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Chondroitin sulphate proteoglycan and embryonic brain enlargement in the chick

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Abstract. Previous studies of the early development of the neural tube have shown the existence of an intraneural fluid, which causes a positive pressure inside this primordium, and seems to play a key role in the early development of the central nervous system. In the present study we investigated the composition and synthesis of this intraneural fluid. By using a sequential method, which includes fixation with glutaraldehyde plus cetylpyridinium chloride, opening the neural cavity after critical point drying and scanning electron microscopy analysis, we found a water-soluble extracellular matrix that filled up the brain vesicles of chick embryos at the earliest stages of the neural tube. An ultrastructural study of the neural epithelium during these stages revealed the existence of a secretion process in the neural cells toward the apical side, the future neural cavity. An immunocytochemical study to asses the nature of the secreted material has shown that the intraneural matrix contains chondroitin sulphate proteoglycan, which appeared homogenously distributed throughout the neural cavity. Our findings demonstrate that the intraneural liquid is a fluid of complex composition and includes chondroitin sulphate proteoglycan as an osmotically active molecule. This suggests a morphogenetic role for the proteoglycan during early brain enlargement. The neural ectoderm is a polarized epithelium from early developmental stages and secretes the intraneural matrix.

Key words: Neural tube – Extracellular matrix – Proteoglycans – Brain development – Chick embryo

Introduction

Brain formation begins early in development with neurulation, a process leading to the formation of the neural tube (Schoenwolf and Smith 1990). After closure of the anterior neuropore and occlusion of the spinal cord lumen at stage 12 (Schoenwolf and Desmond 1984), neurulation is followed by a period of rapid growth and enlargement at the anterior end of the neural tube. This period of brain development is characterized by a large increase in size of the neural vesicles, about 30-fold in only 48 h. About 30% of this increase is due to a change in tissue volume, and 70% to an increase in cavity volume (Desmond and Jacobson 1977). Although the morphological events involved in the formation and enlargement of the neural vesicles are well known, the morphogenetic mechanisms which support these processes are not well understood.

It has been shown that the neural vesicles are filled with a fluid, namely neural tube fluid (NTF), which exerts a positive pressure against the neural wall (Jelineck and Pexieder 1968, 1970). The experimental decrease of this pressure leads to severe dysmorphogenesis of the anterior end of the neural tube, including abnormal epithelial folding and collapse of the developing neural cavity. This suggest a morphogenetic role for the NTF (Pexieder and Jelineck 1970; Desmond and Jacobson 1977). Moreover in other embryonic primordia, such as the inner ear and eye, it is been shown that the fluid filling the embryonic cavities is related to growth and enlargement of the rudiments (Represa et al. 1986; Coulombre 1956). Nevertheless, the biochemical composition of the NTF remains to be elucidated.

It has been proposed that NTF may be secreted from the neuroepithelial cells lining the neural tube cavity, instead of being amniotic fluid trapped in the cavity after neurulation (Jelineck and Pexieder 1968). Recent observations of the otic vesicle and the neural tube show that the ectodermal wall is involved in transporting ions and water toward the embryonic cavities (Barbosa et al. 1985; Giraldez et al. 1987, and unpublished observation from the authors). However, there is as yet no ultrastructural evidence of a secretory activity in the neural wall during this period of active growth and enlargement.

Materials and methods

Chick embryos between the stages 12 and 24 H.H. (Hamburger and Hamilton 1951) were used.

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Scanning electron microscopy (SEM). We used 40 embryos at stage 23 (10 embryos for each fixative) and 4 embryos for each of the remaining stages ranging from 12 to 24 for SEM. Embryos were freed from extra-embryonic membranes and then immersed for 24 h at room temperature in: (1) 4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4; or (2) 4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and 0.5% cetylpyridinium chloride (CPC); or (3) Bouin's fluid; or (4) Carnoy's fixative. After fixation, the embryos were dehydrated in graded ethanols and critical point dried from CO_2, after which a window was opened in the mesencephalic vesicle with tungsten needles. Specimens were then sputter-coated with gold and viewed with a JEOL T300 Scanning Electron Microscope. For each different fixative, dehydration and critical point drying were also applied to previously opened neural tubes, as controls.

