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Involvement of Sulfated Proteoglycans in Embryonic Brain Expansion at Earliest Stages of Development in Rat Embryos

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Key Words

Extracellular matrix · Chondroitin sulfate proteoglycan · Brain development · β-*D*-Xyloside · Rat

Abstract

The expansive force generated by the positive pressure of the neural tube fluid confined inside the brain vesicles has been shown to be a key factor during the earliest stages of brain morphogenesis and development of chick embryos. In a previous study, we demonstrated the existence of an intracavity extracellular matrix rich in condroitin sulfate in this species, which could be involved in the regulation of the expansive process. In this report, scanning electron microscopy and immunohistochemistry show that, after neurulation, a similar extracellular matrix rich in chondroitin sulfate is present inside the brain vesicles of rat embryos during early enlargement of the brain anlage. In vitro treatment of rat embryos with β-*D*-xyloside (a chemical compound which disrupts chondroitin sulfate synthesis) shows that changes in intralumen chondroitin sulfate concentration are accompanied by significant changes in brain anlage growth. These results support the hypothesis that intracerebral chondroitin sulfate plays a relevant role in the regulation of the expansive process of the brain anlage of rat embryos, and could represent a general mechanism in the early brain development of avian and mammalian embryos.

Abbreviations used in this paper

CPC	cetylpyridinium chloride
CSPG	chondroitin sulfate proteoglycan
GAGs	glycosaminoglycans
NTF	neural tube fluid
PBS	phosphate-buffered saline
SEM	scanning electron microscopy

Introduction

The morphological differentiation of the embryonic brain begins early in development with an explosive increase in the volume of the anterior end of the neural tube. Previous studies have been carried out in chick embryos in order to quantify this dramatic process, and Pacheco et al. [1986] described an initial period of moderate cerebral growth from HH [Hamburger and Hamilton, 1951] stages 11–18, followed by a period of rapid growth between stages 19 and 26 in which Desmond and Jacobson [1977] measured a 30-fold increase in the size of the brain vesicles in only 48 h, more than 70% of it being accounted for by cerebral cavity growth.

The development mechanisms involved in this dramatic growth of the brain vesicles are not well understood

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to date. However, studies carried out in chick embryos indicate that the expansive process starts with the occlusion of the spinal cord lumen and the closure of the anterior neuropore; this transforms the brain vesicle cavity into a physiologically sealed system [Schoenwolf and Desmond, 1984; Desmond et al., 1993], inside which the neural tube fluid (NTF) exerts a positive pressure against the neuroepithelial walls and generates an expansive force, described and quantified by Jelinek and Pexieder [1968, 1970]. Desmond and Jacobson [1977] demonstrated that the experimental decrease in NTF pressure leads to severe dysmorphogenesis and brain collapse, thus revealing the involvement of this fluid's pressure in brain anlage growth in the chick embryo.

As regards the generation and regulation of this pressure, in a previous study [Gato et al., 1993], we demonstrated the presence of an extracellular matrix inside the chick embryo brain vesicles cavity, at the early stages of development in which chondroitin sulfate proteoglycan (CSPG) is a ubiquitous component. Regarding the special osmotic properties of these molecules, described by Comper and Laurent [1978], we proposed that the secretion of osmotically active molecules in the brain cavity helps to retain water inside this closed system, thus generating and regulating hydrostatic pressure. Recently [Alonso et al., 1998] we tested this theory by inducing with β-*D*-xyloside an increase in NTF chondroitin sulfate concentration, which leads to a specific hyperenlargement of the brain vesicle cavity in chick embryos.

Although there are no quantitative studies regarding the embryonic brain enlargement in mammals, a notable increase in the volume of the brain vesicles of rat embryos after neurulation is evident, which suggests that rapid brain enlargement, due to intraluminal expansive force, takes place.

In order to test the hypothesis that the presence of intraluminal CSPG in brain vesicles signifies involvement in brain enlargement, and that this could be a common factor in higher vertebrates, we investigated by scanning electron microscopy (SEM) and by immunohistochemistry the presence of CSPG inside the brain cavity of rat embryos at the earliest stages of brain development. Similarly, the functional involvement of this proteoglycan in brain enlargement was studied by means of selective disruption in CSPG synthesis induced by β-*D*-xyloside in rat embryo culture.

β-*D*-Xyloside is a chemical that specifically disrupts the synthesis of sulfated proteoglycans, especially chondroitin-dermatan sulfate [Sobue et al., 1987; Lugemwa and Esko, 1991], acting as an initiation point for sulfated glycosaminoglycan synthesis; therefore, β-*D*-xylosides increase the synthesis of free chains of chondroitin sulfate and induce alterations in normal CSPG [Schwartz et al., 1974; Robinson et al., 1975]. β-*D*-Xylosides have been widely employed in demonstrating the roles played by chondroitin sulfate in several developmental mechanisms in rat [Morriss-Kay and Crutch, 1982], chick [Gibson et al., 1978, 1979; Kanke et al., 1982; Segen and Gibson, 1982; Alonso et al., 1998; Moro Balbos et al., 1998], sea urchin [Kinoshita and Saiga, 1979] and *Xenopus laevis* [Yost, 1990] embryos.

