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Enzymatic digestion of neural tube fluid proteoglycans leads to brain growth disruption

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SUMMARY

The expansive force generated by the positive pressure of the neural tube fluid confined inside brain vesicles has been shown to be a key factor during the earliest stages of brain morphogenesis and development of chick and rat embryos. In previous studies, we demonstrated the existence in these species of an intracavity extracellular matrix rich in proteoglycans that could be involved in the regulation of the expansive process.

Our results show that the enzymatic digestion of the intracavity proteoglycans by testicular hyaluronidase selectively disrupts the expansive process of brain vesicles, significantly reduces the rate of mitosis in the brain neuroepithelium, and increases the number of the apoptotic cells, leading to a decrease in neuroepithelial volume. These results support the hypothesis that intracerebral proteoglycans play a relevant role in the regulation of the expansive process of the brain primordium in rat embryos that they could be involved in regulating the survival and replication of neuroblasts.

Key Words: Extracellular matrix – Rat embryo – Brain development – Neural tube – Proteoglycans

INTRODUCTION

One of the first signs of morphological differentiation in the embryonic brain takes place early in development, with an explosive increase in the volume of the anterior end of the neural tube. In chick embryos, Desmond and Jacobson (1977) reported a 30-fold increase in the size of the brain vesicles in only 48 hours, more than 70% being accounted for by cerebral cavity growth. In chick embryos, the expansive process starts with the occlusion of the spinal cord lumen and closure of the anterior neuropore; this transforms the brain vesicle cavity into a hermetic system (Desmond et al., 1993; Schoenwolf and Desmond, 1984), inside which the Neural Tube Fluid (NTF) exerts a positive pressure against the neuroepithelial walls and generates an expansive force; this has been described and quantified by Jelinek and Pexieder (1968, 1970). The involvement of this fluid in brain expansion was demonstrated by Desmond and Jacobson in 1977, when they showed that an experimental decrease in NTF pressure in chick embryos leads to severe dysmorphogenesis and brain collapse. Furthermore, it has recently been suggested that this fluid could be responsible for many other aspects of the development of the Central Nervous System (Van Essen, 1997).

Although there are no quantitative studies regarding the expansive process of the embryonic brain in mammals, the notable increase in volume of rat embryo brain vesicles after neurulation is evident. This suggests that, among higher vertebrates, rapid brain enlargement is a common factor in early brain development.

As regards the generation of this pressure, in a previous study (Gato et al., 1993) we showed that Chondroitin Sulphate Proteoglycan (CSPG) is a major component of the extracellular matrix in chick embryo brain vesicle cavities, and, as a

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result of their special osmotic properties (Compert and Laurent, 1978), we proposed that the secretion of osmotically active molecules in the brain cavity facilitates water retention inside this closed system; in this way hydrostatic pressure is generated and regulated. We later reported experimental support for this theory, showing that an increase in neural tube fluid CSPG leads to an increase in intra-cavity pressure and to an over-expansion of the brain primordium in both chick (Alonso et al., 1998) and rat embryos (Alonso et al., 1999). In these studies, we reported the presence of at least two proteoglycans, CSPG and Hyaluronic Acid, in neural tube fluid.

The present study, using in vivo digestion with specific enzymes in rat embryos, we attempted to confirm the hypothesis that the proteoglycans of the neural tube fluid are directly involved in the brain's expansive process and in neuroepithelial behaviour.

Materials and methods

"In vitro" rat embryo culture and testicular hyaluronidase treatment

Rat embryos at 9.5 days were explanted in Hank's saline following New's method (1978). After removing Reichert´s membranes, the embryos were placed in glass culture bottles containing 4 ml of heat-inactivated rat serum, 50 I.U. of streptomycin, and a gas phase with 20% oxygen. The bottles were placed in an incubator at 38ºC and rotated continuously. The gas phase was renewed every 12 hours and the culture medium every 24 hours. At 10.7 days of development, 50nl of sterile Hank's solution containing testicular hyaluronidase type I-S from Sigma (an enzyme that digests both chondroitin sulphate and hyaluronic acid) was injected into the midbrain cavity with a micropipette (10 µm inner tip diameter) connected to a microinjector (Medical System Corporation PLI 100) and the embryos were incubated again up to 11.7 days of development. In order to establish the optimal dose of enzyme necessary to avoid their diffusion outside the neural cavity, we previously checked different doses (50, 25, 12.5, 6.25, and 3.12 ng) in several embryos. Finally a dose of 6.25 ng was selected. Thirty embryos were treated with this dose, and the same number of control embryos were injected with a solution of the enzyme inactivated by boiling for 20 min.

