Prorhombomeric subdivision of the mammalian embryonic hindbrain: is it functionally meaningful?

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ABSTRACT The technique of whole embryo culture has made significant contributions to understanding the mechanisms of morphogenesis in mammalian embryos, especially with respect to cranial neurulation and neural crest cell migration. This study traces the fate of two specifically mammalian structures, the preotic and otic sulci. Their formation at the 1/2- and 3-somite stages respectively, divides the hindbrain neuroepithelium into prorhombomeres A, B and C. The preotic sulcus is a deeply recessed structure that forms the rostral boundary of expression of both *Hoxb-2* and the first domain of *Krox-20*. The otic sulcus is a shallow concavity in which the second *Krox-20* domain is expressed. DiI labeling followed by whole embryo culture confirmed that the later fate of the preotic sulcus is the rhombomere 2/3 boundary, and the fate of the otic sulcus is the cranial part of rhombomere 5. Structurally, the preotic and otic sulci show no specialization with respect to actin, tubulin or proteoglycans, but their maintenance depends on contact with the subjacent mesenchyme. Their formation is inhibited by exposure of embryos to retinoic acid prior to the onset of somitic segmentation, indicating that the molecular events governing prorhombomeric subdivision of the hindbrain are retinoic acid-sensitive. The preotic sulcus may be essential for neuroepithelial cell movement towards and into the rapidly enlarging forebrain; the otic sulcus may simply delineate the caudal boundary of prorhombomere B, an area with a discrete neural crest cell population discontinuous with those rostral and caudal to it. Understanding the positional relationships of the preotic and otic sulci to later rhombomeric segments makes them useful landmarks for experimental purposes, but there is no evidence that prorhombomeres are functionally significant as the precursors of rhombomeric segments.

KEY WORDS: *neuroepithelium, segmentation, preotic sulcus, otic sulcus, prorhombomere*

Introduction

The technique of whole rat and mouse embryo culture pioneered and refined by Denis New (1978 and references therein) has provided the basis for a wide spectrum of experimental approaches to mammalian development. Studies aimed at improving the technique have themselves led to new insights into developmental mechanisms: e.g. the finding that a low oxygen gas phase is required for normal cranial neurulation in rat embryos (New *et al.*, 1976), led to the discovery that emigration of neural crest cells from the midbrain and rostral hindbrain neural folds is essential for neural tube closure (Morriss and New, 1979).

The early postimplantation stage of mammalian development is the period during which the body plan is established. Understanding the developmental processes that unfold during this period is fundamental to understanding how the embryo is organized as a whole. During organogenesis stages, many developing systems can be separated from the embryo and studied in organ culture, but during early postimplantation stages the embryo is undergoing fundamental morphogenetic processes including gastrulation, neurulation, neural crest cell migration, somitogenesis, and cardiogenesis. Culture of isolated component parts of the embryo at this stage is of limited usefulness: each process has some degree of autonomy, but interdependence and integration of the whole is essential to the intrinsic development of each part. Because mouse and rat embryos grow and develop normally *in vitro* during this period, and can be physically and chemically manipulated after dissection from the uterus, the technique of whole embryo culture has made a very significant contribution to understanding morphogenesis in both normal and mutant embryos. Studies carried out using this technique have provided

Abbreviations used in this paper: TEM, transmission electron microscopy; SEM, scanning electron microscopy; RA, retinoic acid, r, rhombomere; pr, prorhombomere.

