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Chondroitin Sulphate-Mediated Fusion of Brain Neural Folds in Rat Embryos

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Key Words
Rat embryos · Neural tube closure · Chondroitin sulphate · Epithelial fusion · Anterior neuropore

Abstract
Previous studies have demonstrated that during neural fold fusion in different species, an apical extracellular material rich in glycoconjugates is involved. However, the composition and the biological role of this material remain undetermined. In this paper, we show that this extracellular matrix in rat increases notably prior to contact between the neural folds, suggesting the dynamic behaviour of the secretory process. Immunostaining has allowed us to demonstrate that this extracellular matrix contains chondroitin sulphate proteoglycan (CSPG), with a spatio-temporal distribution pattern, suggesting a direct relationship with the process of adhesion. The degree of CSPG involvement in cephalic neural fold fusion in rat embryos was determined by treatment with specific glycosidases. In vitro rat embryo culture and microinjection techniques were employed to carry out selective digestion, with chondroitinase AC, of the CSPG on the apical surface of the neural folds; this was done immediately prior to the bonding of the cephalic neural folds. In all the treated embryos, cephalic defects of neural fold fusion could be detected. These results show that CSPG plays an important role in the fusion of the cephalic neural folds in rat embryos, which implies that this proteoglycan could be involved in cellular recognition and adhesion.

Introduction

Neural fold fusion is a key step between primary neurulation and neural tube formation. Although many congenital malformations of the central nervous system, such as anencephaly, spina bifida and other dysraphic defects, appear to derive from a defective process of neural fold fusion, the developmental mechanisms involved still remain largely unknown.

Macroscopically, neural fold fusion is a non-homogeneous process. It starts at several specific points of de

Abbreviations used in this paper

CSPG	chondroitin sulphate proteoglycan
HSPG	heparan sulphate proteoglycan
PBS	phosphate-buffered saline
SEM	scanning electron microscopy

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novo contact between both folds, the number and location of which are species specific, and progresses cranially and caudally. The last areas of fusion are the cranial and caudal extremity of the embryo, classically termed 'cranial and caudal neuropores'. However, today, the existence of 2 cranial neuropores has been shown in mammals [Golden and Chernoff, 1993].

At brain level, fusion of the neural folds requires prior concurrency, by shaping and bending, to approximate both neural tips. Thereafter, specific changes take place on the surface of the apical cells involved in the processes of recognition and adherence of cells in both folds. Several studies have explored changes in cellular polarity, specific cell recognition, changes in cellular adhesion molecules, cellular migration and reorganisation, cellular apoptosis and other mechanisms [Waterman, 1975; Geelen and Langman, 1979; Copp et al., 1990; Hoving et al., 1990; Fleming et al., 1997; Colas and Schoenwolf, 2001]. Yet the relevance of such mechanisms needs to be supported by experimental evidence. At brain level, one of the changes described as occurring prior to fusion affects both the surface of the cells about to fuse (the appearance of cytoplasmic prolongations of the same type as the pseudopodia and extracellular vesicles), as well as their internal morphology (the appearance of many apical intracellular vesicles) [Mak, 1978]; similar changes have been reported during palatal fusion [Souchon, 1975; Schupbach et al., 1983; Taya et al., 1999; Martínez-Álvarez et al., 2000a, b]. Furthermore, there has been a report on an extracellular matrix interposed between the apical neural folds immediately prior to contact between the neural folds on both sides [Mak, 1978; Sadler, 1978]. The presence of this surface coat material in the fusion of the neural folds has been shown to be a phylogenetic constant, having been described in the embryos of amphibians [Rice and Moran, 1977; Mak, 1978], birds [Lee et al., 1976; Rovasio and Monis, 1981; Schoenwolf and Fisher, 1983] and mammals [Sadler, 1978].

This material, which is apparently secreted by the neuroblasts which are themselves about to fuse, has been proposed as a key factor in the fusion process, acting as a transitory linkage medium between both neural folds while definitive cellular joining is established [Sadler, 1978]. Other authors have suggested that this apical surface coat material could play a role in specific cellular recognition between fusing cells [Smits-van Prooije et al., 1986]. Moreover, the fusion processes between epithelial cells are common at early stages of embryo development, and the presence of a cell coat material on the apical surface of the cells about to join has also been reported during formation of the palate [Greene and Pratt, 1976], internal and external nasal processes [Geelen and Langman, 1977], the otocyst [Sinning and Olson, 1988] and the crystalline vesicle [Van Rybroek and Olson, 1981].

The precise nature of such a cell surface coat-mediating fusion of the neural folds remains to be determined. However, a glycosidic composition has been suggested, and various studies have been carried out with cationic dyes or lectins showing the glycosidic nature of this material [Lee et al., 1976; Sadler, 1978; Currie et al., 1984; Smits-van Prooije et al., 1986; Wilson and Wyatt, 1995]. In addition, experimental studies conducted involving enzymatic digestion with glycosidases [Schoenwolf and Fisher, 1983; Morriss-Kay et al., 1986; Morriss-Kay and Tuckett, 1989; Tuckett and Morriss-Kay, 1989] or with inhibitors of the synthesis of proteoglycans [Morriss-Kay and Tuckett, 1989; Alonso et al., 1999] have shown that the disruption of the glycoconjugated components during neurulation causes severe defects in neural tube closure. However, there are no studies to identify specific molecules involved in the neural fold fusion process. On the other hand, we have previously demonstrated the involvement in palatal fusion [Gato et al., 2002] and the crystalline vesicle [Gato et al., 2001] of a CSPG-rich extracellular matrix coating the epithelial apical surface.