Immunohistochemistry

After culture, the embryos were immersed for 4 hours in Carnoy´s fixative at room temperature, followed by dehydration in graded ethanol series and embedding in paraplast. Deparaffinized sections of 8 µm were washed in phosphate buffered saline (PBS), pre-incubated with normal horse serum (1/20 in PBS) and incubated overnight with anti-chondroitin sulphate monoclonal antibody CS-56 (Sigma). After washing twice in PBS, the sections were re-incubated for 30 minutes in fluorescein-conjugated goat antimouse Ig M (Vector lab.) as secondary antibody, mounted in Aquamount (Gurr lab.), and observed under a Zeiss LSCM 310 Laser Confocal Microscope. Control sections were prepared as described above but with pre-immune serum as the primary antibody.

Light Microscopy and Morphometric Analysis

After *in vitro* culture, 8 control and 8 hyaluronidase-treated embryos were removed from the extra-embryonic membranes and fixed for 6 hours in Bouin's fluid at room temperature. After dehydration in graded ethanol series and embedding in paraplast, 8 µm transversal sections were stained with haematoxilin-eosin. In these embryos, brain vesicle volume was calculated from the cranial extremity up to the first section in which the otic vesicle appeared, thus including forebrain, midbrain and part of the hindbrain. In one out of every three sections, the inner and the outer limits of the neuroepithelium were outlined with a Leitz SM Lux microscope equipped with a drawing tube and the corresponding areas were calculated with a Videoplan (Kontron Elektronic GMBH) computerised image-analysis system; a VIDAS 2.1 stereology program was used to integrate the areas. In each embryo, the volume of the brain, 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 (number of assimilated sections). Final volumes were expressed as the arithmetic mean of the measured values ± standard error. As regards the statistical significance of our results, a two-tailed Student´s test for independent samples was applied and p values below 0.01 were interpreted as indicating statistical significance.

Results

Enzymatic digestion of Neural Tube Fluid Proteoglycans

The results of the preliminary study show that intra-cerebral injection of testicular hyaluronidase has a dose-dependent effect on brain enlargement and on embryonic development. Doses over 12.5 ng of the enzyme had a deleterious effect, producing severe alterations in the morphology of most of the embryonic primordia, a significant decrease in growth rate, and a disruption of embryonic curvature. Low doses (under 6.25 ng) of testicular hyaluronidase did not seem to affect overall embryo development; however, no clear signs of brain volume being affected

were observed (data not shown). In addition, an immunohistochemical study with antichondroitin sulphate antibody revealed (Figure 1) that intra-cavity injection of doses of 6.25 ng or less did not affect the intensity on the pattern of CSPG expression in the neuroepithelial wall (Figures 1-A and 1-B); by contrast, doses above 6.25 ng significantly reduced CSPG expression in the neuroepithelium (Figure 1C). These data show that with a 6.25 ng dose there is no significant enzymatic digestion of proteoglycans outside the brain cavity and that neuroepithelial proteoglycans are not affected (Figure 1B): Therefore, the effect of testicular hyaluronidase on brain vesicle development seems to be exclusively due to the digestion of neural tube fluid proteoglycans.

From a morphological point of view, most of the rat embryos injected with a 6.25 ng dose (Figure 2) seemed to achieve a normal degree of development, similar to that of control embryos. However, there was a notable reduction in their cephalic volume with no loss of normal morphology. This dramatic reduction in volume was largely due to a defective expansion of the brain vesicle, apparently affecting the fore and midbrain in a selective manner, although the morphology and the limits between the brain vesicles seemed to be preserved. In treated embryos, the eye primordium was also affected and seemed to have undergone a notable reduction in size.

