeed, the importance of CS for cell movement has been established in other systems. For instance, when stationary vascular endothelial cells are induced to migrate, they switch from synthesising an HSPG to CSPG (Kinsella and Wight, 1986). Also, removal of CSPG from the surface of mouse melanoma cells prevents their migration (Faassen et al., 1992).

As an alternative and more attractive interpretation, the effect of CS on thalamic neurone morphology could be mediated by specific and stable binding of this GAG to a receptor, which cannot bind DS and is not present on thalamic glial cells or hippocampal neurones. This view is supported by the finding that labelled CS bound specifically to the surface of thalamic neurones, while they were still round cells, and thereafter to processes and growth cones. Binding was followed by accumulation inside the cells and, upon subcellular fractionation, the labelled GAG was found in endoplasmic reticulum, lysosomes and Golgi apparatus. CS did not interact with hippocampal neurones in the same manner. Labelled CS bound to the cell body surface, as shown by confocal microscopy, but it was not strongly accumulated inside the cells. Fractionation experiments, however, showed GAG label in all subcellular compartments, including the soluble fraction and the nuclear pellet. A likely explanation for this observation is that CS, like DS, loosely bound to the surface of hippocampal neurones, was released during the processes of lysis and subfractionation of the cells, becoming evenly dispersed over all fractions. In thalamic neurones, once bound to a cell surface receptor, CS could be internalised and degraded into smaller oligosaccharides by specific endoglycosidases in endosomes and lysosomes. Products of GAG degradation appear to be transferred to the nucleus, possibly regulating cell activity (Busch et al., 1992; Yanagishita and Hascall, 1992). Alternatively, binding of CS to a specific receptor could activate a second messenger that ultimately will influence axon outgrowth.

Binding of GAGs to specific surface receptors and internalisation have been shown in primary cultures of muscle cells and sequestration of GAGs in hepatocytes results in cell cycle arrest (Castellot et al., 1985; Ishihara et al., 1986; Resink et al., 1989). HS and DS appear to promote axon and dendrite growth, respectively, effects that are mediated by surface binding and translocation to the nucleus of these molecules (Lafont et al., 1992). As a general hypothesis, different kind of neurone could bind different GAGs, possibly through a specific receptor. Interactions of this type could easily explain why different GAGs have different effects on different types of neurones and why different neurones respond differently to a given GAG.

Many different, developmentally regulated proteoglycans are expressed in the mammalian brain, either in the extracellular matrix or associated with cell plasma membranes (Margolis and Margolis, 1989; Herndon and Lander, 1990). Besides being capable of inhibiting neurite outgrowth, PGs can also promote growth cone extension in vitro (Lander et al., 1985; Iijama et al., 1991; Lafont et al., 1992). HSPGs are present in developing axon pathways and enzymic removal of HS disturbs directed growth of axons (Wang and Denburg, 1992; Halfter, 1993). CSPGs with a spatially and temporally restricted distribution have been also recently characterised in the mammalian brain (Hendry et al., 1988; Maeda et al., 1992). One of them, 6B4 CSPG, might be selectively involved in the development and maintenance of the cerebellar mossy fibre system in the rat (Maeda et al., 1992). The molecular identity of the C6SPG expressed in the thalamus and the subplate, as well as its possible involvement in the modulation of thalamic axon growth through the subplate, remains to be established. However, the spatial and temporal constraints on the distribution of C6S but not C4S epitopes in this system, together with our in vitro findings that only chondroitin type C (6 sulphated) but not chondroitin type B (4 sulphated) can enhance thalamic axon growth, suggest that C6SPG could play an in vivo role in this process.

We thank Dr F. Wandosell for the help with iodination procedures and for the many helpful suggestions throughout this work. We are also indebted to Dr Marina Sánchez for participating in some of the initial experiments on cell adhesion and to Dr Regina García at the Centre for Molecular Biology, Universidad Autónoma de Madrid, for helping us with confocal microscopy. We are grateful to Dr Jane Dodd for the gift of the 3A10 monoclonal antibody. This work was supported in part by grants FAR 89-0683, INF 90-0179 and INF 91-0173, from the National Program for Pharmaceutical Research, which also provided a doctoral fellowship to I.F.E.

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