The above findings lead to the hypothesis that sulphated proteoglycans of the chondroitin sulphate type could be a component of the surface coat material in the cells of the apical neural folds, and that they might play an important role during neural tube fusion.

Material and Methods

In vitro Rat Embryo Culture

Wistar strain rats were mated overnight and examined for a sperm-positive vaginal plug the following morning, which was considered gestational day 1. Rat embryos, 10.2 days old, in which neural fold elevation was evident, were explanted in Hanks' saline following the method by New [1978]. After removing Reichert's membranes, the embryos were placed in glass culture bottles containing 4 ml of heat-inactivated rat serum, 50 IU of streptomycin, and a gas phase with 20% oxygen. The bottles were placed in an incubator at 37°C and rotated continuously. The gas phase was renewed every 12 h and the culture medium every 24 h. By periodic visual monitoring, we selected embryos, in which convergence and fusion of the neural folds had begun at midbrain level, in order to avoid interferences in other neurulation mechanisms previous to neural fold fusion. These embryos were injected in the amniotic cavity with a micropipette (10 μm inner tip diameter), connected to a microinjector (Medical System Corporation PLI 100), with 40 nl of a solution of chondroitinase AC (5 IU in 250 μl of phosphate-buffered saline, PBS; Sigma), which specifically di-

gests chondroitin sulphate A and C but not its epimere dermatan sulphate, or streptomyces hyaluronidase type IX (523 units in 200 μl of PBS; Sigma), which specifically digests hyaluronic acid. (The substrate specificity of both enzymes is supported by Sigma manufacturer specifications and by Beeley, 1985.) We had previously verified that these amounts of enzymes are sufficient to digest the proteoglycans of the embryonic tissues [Alonso et al., 2000]. Injection of the enzymes was additionally repeated twice at an interval of 2.5 h in order to protect enzymatic activity, and the embryos were re-incubated up to 11.7 days of development. In order to evaluate non-specific changes in the composition of the surface extracellular matrix shortly after enzymatic treatment (not detectable after 36 h of culture), we did short cultures (8 h) with a single enzymatic dosage in the same conditions as described before; we evaluated the presence of ubiquitous components of extracellular matrix in the fusion area other than chondroitin sulphate, as described below.

A total of 44 embryos were treated with chondroitinase AC, the same number being injected with a solution of the enzyme inactivated by boiling for 20 min, and 25 embryos were treated with streptomyces hyaluronidase.

Following culture, the embryos were fixed and then prepared for scanning electron microscopy (SEM), histology and immunochemistry.

Scanning Electron Microscopy

Subsequent to fixing in Carnoy's fixative, the embryos were dehydrated in acetone and dried with liquid CO_2 at the critical point in a Balzers CPD device. Once the embryos had been dried they were mounted on a metal pedestal and covered with gold film with a Balzers SCD-310 system. A Jeol T-300 scanning electron microscope was used to visualise and photograph the samples.

Histological Study

The embryos were fixed for 4 h in Carnoy's fixative at room temperature, dehydrated in a graded ethanol series and embedded in paraplast. Deparaffinised sections of 8 μm length were stained with haematoxylin-eosin according to standard procedure.

Immunohistochemistry

The embryos were immersed 4 h in Carnoy's fixative, which preserves the proteoglycans of the extracellular matrix [Gato et al., 1993] at room temperature, dehydrated in a graded ethanol series and embedded in paraplast. Deparaffinised sections of 8 μm length were washed in PBS, pre-incubated with normal horse serum (1/20 in PBS) and incubated overnight with an anti-chondroitin sulphate monoclonal antibody (CS-56, Sigma). After 2 washings in PBS, the sections were re-incubated for 30 min in fluorescein-conjugated goat antimouse IgM (Vector) as a secondary antibody, mounted in Aquamount (Gurr) 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.

Occasionally, we pre-incubated histological sections with normal horse serum (1/20 in PBS) and incubated them overnight with antifibronectin polyclonal antibody (Sigma), anticollagen type VI monoclonal antibody (Serotec) and antiheparan suphate monoclonal antibody (Upstate Biotechnology). After 2 washings in PBS, the sections were incubated for 30 min in fluorescein-conjugated secondary antibody and processed as described above.

Immunolabelling of Whole Embryos

Embryos fixed in Carnoy's solution for 2 h at room temperature were washed and continuously agitated 3 times, for 5 min each time, in a 0.05% solution of PBS-Tween 20. As a blocking solution, we used 1% PBS-BSA (Sigma) for 20 min at room temperature.

The embryos were incubated with an anti-chondroitin sulphate antibody (CS-56, Sigma; 1:200 in 0.1% PBS-Tween 20) all night long at room temperature. Following this, they were washed and continuously agitated 3 times in succession, for 5 min each time, in 0.05% PBS-Tween 20, and then incubated for 2 h with an anti-mouse IgM FITC conjugated antibody (Sigma; 1:128 in 0.1% PBS-Tween 20) at room temperature in the dark.