Morphometric Analysis

With morphometric analysis of the volume of brain vesicles, we attempted to quantify the reduction in the volume of treated embryos and to test the degree of statistical significance of the data obtained. We also attempted to evaluate to what extent variations in volume were due to brain cavity or neuroepithelial expansion. Our

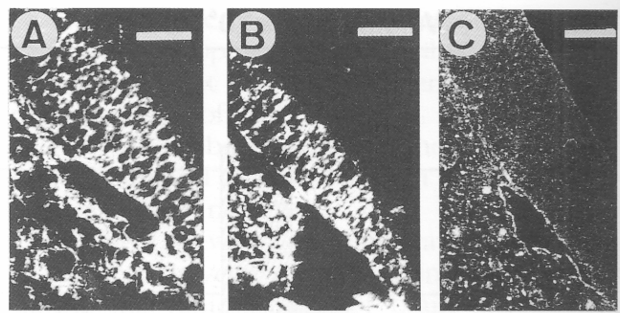

Fig. 1.- Effect of testicular hyaluronidase on the neuroepithelial distribution of chondroitin sulphate. Sections of lateral diencephalic neuroepithelium of a 11.7-day rat embryo immunostained with antichondroitin sulphate monoclonal antibody. **A**: Control embryo showing a diffuse immunolabelling of the extracellular space from the basal to the apical side of the neuroepithelium. **B**: Rat embryo microinjected with a dose of 6.25 ng of testicular hyaluronidase in the brain vesicles. The neuroepithelial wall seems to be thinner than in the control embryos, although the immunostaining pattern of chondroitin sulphate does not undergo significant changes. **C**: Rat embryo microinjected with a dose of 25 ng of testicular hyaluronidase in the brain vesicles, showing enzyme diffusion across the neuroepithelium and the perineural mesenchyme and digestion of most of the chondroitin sulphate. Scale bar: 50 μm in A, B and C.

results (Figure 3) revealed that an injection of testicular hyaluronidase brings about an overall 30% decrease in the rate of brain expansion of the treated embryos with respect to the controls; this was statistically significant ($p<0.01$). As shown in Figure 3, in control embryos 72% of the brain primordium volume was due to the brain cavity and 28% to the neuroepithelial wall. Our results show that the decrease in brain volume induced by testicular hyaluronidase affects both the brain cavity and neuroepithelial volume. However, the decrease in brain cavity size was more intense, reaching 34% with respect to control, and was statistically significant ($p<0.01$). By contrast, neuroepithelial wall volume decrea-

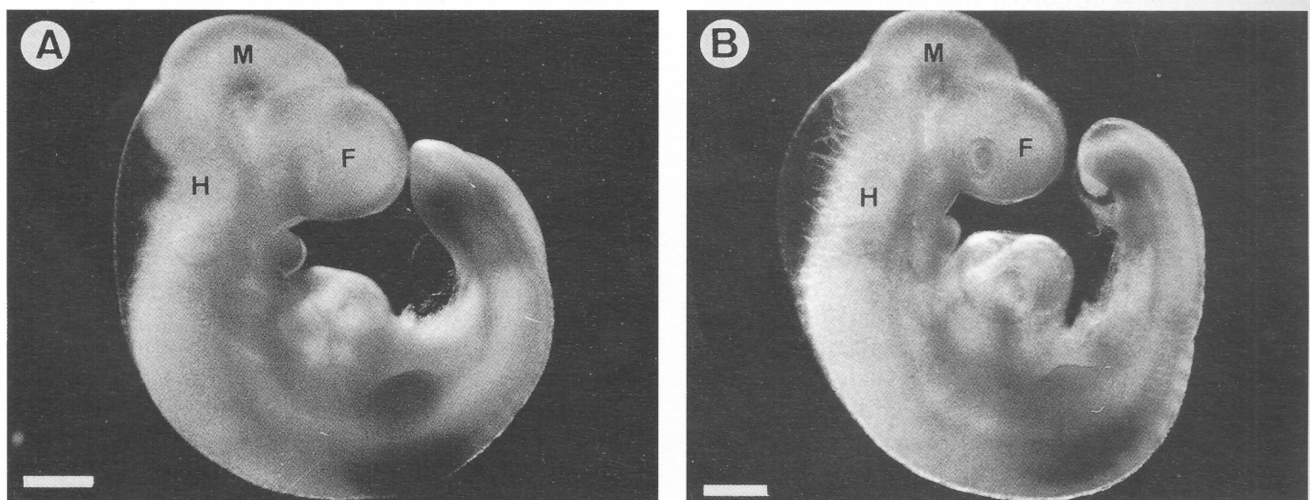

Fig. 2.- Photomicrographs of 11.7-day rat embryos after 24-hour culture in vitro. **A**: Control embryo showing normal morphology and developmental rate. Note the voluminous cephalic end in which fore- (F), mid- (M) and hindbrain (H) can be clearly distinguished. **B**: Embryo treated with testicular hyaluronidase showing a significant decrease in brain enlargement which mainly affects the fore- and midbrain. There are no other evident morphological alterations apart from a reduction in the size of the eye primordia. Scale bar in A and B = 400 μm.

