Original Article

Local increase level of chondroitin sulfate induces changes in the rhombencephalic neural crest migration

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ABSTRACT Numerous studies suggest that chondroitin sulfate proteoglycan (CSPG) inhibits neural crest cells (NCC) migration at the trunk level. However, its action on the cephalic neural crest is not clear. To determine this action, we have microinjected 0.5 nl of different concentrations of chondroitin sulfate (CS) at the anterior rhombencephalon level in 9 stage chick embryos, as well as subgerminally administering β-D-xyloside to 8 stage chick embryos. β-D-xyloside disrupts CSPG synthesis, producing an increase in CS free chains in several embryonal anlages. Chondroitin sulfate microinjected embryos and β-D xyloside treated embryos were reincubated until attaining 12 stage. Results obtained for both experimental groups were similar. Immunoreactivity with HNK-1 antibody revealed that NCC did not migrate, remaining near the rhombencephalon dorsal wall; in addition, several NCC did not separate from the neural fold, becoming invaginated towards the rhombencephalon cavity. Our findings indicate that an increase in CS free chains in cephalic neural crest migratory routes not only disrupts their migration, but also impedes delamination and detachment of the rhombencephalic neuroepithelium NCC. These data suggest that the inhibitory action upon the neural crest migration attributed to CSPG may rest on its glycosaminoglycan (GAG). We cannot, however, rule out the possibility that increases in other GAGs apart from CS, may produce similar effects on neural crest migration.

KEY WORDS: *rhombencephalon, glycosaminoglycans, cell migration, proteoglycans, neural crest*

Introduction

In chick embryos cephalic neural crest cells emerge from the neuroepithelium dorsal surface and initiate their migration coinciding with neural tube closure. Migration begins in the mesencephalon anterior region at 8 stage of Hamburger and Hamilton (1951) and then continues its caudal direction to the metencephalon (rhombomeres 1, 2 and 3) and myelencephalon (rhombomeres 4 and 5), this process lasting between 24 and 36 h (Lumsden *et al.*, 1991; Bronner-Fraser 1994). Neural crest cells (NCC) of the caudal region of the mesencephalon and the rostral area of the rhombencephalon (rhombomere 1), colonize the first branchial arch. Cells migrating from rhombomeres 2, 4 and 6 invade the first, second and third branchial arches, respectively (Lumsden *et al.*, 1991). Discussion nowadays concerns whether rhombomeres 3 and 5 carry cells to the cephalic neural crest. Graham *et al.* (1993) consider that the cells of these rhombomeres are eliminated by apoptosis, whilst Birgbauer *et al.* (1995) maintain that the cells of these rhombomeres migrate alongside those of the adjacent rhombomeres.

Neural crest migratory routes contain an intricate extracellular matrix which seems to play an important role in controlling the motility and migration course of NCC, (for rewiew, see Perris 1997). *"In vitro"* these cells migrate widely in substrates containing certain extracellular matrix molecules, such as fibronectin and laminin (Rovasio *et al.*, 1983; Newgreen 1984; Perris *et al.*, 1989) and various types of collagen (Perris *et al.*, 1991a); this suggests that individual components of the extracellular matrix may act as permissive migratory substrates. Cell-matrix interactions appear to be brought about by integrins receptors situated on the surface of the NCC (Lallier and Bronner-Fraser 1992,1993; Lallier *et al.*, 1994).

In correlation with experiments carried out *"in vitro"*, high levels of certain molecules have been shown to exist in the extracellular matrix along the neural crest migratory pathways; more specifically, these are hyaluronate, fibronectin, laminin, tenascin/cytotactin and collagens (for review, see Bronner-Fraser 1994). The role of several of these molecules in neural crest migration is fundamental, as shown by the fact that experimental disruption by means of enzymes or antibodies alters migration of NCC (Bronner-Fraser

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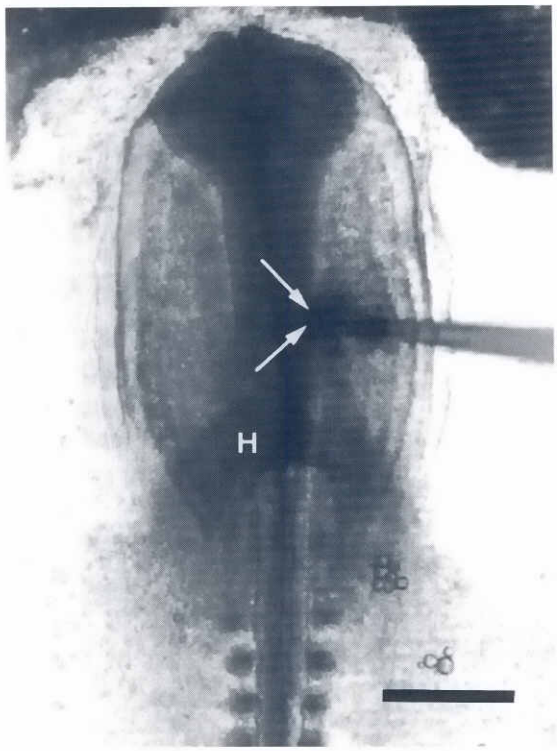

Fig. 1. Microinjection of a coloured solution with blue toluidin (arrows), showing the level at which chondroitin sulfate injections were carried out, in the rhombencephalon anterior portion, near the neural folds, cranial to the heart primordium (H). *Bar, 400 μm.*

1985,1986; Bronner-Fraser and Lallier 1988; Morriss-Kay and Tuckett 1989). Yet although we have a quite ample understanding of how the extracellular matrix regulates migration of NCC, a point mentioned earlier, little is known concerning the role it plays in inhibiting or stopping migration. Some candidate inhibitory molecules are selectively distributed in the areas in which neural crest cells do not penetrate at trunk level; namely, chondroitin sulfate proteoglycan (CSPG) and carbohydrates detected by means of peanut agglutin (Perris *et al.*, 1991b; Oakley *et al.*, 1994; Krull *et al.*, 1995), T-cadherin (Ranscht and Bronner-Fraser 1991), collagen IX (Ring *et al.*, 1996) and versican (Landolt *et al.*, 1995), a chondroitin sulfate (CS) with a high molecular weight core proteins (Reichardt, 1991). Wang and Anderson (1997) have recently found that transmembrane ligands from the Eph family, specifically Lerk2 and HtkL, appear in the sclerotome caudal half in rat and chick embryos. This region does not have neural crest migratory cells, so these ligands may behave as chemorepellent molecules for the neural crest migration.