After 3 successive washes in PBS, the embryos were made transparent in glycerol and visualised with a Zeiss LSM-310 confocal microscope.

Detection of Apoptotic Cells

Apoptotic cells were detected by the TUNEL assay on paraffin sections from formalin-fixed embryos. Apoptotic cells were detected using the Apoptosis Detection System Fluorescein Kit (Promega) following the manufacturer's instructions. Visualisation was made with a confocal microscope (Zeiss LSM-310). A quantitative analysis was performed by counting the number of apoptotic nuclei in 8 microscopic fields of 1,400 μm^2 in each experimental condition. The average of each condition and the standard error were plotted (table 2), and their significance was tested by a 2-tailed Student's t test.

BrdU Incorporation

Occasionally, we did short cultures in which the embryos were exposed to BrdU by microinjection in the vitelline cavity (2 μl of a 10-mM solution in PBS) during the last hour of culture, in order to evaluate their DNA synthetic activity. BrdU was detected following standard procedures: sections were incubated in a solution containing a monoclonal antibody to BrdU (Dako) at 1/100 for 30 min. To detect the primary antibody, we used the avidin-extravidin system conjugated to peroxidase (mouse anti-rabbit 1/20 for 30 min and extravidin 1/20 for 10 min; Sigma), and staining was undertaken with DAB. A quantitative analysis of nuclear BrdU incorporation was performed by counting the number of BrdU-positive nuclei in 20 microscopic fields of 1,400 μm^2. The average of each condition and the standard error were plotted (table 2) and their significance was tested by a 2-tailed Student's t test.

Results

Study of the Extracellular Material in the Fusion Area with SEM

At the forebrain level of rat embryos, convergence and apposition of the neural folds took place between 10.5 and 10.9 days of development; consequently, at 11 days, neural tube fusion was complete.

Our study with SEM in rat embryos showed that prior to neural fold apposition in the forebrain, the thin layer of extracellular material which covered the apical surface of the cells, as previously described (fig. 1a, b), underwent a notable increase before initial contact, forming an abundant extracellular matrix interposed between both neural folds (fig. 1c, d).

In the areas of forebrain neural folds where preservation of the matrix had not taken place, certain protruding cellular formations could be seen on the apical surface of the cells (fig. 1b, d), which, due to their morphological appearance, might coincide with the apoptotic cells detected by TUNEL (fig. 2i).

In the regions where apposition between both neural folds had commenced, this extracellular matrix was not preserved (fig. 1e), and in the depth of the fusion zone, numerous filament-like bridges appeared between the apical surfaces of the neuroepithelial cells of both neural folds (fig. 1f), suggesting that fusion progresses from below the surface to the surface. Subsequently, the continuity of the ectoderm of both folds was established, with no evidence of fusion at the superficial level (fig. 1g).

Demonstration of the Presence of CSPG in the Region of Neural Fold Fusion

Firstly, we used in our study rat embryos of 10.5–10.9 days postcoitum. Whole embryos were stained with the CS-56 antibody, which recognises the glycidic portion of the CSPG. These embryos were studied by confocal laser microscopy, the 100-μm optical sections showing a discrete immunostaining at the ectoderm level of the forebrain surface which intensified on the vertex of the neural folds during approximation (fig. 1h).

At stages immediately before and during contact of the neural folds at forebrain level, a CSPG-positive material could be appreciated (fig. 1i), which, on account of its location, corresponded to the extracellular matrix described with the SEM vertex of the neural folds (fig. 1c, d).

Table 1. Enzymatic treatment of cultured embryos

Experimental condition	Number of embryos treated	Neural fold fusion disruption
Chondroitinase heat inactivated	44	0%
Chondroitinase AC	44	100%
Streptomyces hyaluronidase	25	<10%