Materials and Methods

Scanning Electron Microscopy

Rat embryos at different development stages, ranging from 10.5 to 12.5 days (calculated from the 1st day of positive vaginal plug), were explanted from pregnant Wistar rats and removed from their extraembryonic membranes.

In order to test the influence of the fixative used to preserve the intracavity content of the brain vesicles, we resorted to different types: Bouin, 4% paraformaldehyde, 4% paraformaldehyde plus 0.05% cetylpyridinium chloride (CPC), Carnoy, glutaraldehyde plus ruthenium red with osmium postfixation as established by Cohn [1977]. The best results with regard to preserving the intracavity matrix of the brain vesicles were obtained with Bouin, despite its shortcomings as a fixative for studying cell structure with SEM.

After fixation for 6 h at room temperature, embryos were dehydrated in graded ethanol series and critical point-dried with CO_2; the embryos were transversally cleaved with tungsten needles, at different brain levels, to display the content of the neural tube cavity. Finally, the specimens were sputter-coated with gold and observed under a JEOL T-300 SEM.

Immunohistochemistry

Rat embryos of similar development stages to those used for the SEM study were immersed for 4 h in Carnoy's fixative at room temperature, dehydrated in graded ethanol series and embedded in paraplast. Carnoy fixative was the best as regards maintaining the carbohydrate epitope recognized by the CS-56 antichondroitin sulfate antibody.

Deparaffinized sections of 8 μm from the different brain vesicles of each stage were washed in phosphate-buffered saline (PBS), preincubated with normal horse serum (1/20 in PBS) and incubated overnight with anti-chondroitin sulfate monoclonal antibody CS-56 (Sigma). After washing twice in PBS, the sections were reincubated for 30 min in fluorescein-conjugated goat antimouse IgM (Vector) as a secondary antibody, mounted in Aquamount (Gurr) and studied under a Laser Confocal Microscopy Zeiss LSCM 310. Control sections were prepared as described above but using preimmune serum as the primary antibody.

β-D-Xyloside Treatment of Rat Embryos in Culture

Rat embryos at 9.5 days were explanted in Hanks' saline according to the method of New [1978]. After removing the Reichert's membrane, the embryos were placed in glass culture bottles contain-

ing 4 ml of heat-inactivated rat serum, 50 IU of streptomycin and a gas phase with 20% of oxygen. The bottles were placed in an incubator at 37°C and underwent permanent rotary movement. The gas phase was renewed every 12 h and the culture medium every 24 h.

In order to determine the optimal β-*D*-xyloside (*p*-nitrophenyl-β-*D*-xylopyranoside from Sigma) concentration which has an influence on brain vesicle growth without having a general effect on the embryo, we carried out a dose response study, between days 10.7 and 11.7 of development, with series of 6 embryos per dose of β-*D*-xyloside dissolved in Hanks' saline; it was found that the optimal dose was a 1 m*M* concentration. A total of 56 embryos were treated with this dose of β-*D*-xyloside with the same number of control embryos divided into two series: in the first series the same amount of Hanks' saline as for the treated embryos was added to the culture medium; in the second series, and so as to eliminate possible direct toxic effects of the xylosides on embryo growth and development, the same concentration of α-*D*-xyloside (an inactive anomer of β-*D*-xyloside) was added to the culture medium. Afterwards, cultured embryos were classified in accordance with the criteria of Brown and Fabro [1981].

Light Microscopy and Morphometric Analysis

After culture, 8 control and 8 β-*D*-xyloside-treated embryos were removed from the extraembryonic membranes and fixed for 6 h in Bouin's fluid at room temperature. After dehydration in graded ethanol series and embedding in paraplast, 8-μm transversal sections were stained with hematoxylin and eosin.

The volume of the brain vesicles was calculated from the cranial extremity until the first section in which the otic vesicle appeared, including forebrain, midbrain and the anterior part of the hindbrain. The inner and outer limits of the neuroepithelium were drawn in one of every three sections (section factor = 3) using a Leitz SM Lux microscope equipped with a drawing tube (total magnification 60×), and the corresponding areas integrated with a Videoplan (Kontrol Elektronic) computerized image analysis system, via the VIDAS 2.1. stereology program. In each embryo, the volume of the brain, of its cavity, and that of the neuroepithelial wall were obtained by adding the corresponding sectional areas (in μm^2) multiplied by 8 μm (thickness) and by 3 (section factor).

According to Desmond and Jacobson [1977] and Pacheco et al. [1986], the measurement of every fifth section differs less than 10% from the measurement of every section in HH stage 23 chick embryos, and this error can be regarded as acceptable. The final volumes are expressed as the arithmetic mean of the values of the eight embryos ± standard error. For comparison of the average volumes (total, cavity, and tissue) between experimental and control embryos, a two-tailed Student t test for independent samples was applied and p values below 0.001 were interpreted as indicating statistical significance.