Transmision electron microscopy (TEM). Three embryos from alternate stages ranging from 12 to 24 were fixed overnight at room temperature in Karnosky's fixative with 2% tannic acid, according to the methods of Singley and Solursh (1980), and then postfixed in 1% osmium tetroxide (1 h at room temperature). The embryos were dehydrated in a graded series of acetones and embedded in plastic (Spurr 1969). Transverse thin sections were taken at various levels along the brain vesicles from embryos at different development stages. Sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined under a JEOL 1200EX electron microscope at 80 kV.

Immunohistochemistry. Three embryos of alternate stages ranging from 12 to 24 were fixed for 24 h in Carnoy's fixative, dehydrated in ethanol and embedded in Paraplast. Immunohistochemistry was carried out in 5-μm sections taken from different brain vesicles at each stage. Sections were deparaffinized, washed in phosphate-buffered saline (PBS), preincubated with normal horse serum (1/20 in PBS) and incubated overnight with an anti-chondroitin sulphate monoclonal antibody (CS-56 from Sigma). After washing twice in PBS, sections were reincubated in a secondary antibody (fluorescein-conjugated goat antimouse Ig M from Vector Lab.), for 30 min. Sections were mounted in Aquamount (Gurr Lab.) and viewed with a Leitz Laborlux 12 microscope with a fluorescence attachment. Control sections were prepared as above but substituting for the primary antibody with preimmune serum.

Results

Scanning electron microscopy

Our findings revealed the existence of an abundant precipitated material occupying the neural cavity in all the embryonic stages studied (Fig. 1d, h shows embryos of stages 13 and 23). This material appeared when the cavity was opened after critical point drying, according with out method illustrated in Fig. 1b. In contrast, the normal processing for SEM in the control embryos, with opening of the neural tube cavity after fixation, leads to the loss of the intraneural matrix (Fig. 1a).

The quantity and placement of the precipitated material in the neural cavity varies considerably, depending on the fixative used. The addition of CPC to glutaraldehyde substantially improved the preservation of such material (Figs. 1c, d). The utilization of other fixatives, such as Bouin's fluid (Fig. 1e) or Carnoy's fixative (Fig. 1f) partially preserved the material; however, even in this case, clear signs of material retraction are present.

Observation at high magnification of the material precipitated by the glutaraldehyde-CPC in the mesencephalic cavity (Fig. 1g) reveals the existence of a structure similar to a matrix, formed by a threedimensional fibrous net, to which abundant formations of a floccule appearance adhere. This resembles very closely the appearance of water-soluble matrices in other embryonic primordia (Bard and Abbott 1979).

Transmision electron microscopy

Ultrastructural study of the epithelial cells of the cerebral vesicles, during the embryonic stages studied, reveals the existence of the following morphological features. At the apical pole of the cells (Fig. 2), numerous vesicles of large size (approximately 500 nm in diameter) can be seen grouped together near the cell surface (Fig. 2a, c). These large vesicles were bounded by a membrane, and their content was moderately electron dense. Occasionally, the lumen of the vesicles was seen in wide communication with the neural cavity, which may reflect their opening towards this cavity. We also detected the presence of other vesicular formations of smaller size (diameter approximately 75 nm), whose membranes present a high electron density and an electron-lucent interior (Fig. 2b). These smaller vesicles are often clatharin-coated and usually considered endocytotic.

A further ultrastructural characteristic of the neuroepithelial wall is the presence of abundant intracellular membranes at the apical end of the cells. These appear to be mainly Golgi apparatus (Fig. 2c).

The material precipitated in the cavity appears with TEM as a series of spherical formations, with an electron-dense wall and electron-lucent interior; these formations are connected to fibrous material (Fig. 2d).