Table 2. Quantification of cellular death and replication

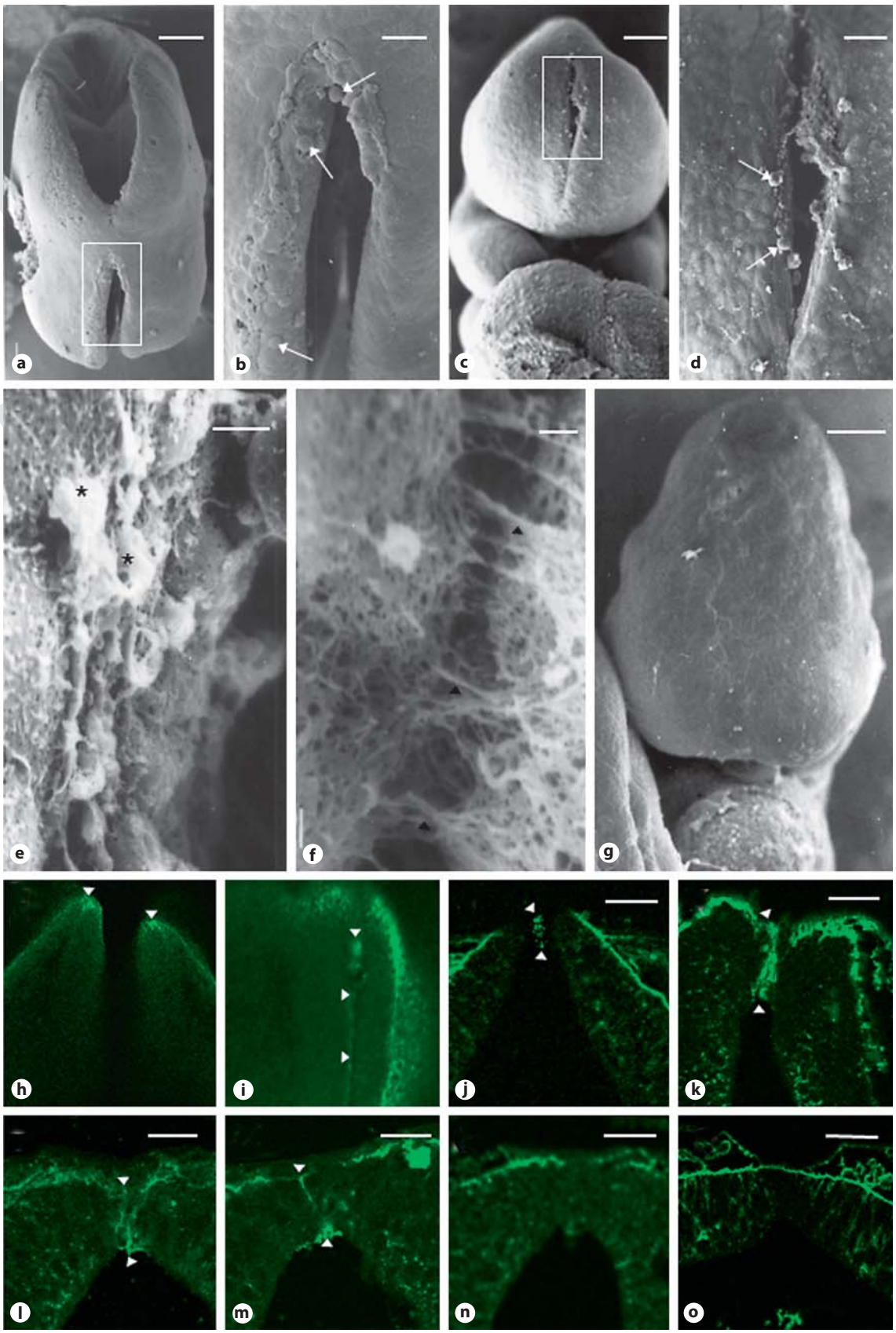
Experimental condition	Apoptotic-labelled nucleus	BrdU-labelled nucleus
Chondroitinase heat inactivated	1 ± 1.41	22.9 ± 6.12
Chondroitinase AC	1.25 ± 1.28	26 ± 6.36
	n = 8, p > 0.05	n = 20, p > 0.05

Measurements are means ± standard error.

Secondly, with a view to confirming these findings and pinpointing the location of the CSPG-positive material during apposition of the forebrain neural folds, we carried out an immunohistochemical study on transversal histological sections. This study confirmed the presence of a CSPG-positive extracellular matrix interposed between both neural folds at the moment immediately prior to contact (fig. 1j). The development of this CSPG-positive substance in these embryos during different phases of adhesion and fusion shows that this matrix remains whilst initial contact is established between both neural folds (fig. 1k). Later, it breaks up and appears as dispersed intercellular accumulations (fig. 1l, m), before disappearing totally once fusion is complete (fig. 1n, o).

The spatio-temporal pattern of CSPG expression, described as coinciding with the process of apposition-fusion of forebrain neural folds, suggests that this proteoglycan might be involved in the process.

Fig. 1. Rat embryos of 10.2–10.9 days of development showing neural fold fusion in the rostral [illegible]on of the cephalic extremity. SEM images (**a**–**g**) reveal the tri-dimensional appearance of the apical surface of the cells about to join together, showing globular images (arrows in **b** and **d**). An extracellular matrix can also be observed which covers the apical surface of these cells (asterisks in **e**) and which becomes particularly abundant when contact between both neural folds is imminent (**c**, **d**). Occasionally, the disappearance of the surface matrix makes it possible to appreciate in the depths of the adhesion zone of both folds threa[illegible] tracts which seem to join cells of the latter (arrowheads in **f**). Immunohistochemistry with anti-CSPG Ac., employing both in toto (**h**, **i**) labelling as well as histological sections (**j**–**o**), reveals the presence of CSPG (arrowheads) in the matrix covering the apical surfaces of the neural folds and its evolution during their fusion. Bars = 100 μm (**a**, **c**, **g**), 30 μm (**b**, **d**), 25 μm (**h**–**o**), 3 μm (**e**), 1 μm (**f**).



Effect of Chondroitinase AC Treatment on Neural Fold Fusion

In order to assess the importance of the extracellular CSPG matrix interposed between the prosencephalic neural folds we detected during fusion, we performed CSPG enzyme degradation in a culture of in vitro embryos. Chondroitinase AC (a glycosidase which specifically digests chondroitin and dermatan sulphate) was microinjected into the amniotic cavity. Treatment was administered following fusion at midbrain level, progressing cranially towards the forebrain. In the control embryos (injected with the heat-inactivated enzyme), the process of neural fold fusion developed in all cases similarly to the untreated embryos (fig. 1g), with continuity in the surface ectoderm at the level of the middle line of the embryo's most cranial region (fig. 2a).

