Neural tube defects induced by $\beta$-D-xyloside: evidence of a role for sulfated proteoglycans in neural fold fusion in rat embryos

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SUMMARY

Neural fold fusion is considered the final step in primary neurulation. Although a failure in neural fold fusion has been suggested to be responsible for many neural tube defects, one of the most prevalent congenital malformations in humans, to date the developmental mechanisms involved in this process remain unclear. In this paper we explore how sulfated proteoglycans might play a key role in this important process.

Rat embryos whose neural folds were in close proximity were explanted and cultured in vitro for 24 hours, either with or without $\beta$-D-xyloside, a chemical that selectively disrupts sulfated proteoglycan biosynthesis.

Our results show that, under these particular conditions, neural fold fusion is disrupted by the presence of $\beta$-D-xyloside. Treated embryos displayed neural tube defects, normally located at the mesencephalo-rhomencephalic roof. These embryos featured subsequent defects in brain development, with a generalized failure in brain vesicle expansion and abnormal infoldings of neuroepithelial walls inside the brain cavity.

These results provide experimental support for the hypothesis that sulfated proteoglycans, probably associated with the extracellular matrix intercalated among neural folds, play a key role in the fusion process.

Key Words: Rat embryo - Neural fold fusion - Neurulation - Sulfated Proteoglycans - $\beta$-D-xyloside.

INTRODUCTION

Neural fold fusion is an interesting developmental process which takes place at the end of primary neurulation and which concludes with neural tube formation and isolation from surface ectoderm. Although many congenital malformations of the central nervous system, such as anencephaly, spina bifida and other disraphic defects, seem to stem from a defective neural fold fusion process (for revision see Coop et al., 1990 and Hall, 1994, CIBA), until now the developmental mechanisms involved remain largely unknown.

Epithelial fusion requires the previous concurrence of morphogenetic mechanisms which place both neural folds tips in proximity. The origin of the mechanical forces involved in this process has been widely studied and there is some controversy about the relevance of intra or extraneural mechanisms (for a review, see Schoenwolff and Desmond, 1984; Copp et al., 1990). Furthermore, sulfated proteoglycans have been directly implicated in some of these morphogenetic changes (Morriss-Kay and Crutch, 1982).

Neural fold fusion is a complex process which involves the coordination of several cellular and molecular mechanisms in order to fuse the neural fold apices and to establish a dorsal continuity in neuroectoderm and surface ectoderm, with independence between both epithelial layers. Several studies have explored changes in cellular polarity, specific cell recognition, changes in cellular adhesion molecules, cellular migration and reorganization, cellular apoptosis and other mechanisms (for an extensive review, see Copp et al., 1990 and Fleming et al., 1997). It has been claimed that immediately prior to
contact between neural folds from both sides an extracellular matrix arises, interposed between the apical neural folds (Sadler, 1978; Mak, 1978). This material, which seems to be secreted by neuroblasts themselves (which later fuse) has been proposed as a key factor in the fusion process, in which it as a transitory linkage medium between both neural folds until definitive cellular union has been established (Sadler, 1978). Other authors have suggested that this apical surface coat material could play a role in specific cellular recognition among fusing cells (Smits Van Prooije et al., 1986). The precise nature of these cell surface molecules that mediate the fusion of neural folds remains to be determined; however, their glycosidic nature has been demonstrated and sulfated proteoglycans have been proposed as a functionally relevant constituent (Sadler, 1978; Smits Van Prooije et al., 1986). Despite this, there is to date no experimental evidence to support the direct involvement of sulfated proteoglycans in neural fold fusion.

In past decades, β-D-xylosides have been used as a useful tool to test the biological role of sulfated proteoglycans (mainly chondroitin sulfate proteoglycan) in embryonic development (Gibson et al., 1978; Kanke et al., 1982; Kinoshita and Saiga, 1979; Morriss-Kay and Grutch, 1982; Morriss-Kay and Tuckett, 1989; Segen and Gibson, 1982). β-D-xylosides selectively disrupt the glycosylation sequence of sulfated proteoglycan core protein, acting as an artificial substrate for the glycosyltransferase enzyme. The result of this alteration is the synthesis of large amounts of chondroitin sulfate free chains and the synthesis of anomalous molecules of chondroitin sulfate proteoglycans (Roden, 1980).