Different factors exist which point to the inhibitory role of CSPG in neural crest migration. It has been revealed that CSPG derived from cartilage are nonpermissive substrate for chick neural crest cell-substrate adhesion (Newgreen 1982), quail NCC migration (Tan *et al.*, 1987) and axolotl NCC migration "*in vitro*" (Perris and Johansson, 1987). It has also been tested that collagen IX, whose α2(IX) subunit shows CS chains covalently attached, inhibits NCC migration in neural tube explants cultures (Ring *et al.*, 1996). These experiments carried out with culture of NCC have been supported by others taking place "*in vivo*"; it has been proven that CSPG decreases and even disappears when trunk neural crest cells begin to migrate towards the dorsolateral path in chick embryos (Perris *et al.*, 1991b; Oakley *et al.*, 1994). In addition, dermomyotome deletion which specifically abolishes expression of CSPG causes NCC to enter this path precociously (Erickson *et al.*, 1992; Oakley *et al.*, 1994). Following microsurgical implantation, in chick embryos, of notochords laterally to the neural tube, NCC do not approach the ectopic notochord (Pettway *et al.* 1990; Stern *et al.*, 1991; Bronner-Fraser 1994); CSPG is known to be plentiful at the level of the perichordal sheath and the extracellular matrix surrounding the notochord (Hennig and Schwartz 1991).

A curious phenomenon is observed at the dorsolateral migration path level of the trunk neural crest when comparing black (normal) axolotl embryos with white (mutant) axolotl embryos: Perris *et al.* (1990), Olsson *et al.* (1996) and Epperlein *et al.* (1996) revealed that in white mutant embryos there was abundant CSPG and Keratan sulfate proteoglycan on this path and that NCC did not enter it, and consequently no pigmentation was developed. In normal (black) embryos NCC did invade the dorsolateral path and their CSPG content was considerably less. All the points previously mentioned suggest that CSPG impedes NCC migration, however, the experiments cited refer exclusively to the trunk neural crest, and we have found only one reference to the role of CSPG in cephalic neural crest migration: Morriss-Kay and Tuckett (1989), who assign to it a facilitating role of the migration in rat embryos.

CSPG consists of repeated units of dissacharides (glycuronic acid and N-acetylgalactosamine) linked to a protein core by a union of serine-xylose-galactosamine-galactosamine; sulfation may take place in positions 4 or 6 of N-acetylgalactosamine residue. The glycidic part is generically referred to as glycosaminoglycan (GAG)(Goetinck 1991). It is known that at least two different CSPGs have an inhibitory effect upon the neural crest migration "*in vitro*", specifically versican and aggrecan (Perris *et al.*, 1996). Collagen IX, which carries CS chains, causes a similar effect (Ring *et al.*, 1996). The inhibitory effects of these molecules seem to depend on the proteoglycan integrity, however, whether the inhibitory faculties rest on the protein core or on the GAGs chains is still unknown. In this study it is shown that the administration via microinjection of CS free chains in the rhombencephalic migration path paralyzes NCC migration and even prevents these cells from separating from the neuroepithelium. We have also produced a similar effect following treatment with β-D-xyloside, which, despite its capacity to reduce the amount of CSPG, considerably increases CS free chains concentration (Gibson *et al.*, 1979; Kanke *et al.*, 1982; Sobue *et al.*, 1987). Our results show that the inhibitory effect of CSPG upon the neural crest migration might rest on the GAGs chains.

Results

Light microscopy and scanning electron microscopy

Microinjected embryos

After microinjection at the 9 stage (Hamburger and Hamilton 1951) and subsequent reincubation in Britt and Herrman (1959) culture medium, both control embryos and those microinjected with CS and HS reached a similar developmental level, namely, stage 12.

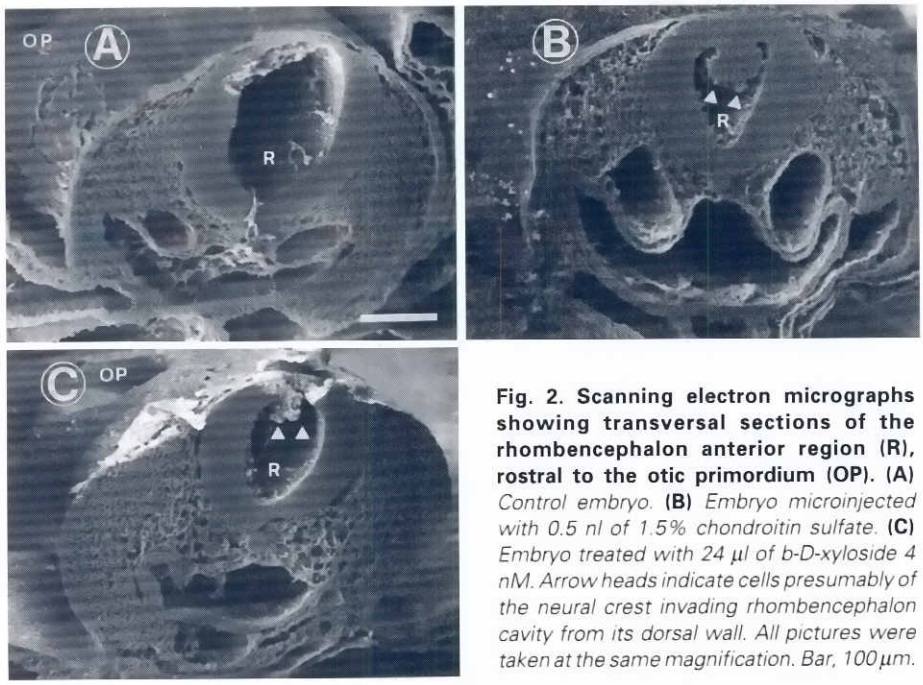

Fig. 2. Scanning electron micrographs showing transversal sections of the rhombencephalon anterior region (R), rostral to the otic primordium (OP). (A) *Control embryo.* **(B)** *Embryo microinjected with 0.5 nl of 1.5% chondroitin sulfate.* **(C)** *Embryo treated with 24 μl of b-D-xyloside 4 nM. Arrow heads indicate cells presumably of the neural crest invading rhombencephalon cavity from its dorsal wall. All pictures were taken at the same magnification. Bar, 100 μm.*