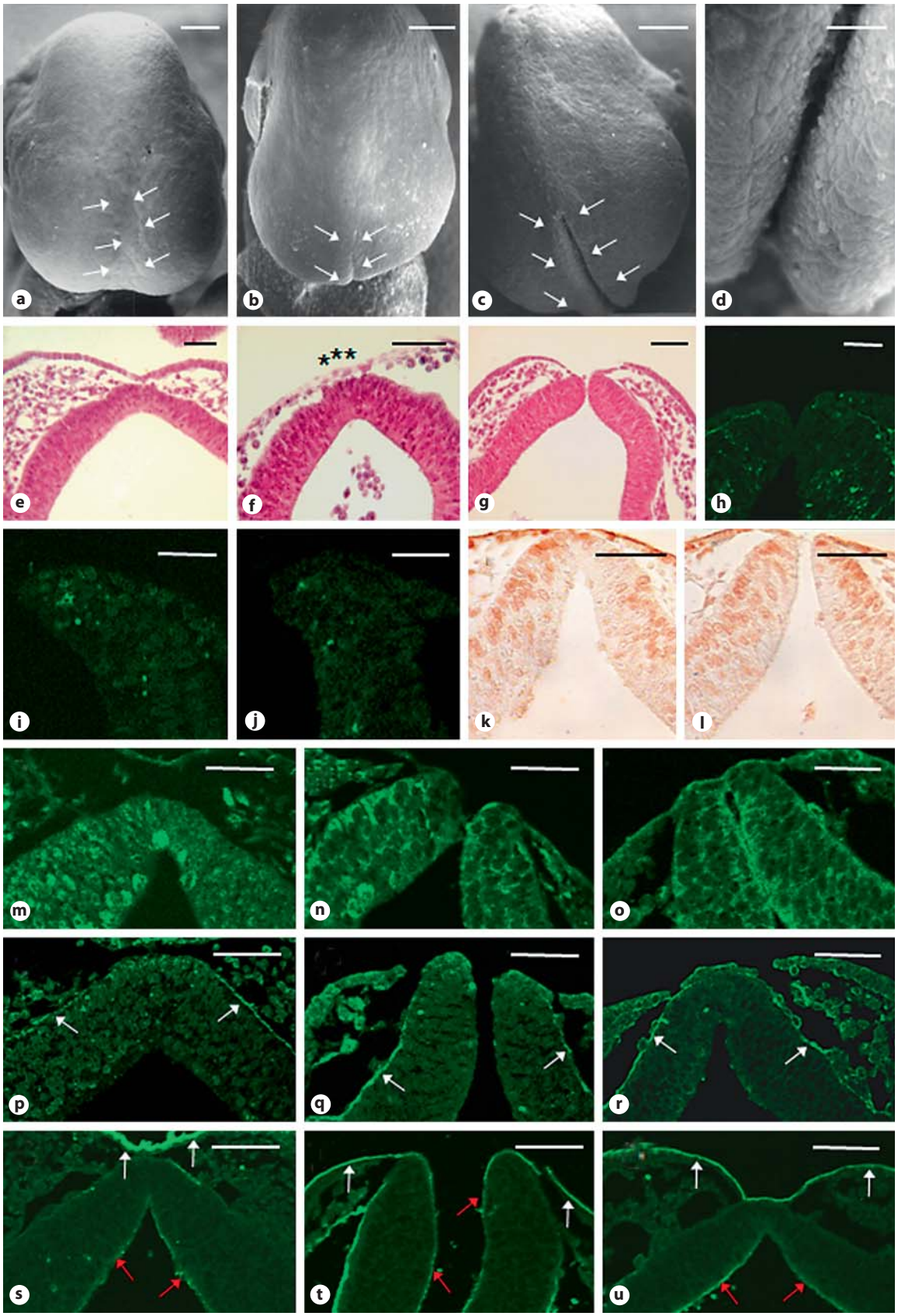
In all cases (table 1), embryos treated with chondroitinase AC showed neural tube closure defects (fig. 2c, d, g). The rostral disposition of these defects suggests that chondroitinase AC checked the cranial progression of neural fold fusion, affecting fusion in the forebrain region. A large amplification SEM study in the area of the fusion defect revealed unbroken continuity between the ectoderm and neuroectoderm, despite both epithelia being easily recognisable (fig. 2d). It should be mentioned that after 36 h of culture, no extracellular matrix could be appreciated covering the apical surface in the open neural tube defect area. This was probably due to a non-replacement of extracellular matrix after fusion time and dragging during culture. As a consequence, complementary experiments to test the composition of the matrix after chondroitinase AC treatment were performed in short-time cultures (8 h).

To check the specificity of enzymatic treatment in neural fold fusion, a group of embryos were microinjected with hyaluronidase of streptomyces (an enzyme which specifically digests hyaluronic acid and not chondroitin sulphate). The results demonstrated that in more than 90% of the embryos (table 1) this enzymatic treatment did not disrupt neural fold fusion at forebrain level, which developed similarly to that of the controls; however, occasionally, we could observe a slight delay in this process (fig. 2b, f).