In this paper we attempt to demonstrate a specific role for sulfated proteoglycans in neural fold fusion of rat embryos by administering a β-D-xyloside to rat embryos cultured in vitro just before neural fold fusion takes place.

were placed in culture in 30 ml. glass culture bottles. The culture medium was 4 ml. of rat serum (1 ml/embryo) obtained from immediately-centrifuged blood that was heat-inactivated at 56°C for 30 min, containing 50 µg/ml streptomycin. The bottles were rotated at 30 r.p.m. at 38°C. Every 12 hours, the culture bottles were gas-flushed for 10 min. with a mixture containing 5% oxygen for the first 24 h. and 20% thereafter.

**Exposure to p-nitrophenyl-β-D-xylopyranoside and subsequent processing**

A total of 51 embryos were cultured under these conditions and the appearance and convergence of neural folds were controlled directly under a binocular microscope until the mesencephalic neural folds were in clear proximity. Then, 100 µl. of a solution of p-nitrophenyl-β-D-xylopyranoside 1 mM in Hank’s sterile medium was added to the culture medium of 31 embryos. Twenty embryos were used as controls, divided into two groups: the first group was only treated with saline solution (Hank). The second control group was treated with p-nitrophenyl-β-D-xylopyranoside (α-D-xylose) at the same concentration as that used for β-D-xylose; α-D-xylose is an inactive anomer of β-D-xylose. This control group was employed to rule out possible direct toxic effects of the xylosides on embryonic development and growth. The total time of culture was 48 hours (from 9.7 to 11.7 days of development).

At the end of the culture, extraembryonic membranes were stripped and embryos were washed in Hank’s solution and were staged with the scoring system described by Brown and Fabro (1981). Afterwards, the embryos were fixed in Bouin solution for 6 hours at room temperature, dehydrated in a graded ethanol series and embedded in paraplast. The embryos were transversally oriented and serially sectioned at 8 mm. Sections were stained with haematoxylin-eosin. The most representative sections were photographed with a Nikon microphot-FX-A photomicroscope.

**Materials and Methods**

**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. The in vitro culture system was based on the technique described by New (1978). On the afternoon of day 9 of pregnancy, the uterus was excised from ether-anesthetized rats and individual implantation sites were collected in a sterile Petri dish containing Hank’s balanced salt solution. The egg cylinders were dissected under a dissecting microscope and after removal of Reichert’s membrane the embryos

**Results**

As described above, rat embryos were explanted at the 9.7 day stage and cultured in vitro until the 11.7 day stage, with or without β-D-xylose in the culture medium. Only those embryos which showed a clear process of neural fold proximation in the mesencephalic mid dorsal line at the beginning of culture were selected for study; the aim of this was to perform an accurate study of the effect of β-D-xylose during neural fold fusion.

After 48 hours of in vitro culture, control and α-D-xylose-treated embryos showed an adequate morphological degree of development for 11.7 day embryos according to the criteria of
Brown and Fabro (1981). As can be seen in Fig. 1-A, control embryos showed a completely fused neural tube. The prominent cephalic end revealed that brain expansion was in progress, and the morphological changes undergone by the neuroepithelium pointed to the formation of secondary brain vesicles such as the diencephalon. A normal development of other cephalic primordia, such as the optic and otic vesicles, was also seen and three branchial arches had developed.

Most of the embryos treated with β-D-xyloside showed a clear disruption in normal development. Treated embryos were smaller than controls and, to a large extent, spiral torsion had been lost. However, the most severe embryonic alterations seemed to be localized at cranial level, including a noteworthy alteration in the growth and morphogenesis of the embryonic cephalic extremity (Fig. 1-B), whose cephalic volume/embryonic volume ratio was considerably decreased with respect to control embryos.

The development of other cephalic anlagen, such as the otic primordium, was also severely affected. In control embryos the otic anlagen appeared as a vesicular formation under the surface ectoderm, dorsal to the second branchial arch (Fig. 1-A). In β-D-xyloside treated embryos, the otic primordium appeared on the surface as a slightly invaginated rudimentary otic plate (Fig. 1 B, C and D).