In the histological sections and scanning electron microscopy images, at the anterior rhombencephalon level (rhombomeres 2-3 area), and rostral to the otic primordium (where microinjection took place), control embryos had a normal morphological appearance (Figs. 2A and 3A). The neural tube was closed and its dorsal wall had become very thin. In the area ventro-lateral to the pharynx, corresponding to the branchial region, there was greater cellular density than in the rest of the mesenchyme (Fig. 3A), presumably this region had already been invaded by cephalic NCC.

Embryos microinjected with CS concentrations under 1.5% showed no morphological disruptions in relation to control embryos, whilst concentrations greater than 3% produced quite considerable vascular dilations at dorsal aortas level (Fig. 3B); consequently, neither group of embryos was included in our study.

Embryos microinjected with 1.5% and 3% CS concentrations showed alterations exclusively in the injected area, the rhombencephalon anterior region (rhombomere 2), rostral to the otic primordium. In these embryos, as in the control embryos, the rhombencephalon was also closed, but there was no characteristic thinning of the dorsal wall and in most cases a compact cellular mass could be observed which were invaginated from this wall towards the neural tube lumen (Fig. 3C). This invagination might, in view of its location, be formed by rhombencephalic NCC not yet having commenced migration and maintaining contact with neuroepithelium; in its interior some cells could be seen with intensely stained picnotic nuclei (Fig. 3D), which may imply apoptosis at this level. Laterodorsal to the rhombencephalon and bilaterally, cellular grouping was observed (Fig. 3C), which did not appear in control embryos (Fig. 3A) and could, judging from its situation, correspond to cephalic NCC which had disrupted migration, the arrangement being reminiscent of this formation's initial migration phases. Around the branchial region (ventrolateral to the pharynx) cellular density was considerably lower in CS microinjected embryos than in control specimens (compare Fig. 3A and C), which appears to indicate that NCC have not invaded this region.

Embryos microinjected with 1.5% and 3% HS concentrations, revealed similar alterations in rhombencephalon neural crest migra-

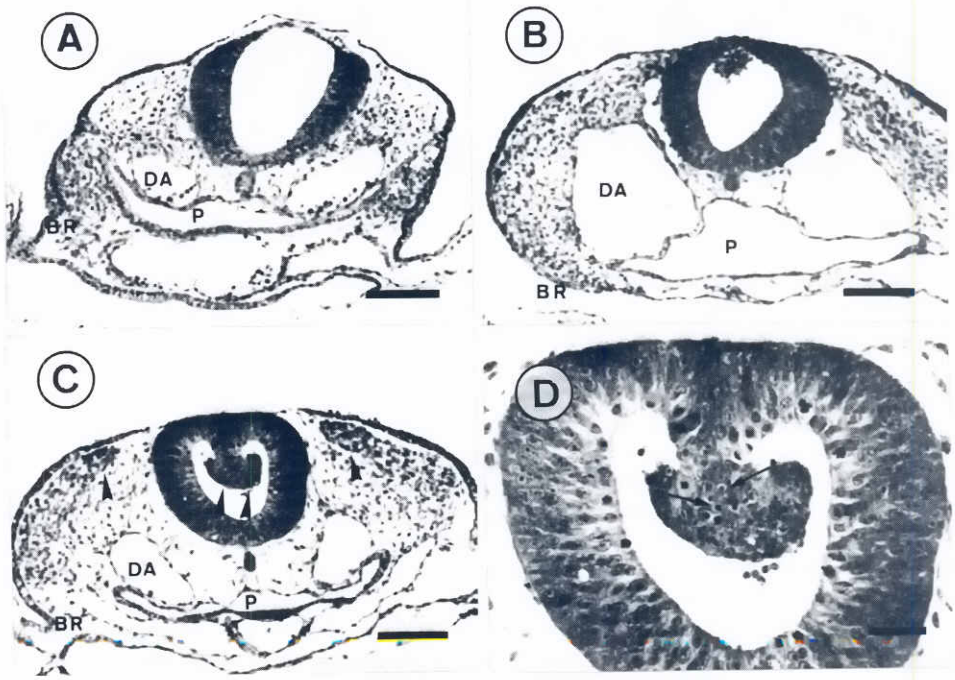

Fig. 3. Transversal sections at the anterior rhombencephalon level (rhombomere 2) of a control embryo (A), embryo microinjected with 0.5 nl of 6% chondroitin sulfate (B) and embryo microinjected with 0.5 nl of 3% chondroitin sulfate (C). *Arrow heads in C show cells presumably of the neural crest near the rhombencephalon dorsal wall and invading its cavity.* **(D)** *Higher magnification of C corresponding to the rhombencephalon; intensely stained picnotic nuclei can be observed (arrows), in cells invaginated into the neural cavity. DA, dorsal aorta; P, pharynx; BR, branchial region. Bars, 100 μm (A, B and C) and 30 μm (D).*