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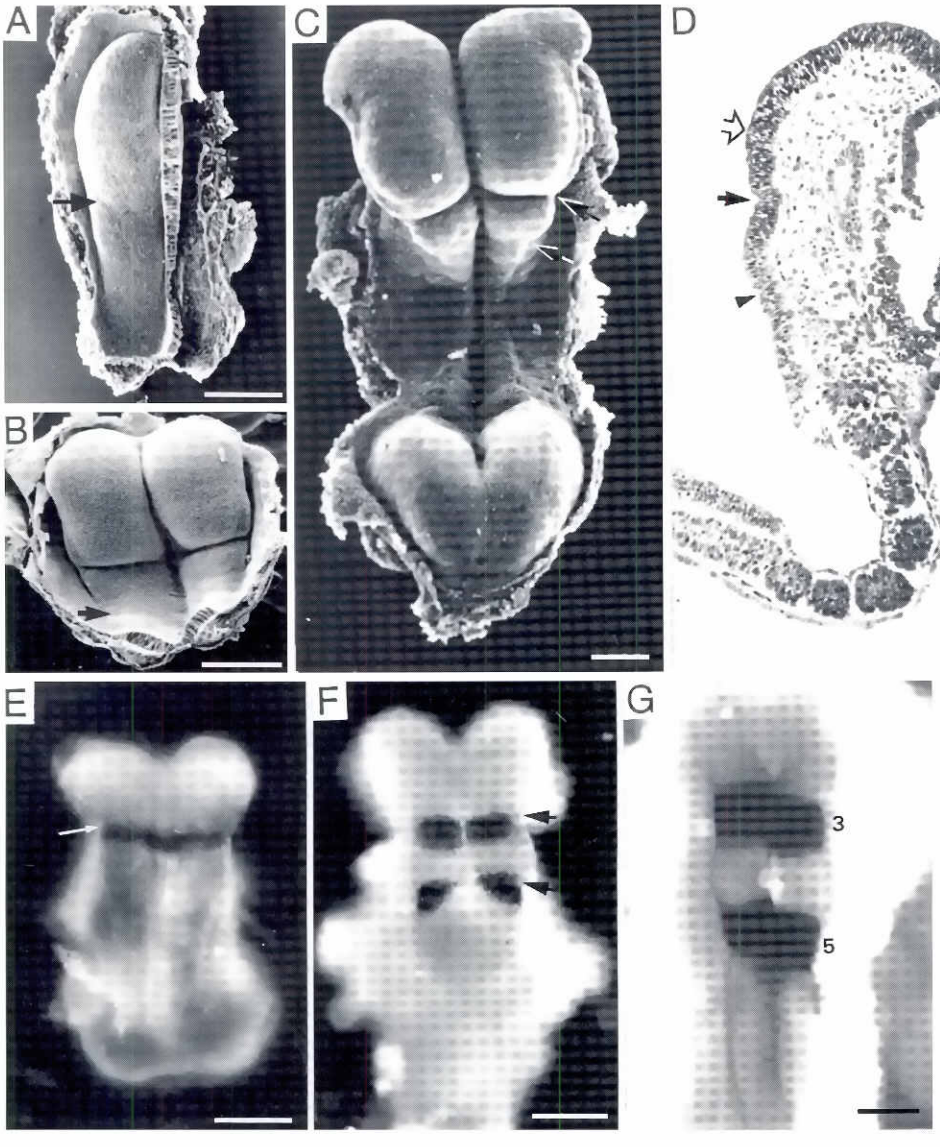

Fig. 1. Scanning electron-micrographs showing development of the preotic and otic sulci, and their relationship to *Krox-20* expression. **(A)** *SEM of a late presomite-stage rat embryo showing a slight depression (arrow) in the neuroepithelial surface where the preotic sulcus will develop.* **(B)** *SEM of a 3-somite stage embryo with a fully-developed preotic sulcus but only a slight depression (arrow) where the otic sulcus will form.* **(C)** *SEM of a 6-somite stage embryo with fully-formed preotic and otic sulci. Bar for A-C, 200 μm.* **(D)** *Semithin plastic section through the head of a 7-somite stage mouse embryo showing the preotic (arrow) and otic (arrowhead) sulci. Bar, 100 μm.* **(E-G)** *Mouse embryos at 4-, 7-, and 11-somite stages showing in situ hybridization for Krox-20. Bar, 200 μm.*

insights that are important for the functional interpretation of observations using fixed whole and sectioned embryos, e.g. in studies using electron microscopy or *in situ* hybridization.

Mammalian embryos have important differences from other vertebrate embryos, and it is not always safe to extrapolate from the results of experimental morphogenetic studies in avian or amphibian embryos to make functional interpretations of mammalian development. In contrast, rodent and human embryos are very similar to each other during early postimplantation stages, with respect to both morphogenesis and genetic conservation; research on early rat and mouse embryos can provide an important basis for understanding mechanisms of normal and abnormal human development. The potential of the whole embryo culture technique to contribute to our understanding of the developmental processes and mechanisms of mammalian cranial neurulation and neural crest cell migration has been exploited extensively by three laboratories (e.g. Tan and Morriss-Kay, 1986; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1996). The present study adds a small detail to this series, by tracing the fate of the prorhombomeric subdivisions of the early hindbrain neuroepithelium to the rhombomeres of the closed hindbrain.