NTF Immunohistochemical and Biochemical Analysis

In order to test whether β-*D*-xyloside produces changes in the NTF CSPG, microaspiration of this fluid was performed as follows. Both control and β-*D*-xyloside-treated embryos were released of their extraembryonic membranes, rinsed twice in sterile saline solution, and placed in a dry Petri dish; the surrounding fluid was aspirated. A micropipette connected to a microaspirator (Medical System PLI 100) was carefully placed into the middle of the mesencephalic cavity under dissecting microscope control, avoiding contact with the neuroepithelial wall. The NTF was slowly aspirated, until a sufficient amount for biochemical and immunohistochemical analysis was obtained, aliquoted and frozen at –40°C until use.

Changes in chondroitin sulfate concentration induced by β-*D*-xyloside in the NTF were assessed by dot-blot. 5-μl aliquots of NTF from control embryos and those treated with β-*D*-xyloside were placed on nitrocellulose membranes and left to dry at room temperature. The membrane with the samples was incubated for 2 h at room temperature in the blocking solution (PBS containing 5% skimmed milk), and was then exposed overnight, at 4°C, to anti-chondroitin sulfate monoclonal antibody (CS-56 from Sigma), which recognized the CSPG glycosidic fraction [Avnur and Geiger, 1984], and identified with a peroxidase-labeled mouse IgM PK 4010 Vectastain ABC kit (from Vector). The peroxidase activity was revealed with diaminobenzidine (0.5 mg/ml in PBS with 0.02% hydrogen peroxide). The images were scanned and processed by the Biosoft Quantiscan program to assess color intensity in each sample. So as to check the sensitivity of this technique, we carried out a dot-blot on samples with different amounts of 12-day-old rat embryo NTF and on samples with different concentrations of bovine trachea chondroitin sulfate-free chains (from Sigma).

β-*D*-Xyloside-induced differences of chondroitin sulfate were rated by electrophoretic separation and enzyme digestion of the NTF in the following way: NTF from control and treated embryos as well as standard solutions of glycosaminoglycans (GAGs) from Sigma were applied to cellulose acetate strips (cellogel 2.5 × 17), and electrophoresed in 0.1% formic acid-pyridine buffer, pH 3.1, at 270 V for 1 h in accordance with Beeley [1985]. After electrophoresis, the strips were stained with 1% Alcian blue 8GX (from Gurr) in 0.05 *M* acetate buffer, pH 5.8, and ethanol (v/v). For enzymatic identification of NTF GAGs, chondroitinase AC from *Flavobacterium heparinum* (Sigma) was used; this enzyme specifically digests chondroitin 4,6-sulfate [Yamagata, 1968]. The digestion procedure was based on that of Breen et al. [1976]. Briefly, the activity of the enzyme was previously checked with standard GAGs; afterwards, aliquots of NTF from β-*D*-xyloside-treated embryos were diluted in 50 μl of 0.15 *M* Tris chloride acetate buffer pH 7, containing 0.5 IU of chondroitinase AC. The mixtures were incubated at 37°C for 3 h under gentle shaking, and the digestion product was electrophoresed as described above.

Results

Scanning Electron Microscopy

Our results show that the amount and appearance of material inside the brain vesicles vary according to the fixative employed (fig. 1). The use of classic fixative for GAG preservation, such as paraformaldehyde with CPC and glutaraldehyde with ruthenium red (fig. 1E), preserves only slightly brain material found on the apical surface of the neuroepithelium. Bouin preserves more precipitated material, which occupies a large part of the lumen of the brain vesicles (fig. 1A, C). Carnoy fixative (fig. 1D) also preserves an abundant amount of intracavitary extracellular matrix, albeit to a lesser extent than Bouin fixative.