Immunohistochemistry

Positive chondroitin sulphate proteoglycan (CSPG) immunoreactivity was detected with the CS-56 antibody labelling in the neuroepithelial wall and the intraneural matrix (Fig. 3). The epitrope bound by CS-56 antibody is associated with the glycosaminoglycan moieties of native CSPG (Avuur and Geiger 1984). CSPG-like immunoreactivity shows the presence of CSPG, as a component widely distributed in the intracavity matrix of the cerebral vesicles in all the stages studied. The morphological pattern of arrangement of CSPG immunoreactivity consists of abundant formations of a flocculent aspect (Fig. 3c), which resemble the arrangement observed by SEM. CSPG-like immunoreactivity was also detected within the neuroepithelium of the brain vesicles, with higher positive immunolabelling located at the apical end of the cells (Fig. 3c).

Discussion

We have shown that the neural vesicles of the chick embryo contain water-soluble extracellular matrix that is precipitated by fixation and contains chondroitin sul-

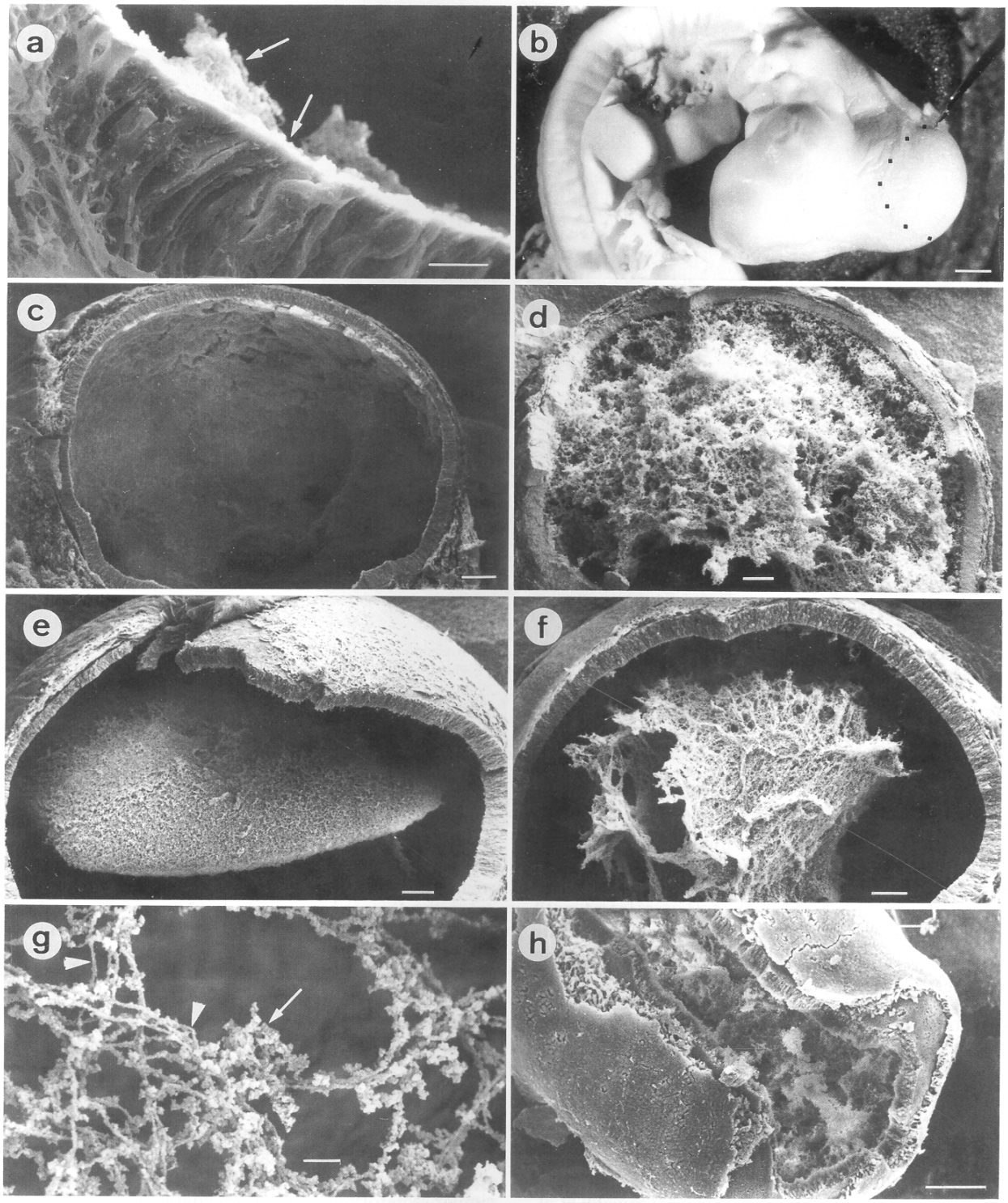

Fig. 1 a–h. Photomicrographs from stage 23 (**a, b, c, d, e, f, g**) and stage 13 (**h**) chick embryos processed for SEM. The existence of a precipitated material occupying the neural cavity can be seen in all the embryos in which the cavity was opened after the critical point drying (**c, d, e, f, g, h**). In the control embryos, with opening of the N.T. cavity inmediately after fixation, the loss of the intraneural matrix can be observed (**a**). Circle of dots in **b** shows the dissection line for opening the neural cavity. Notice that fixation with cetylpyridinium chloride (CPC) plus glutaraldehyde improves the preservation of intraneural material (**d**). Results from other fixatives, i.e. glutaraldehyde alone, Bouin's fluid and Carnoy's fixative are illustrated in **c**, **e** and **f** respectively. **g** The material precipitated by the Gluta-CPC in the mesencephalic cavity is shown at high magnification. It is formed by a three-dimensional fibrous net (*arrowhead*) and abundant formations of a flocculant appearance (*arrow*). Bars 10 μm (**a**), 500 μm (**b**), 100 μm (**c, d, e, f, h**) and 1 μm (**g**)