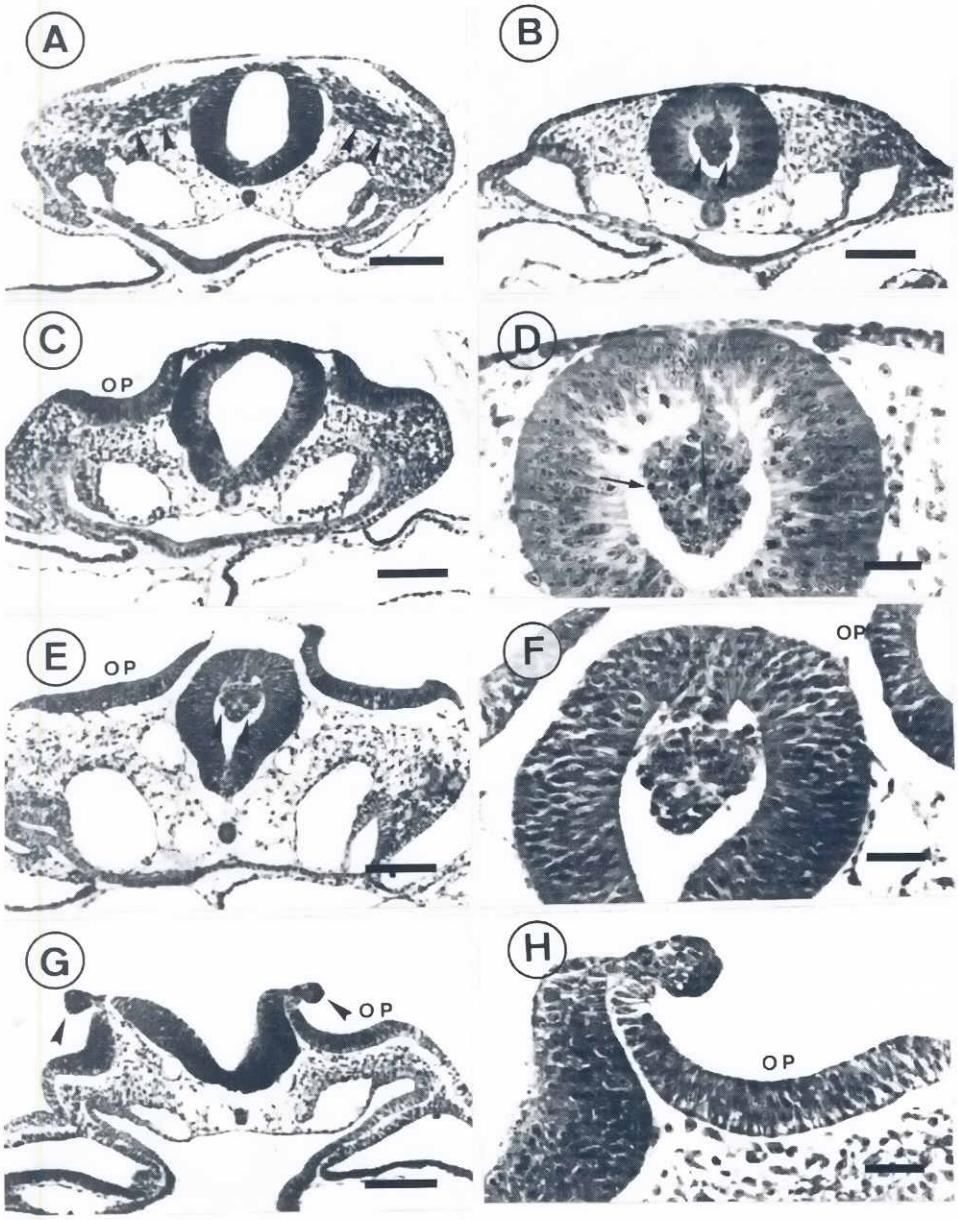

Fig. 4. Comparison between β-D-xyloside treated embryos and non-treated control embryos. (A) *Transversal section of a control embryo, immediately cranial to the otic anlage (rhombomere 4); arrow heads show cells presumably of the neural crest lateral to the rhombencephalon.* **(B)** *Transversal section of a embryo treated with 24 μl of β-D-xyloside 4 mM, at a similar level to the embryo represented in A; arrow heads show presumably neural crest cells invaginated into the rhombencephalic cavity; magnified* **(D)**, *picnotic nuclei may be observed in the interior (arrows). Similar effects are apparent more caudally at the otic primordium (OP) level (rhombomere 5), comparing a control embryo* **(C)** *with a treated embryo* **(E)**; *arrow heads mark crest cells invaginated into the rhombencephalon cavity from its dorsal wall; in* **(F)** *this region is magnified.* **(G)** *Transversal section at the otic primordium (OP) level of a β-D-xyloside treated embryo in which defective rhombencephalon closure is evident; arrow heads show a conglomeration of presumably neural crest cells, evaginating towards the exterior between the neural folds and otic primordium.* **(H)** *Detail of transition zone between the neural fold and otic primordium (OP) of embryo shown in G. Bars, 100 μm (A, B, C, E and G) and 30 μm (D, F and H).*

tion to those of embryos microinjected with the same concentrations of CS.

All these data suggest that certain sulfated GAGs might alter not only cephalic NCC migration, but also delamination and detachment of these rhombencephalic neuroepithelium cells.

β-D-xyloside treated embryos

In this experimental group injection was undertaken "*in ovo*", subgerminally and at an earlier stage (8 stage, Hamburger and Hamilton 1951) than the CS microinjected group of embryos, since the maximum effect of β-D-xyloside is obtained 3-6 h following its administration (Schwartz *et al.*, 1974). Subsequent to reincubation, both control and experimental embryos attained a similar developmental level, which corresponded to stage 12.

Embryos treated with α-D-xyloside and those injected with Ringer solution (control groups) showed no sign of morphological alteration (Fig. 4A and C), whereas β-D-xyloside treated embryos revealed different degrees of malformation. Most affected embryos (29.2%) had intense vascular dilations at dorsal aortas level, similar to those described with microinjection of over 3% CS concentrations. Moreover, the neuroepithelium showed abundant signs of cellular necrosis (data not provided); these embryos were not included in our study. A small embryo group (20.8%) revealed no evident morphological changes, whilst a wider group (50%) had specific cephalic neural crest changes . These changes took place cranial to and at otic anlage level. These differences in the results may be due to the fact that the means by which administration was effected did not permit a strict control of β-D-xyloside absorption, which might have meant variations in its intraembryonal concentration.