The branchial arch in β-D-xyloside treated embryos was smaller than that of the controls (Fig. 1-B). Regarding this, in histological sections a notable reduction in cellular density was seen at the level of the first branchial arch (compare Fig. 3-C, showing a control embryo, with Figs. 3-F and 3-I, corresponding to β-D-xyloside-treated embryos).

Effects induced by β-D-xyloside on neural fold fusion

One of the most interesting developmental alterations in the treated embryos was the consistent presence of macroscopic defects in neural fold closure located in the cephalic region; these did not seem to affect the spinal part of the neural tube except for some delay in caudal neuropore closure. The degree of neural tube defects varied in extent between a small fissure located normally in the mesencephalon (Fig. 1-D) to broad apertures normally located in the rhombencephalon and sometimes affecting more than one brain vesicle (Fig. 1-B-C), exposing the apical surface of the neuroepithelium to the amniotic cavity.

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**Fig. 1.** Macroscopic view of 11.7 day rat embryos. Control embryos **A**, show normal development, with a prominent cephalic end, due to brain vesicle expansion. β-D-xyloside-treated embryos **B, C and D** show severe alterations in the brain expansion process and neural tube closure defects at the dorsal mid line (arrow heads). β-D-xyloside treatment also disrupts the normal development of the optic and otic primordia as well as in the branchial arches. Calibration bar A-B: 400 μm and C-D: 420 μm.
Histological study of the fusion zone in control embryos after culture revealed (Fig. 2-A) the integrity and independence of the neuroepithelium and ectodermal layer with a mesenchymal layer interposed. The neuroepithelium at the mid dorsal line had the characteristic morphology of the roof plate. In β-D-xylene treated embryos, the histology of the neural fold fusion zone appeared to be seriously affected. Fig. 2-B shows an unfused neural tube in which a thick neuroepithelium abruptly continues with a cell monolayer ectoderm. In other cases (Fig. 2-C) neural folds remained together and seemed to have an incomplete fusion zone near the dorsal border of the neuroepithelium; however, no differentiation nor independence of ectodermal and neuroepithelial cells were observed. In other cases (Fig. 2-D) there seemed to be a complete fusion of the neuroepithelium at the dorsal midline and the surface ectoderm was continuous and independent. However, in the fusion zone the cells of each neural fold failed to mix and a clear division remained between both sides of the neuroepithelium in the mid dorsal line. The pattern of complete disruption (Fig. 2-B) usually appeared at mesencephalon-rhombencephalic level, whilst patterns of incomplete or aberrant fusion (Figs. 2-C and 2-D) were more commonly found at prosencephalic level. These patterns coexisted in the same embryo or appeared isolated in different embryos.

Effects of β-D-xylene on brain expansion and vesiculation

Another major disruption induced by β-D-xylene in embryonic brain development affected brain expansion. Fig 1-B shows that the cephalic volume of treated embryos was clearly smaller than that of control embryos (Fig. 1-A), and severe alterations in brain vesicle morphogenesis were observed (Fig. 1-C and D). Histological study confirmed these observations and revealed (Fig. 3) that the brain vesicle cavity was dramatically smaller in all treated embryos (Fig. 3 D to I) than in control embryos (Fig. 3 A to C). The morphology of the neuroepithelial wall was also abnormal and showed several abnormal infoldings into the neural cavity that sometimes completely filled the brain lumen. These abnormal infoldings were normally located in the prosencephalic and mesencephalic vesicles and the intervesicular limits were also abnormal.

Histological sections from treated embryos revealed that the optic primordia had a similar developmental rate to those of the control embryos, but their morphology was seriously affected. In control embryos (Fig. 3-B) these primordia appeared as a lateral evagination of the diencephalon (optic vesicle) with a broad optic stalk. In β-D-xylene-treated embryos, the optic vesicle appeared with a collapsed cavity vesicle and its epithelial wall was abnormally developed (Fig. 3-E and H), in most cases the optic vesicle failing to come into contact with the surface ectoderm.