Early subdivisions of the human hindbrain have been termed rhombomeres A-C (Bartelmez, 1923) or rhombomeres A-D (Müller and O'Rahilly, 1983). Here we use the term prorhombomere (pr) A, B and C, in order to distinguish these early subdivisions from the seven rhombomeres that form later, and the term "occipital hindbrain" for the region level with the occipital somites (Müller and O'Rahilly's rhombomere D). Prorhombomere B is first observed in human embryos with one or two pairs of somites (Bartelmez, 1923), although in rodents the prB/C boundary is not distinct until the 3-somite or 4-somite stage (Morriss-Kay, 1981). The sulcus dividing prA and prB is the preotic sulcus (Adelmann, 1925). Throughout this report, we refer to the prB/C sulcus as the otic sulcus. The area between the two sulci has been termed the otic segment (Bartelmez, 1923) or "otic rhombomere" (Adelmann, 1925), because the thickened area of surface ectoderm lateral to

it is identified as the "otic disc". However, early human embryonic material is rare and difficult to study, and the position of the ectodermal thickenings varies between the embryos so far described, extending caudally almost to the first pair of somites in the Müller and O'Rahilly (1983) embryo Da 1. Since the position of the closed otic vesicle of later embryos indicates that the fate of the so-called otic segment is preotic, the neutral term prB is more appropriate.

There is no consensus as to the relationship between the sites of the prorhombomeric subdivisions and later rhombomeric segmentation. Bartelmez (1923) and Adelmann (1925) considered the preotic sulcus to be the future boundary between rhombomere (r)3 and r4. Bartelmez and Evans (1926) later published a revised assessment, identifying the preotic sulcus as the future boundary between r2 and r3, but it is the earlier reports that have been quoted in recent studies. The r3/4 interpretation was used by us in a description of the changing domains of expression of the retinoic acid binding proteins CRABP I and CRABP II (Ruberte *et al.*, 1992). This interpretation places the preotic sulcus at the caudal boundary of the r3 *Krox-20* domain, and at the rostral boundary of the *Hoxb-1* and *Hoxa-1* domains, an interpretation that has also been made by Murphy and Hill (1991) and by Conlon and Rossant (1992). Careful comparison, using superimposed tracings, of the morphology and gene expression patterns in these and other publications (Wilkinson *et al.*, 1989a,b; Marshall *et al.*, 1992) showing relevant sections, and our own unpublished material, suggests that this is a misinterpretation and that the preotic sulcus coincides with the rostral boundary of both *Hoxb-2* and the first of the two *Krox-20* domains. This would be consistent with a fate at the r2/3 boundary. In order to settle this uncertainty, and to identify the fate of the prB/C boundary, we have labeled the preotic and otic sulci of mouse and rat embryos with DiI and allowed them to develop in culture until rhombomeres are well formed.