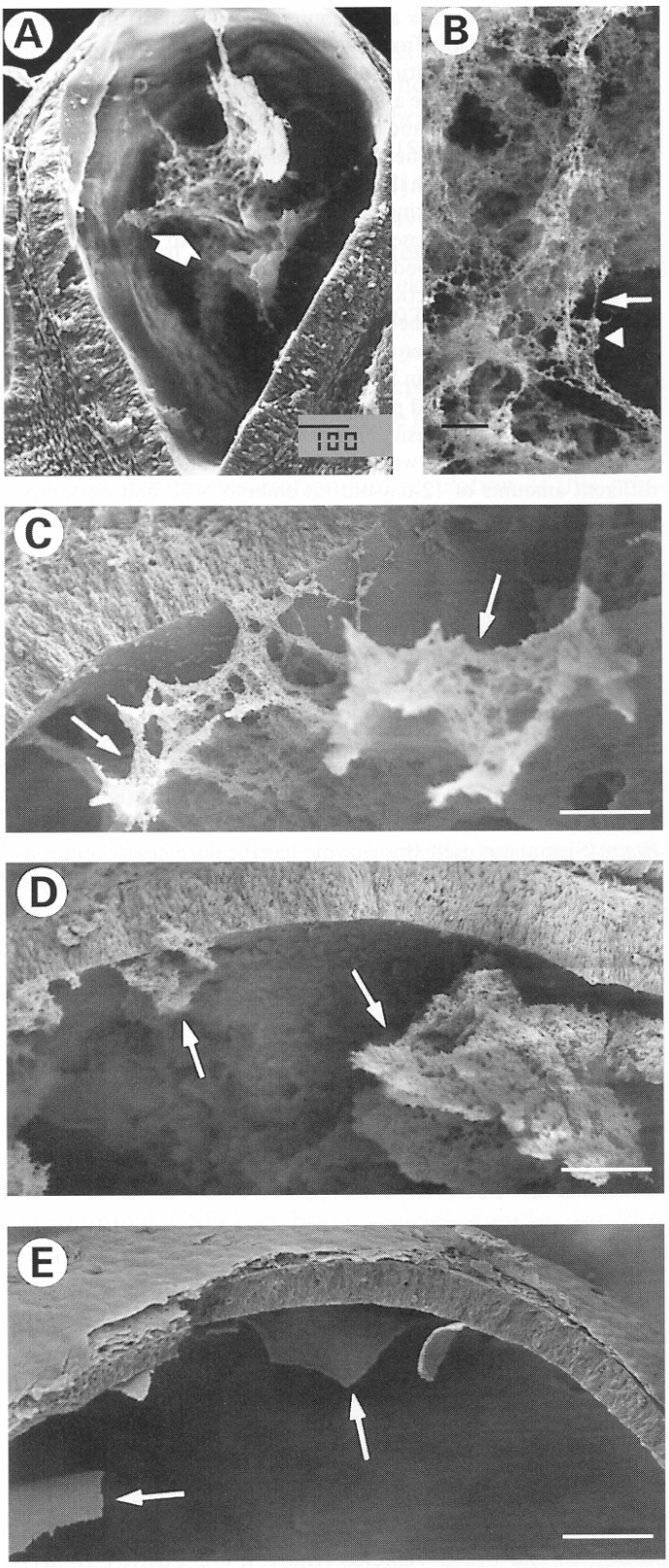

Fig. 1. Brain cavity of 11.7-day rat embryos cleaved after critical point drying. **A** Panoramic view of rhombencephalic cavity showing the precipitated material inside by Bouin's fluid fixation (arrow). **B** At higher magnification, the material shown in **A** appears to be made up of an amorphous component (arrowhead) arranged in a fibrous three-dimensional net (arrow). The capacity of different fixatives to preserve the extracellular matrix inside the midbrain cavity (arrows) is shown: Bouin's fluid (**C**), Carnoy (**D**) and glutaraldehyde plus ruthenium red (**E**). **A**, **C–E** Bar 100 μm. **B** Bar 10 μm.

The observation of this material at high magnification (fig. 1B) revealed that its organization consisted of a fibrous component arranged in a three-dimensional net to which a globular and amorphous component adheres, occupying the empty spaces, in a structure similar to that of the extracellular matrices. At the stages studied no cellular components inside the neural cavity could be observed. Variable amounts of this material were present at all the stages studied and in all the brain vesicles.

Our findings reveal that at these stages the brain vesicle cavity of rat embryos contains a complex extracellular matrix, the preservation of which largely depends on correct fixation and processing.

Immunohistochemistry

Immunohistochemical study with CS-56 monoclonal antibody, which recognizes the glycosaminoglycan moiety of native chondroitin sulfate proteoglycan [Avnur and Geiger, 1984], revealed intense immunoreactivity in the material contained inside the brain cavity (fig. 2), which was maximal in the embryos processed with Carnoy fixative. Although this fixative seems to be the best for the immunodetection of glycosaminoglycans in embryonic tissues, inasmuch as it better preserves the chondroitin sulfate epitope, the material remaining after immunohistochemical staining was scarce, which suggests that a part of it had been eliminated during the procedure. Nevertheless, our results show the constant presence of CSPG in the intracavity matrix of the brain vesicles of rat embryos at all the stages studied.

The brain neuroepithelium was also CSPG-positive, but in a heterogeneous manner; the CSPG seems to be extracellular, surrounding the neuroblasts, and maximum immunolabeling was detected in the basal membrane and especially in the apical end of the neuroepithelium. Immunolabeling was very low in the optic stalk and on the floor of the brain vesicles.

Treatment with β-D-Xyloside

Macroscopic Study. Our findings demonstrate that 0.5 m*M* or lower concentrations of β-*D*-xyloside do neither modify the expansion of brain vesicles nor affect embryo development and overall growth. 2 m*M* or higher

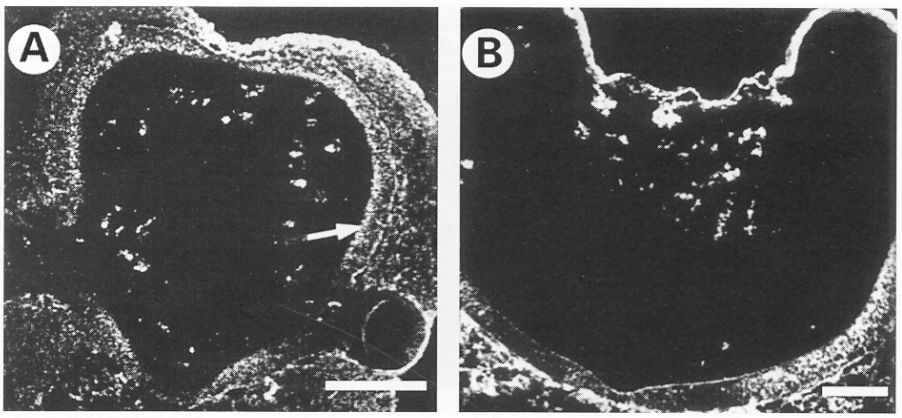

Fig. 2. Sections of an 11.7-day rat embryo immunostained with anti-chondroitin sulfate monoclonal antibody. Several accumulations of strongly stained extracellular material can be seen inside the neural cavity of the prosencephalon (**A**) and the rhombencephalon (**B**). The labeling of the neuroepithelial wall is not homogeneous, being more pronounced at the apical end of the lateral neuroblasts (arrow in **A**). **A** Bar 250 μm. **B** Bar 100 μm.