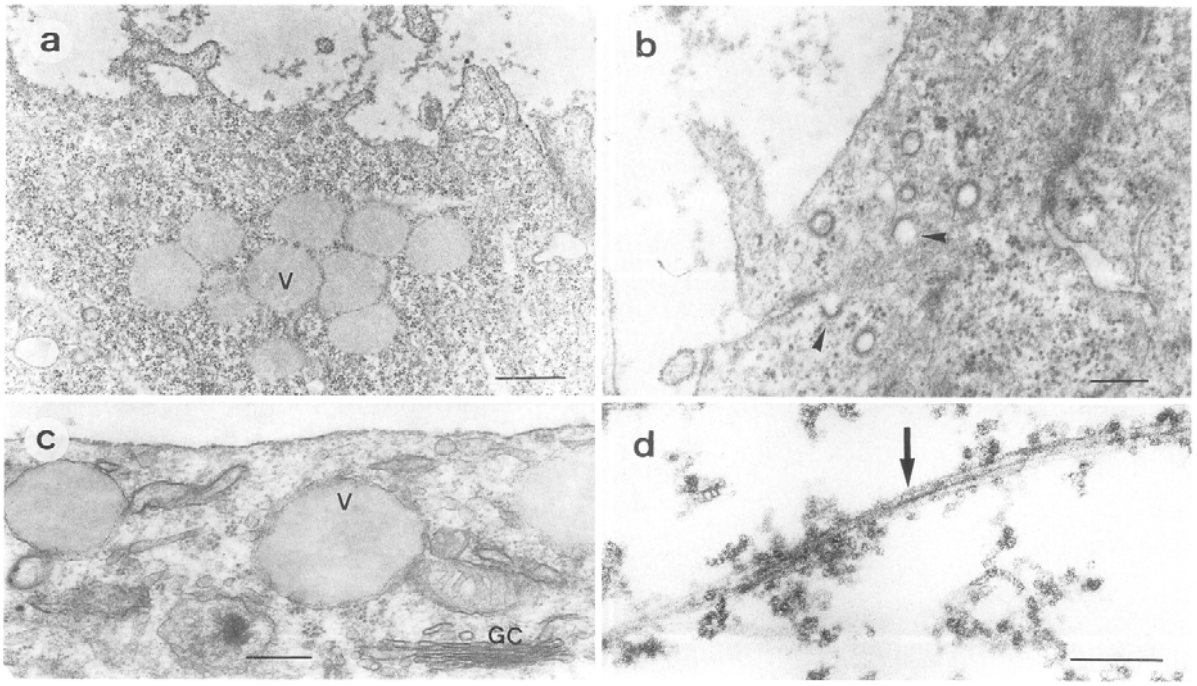

Fig. 2a–d. Transmision electron micrographs of neural epithelium from stage 16 (**a**), stage 13 (**b**) and stage 23 (**c**) chick embryos fixed in Karnovsky's with 2% tannic acid. Notice the presence of large vesicles (*V*) localized at the apical pole of the cells (**a, c**). The presence of other vesicular formations of small size and electron-lucent interior can also be observed (**b**, *arrows*), as well as an abundant Golgi complex (*GC*). The micrograph in **d** shows a high-power view of the intraneural flocculant material in a stage 23 chick embryo. This appears in addition to fibrous formations, and may be attached to them (*arrow*). Bars 500 nm (**a**), 200 nm (**b, d**) and 250 nm (**c**)

phate proteoglycan (CSPG). This CSPG-containing matrix forms part of the intraneural fluid.

Scanning electron microscopy shows that for the appropriate preservation of the intraneural matrix, the walls of the cerebral vesicles must remain intact until after the critical point drying. Such a procedure prevents the matrix from being lost by fluid interchanges during the process of dehydration (Waterman 1980). The degree of preservation of the matrix depends upon the biochemical features of the fixatives. The improvement in the preservation of the matrix with CPC suggests that most of the matrix components possess a marked polyanionic character (Scott 1955). The use of this fixative was foreseen by Waterman in 1980, for the preservation of water-soluble matrix moieties not conjugated by aldehyde fixatives. Similarly, it has been employed widely in the preservation of the different matrices and surface coat materials during embryonic development (Markwald et al. 1978; Bard and Abbott 1979; Hilfer and Yang 1980; Sinning and Olson 1988).

Transmission electron microscopy revealed an abundant number of large vesicles and Golgi apparatus polarized towards the apical side in the neuroepithelial cells. This ultrastructural morphology is compatible with a secretory activity in the neural epithelium. Moreover, these ultrastructural features detected at the apical pole of neural cells resemble very closely those described by Hascall et al. (1991) during the process of active secretion of proteoglycans in chondrocytes. On the other hand, the presence at the apical pole of neural cells of small vesicles with intensely stained membranes calls to mind the "coated vesicles" that are often considered endocytotic. These are thought to be a compensatory recuperation mechanism of intracellular membranes, characteristic of cells that have an intense secretory activity (Herzog and Farquhar 1977; Snider and Rogers 1986).

Although the ultrastructural features by themselves do not prove the existence of a secretory process, taken together with the presence of chondroitin sulphate proteoglycan immunoreactivity at the apical side of the neuroepithelium and also in the neural cavity, they suggest that the neuroepithelial cells may be responsible for secreting this proteoglycan. Other authors have described similar secretory ultrastructural characteristics in the epithelium of the neural folds prior to their fusion (Mak 1978) and coinciding with the appearance of a surface coat material rich in glycoconjugates (Smits-Van Prooije 1986).