Figure 4A shows a control embryo histological section immediately rotral to the otic primordium (this region corresponds to

rhombomere 4). NCC form a stripe at both sides of the rhombencephalon dorsal surface, having become detached from the neuroepithelium. The neural crest migration in rhombomere 4 happens later than that of rhombomeres 2-3, which accounts for the difference between the disposition of the presumptive NCC in the control embryos of Figure 3A and that of Figure 4A. Histological sections of β-D-xyloside treated embryos (Fig. 4B) at the same level as the control embryo of Figure 4A, look very much like those observed in embryos microinjected with 1.5% and 3% CS or HS concentrations. Also visible is a cellular mass which, from the neural tube dorsal surface, is invaginated into its cavity and remains in contact with the neuroepithelium. In addition, it is possible to observe abundant picnotic nuclei inside the invaginated cellular mass (Fig. 4D), perhaps corresponding to dead cells. Among β-D-xyloside treated embryos, there is no evidence of NCC migrating laterally to the rhombencephalon, in contrast to control embryos (compare Fig. 4A and B).

Crest migration alteration extends posteriorly towards otic anlage level (Fig. 4E and F), which is invaginating at this moment. This rhombencephalic region corresponds to rhombomere 5; these changes are identical to those already described in more cranial regions of the rhombencephalon. Pictures showing inhibition of rhombencephalic neural crest migration similar to those shown in Figure 4E and F, have been obtained by Gerchman *et al.* (1995) following microinjection with β-D-xyloside at otocyst level, yet the authors make no reference to these data in their study.

Occasionally neural tube closure was defective in β-D-xyloside treated embryos. In such cases, neural crest cells appear as cellular conglomerates situated between the neural folds and the otic placode, with projection outwards (Fig. 4G and H).

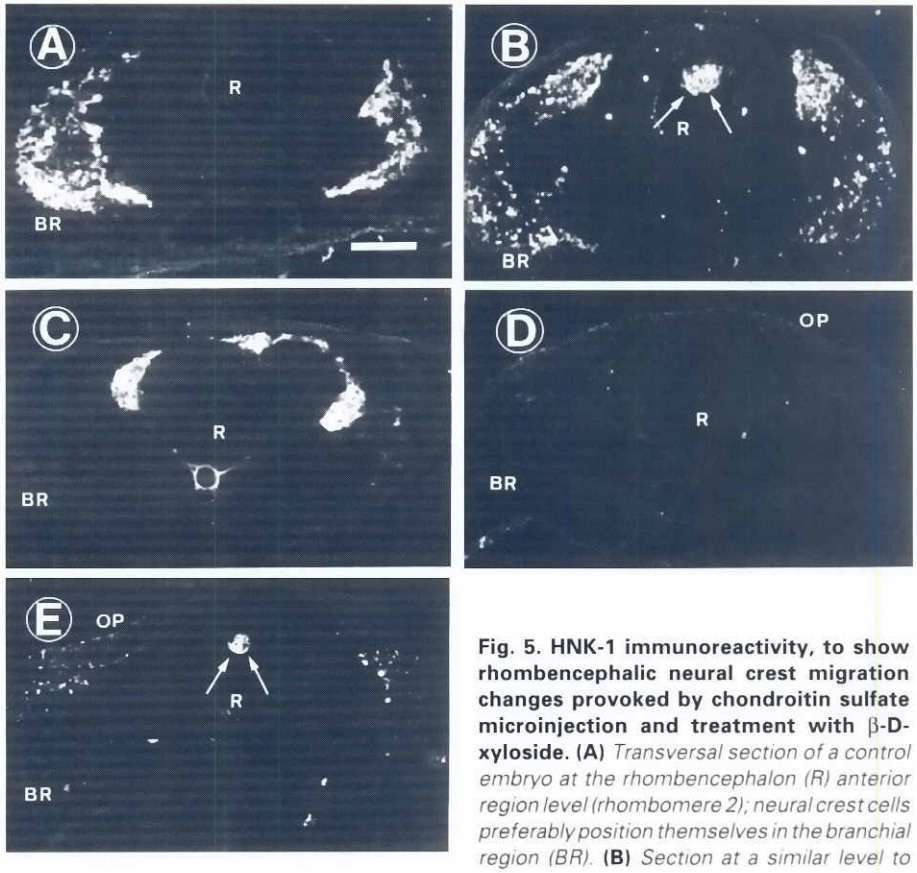

Fig. 5. HNK-1 immunoreactivity, to show rhombencephalic neural crest migration changes provoked by chondroitin sulfate microinjection and treatment with β-D-xyloside. (A) *Transversal section of a control embryo at the rhombencephalon (R) anterior region level (rhombomere 2); neural crest cells preferably position themselves in the branchial region (BR).* **(B)** *Section at a similar level to that of A of an embryo microinjected with 0.5 nl of 3% chondroitin sulfate; immobile neural crest cells can be observed in the proximity of the rhombencephalon (R) and invading neural tube cavity (arrows).* **(C)** *Transversal section at the same level as the previous ones of embryo microinjected with 0.5 nl of 1.5% chondroitin sulfate; neural crest cells have halted on the rhombencephalon dorsal surface (R).* **(D)** *Transversal section of a control embryo at the otic primordium level (OP); this region corresponds to rhombomere 5; no immunoreactivity can be observed in this region.* **(E)** *Transversal section at the same level as before of an embryo treated with 24 μl of β-D-xyloside 4 mM; neural crest cells have invaded rhombencephalic cavity (arrows) and some have stopped below the otic primordium (OP). All pictures have been taken with the same magnification. Bar, 100 μm.*

HNK-1 immunoreactivity

We used Ac. HNK-1, which recognizes NCC (Tucker *et al.* 1984), in order to confirm rhombencephalic neural crest migration changes engendered after CS microinjection or treatment with β-D-xyloside.

Embryos microinjected with chondroitin sulfate

In control embryos which had reached Hamburger and Hamilton stage 12, there was evidence, in the sections carried out cranial to the otic anlage on the level of rhombomere 2 (microinjection area), of subectodermical positive immunoreactivity laterally to the dorsal aortas and pharynx. Many labeled cells which had reached the branchial region (Fig. 5A) were also present. In no case was it possible to witness positive marking either in the rhombencephalon walls nor on its dorsal surface. These immunoreactivity patterns at this level corresponded with the typical example described by other authors in chick development (for review, see Maderson 1987).