concentrations seriously affect overall embryo development. Maximum effect on brain vesicle expansion with little effect on embryo growth and development was obtained with concentrations around 1 m*M* and, consequently, this was the dose chosen to determine the role of sulfate proteoglycans in brain vesicle expansion.

The two groups of control embryos, α-*D*-xyloside and Hanks saline-treated embryos (fig. 3A), cultured between 9.5 and 11.7 days, showed a normal level of development and growth in accordance with the developmental criteria of Brown and Fabro [1981].

Embryos cultured during the same period with 1 m*M* of β-*D*-xyloside developed in a similar way to control embryos. However, selective alterations were present at the cranial extremity level, the latter appearing notably dilated in relation to control embryos (fig. 3B). This dilation is due to an enlargement of the brain vesicles, chiefly the forebrain and the hindbrain. Nevertheless, the basic morphology of the embryonic brain does not appear to be greatly affected; the limits between the different brain vesicles are clearly observed, but the limit between the telencephalon and the diencephalon has disappeared.

Macroscopic appearance of cranial extremity anlagen in 1 m*M* β-*D*-xyloside-treated embryos is normal, although the eye anlage appears notably dilated (fig. 3B) in relation to control embryos (fig. 3A). In addition, otic placode invagination and closure is clearly delayed.

Light Microscopy. Histological sections confirm the findings mentioned above: the cavities of the forebrain (fig. 3D) and the hindbrain (fig. 3F) appear notably dilated with respect to the controls (fig. 3C, E), but the dilation of the mesencephalic cavity seems to be smaller (compare fig. 3C, D).

The morphological pattern of brain vesiculation appears unaltered in 1 m*M* β-*D*-xyloside-treated embryos; however, the neuroepithelial wall is significantly thinner than that of control embryos, particularly at the anterior brain (compare fig. 3C and D) and posterior brain (compare fig. 3E and F) levels. This thinning is especially noticeable in the hindbrain lateral wall; at this level the neuroepithelium in control embryos (fig. 3G) appears characteristically pseudostratified with numerous nuclear layers at different levels; on the other hand, in 1 m*M* β-*D*-xyloside-treated embryos (fig. 3H) there are considerably fewer nuclear layers.

The eye anlage in treated embryos (fig. 3D) shows alterations similar to those of the brain vesicles with a notable dilatation of its cavity and of the optic pedicle as well as a considerable thinning of the epithelium compared with the control embryos (fig. 3C).

Morphometric Analysis. In order to evaluate to what extent variations in the size of brain vesicles are due to a larger vesicular cavity or to an increase in the size of the wall, we have quantified the total volume of the brain vesicles, that of the cavity and the wall, and have carried out a Student test with the data in order to test the degree of statistical significance. These findings (fig. 4) show that there is a notable increase (136%) in the total brain anlage volume of treated embryos compared with controls, and that this was statistically significant ($p < 0.001$). Volume measurements of the brain vesicles cavity revealed a 229% increase in treated embryos with regard to controls, which was also statistically significant ($p < 0.001$). In β-*D*-xyloside-treated embryos, tissue volume increased by 37% compared with controls, but this was not statistically significant ($p > 0.05$). This data demonstrate that β-*D*-xyloside-induced hypergrowth in the embryonic cranial extremity is due fundamentally to hyperexpansion of the brain vesicle cavity, and that no obvious modifications are found in neuroepithelial volume.