In order to evaluate the effectiveness of digestion of the CSPG matrix interposed during fusion, we performed CSPG detection with the CS-56 antibody in the fusion region of the treated embryos. The results show that in the embryos treated with chondroitinase AC (fig. 2h), the fusion zone (which, in the control embryos, showed intense immunostaining) appeared totally lacking in CSPG both in long- (36 h) and short-time (8 h) cultures (data not shown). The process of CSPG digestion seemed to have spread to the neuroepithelial wall as well, since the neuroepithelium of chondroitinase-AC-treated embryos showed less staining than in the control embryos.

We tried to discard the possible interference of CSPG digestion in cellular behaviour such as cellular survival and/or replication, which could lead to the non-specific disruption of neural fold fusion. Consequently, we made a study of the apoptotic cells in the neural folds by the TUNEL method. This revealed that there were no significant differences between the control (fig. 2i) and chondroitinase-AC-treated embryos (fig. 2j) in terms of the number of apoptotic cells in the neural fold fusion area (table 2). Also, we labelled the nuclei of replicating cells in the fusion area of the control and chondroitinase-

Fig. 2. Rat embryos cultured in vitro from 10.2 to 11.7 days of development. SME (**a**) and haematoxylin-eosin (**e**) images of 10.7-day non-cultured embryos showing (arrows) that the adhesion/fusion process of the neural folds is complete at the cephalic level. SEM (**b**) and haematoxylin-eosin (**f**) images of an embryo cultured with an intra-amniotic microinjection of hyaluronidase of streptomyces (which only digests hyaluronic acid), showing (arrows) that fusion of the neuroepithelium is normal, whilst fusion of the ectoderm and its isolation appears to be affected (asterisks). SEM (**c**, **d**) and haematoxylin-eosin (**g**) images correspond to an embryo cultured with an intra-amniotic microinjection of chondroitinase AC (which digests CSPG) and reveal a total disruption of the fusion process from the moment the enzyme is administered; note the complete disappearance of the extracellular matrix from the apical surface of the neural fold cells (**d**), coinciding with the disappearance of local CSPG expression detected by immunohistochemistry (**h**). The presence of apoptotic cells, revealed by TUNEL, was similar in the control (**i**) and the chondroitinase-AC-treated embryos (**j**), as shown in table 2. BrdU-positive nuclei, revealed by immunolabelling, showed a similar pattern in control embryos (**k**) and those treated with chondroitinase AC (**l**), as shown in table 2. Fibronectin immunolabelling showed a similar pericellular pattern in the fusion area of the neuroepithelium in control embryos (**m**) as well as in those treated with chondroitinase AC (**n**) and hyaluronidase (**o**). Type IV collagen appeared in the basal membrane (arrows) of the neuroepithelium in control embryos (**p**), and no differences could be appreciated in embryos treated with chondroitinase AC (**q**) or hyaluronidase (**r**). In the control embryos (**s**), HSPG distribution in the fusion area was restricted to the ectodermal surface (white arrows) and the apical surface of the neuroepithelium (red arrows), whereas no changes in this pattern could be observed in embryos treated with chondroitinase AC (**t**) and hyaluronidase (**u**). Bars = 100 μm (**a**, **b**, **c**), 50 μm (**e–g**, **k–u**), 25 μm (**d**, **h–j**).



AC-treated embryos with BrdU; as shown (fig. 2k, l; table 2), there were no statistical differences between both groups in the number of BrdU-positive nuclei. Both results (apoptosis and BrdU-positive nucleus) support our theory concerning the direct involvement of CSPG in cellular adhesion between neural fold cells to initiate the fusion process.

Moreover, to rule out the interference of chondroitinase AC treatment in components of the extracellular matrix other than chondroitin sulphate, which disrupts fusion of the neural folds, we made short-time cultures (8 h) and, by immunohistochemistry, evaluated the expression pattern of other ubiquitous components of extracellular matrix in the fusion zone. As our results show, in the fusion area, no detectable changes can be appreciated in the expression pattern of fibronectin (fig. 2m–o), type VI collagen (fig. 2p–r) and heparan sulphate (fig. 2s–u), between control, chondroitinase-AC-treated and streptomyces hyaluronidase-treated embryos. These data support the specificity of enzymatic treatment with chondroitinase AC and the specific involvement of CSPG in neural fold fusion.

Discussion

Our results in this paper show the following: (1) in rat embryos, an increase in apical secretion of extracellular matrix is associated with cephalic neural folds fusion; (2) this matrix has a high CSPG content; (3) this CSPG content plays a key role in intercellular recognition and adhesion of both neural folds.