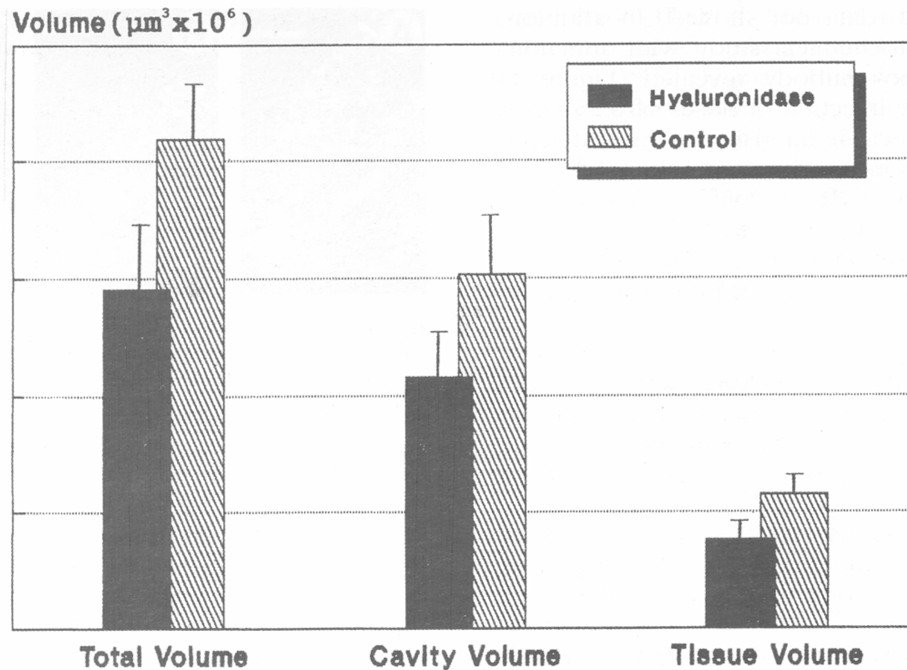

Fig. 3.- Brain size comparison of 11.7-day control and hialuronidase-treated rat embryos shows a significant disruption of brain enlargement. Total brain primordium volume of treated embryos is 30% lower than that of control embryos. This decrease is principally due to brain cavity volume, 34% less than that of the control specimens, while brain tissue mass was only 29% less. Measures are means ± standard error (n=8). In all cases statistical significance ($p<0.01$) was found between control and treated embryos.

sed by 29% and this also was statistically significant ($p<0.01$).

These data indicated that enzymatic digestion of intra-cerebral proteoglycans induces a significant reduction in embryonic brain growth, mainly due to interference in brain cavity expansion.

Histological study

Observation of the histological sections obtained reveals that enzyme treatment appears to affect only the brain primordium, with no apparent disruptions in other primordia of the cephalic extremity of the embryos, as can be seen in Figures 4A and 4B, which correspond to a transversal section at forebrain level. This indicates that the effect of treatment with hyaluronidase was limited to the brain primordium cavity.

Our study demonstrates that two types of disruptions are induced by enzymatic degradation of NTF proteoglycans. First a notable reduction occurs in brain cavity volume among treated embryos in relation to the controls; this reduction was quantified in the above morphometric study. Secondly, alterations were observed in the neuroepithelium of the treated embryos, which at all times were more evident at forebrain and midbrain, as compared with hindbrain levels. In this regard, we observed disruptions both in the structure of the neuroepithelium and in its cellular behaviour. In control embryos, the neuroepithelial structure appeared to be pseudo-stratified, whereas the neuroepithelium of embryos treated with testicular hyaluronidase was remarkably thin in relation to the controls (Figure 4). Occasionally we also observed an apparent loss of pseudo-stratification, with no any alterations in the borders of the neuroepithelium.