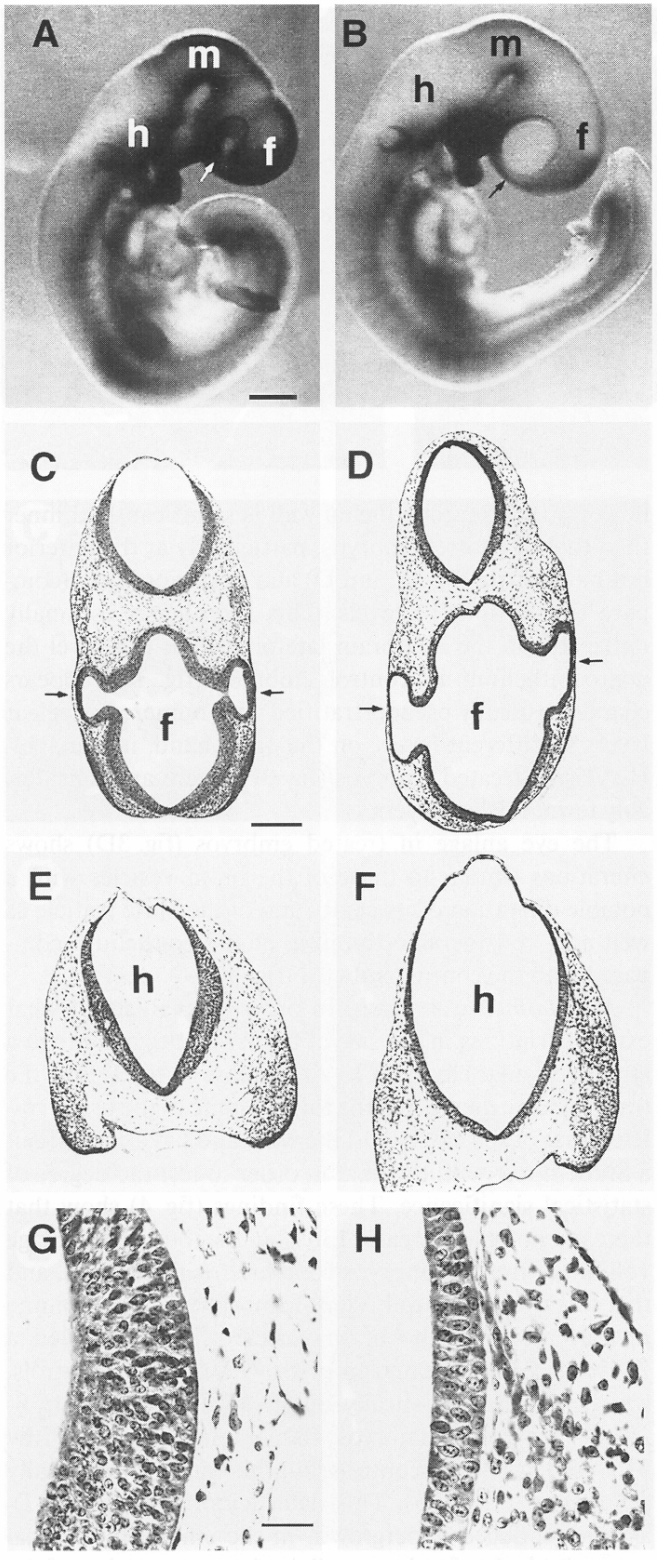

Fig. 3. Macroscopic and histological comparison of 11.7-day-old rat embryos: controls treated with Hanks' saline (**A**, **C**, **E**, **G**) and 1 m*M* β-D-xyloside-treated (**B**, **D**, **F**, **H**). Observe the hyperexpansion of the brain vesicles and eye anlagen in β-*D*-xyloside-treated embryos (**B**) compared with controls (**A**). Histological sections at the forebrain (**C**, **D**) and hindbrain (**E**, **F**) levels show a considerable increase in intralumen volume in β-*D*-xyloside-treated embryos (**D**, **F**) in respect of controls (**C**, **E**); similarly, in β-*D*-xyloside-treated embryos (**H**), as compared with controls (**G**), it is possible to observe a generalized thinning of the neuroepithelial wall, more evident at hindbrain level. **A**, **B** Bar 400 μm. **C**–**F** Bar 400 μm. **G**, **H** Bar 200 μm. Arrows show the optic anlage. f = Forebrain; m = midbrain; h = hindbrain.

β-D-Xyloside-Induced Alterations in NTF Proteoglycans

With a view to determining whether β-*D*-xyloside induces changes in NTF CSPG/CS concentration, we performed a dot-blot with CS-56 antichondroitin sulfate antibody. Previously, we proved that this technique with CS-free chains implies the loss of some of these, probably as a result of retention difficulties in the nitrocellulose

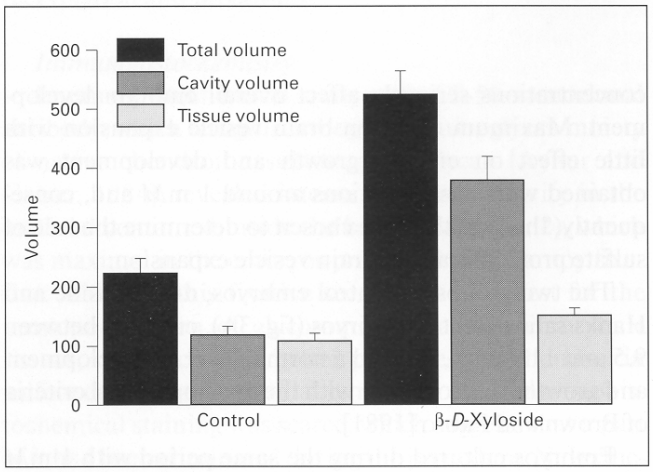

Fig. 4. Brain size comparison of 11.7-day control and β-*D*-xyloside-treated rat embryos shows a significant disruption of brain enlargement. Total cerebral anlage volume of the treated embryos is 136% higher than that of the controls. This increase is due to cerebral cavity volume, 229% greater than that of control specimens, whilst brain tissue mass increased by 37%.