These results confirm the presence of an extracellular matrix interposed between the apical surfaces of neural fold cells, which increases significantly prior to contact between the 2 folds. Therefore, they coincide with the description of an extracellular material lying between the neural folds given in histological sections by Lee et al. [1976] and Sadler [1978], and also agree, at least in part, with the descriptions involving the use of SEM and transmission electron microscopy given by Rice and Moran [1977] and by Rovasio and Monis [1981]. Our findings are based on a technique of fixing with Carnoy's solution which has shown itself to be most effective in terms of preserving embryonal extracellular matrixes of this type [Gato et al., 1993; Alonso et al., 1999]. They confirm the presence of such a matrix whilst at the same time supporting the idea of dynamic behaviour in the secretion process, related with that of neural fold fusion. Several studies have shown that similar extracellular matrices exist, covering the apical surface of epithelial cells in various embryonal primordia at moments prior to epithelial fusion, for example, in the nasal folds [Gaare and Langman, 1977; Burk et al., 1979], the otic vesicle [Sinning and Olson, 1988], the lens vesicle [Gato et al., 2001] and the palate [Greene and Kochhar, 1974; Pratt and Hassell, 1975; Souchon, 1975]. In this regard, it has been shown that these neuroepithelial cells on the tip of the neural folds or other primordia have ultra-structural characteristics compatible with active secretion processes occurring before fusion [González Santander and Martínez Cuadrado, 1976; Mak, 1978; Rovasio and Monis, 1981; Sinning and Olson, 1988; Alonso et al., 1998a].

All of the above findings support the theory that the secretion of an apical cover matrix by epithelial cells in embryonal primordia immediately prior to homologous fusion is a common morphogenetic mechanism which might be involved in cellular recognition and adherence.

Furthermore, our study with SEM reveals that in the deepest fusion zones, there are numerous filament-like bridges, although we cannot rule out a fixation artefact with the surfaces pulling apart, most likely due to the presence of filopodia-like cytoplasmic prolongations; however, in both cases, they reflect that the fusion process has already commenced. Thus, it has been proposed that the fusion between homologous epithelia, such as the case of dorsal closure in drosophila [Jacinto et al., 2000; Jacinto and Wolpert, 2001] or the midbrain in chick embryos [Van Straaten et al., 2002], is related with the extension of filopodia, generated by actin microfilaments which cause cytoplasmic bridges to be established. These findings suggest that the adhesion/fusion process of neural folds takes place in 2 stages: one initial phase of recognition and labile adhesion, mediated by the cell cover matrix, and a second step based on the formation of more stable unions by means of cytoplasmic bridges progressing from deep to superficial areas, stabilising and consolidating the fusion process.

There are numerous references in the literature which, based on histochemical methods or staining with lectins, describe the intercellular matrix interposed between the neural folds, both in mammals and birds, as one rich in glycoconjugates [Hay and Meier, 1974; Smits-van Prooije et al., 1986; Zalik et al., 1994; Wilson and Wyatt, 1995]. Our results show that CSPG, a markedly glycidic molecule, is a fundamental component of this matrix and, on the basis of our previous studies showing that CSPG is present during the fusion of the crystalline in chick embryos [Gato et al., 2001] and palate formation in rat em-

bryos [Gato et al., 2002], its presence seems to be a constant in the interposed matrix during the fusion of distinct embryonal primordia. Experimental studies carried out involving disruption of the synthesis of glycoconjugates [Rovasio and Monis, 1981; Alonso et al., 1998a, b] or the use of glycosidases [Morriss-Kay and Tuckett, 1989] have shown a disruption of neural fold elevation and fusion.

Although we used an experimental approach similar to the previously cited works, our study revealed significant differences. First, Morriss-Kay and colleagues [1986] used chondroitinase ABC, which digests chondroitin sulphate, dermatan sulphate and some hyaluronic acid [Beeley, 1985; Sigma manufacturer specifications] and, as a consequence, is not specific to chondroitin sulphate. In contrast, we carried out a specific digestion of CSPG by chondroitinase AC, which selectively digests chondroitin 4 and 6 sulphate [Beeley, 1985; manufacturer specifications] and we contrasted its effect with digestion with streptomyces hyaluronidase, which only digests hyaluronic acid [Beeley, 1985; manufacturer specifications]. This enzyme combination allowed us to identify CSPG as responsible for the induced fusion defects.

The second difference was enzyme administration time, at the beginning of neurulation by the Morriss-Kay group [1986], which allowed the enzymes to digest proteoglycans during elevation and convergence of neural folds, indirectly avoiding fusion. However, in our study, we paid great attention to the exact moment of enzyme administration, immediately prior to contact between the neural folds (performed by visual control), in order to avoid interferences with other phases of neurulation (like elevation or convergence of neural folds), in which CSPG or other proteoglycans could be involved. Moreover, in the studies previously cited, despite the observation of CSPG expression during the entire neurulation period, there is no description of the presence of CSPG being related with neural fold fusion. In fact, their conclusion is that proteoglycans (especially CSPG) are involved in neural fold elevation and convergence as well as in the migration processes of neural crest cells, and no reference is made in the fusion process. Our results are supported by a recent study [Salgueiro et al., 2006] which demonstrated the selective expression, on the tip of mouse neural folds immediately prior to their fusion, of specific enzymes involved in CSPG synthesis. In addition, the selective digestion of CSPG with a specific glycosidase (chondroitinase AC) causes fusion defects in the otic vesicle [Gerchman et al., 1995], the crystalline vesicle [Gato et al., 2001] and the palate [Gato et al., 2002]. This shows that in these primordia, the CSPG of the interposed matrix plays a key role in epithelial fusion. In this paper we show, by means of the same experimental approach (digestion with chondroitinase AC), that the CSPG of the matrix interposed between the neural folds at cephalic level has a significant role in the fusion process, supporting our hypothesis that a matrix rich in CSPG might be involved as a general development mechanism in epithelial recognition and adhesion. As our results show, in the fusion area shortly after initiation of the fusion defect, the embryos treated with chondroitinase AC did not reveal substantial changes in the expression of other ubiquitous molecules of the extracellular matrix such as fibronectin, or basal membranes such as type VI collagen. This result suggests that treatment with chondroitinase AC does not induce a global and non-specific destructuring in the extracellular matrix interposed between both neural folds during their initial contact, thereby highlighting the specific role of CSPG in this process.