Among embryos microinjected with 1.5% and 3% CS concentrations, the immunoreactivity pattern with Ac. HNK-1 differed considerably in relation to control specimens. In embryos having undergone cellular invagination from the rhombencephalon dorsal wall towards the neural tube lumen, a large part of invaginated cellular mass showed HNK-1 positive labeling (Fig. 5B), indicating that NCC had been enclosed in the neuroepithelial wall during neural folds fusion and subsequently invaded the neural tube cavity. In Figure 5B, it is also possible to observe a greater proportion of cells marked with Ac. HNK-1 near the rhombencephalon dorsal wall and a smaller immunoreactivity in the branchial region when compared with control embryos (Fig. 5A), which shows an interruption or a delay in the neural crest migration on this level.

Experimental embryos among which rhombencephalon dorsal wall invagination was less evident (Fig. 5C) showed no signs of

HNK-1 immunoreactivity at neuroepithelium level. Nevertheless, clearly visible were HNK-1 positive NCC which had stopped on the neural tube dorsal and lateral surface.

The existence of these two distribution patterns for NCC in experimental embryos (Fig. 5B and C), leads us to believe that at the moment of CS microinjection there might have been a slight asynchrony. If microinjection were carried out too soon, when neural fold fusion begins, the non-migrating NCC would remain in the neuroepithelium and would subsequently be invaginated into the neural cavity (Fig. 5B). Should microinjection take place a little too late, the neural crest would become totally isolated from the neuroepithelium and NCC halt around the rhombencephalon dorsal wall (Fig. 5C).

β-D-xyloside treated embryos

β-D-xyloside treated embryos revealed similar changes in Ac. HNK-1 immunoreactivity patterns to those described in the last paragraph.

These changes took place cranial to and at the otic anlage level. Figure 5D shows a section of a control embryo at initial otic primordium level (this region corresponds to rhombomere 5). There is a total absence of immunoreactivity in this area, maybe because NCC have already craneally migrated to join the rhombomere 4 migratory stream, as indicated by Birgbauer *et al.*(1995). In β-D-xyloside treated embryos (Fig. 5E), HNK-1 positive cells can be seen at cellular mass level invaginated from the rhombencephalon dorsal wall. It is also possible to observe marked cells ventral to the otic placode: in the first case, these are neural crest cells which have remained joined to the neuroepithelium, and in the second, crestal cells which, as was stated above, have interrupted migration in cranial direction.

Biochemical analyses

Protein calculation

The average number of total proteins per control embryo was estimated to be 138 μg, whilst for β-D-xyloside treated embryos the figure was 146 μg. Data indicate that there are no significant variations between both groups as regards total protein synthesis. This would suggest that, at the doses employed, β-D-xyloside does not affect embryonal growth.

Quantitive analysis of GAGs

Uronic acid is a specific component of most GAGs and its measuring is a parameter for evaluating total concentration in embryonal tissues. Average uronic acid concentration per control embryo, gauged by means of the carbazole technique, was 1.37 μg, compared with average values of 3.51 μg per embryo obtained in the case of β-D-xyloside treated embryos; this constitutes a 156% increase in uronic acid concentration among β-D-xyloside treated embryos in relation to control specimens. This indicates that β-D-xyloside considerably increases total GAGs synthesis, although it does not permit us to estimate which specific GAG increases.

Discussion

In our experiments we managed to inhibit rhombencephalic NCC in chick embryos, increasing by microinjection sulfated GAGs concentrations in the extracellular matrix around the neural folds, at the moment these were about to fuse. NCC became paralyzed in the vicinity of the rhombencephalon dorsal wall, and the majority were enclosed in the neuroepithelium, invaginated into the neural tube cavity. The CS microinjection effect was bilateral, even though the injection was only applied on one side. This can be explained having in mind that the time of microinjection (9HH stage) almost coincides with the neural tube fusion, that in embryos between 9 and 11 somites happens on the rhombencephalon level (Van Straaten *et al.*, 1996), which corresponds to a 9-10 stage of Hamburger and Hamilton (1951). As the neural tube closes, a little mesenchyme fringe appears on the dorsal rhombencephalic surface connecting both sides of the embryo; this might allow the CS spreading, having an effect on both sides of the neural crest. On the other hand, Couly *et al.* (1996) have tested that in one-sided neural crest heterografts from quail to chicken, quail NCC appear in the migratory routes of both sides of the embryo. The bilateral changes in the neural crest emigration produced after the CS microinjection may also be explained on the same basis.

Similar results to those obtained with CS microinjection were obtained following treatment with β-D-xyloside, although in these embryos lesion extent was greater. The changes in the neural crest migration in embryos treated with β-D-xyloside reach in the caudal direction to the otic placode; this region corresponds to rhombomere 5. Nowadays it is being discussed whether rhombomeres 3 and 5 can produce neural crest migratory cells. Graham *et al.* (1993) argue that the NCC of these rhombomeres are removed by means of apoptosis and no cell comes out of the neural crest on these levels. However, Birgbauer *et al.* (1995) have tested by means of DIL marking, that these rhombomeres can produce NCC and these cells migrate both rostral and caudally to join the migratory streams of adjacent rhombomeres. Our data suggest that rhombomere 5 can produce NCC, as in the embryos treated with β-D-xyloside appear HNK-1 positive cells on this rhombomere on the dorsal rhombencephalic wall and near the otic placode (see Fig. 5E). These cells should join rhombomere 4 migratory route, but their migration has been delayed in relation to control embryos (see Fig. 5D), in which there is not any NCC marked with Ac. HNK-1, because in stage 12 HH these cells would have already moved towards more cranial regions.

β-D-xylosides have frequently been employed to study the role played by sulfated proteoglycans in embryonal development among different species of animals: in rat embryos (Morriss-Kay and Crutch 1982), chick embryos (Gibson *et al.*, 1979; Kanke *et al,*. 1982; Segen and Gibson 1982), sea urchin embryos (Kinoshita and Saiga 1979) and in *Xenopus laevis* embryos (Yost 1990). In order to assess the action of β-D-xyloside on embryonal GAGs, we determined their total concentration per embryo by quantifying the uronic acid; we found that this increases by 156% in relation to control embryos. Determining the total number of proteins did not reveal significant differences between control embryos and those treated with β-D-xyloside. This appears to indicate that, at the dosages we employed, β-D-xyloside acts specifically on GAGs synthesis. Other toxic effects xylosides might have on embryo development, were ruled out by using as controls embryos treated with α-D-xyloside (an inactive anomer of β-D-xyloside). In this control group none of the morphological changes brought about by β-D-xyloside were detected.