Chondroitin sulphate is a proteoglycan whose elevated osmotic power enables it to retain large quantities of water (Compert and Laurent 1978; Compert and Zamparo 1990). In this respect, numerous matrices containing proteoglycans have been implicated, during em-

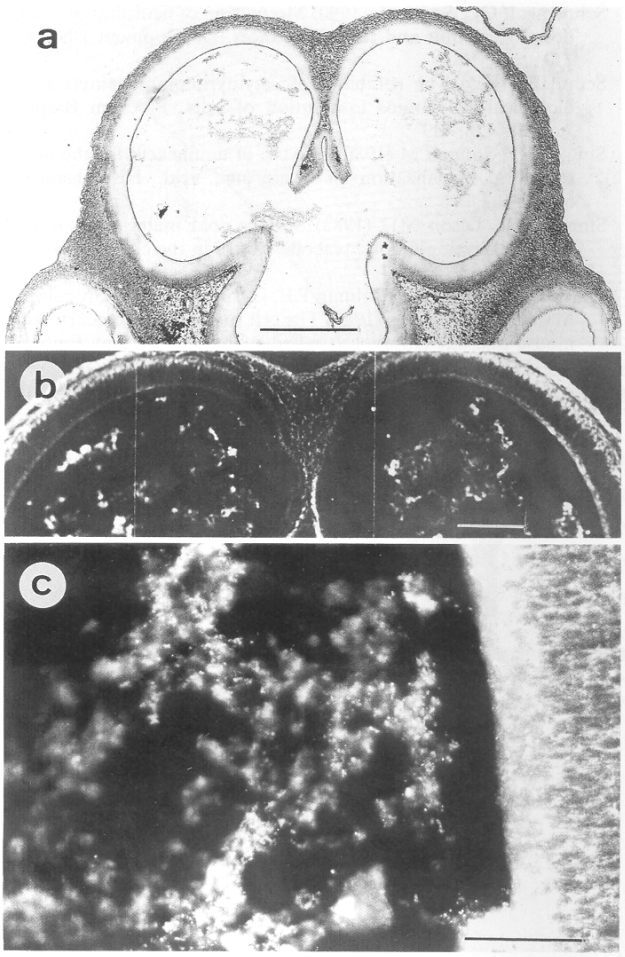

Fig. 3a–c. Micrographs from transverse sections through the prosencephalic vesicles of a stage 23 chick embryo. Both phase contrast (**a**) and FITC fluorescence (**b, c**) are shown. Positive chondroitin sulphate proteoglycan (CSPG) immunoreactivity can be seen labelling the intraneural matrix as well as the neural epithelial walls. Notice that CSPG-like immunoreactivity is higher at the apical ends of neuroepithelial cells. Bars 500 μm (**a**), 250 μm (**b**), and 50 μm (**c**)

bryonic development, in the creation of propellent forces with morphogenetic character, as in the formation of the semicircular canals of the inner ear (Haddon and Lewis 1991), or in the cephalic fold of the neuroepithelial walls during neurulation (Morris and Solursh 1978). Likewise, the growth of embryonic cavity structures based on the expansive force created by intracavity PG has been suggested in the vitreous (Coulombre 1956) and the anterior cavity of the eye (Bard and Abbott 1979). The presence of CSPG in the cavity of the cerebral vesicles could, therefore, play a decisive role in the formation of neural tube fluid.

Although direct physiological evidence of secretion is still lacking our data suggest that the secretory activity of the neuroepithelial cells extends over the whole neural epithelium of the cerebral vesicles during the rapid period of brain enlargement. Our results contribute to the clarification of the origin and composition of NTF. We propose that this fluid is generated by the neuroepithelial secretion of osmotically active compounds towards the cavity of the cerebral vesicles and is capable of trapping within them a volume of water with sufficient pressure to maintain the neuroepithelial walls in a state of continuous expansion. The presence of CSPG in the intracavity matrix, due to its physical characteristic, could be mainly responsible for this osmotic mechanism; however, the existence of other compounds also implicated in this process cannot be discounted.

Cerebral morphogenesis, in early stages, depends to a large degree on mechanisms that lie in the cells themselves, such as cell proliferation. However, our results suggest that there is an additional mechanism that involves the cavity and its contents. This mechanism may be coordinated with the cell proliferation and migration taking place in the wall of the neural vesicles to build up the three-dimensional shape of the early developing brain.

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