As regards its cellular components, the neuroepithelium of control embryos had many mitotic figures at the apex (Figures 4C and E), as is typical of normal development of the neural tube at this stage. By the contrast, embryos treated with testicular hyaluronidase had a dramatically lower number of mitotic figures at neuroepithelium level (Figures 4D and F). This suggests that enzymatic digestion of NTF proteoglycans affects the replication of neuroblasts in brain vesicles. Moreover, the histological sections of the embryos treated with testicular hyaluronidase had abundant pyknotic nuclei (Figures 4D and F) as compared with the control embryos. This might be a reflection of abnormal apoptosis, suggesting the possibility that NTF proteoglycans could also be involved in the survival of cerebral neuroblasts in early developmental stages.

The eye vesicles of the embryos treated with testicular hyaluronidase showed decreased evagination with respect to control embryos (compare Figure 4A and 4B) as well as a reduction in size, an abnormal morphology and a widening of the eye pedicle. We also observed histological alterations in the walls of the eye vesicles similar to those described in the brain neuroepithelium.

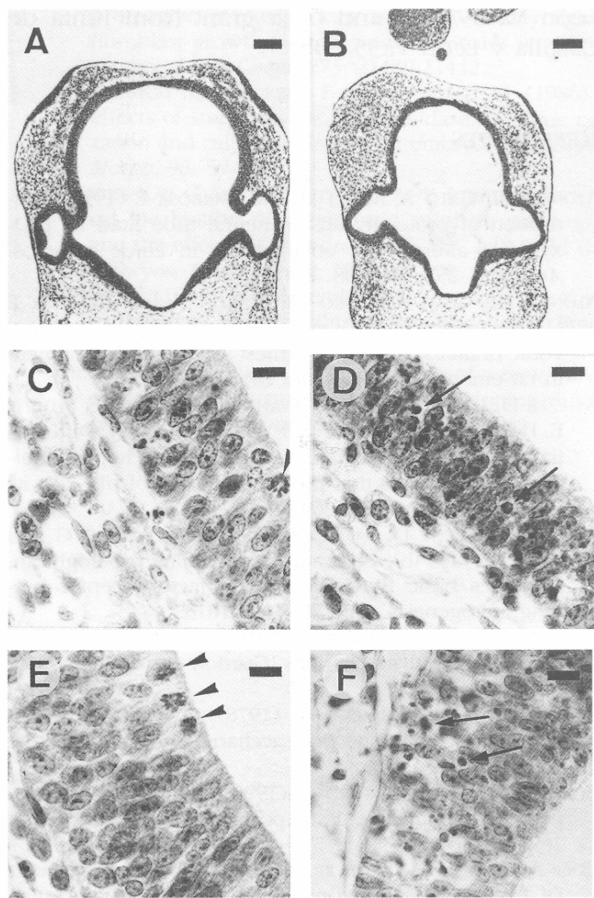

Fig. 4.- Histological comparison of 11.7-day-old rat embryos, at forebrain level, of control (treated with heat-inactivated enzyme) (**A-C-E**) and experimental embryos (treated with 6.25 ng of testicular hyaluronidase) (**B-D-F**). Note the reduction in size of the forebrain vesicle and eye primordium in the treated embryos (B) as compared with controls (A). This reduction is due to a decrease in cavity volume and also in neuroepithelial thickness. In testicular hyaluronidase-treated embryos, it is possible to observe abundant pyknotic nuclei (arrows in D and F) and the absence of mitotic figures, which are abundant in the control embryos (arrowheads in C and E). Scale bar in A and B: 200 µm, and in C, D, E and F: 20 µm.

Discussion

Since the eighties, no scientific studies have been carried out in order to clarify the intrinsic mechanisms involved in the regulation of brain expansion during the earliest stages of development, although Jacobson (1991) and, more recently, Van Essen (1997) have claimed that intra-cavity pressure is the basis of several relevant aspects of Central Nervous System development. An understanding of these mechanisms could help to throw light on the origin and subsequent evolution of different brain malformations (Desmond, 1985; Copp et al., 1990).

Previous studies have shown that brain growth is mainly due to the expansion of its cavity (Desmond and Jacobson, 1977), positive pressure of the neural tube fluid being a key factor in this process (Jelineck and Pexieder, 1968).

The presence of CSPG in the earliest stages of rat brain development was described by Morriss-Kay and Tucket (1989), who demonstrated that the synthesis of CSPG by the neuroepithelial cells in rat embryos starts at the beginning of neurulation and increases to high levels after closure of the neural tube.