The P-nitrophenyl β-D-xylopyranoside, used in our experiments, selectively disrupts the synthesis of CSPG in embryonal tissues and cellular cultivations from different sources, without

altering the synthesis of other proteoglycans (Galligani *et al.*, 1975; Spooncer *et al.*, 1983; Sobue *et al.*, 1987; Lugemwa and Esko 1991). The P-nitrophenyl β-D-xylopyranoside produces an increase in CS free chains, in its role as artificial initiator of GAGs synthesis (Robinson *et al.*, 1975; Sobue *et al.*, 1987). According to our data, it may be supposed that the increase in uronic acid we detected in β-D-xyloside treated embryos might be largely due to an increment in CS free chains. However, we cannot rule out the possibility that there may be changes in CSPG concentrations. On this matter, Gibson *et al.* (1979) maintain that β-d-xyloside provokes the synthesis of a shorter proteoglycan, and that the resulting CS chains are less sulfated.

Given that treatment with β-D-xyloside produces alterations in rhombencephalic neural crest migration which closely resemble those induced by the microinjection of sulfated GAGs (CS and HS), it is fitting to propose that an increase in CS free chains at extracellular matrix level might disrupt neural crest migration. In this regard Tucker and Erickson (1986) tested that the addition of CS free chains affects the migration of amphibian pigmentary cells in collagen gels. More recently Pettway *et al.* (1996) observed that chondroitinase treatment significantly reduces the inhibitory effect of an extra notocord implanted in the ventromedial migratory route of the trunk neural crest. This fact suggests that the CSPG glycosylation may be an important factor in the inhibition of the neural crest migration, and the results we have obtained lead also to that conclusion; it could even be necessary a determinate concentration of sulfated GAGs in the extracellular matrix so as to make evident the inhibitory effect, as low CS concentrations (lower than 1.5%) do not affect the migration.

Most authors attribute to CS an inhibiting effect on neural crest migration (see Introduction). Morriss-Kay and Tucket (1989), however, maintain that enzymatic degradation of CSPG by treatment with ABC chondroitinase facilitates cephalic neural crest migration in rat embryos, as adhesivity of NCC to extracellular matrix fibronectin diminishes. Nevertheless, ABC chondroitinase does not cause specific degradation of CSPG since, albeit to a lesser degree, it also has activity on dermatan sulfate and hyaluronate, and the latter molecule appears to have an important facilitating effect on neural crest migration, especially in early stages (Derby 1978; Pintar 1978; Perris *et al.*, 1991b). Furthermore, general via administration of an enzyme may induce embryonal development alterations which in turn disrupt neural crest migration. Enzymatic degradation of HS by treatment with heparitinase does not disrupt cefalic neural crest migration, although cranial neurulation is seriously perturbed in rat embryos (Tuckett and Morriss-Kay 1989). Perhaps for neural crest migration to take place correctly an adequate proportion must be maintained between the different molecules making up the extracellular matrix; along these lines Moro Balbas *et al.* (1993) proved that treatment with retinoic acid causes an increase in hyaluronate together with a decrease in sulfated glycosaminoglycans in the extracellular matrix, provoking changes in rhombencephalic neural crest migration in chick embryos.

The process by which CS may influence neural crest migration is not clear and the matter is one of controversy. Daniels and Solursh (1991) showed, by neural tube explants in culture, that β-D-xyloside paralyzes neural crest migration, and they suggested that β-D-xyloside reduces CSPG on the surface of neural crest cells, disrupting migration. These authors base the decline in CSPG on immunonegativity to the mAb CS56 antibody, which recognizes CSPG's GAG portion but not the protein core; this immunonegativity may, therefore, be the result of GAG loss during tissue process. As stated above, our biochemical results suggest that β-D-xyloside may bring about a rise in CS free chains synthesis and this increase could, may be due to modulation of cell adhesion by steric exclusion, be responsible for neural crest migration disruption. In fact, it is known that GAGs can promote cellular aggregation, preventing cells from dispersing; what is more, certain non-biological polymers such as polyethylene glycol and polyvinyl alcohol possess a similar effect, and the provoking of aggregation does not appear to depend on whether the polymers are neutral or whether they are negatively charged; nor does there seem to be any molecular level cross-linking (Morris 1993). It has been proposed that, as a result of their provoking volume exclusion, polymers might increase cell concentration, making them join and thereby augmenting their natural adhesive potential. Regarding this matter, cell dispersion or migration might be linked to GAGs synthesis regulating cycles. Morris (1993) suggests the capacity of cells to disperse or join might be modulated by polymers like CS; polymers on the cellular surface would tend to force cells to separate, whereas those localized in solution or in the matrix would tend to make them remain joined.

Another possibility is that molecules inhibiting migration may act directly, affecting cellular motility by paralyzing protrusive activity upon contact or by depressing specific motile activities (Oakley *et al.*, 1994). In this regard it is known that the CSPG affects the growing and the axonic guide in neurons of dorsal root ganglia. Snow *et al.* (1994) have tested that the CSPG produces changes in the cytoplasmic Ca^{2+} levels of these neurons and they suggest that this proteoglycan might somehow control the cellular cytoskeleton dynamics. The theory has also been postulated that CS might prevent the access of cells to matrix molecules acting as a substrate for migration, by literally covering these molecules. In relation to this it has been demonstrated that CS is capable of bearing with fibronectin (Ruoslahti, 1988) and hyaluronic acid (Perris and Johansson, 1990). It is known that both molecules (fibronectin and hyaluronic acid) play an important role in neural crest migration.