**Fig. 2**—High magnification photomicrographs from the dorsal midline of the neural tube after fusion in control (A) and β-D-xylene-treated embryos (B, C and D). Treated embryos show different degrees of neural tube fusion disruption, complete (B), partial (C) and aberrant (D). Calibration bar A-D: 50 μm.
Fig. 3.—Histological appearance of 11.7 day control (A, B and C) and β-D-xyloside-treated (D-E-F and G-H-I) rat embryos. Sections were taken at three different levels similar in A-D-G, B-E-H, and C-F-I. Treated embryos show defects in neural fold fusion (arrow heads in G and E), a reduced brain cavity volume that affects the optic vesicle and several neuroepithelial infoldings. Branchial arches are atrophic. Calibration bar in all figures: 250 μm.

DISCUSSION

Sulfated proteoglycans play a role in the development of numerous embryonic anlagen owing to their nature as fundamental components of the extracellular matrix. As a result, disruption of synthesis by treatment with β-D-xyloside may interfere with the development of different anlagen. Our results point to a reduction in the size of β-D-xyloside-treated embryos, which may to a large extent be due to the lack of expansion of the neural tube's cephalic extremity. However, we cannot rule out overall growth disruption, since some authors have included CSPG in embryo cell proliferation (Morriss-Kay and Crutch, 1982), whereas Comper and Laurent (1978) and Comper and Zamparo (1990) argue that sulfated proteoglycans are molecules which play an important part in regulating embryo tissue volume, as a result of which their disruption might imply changes in extracellular space volume. Nevertheless, per se this reduction in growth does not imply a generalized delay in development. In fact, we did not observe a similar alteration of the embryos' different anlagen, suggesting that certain anlagen could be more
susceptible to CSPG disruption at the precise moment when β-D-xyloside is administered. Concerning this, we believe that the alteration in the invagination and closure of the otocyst must be due to a specific disruption of the extracellular matrix sulfated proteoglycans rather than to a generalized delay in development. In the study performed by Gerchman et al. (1995), it was shown that local administration, by microinjection, of β-D-xyloside produced an effect similar to that described by us concerning the invagination of the otic placode.

Our results reveal hypoplasia of the branchial arches with a notable reduction in cells, an effect which may also be explained in terms of the ability of β-D-xyloside to disrupt CSPG synthesis. Regarding this point, Daniels and Solursh (1991) showed that the administration of β-D-xyloside to in vitro chick embryo neural tube explants seriously delayed cephalic neural crest migration; likewise, Moro-Balbás et al. (1998) obtained a similar effect by subgerminal β-D-xyloside treatment in chick embryos, at the same time eliciting cellular depletion at branchial arch level.

Our results show that selective disruption of sulfated proteoglycan synthesis in rat embryos, immediately prior to neural folds fusion, leads to a specific disruption of the fusion process, with minor alterations in the convergence of neural folds. These data suggest that sulfated proteoglycans could play a key role in the fusion of neural folds.

Neural tube defects are normally located at cranial rhombencephalic level. Neural fold closure has been described as a multistep process with some places of “novo contact”; fusion proceeds from these initiation sites in both cranial and caudal directions to form the last areas to fuse, named neuropores. The cranial-rhombencephalic level coincides with the point of convergence of two fusion waves (Copp and Bernfield, 1994) and could explain the preferential location of neural tube defects induced by later treatment with β-D-xyloside. By contrast, in this study neurulation at spinal level did not seem to be affected and showed normal progress in the presence of β-D-xyloside, suggesting that sulfated proteoglycans are not involved at this level. These data support the hypothesis of Fleming et al. (1997) that dramatic variations occur in the timing and mode of neural tube closure at different levels of the body axis and that there are fundamental variations in the mechanism of neurulation along the body axis.

Some embryos showed clear signs of affected neural fold convergence especially in the rhombencephalic vesicle; however, it is not possible to differentiate between a primary failure in neural fold approximation and suspension of the action of the approximative forces after defective neural fold fusion.