	Control, $\mu m^3 \times 10^6$	β-*D*-Xyloside, $\mu m^3 \times 10^6$
Total volume	221 ± 28.6*	522 ± 49.4*
Cavity volume	114 ± 19.6*	375 ± 45.6*
Tissue volume	107 ± 8.7	147 ± 16.4

Measurements are means ± standard error (n = 8). * $p < 0.001$.

membrane. What is more, a dot-blot performed on different amounts of control rat embryos NTF (not shown) suggests that this technique can detect an increase in the epitope's concentration but, since it is not proportional, does not permit suitable quantification.

Figure 5 shows the result of dot-blot on the NTF of control embryos and those treated with β-*D*-xyloside; this test was repeated twice and, on both occasions, we observed a clear increase in epitope concentration in treated embryos which, for reasons stated before, we did not quantify.

Qualitative changes in NTF CSPG have been rated by electrophoretic separation of native state proteoglycans and enzyme digestion with chondroitinase AC. In native conditions (with no previous treatment), there is an absence of electrophoretic separation of control embryo NTF proteoglycans, which appear as a stain of Alcian blue-positive material in the place where the sample was put (fig. 6D). In the NTF of β-*D*-xyloside-treated embryos, electrophoretic separation in conditions identical to controls shows the existence of two quite distinct components (fig. 6B): as in the case of control embryos, some of the proteoglycans remain in the place where the sample was put; however, another component capable of migration and similar to that of the sulfated GAGs used as standard (fig. 6A) can be perceived. Both these components were digested by the chondroitinase AC which specifically digests chondroitin sulfate A and C (fig. 6C).

These data suggest that in rat embryo NTF, β-*D*-xyloside induces an increase in chondroitin sulfate, most of this as free chains of GAG.

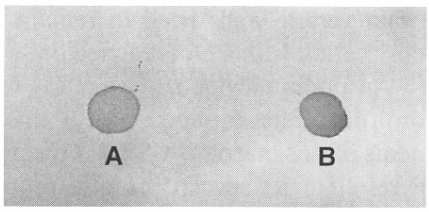

Fig. 5. Immunomarking with CS-56 anti-chondroitin sulfate antibody on 11.7-day rat embryo; 5-µl NTF aliquots placed on nitrocellulose membrane (dot-blot): control embryos (**A**) and β-*D*-xyloside-treated (**B**).

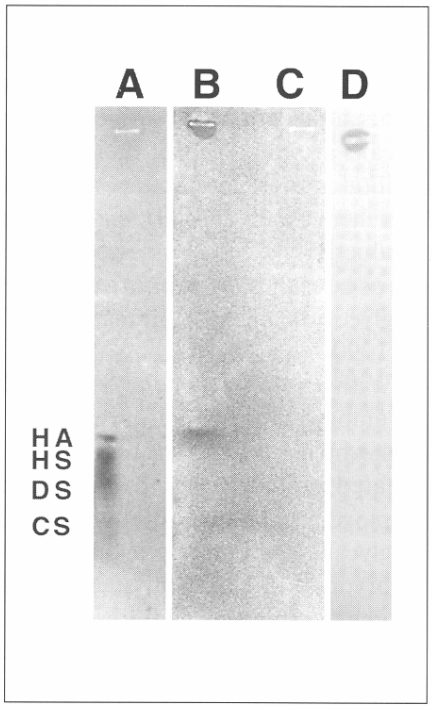

Fig. 6. Electrophoretic separation of 11.7-day rat embryo NTF proteoglycans. Band A corresponds to the migratory pattern of standard GAGs. HA = Hyaluronic acid; HS = heparan sulfate; DS = dermatan sulfate; CS = chondroitin sulfate. Band B shows the migratory pattern of NTF PGs in 1 m*M* β-*D*-xyloside-treated embryos in native conditions; notice an Alcian blue-positive stain where the sample was placed and an Alcian blue-positive band with a similar capacity to that of the standards. In band C, these proteoglycans have been digested with chondroitinase AC, which indicates that the Alcian blue-positive material on band B corresponds to chondroitin sulfate. The existence, in native conditions, of a band with a similar migratory capacity to that of standards and susceptible to the action of chondroitinase AC indicates the presence of free chondroitin chains in the NTF of β-*D*-xyloside-treated embryos. Band B corresponds to control embryo NTF in which all Alcian blue-positive material remains where the sample was placed, indicating that free chondroitin sulfate chains are not present.

Discussion

Our findings reveal the presence of a precipitated material inside the brain vesicle cavity; the amount and distribution vary in accordance with the fixative used. Those fixatives which best preserve this material are Bouin and Carnoy, whilst the ones commonly used for preserving carbohydrate molecules, such as CPC and ruthenium red, only partially conserve this material appearing on the apical surface of the neuroepithelium. This suggests the existence of an extracellular matrix in the brain vesicle cavity during the period of rapid brain growth; its composition seems to be heterogeneous. To this effect, Gato et al. [1993] demonstrated the presence of a similar matrix in the brain cavity of chick embryos during the same period of development. In this study the difficulty of preserving this matrix in SEM was shown; the

brain vesicle walls need to remain intact until embryo desiccation with CO_2 is carried out.