We have previously demonstrated the presence of proteoglycans in the brain vesicle cavity during the rapid period of brain enlargement that follows to neurulation, and described their involvement in regulating this process in chick and rat embryos (Gato et al., 1993; Alonso et al., 1998; Alonso et al., 1999).

Proteoglycans have been reported to be key molecules in the regulation of the water content of embryonic tissues as a result of their polyanionic nature, which affords them special osmotic properties. Chondroitin sulphate and Hyaluronic Acid have the greatest osmotic capacity in aqueous solutions, and relatively small variations in concentration produce large increases in this capacity (Compert and Laurent, 1978; Compert, 1981; and Compert and Zamparo, 1990). At the stages of development studied here, the brain vesicles constitute a hermetic system, inside which variations in the osmolarity of the fluid contained may lead to changes in water content and to a subsequent modification in pressure and/or brain vesicle expansion. However, the osmotic properties of proteoglycans in biological tissues to a large extent depend on their functional relation with inorganic micro-ions, of which sodium seems to be the most abundant in the neural tube fluid of chick embryos (Alonso et al., 1998). Undoubtedly, the composition and developmental process of neural tube fluid is a matter that requires further investigation.

Our results show that enzymatic digestion of intra-cavity proteoglycans with no apparent degradation of neuroepithelial proteoglycans produces a significant decrease in brain enlargement with no serious alterations in its morphogenesis. This supports the hypothesis that, in rat embryos, a direct relationship exists between intra-cavity proteoglycans and brain expansion.

In addition, the presence of other proteoglycans such as Heparan Sulphate Proteoglycan inside the hindbrain cavity has been described (Ojeda and Piedra, 2000) as part of an extracellular matrix capable of acting as a mechanical support element and perhaps one which regulates neuroblast behaviour. Moreover, previous electrophoretic studies undertaken at our laboratory (Gato et al., 1998) showed, that NTF has a complex protein make up that includes molecules involved in inter-cellular communication phenomena, such as growth factors. All these data suggest that, regardless of its role in the enlargement of brain vesicles, NTF may have a significant part to play in regulating the behaviour of neuroepithelial cells.

As mentioned above, testicular hyaluronidase, which degrades hyaluronic acid and chondroitin sulphate, produces a decrease in total neuroepithelial volume that is accompanied by a decrease in mitotic figures and the presence of apoptotic nuclei in the neuroepithelial wall. In this regard, it has been suggested that certain proteoglycans might be involved in regulating the proliferation of neuroblasts in the early stages of development. This regulation process could occur either by means of direct action on cell replication (Morris-Kay et al., 1986; Ruoslathi, 1989) or via its relationship with growth-factor molecules. It is known that proteoglycans can bind to several growth factors, modulating their activity (Goetinck, 1991; Ruoslahti and Yamaguchi, 1991; Aviezer et al., 1994 a,b; Johnson and Wong, 1994; Schuger et al., 1996; David and Bernfield, 1998 and Milev et al., 1998). This interaction has been studied mainly for fibroblast growth factors (FGFs) and several mechanisms have been proposed (Jaakkola and Jalkanen, 1999) to explain how HSPG may modulate the action of the FGFs. First, the glycosaminoglycans in proteoglycans may induce oligomerization of FGF molecules required for fibroblast growth factor clustering. Second, proteoglycans could protect FGFs from proteolytic degradation and serve as a reservoir for these growth factors. Third, HSPG could simply serve as molecules that have the ability to present FGFs to high-affinity receptors. Finally, it has been proposed that the main function of proteoglycans is to reduce the dimensionality of FGF diffusion. This might explain how enzymatic digestion of NTF proteoglycans is able to alter the mitotic pattern of neuroepithelial cells and, consequently, brain primordium tissue volume, as well as to influence the survival of neuroblasts. This would therefore explain the abundance of pyknotic nuclei, which indicate abnormal apoptosis.

The alterations described at eye primordium level in embryos treated with testicular hyaluronidase perhaps respond to the same mechanisms mentioned previously. At these stages of development, the eye vesicles depend on the forebrain and are directly communicated with its cavity and, hence, with the NTF.

In conclusion, our results lend weight to the hypothesis that the proteoglycans in the NTF are involved in the enlargement of brain vesicles and in the survival and proliferation of neuroblasts during the early stages of rat embryonic development.

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