In previous studies carried out on rat embryos, it has been shown that sulfated proteoglycans are involved in morphogenetic mechanisms related to neurulation. Morriss-Kay and Crutch (1982) reported that precocious disruption of sulfated proteoglycan synthesis by β-D-xyloside at 9 days of development, leads to a failure in the convexo-concave conversion of neural folds with broad neural tube defects. Likewise, Morriss-Kay and Tuckett (1989) described a generalized disruption in the mechanisms involved in cephalic neurulation after chondroitin sulfate proteoglycan digestion with chondroitinase ABC in 9-day old rat embryos. In neither case can the involvement of sulfated proteoglycans in neural fold fusion be proved because the disruption in sulfated proteoglycan biosynthesis was induced in the earliest stages of neurulation and mainly affected the bending and convergence of neural folds, which are an essential step in neural fold fusion. Contrarily, Morriss-Kay and Tuckett (1989) showed that in long-term treatment with chondroitinase ABC neural folds fuse, suggesting that sulfated proteoglycans are not involved in the fusion of neural folds in rat embryos. However, these results may have been due to inactivation or depletion of enzymatic activity after several hours of culture, which allows embryonic cells to raise CSPG levels to those compatible with neural folds fusion.

Epithelial fusion is a morphogenetic mechanism common to different types of embryonic anlage development and has been particularly well studied in the fusion of the primary and secondary palates, nasal folds and neural folds. The presence of extracellular material associated with apical cellular surfaces shortly before and during epithelial fusion are a common factor to all of them (Burk et al., 1979; Smuts, 1977; Gaare and Langman, 1977; and Sadler, 1978, among others). The most widely accepted theory is that these surface macromolecules provide initial adherence between both epithelial sheets until more permanent cell contact can be established. The glycosidic nature of these surface coats has been widely demonstrated in nasal fold fusion (Burk et al., 1979), palate fusion (Smuts, 1977, and Greene and Pratt, 1977) and neural fold fusion (Smits van Prooijen et al., 1986; Copp et al., 1990). This suggests that the glycosidic nature of the surface coat could be due, at least in part, to the presence of proteoglycans. It has been proposed that proteoglycans could be involved in neural fold fusion, in terms of a direct relation between proteoglycans and neural cell adhesion molecules. This theory is supported by the fact that some neural tube defects in avian embryos show a concomitant alteration of sulfated proteoglycans and cell adhesion molecules in the fusion zone (Newgreen et al., 1982). Likewise, sulfated proteoglycans have been described as
conformational modulators of neural cell adhesion molecules in the neural tube immediately prior to neural fold fusion, thus priming cellular adherence (Ruoslalht, 1988 and Cole and Burg, 1989).

Our results show that subsequent brain development in open neural tube embryos is clearly abnormal regardless of the extent of the opening. In all embryos showing a neural tube defect, the neuroepithelium of brain vesicles is morphologically abnormal, with several inclusions that erase the intervesicular limits and with a profound reduction in brain cavity volume. These results are similar to those described by Desmond and Jacobson (1977) in the chick, Harris et al. (1997) in the mouse and Patton (1952) and Desmond (1985) in human embryos with neural tube defects. The common factor to all these embryos is the spontaneous or artificial communication of the brain vesicle cavity with the amniotic fluid.

When the anterior neuropore closes, primary neuroplasia concludes at brain level. Shortly afterwards, there is a transitory occlusion of the spinal cord cavity which transforms the brain vesicles into a physiologically sealed system (Schoenwolf and Desmond, 1984), filled with a neural tube fluid that exerts positive pressure against the neuroepithelial walls (Jelinek and Pexieder, 1970). This pressure seems to be generated by the neuroepithelial cells themselves through the secretion of osmotically active molecules towards the brain cavity (Gato et al., 1993) and has been proposed as a critical factor for subsequent morphological and histological brain development (Desmond and Jacobson 1977, Van Essen 1997). Therefore, the developmental brain alterations induced by us can be interpreted as a failure in intracerebral pressure by neural tube fluid loss throughout the fusion defect. However, in the same context we cannot discard a deprivational effect of the contact of neuroblasts with some components of neural tube fluid.

Since during these developmental stages the eye anlage is extensively connected with the diencephalon cavity, and since its expansion is dependent on the intracavity pressure of the brain vesicles, such a reduction in pressure might also account for the morphological alterations observed by us in β-D-xyloside-treated embryos, which coincide with those described by Desmond and Jacobson (1977).

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REFERENCES