It has been demonstrated that other sulphated proteoglycans such as heparan sulphate proteoglycan (HSPG) and, more specifically, glypican 4, are expressed by neuroepithelial cells of neural folds; this molecule has been involved in convergent extension and subsequent fusion [Tuckett and Morriss-Kay, 1989; Ybot-Gonzalez et al., 2005]. However, our results, which focus only on the fusion process and show non-HSPG disruption by treatment with chondroitinase AC, suggest that HSPG (glypican) are involved in neurulation steps prior to neural fold fusion. In this regard, we have previously shown a specific involvement of basal membrane HSPG in embryonic epithelial folding but not in the fusion of otic vesicle development [Moro et al., 2000].

In order to monitor the specificity of the action of CSPG, we performed complementary studies with hyaluronidase of streptomyces (which selectively digests hyaluronic acid) and, unlike the description given by Schoenwolf and Fisher [1983] in chick embryos, we did not find that this enzyme affected the process of neural fold fusion, an observation which coincides with the results given by Morriss-Kay et al. [1986]. This discrepancy is possibly due, rather than to a species-specific effect, to the fact that in our case, the enzyme was administered once the neural folds were elevated and, in the area of the anterior neuropore, were about to establish contact. This would avoid interference in the processes of elevation and convergence of the neural folds, which could depend on the presence of hyaluronic acid and disrupt the elevation and approximation of the latter, indirectly affecting their fusion.

To discard the possibility that defective fusion of the neural folds is an indirect effect of CSPG deprivation on cell behaviour, we carried out a complementary study of cell survival, testing the presence of apoptotic cells in the neural folds of treated embryos using the TUNEL method. These embryos did not show 'significant' differences with regard to the control embryos, ruling out the possibility that CSPG disruption might modify apoptosis, which could be involved in neural fold fusion. In the same way, we checked changes in the number of BrdU-positive cells in the fusion area of control and chondroitinase-AC-treated embryos and did not find any significant differences. This suggests that treatment with chondroitinase AC has no influence on basic cellular behaviour (cell survival and replication).

Fusion processes among homologous cells such as those of neural folds are based on recognition and adhesion mechanisms between the apical surfaces of the particular cells. It is known that separate cells of diverse embryonal epithelia tend to recognise each other and group together according to tissue identity due to the existence of cellular adhesion molecules. This specific recognition among cells is explained by the action of diverse families of transmembrane glycoproteins such as integrins, cadherins, immunoglobulins, selectins and proteoglycans [Gumbiner, 1996; Hynes and Zhao, 2000]. Several studies exist on the expression of cellular adhesion molecules (some of which may contain CSPG in their molecules) in neuroepithelial cells during development, such as the α_6-integrin [Colas and Schoenwolf, 2001], N-CAM [Kintner, 1988] or N-cadherin [Bronner-Frasser et al., 1992]. Nevertheless, their expression pattern in the neuroepithelium does not coincide with that of CSPG described by us, and the experimental studies by immunoblocking and/or loss of expression show that these molecules are more related with maintaining neuroepithelial identity and integrity than with neural fold adherence.

Nowadays, proteoglycans per se are attributed with the capacity to act as key molecules in the processes of cell recognition and adhesion. More specifically, the proteoglycans of the family of lecticans (aggrecan, versican, neurocan and brevican) contain CSPG in their molecule, and are expressed in the central nervous system in different locations and at different times of development and post-natal life. They are considered to be molecules involved in regulating cellular adhesion processes either directly or by means of their capacity to coordinate the action of other cellular adhesion molecules [Miura et al., 1999; Li et al., 2000; Yamaguchi, 2000; Rauch et al., 2001; Wight, 2002; Schwartz and Domowicz, 2004]. This suggests that the chondroitin sulphate detected by us in the fusion area of the neural folds might form part of some type of proteoglycan, the identification of which requires further study. In this regard, there are recent studies [Bucior et al., 2004; Bucior and Burger, 2004] which prove that the glycidic molecules of the proteoglycans (known as glycosaminoglycans) are capable of generating among themselves strong powers of adhesion (similar to those of an antibody antigen union). This union has high proteoglycan-type specificity, which accounts for the fact that these molecules are believed to be involved in processes of recognition and homophylic adhesion among cells, which would justify their role in specific recognition and intercellular adhesion both in neural fold fusion, that of the crystalline vesicle [Gato et al., 2001] or fusion of the palate [Gato et al., 2002].

To conclude, the incidence of neural tube closure defects has diminished drastically in the last few years as a result of folic acid administration during pregnancy. Several studies exist which show that folic acid has a direct influence on synthesising proteoglycans during development of the embryo [Paynton et al., 1978; Schmidt et al., 1982, 1983]. The findings of this paper might account for the action of folic acid in regulating the synthesis of proteoglycans involved in neural fold adhesion.

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