An immunocytochemical study with CS-56 antichondroitin sulfate antibody proves that one of the components of this matrix is CSPG. This proteoglycan could, as a result of its special osmotic properties [Comper and Laurent, 1978; Comper and Zamparo, 1990], be involved in brain vesicle growth in rat embryos; in fact, Alonso et al. [1998] showed its involvement in this process in the case of chick embryos, proving that an increase in NTF CSPG concentration is accompanied by a rise in this fluid's osmotic pressure which, confined in a closed cavity, results in an increase in hydrostatic pressure leading to hyperexpansion of the brain vesicles.

Proteoglycans have been proposed as fundamental molecules in the regulation of the water content in biological tissues. Certain major biological properties of proteoglycans are the result of their polyanionic nature, which facilitates their interaction with water in biological tissues; hence their special osmotic properties and hydraulic conductivity. CSPG possesses the greatest osmotic capacity, as a result of its uronic acid content and the nature of its glycosidic union [Comper, 1981; Comper and Zamparo, 1990].

With a view to checking whether CSPG plays a role in rat embryo brain vesicle growth, we have used β-*D*-xyloside, which induces a specific alteration in the synthesis of sulfated proteoglycans, β-*D*-xylosides have been widely employed in the study of the roles played by sulfated proteoglycans (particularly chondroitin sulfate) at different embryonic development stages in rat [Morriss-Kay and Crutch, 1982], chicken [Gibson et al., 1978, 1979; Kanke et al., 1982; Segen and Gibson, 1982], sea urchin [Kinoshita and Saiga, 1979] and *X. laevis* [Yost, 1990]. These chemicals specifically alter the synthesis of those sulfated proteoglycans whose glycosylation begins with the sequence xylosyl-galactosyl-galactosyl which is the initiation point for sulfated GAG chain synthesis, including heparan sulfate, heparin, chondroitin sulfate and its epimer dermatan sulfate [Schwartz et al., 1974; Robinson et al., 1975; Roden, 1980]. However, *p*-nitrophenyl-β-*D*-xylopyranoside specifically disrupts the chondroitin/dermatan sulfate synthesis, and several studies have shown its inability to prime the heparan sulfate and/or heparin synthesis in embryonic tissues [Sobue et al., 1987; Lugemwa and Esko, 1991] or in tissue cultures from different sources [Galligani et al., 1975; Spooncer et al., 1983].

The results obtained with dot-blot and electrophoresis in cellulose acetate show that β-*D*-xyloside produces an increase in NTF CS concentration in rat embryos; however, we have been unable to quantify this increase. As can be expected of the way β-*D*-xyloside works, and on the basis of the electrophoretic migration pattern of NTF proteoglycans, this increase appears to be due to the presence of free chondroitin sulfate chains.

This change in NTF composition is accompanied by hypergrowth of the brain vesicles; this is due to an increase in cavity volume given that there are no significant variations in neuroepithelium tissue volume. These findings suggest a direct relation between NTF proteoglycans and expansion of the brain vesicles cavity, and are similar to those of Alonso et al. [1998] in chick embryos; this may be explained by the great osmotic properties generated by CS in solution [Comper and Laurent, 1978]. In addition, Alonso et al. [1998] have shown that a β-*D*-xyloside-induced alteration in NTF CS concentration in chick embryos is accompanied by a significant increase in the total concentration of other osmotically active compounds, such as sodium; similar effect may take place in rat embryos.

We have proved that brain vesicles hypergrowth is not accompanied by obvious alterations in the morphological pattern of brain vesiculation; this suggests that brain expansion and vesiculation are independent, albeit in part simultaneous, phenomena. CSPG appears to play an important role in brain growth, but does not seem to intervene directly in morphological changes related with vesiculation.

Our results demonstrate that β-*D*-xyloside produces no significant variations in neuroepithelium volume ($p > 0.05$), although the latter appears generally thinner than in control embryos; this could be due to a restructuring of the neuroepithelial cells as a consequence of increased intracavity hydrostatic pressure. In this regard, Desmond and Jacobson [1977] and Desmond [1985] have shown that NTF hydrostatic pressure is an important factor in neuroepithelial histogenesis.

Other alterations observed in 1 m*M* β-*D*-xyloside-treated embryos during the period under study concern the development of the eye and ear anlagen. The eye anlage cavity appears considerably dilated and the epithelial wall has undergone a thinning process; these modifications are similar to those described in the neural tube and, given the fact that the eye vesicle and the forebrain communicate via the eye pedicle, we interpret these alterations as responding to some mechanism similar to ones already mentioned. Finally, invagination and closure of the ear placode is delayed, suggesting that sulfated proteoglycans are involved in these processes. In this regard, Gerchman et al. [1995] proved that β-*D*-xyloside local

microinjection disrupting CSPG in chick embryos produced similar effects to those we have described in rat embryos.

On the basis of data included in this paper, we conclude that brain vesicle cavity CS is involved in rapid brain growth in rat embryos, perhaps acting as a regulator of osmotic pressure in the NTF which would modulate intravesicular hydrostatic pressure; yet we cannot rule out the involvement in this mechanism of other molecules present in the NTF. Having demonstrated a similar mechanism in chick embryos, we believe that this is a common process in embryonic brain development in higher vertebrates.

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