Microinjection of over 3% CS concentrations causes serious embryonal vascular alterations, consisting fundamentally of in-

TABLE 1

CONCENTRATIONS OF CS AND HS EMPLOYED AND NUMBER OF EMBRYOS MICROINJECTED

	CS	HS	Ringer solution
0.35%	10	–	10
0.75%	15	–	10
1.5%	30	13	25
3%	25	11	17
6%	15	–	15

Number of embryos utilized in treatment with xylosides

β-D-xyloside	α-D-xyloside	Ringer solution
61	32	38

tense dorsal aortas dilations; we obtained similar effects in some β-D-xyloside treated embryos (29.2%). In this way, Morriss-Kay and Crutch (1982) make reference to cardiovascular alterations following β-D-xyloside treatment in early developmental stage rat embryos.

Occasionally we detected defective neural tube closure in β-D-xyloside treated embryos, reminiscent of those described by Morriss-Kay and Crutch (1982) in rat embryos. Chondroitin sulfate may play an important role in neuroblasts migration within the neural tube as well as in neuroepithelium curvatures formation, engendering a flexibility which could promote elevation and eventual contact with neural folds (Morriss-Kay and Tuckett 1989).

Materials and Methods

Fertile White Leghorn chicken eggs, were incubated at 38°C in a forced draft egg incubator, with 90% relative humidity. Embryos to be microinjected were incubated for 33 h until reaching the 9 stage of Hamburger and Hamilton (1951), whilst those to be treated with β-D-xyloside were incubated for 27 h until they attained the 8 stage.

Chondroitin sulfate microinjection and embryo culture

Embryos were extracted from the yolk and placed in a Petri plate with sterile Ringer at 37°C, at which cleaning and classification took place; only 9 stage embryos being employed. Subsequently, microinjection was effected with microneedles of 10 μm tip diameter. Following the loading of the microneedles, they were connected to a microinjector (Medical Systems Corp., Greenvale, NY 11548, PLI-100) and microinjection began: 0.5 nl of different chondroitin 4-6 sulfate (CS) concentrations taken from bovine trachea (Sigma) or heparan sulfate (HS) from bovine kidney (Sigma), dissolved in Ringer solution. Microinjection was subectodermal and unilaterally in the vicinity of the neural folds at the rhombencephalon anterior portion level, rostral to the cardiac anlage (Fig. 1). The control embryos were microinjected solely with Ringer solution. The different concentrations of CS and HS employed, as well as the number of embryos microinjected, are summarized in Table 1.

After microinjection the embryos were cultivated in a medium made up of homogenized egg and agar for 20 h, in accordance with the technique described by Britt and Herrmann (1959), until reaching the 12 stage of Hamburger and Hamilton (1951).

Treatment of embryos with β-D-xyloside

Subsequent to incubation, a small window was made in the eggshell and embryo classification commenced; only the 8 stage embryos were utilized in our study. Using a Hamilton syringe, we proceeded to inject subgerminally a 24 μl dose of a solution of 4 mM P-nitrophenyl β-D-xylopyranoside (Sigma), dissolved in Ringer. We made two groups of control embryos: The first group was only injected with saline solution (Ringer). The second control group was treated with p-nitrophenyl-β-D-xylopyranoside from Sigma (α-D-xyloside), at the same concentration as the one used for β-D-xyloside; α-D-xyloside is an inactive anomer of β-D-xyloside. This control group was employed to rule out possible direct toxic effects of the xylosides on embryonal development and growth.

Once injection had been completed, we sealed the opening in the shell with adhesive plastic and proceeded to reincubate the embryos for 26 h, until they reached the 12 stage. The number of embryos utilized in each of the series is summarized in Table 1.

Light microscopy

Embryos to undergo light microscopy were fixed in Bouin solution, dehydrated in graded ethanol and embeded in paraplast. The embryos were transversally oriented and serially sectioned at 8 μm. Sections were stained with haematoxylin-eosin. The most representative sections were photographed with a Nikon microphot-FXA photomacroscope.

Scanning electron microscopy

We used the technique described by Schoenwolf (1995) so as to obtain accurate level embryo sections. Following fixing in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, the embryos were dehydrated in acetone and embeded in paraplast. Paraplast blocks were cut until the required level was obtained, at which moment deparaffining was carried out with exposure to xylene and immersion in acetone. Subsequently, the embryo was dried with liquid CO_2 by 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. For visualization and photographing the samples, we used a Jeol T-300 scanning electron microscope.

HNK-1 immunoreactivity

Embryos to undergo this technique were fixed in Carnoy and processed for immunocytochemistry using a HNK-1 antibody (Mouse IgM monoclonal anti-leu 7 HNK-1, dilution 1:50; Becton and Dickinson), which identified NCC (Tucker *et al.*, 1984). Primary antibody was recognized using a FITC antimouse IgM (Sigma), following standard procedures for immunofluorescence. Negative controls were carried out replacing the first antibody with preimmune serum. For visualization and photographing the preparations we used an confocal microscope (Zeiss LSM-310).

Biochemical analyses

A total of 13 β-D-xyloside treated embryos and another 13 α-D-xyloside injected control embryos were used for biochemical considerations. After their securing, the embryos were homogenized in 500 μl. Ringer solution. Following this process, we went on to quantify the total number of GAGs and proteins in the macerated embryos. This would permit us to rule out possible direct toxic effects of β-D-xyloside on embryonal growth.

Isolation and purification of GAGs

The glycosaminoglycans isolation and purification was based in the method proposed by Beely (1985). In summary, aliquots from control and treated embryos, were exahustively digested with Protease (Type XIV from Sigama), GAGs were taking from to the digest by precipitation with 5 volumes of 5% potassium acetate in absolute ethanol at 4°C and centrifugation at 17500 G.

Quantitative analysis of GAGs

Total amount of GAGs isolated from control and β-D-xyloside treated embryos, were estimated by the uronic acid-carbazole reaction according to the method of Bitter ans Muir (1962). Briefly, GAGs were vigorously hydrolyzed in 0.025 M sodium tetraborate in sulphuric acid. Following 200 μl of 0.125% carbazole (from Fluka) in absolute ethanol was added. The optical density was read at 530 nm and the absorbance value was plotted against the optical densities developed by standard solutions of CS.

Protein analysis

Total concentration of proteins from control and β-D-xyloside treated embryos was determined by using a Bio Rad protein assay, based in the Bradford (1976) dye binding procedure.

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