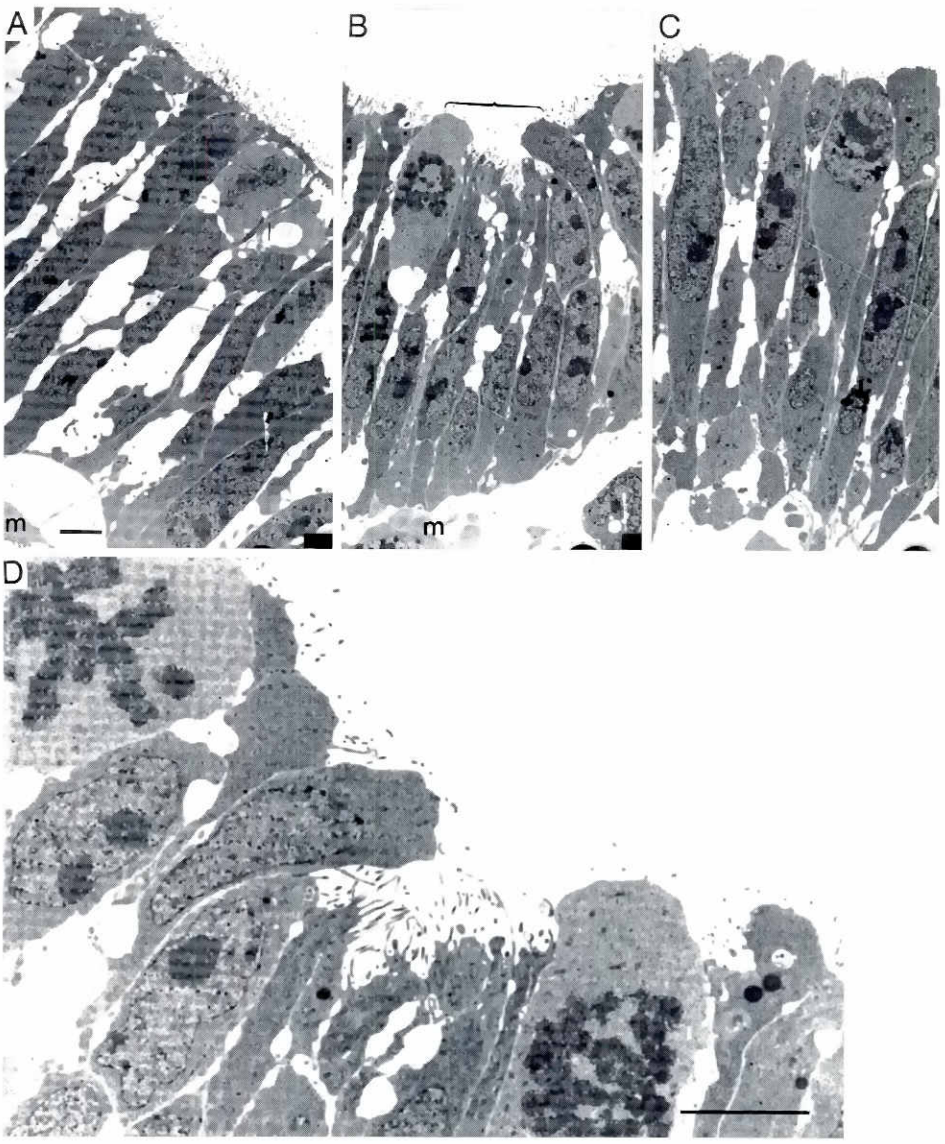

Fig. 2. Transmission electron micrographs at three regions of the neuroepithelium of a 6-7-somite stage mouse embryo: (A) *midbrain, showing cytoplasmic bridges between cells at the apical surface;* **(B)** *preotic sulcus (bracketed), showing recessed, small cells between two projecting cells;* **(C)** *prorhombomere B, showing the neuroepithelial structure seen throughout the hindbrain except for the preotic sulcus (but including within the otic sulcus);* **(D)** *preotic sulcus region at higher magnification (reversed image). m, mesenchyme. Bar, 5 μm.*

Very little information is available in the literature on the fine structure of the preotic and otic sulci, or on the mechanism by which their structure is maintained. We have therefore looked for ultrastructural and molecular specializations using transmission electron microscopy and immunohistochemistry, and have analyzed the importance of microfilaments, microtubules and some components of the extracellular matrix in the structural maintenance of the two sulci. We have also investigated the effects of retinoic acid on their formation.

Results

Normal structure of the neuroepithelium: light microscopy and scanning electron microscopy

In both rat and mouse embryos, freshly-dissected embryos observed by light microscopy and fixed embryos observed by scanning electron microscopy showed the preotic sulcus to appear as the first two pairs of somites are segmenting (Fig. 1A) (these two pairs of somites segment simultaneously in rodents). The otic sulcus was not apparent before the three-somite stage (Fig. 1B). By the 6-somite stage, the preotic sulcus is a clearly delineated transverse depression of the neuroepithelium, and the otic sulcus a shallow transverse concavity (Fig. 1C). After the 8-

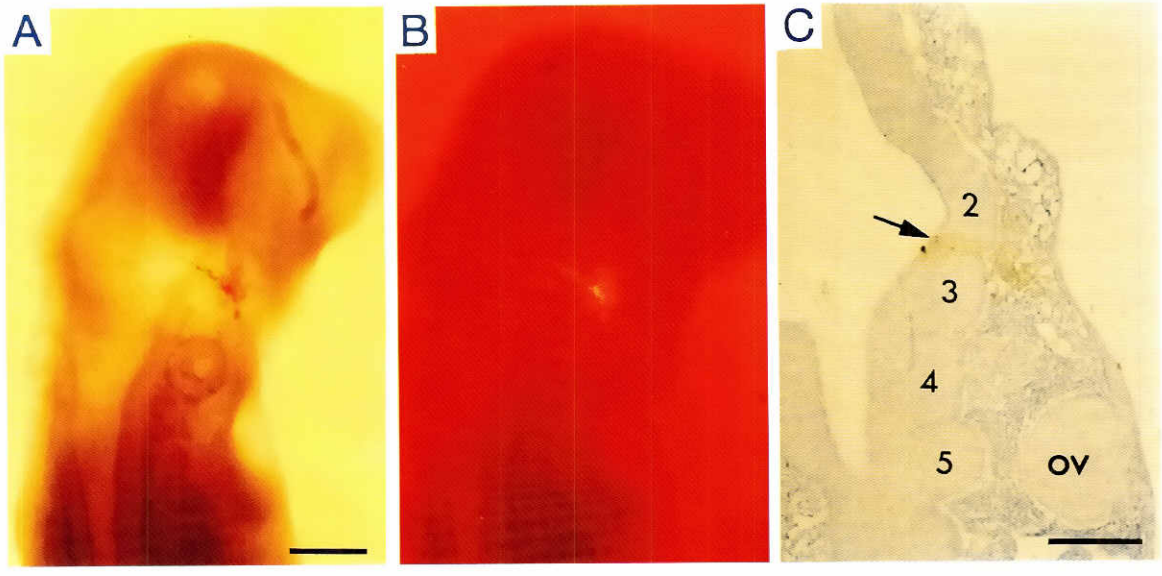

Fig. 3. Rat embryos injected with DiI in the preotic sulcus and cultured to the rhombomeric stage of hindbrain development. (A and B) *show the same embryo in light and fluorescence microscopy. The labeled cells are located between rhombomeres 2 and 3. Bar, 250 μm.* **(C)** *Coronal section through the rhombomeres after photo-oxidation confirming the r2/3 position of the labeled cells (arrow). Bar, 100 μm. ov, otic vesicle.*

somite stage the preotic sulcus becomes less distinct, and it has flattened out by the 10-somite stage; by the 11-somite stage rhombomeric sulci and gyri are well developed (stages not shown here are illustrated in Morriss-Kay, 1981; Morriss-Kay *et al.*, 1991). Light microscopy of 6-7-somite stage embryos (Fig. 1D) showed the preotic sulcus to have a complementary depression of the basal surface of the epithelium into the subjacent mesenchyme.

Krox-20 expression and neuroepithelial morphogenesis

Krox-20 is first expressed in the neuroepithelium at the 2-3-somite stage as a fine transverse stripe four or five cells wide across the whole width of the epithelium, within and just caudal to the preotic sulcus (Fig 1E; a slightly earlier stage is illustrated by Schneider-Maunoury *et al.*, 1993). It broadens during the 3-6-somite stages by extending caudal to the sulcus (Fig. 1F). At the 5-6 somite stage a second stripe appears in the rostral part of the otic sulcus, between prorhombomeres B and C; this domain also extends caudally, the lateral edge being in advance of the medial part of the domain (Fig. 1F). By the 12-somite stage the two *Krox-20* expression domains are clearly located in rhombomeres 3 and 5 (Fig. 1G), as previously described (Wilkinson *et al.*, 1989a).

Transmission electron microscopy (TEM)

Embryos at the 6-7-somite stage were studied in more detail by TEM. The apical surface of the forebrain and midbrain neuroepithelium showed a different cellular organization from that of the hindbrain, having overlapping cell processes joined by adherens junctions at the apical surface (Fig. 2A). Throughout the hindbrain, including the preotic sulcus, the neuroepithelial cells were oriented parallel to each other throughout their length, so that the apical regions did not overlap and the adherens junctions were oriented perpendicular to the surface (Fig. 2B,C). Gap junctions were observed subapical to approximately 1 in 10 of the adherens junctions in all regions. Microfilaments were associated with all of the adherens junctions, but neither in the preotic sulcus nor elsewhere were they seen to project for long distances across the cells as previously described for areas of concave curvature at later stages (Morriss and New, 1979).

The preotic sulcus was seen to involve a recessed transverse line 8-10 cells wide (Fig. 2B). Microtubules were observed with low frequency in both the preotic sulcus and elsewhere. The preotic sulcus cells were rich in apical microvilli, longer than those of adjacent cells (Fig. 2B,D). Their cytoplasm was more heterogeneous than that of the non-sulcus cells, with more mitochondria, in contrast to the more polyribosome-rich cytoplasm elsewhere.

In contrast to the preotic sulcus, the otic sulcus was characterized by a shallow neuroepithelial curvature whose depth was variable between embryos; it showed no detectable structural or ultrastructural differences from the non-sulcus hindbrain neuroepithelium rostral and caudal to it.

DiI injections

3-to 6-somite-stage rat and mouse embryos, in which the preotic sulcus could clearly be seen, were microinjected with DiI solution into the preotic or otic sulcus and allowed to develop in culture for 24 h (mouse) or 36 h (rat). The purpose of this procedure was to determine the position of the labeled sulcus cells in the neuroepithelium at a stage when rhombomeres are clearly visible. After culture the embryos were observed by epifluorescence. In specimens injected into the preotic sulcus, discrete labeling was present in the neuroepithelium level with the caudal border of the first pharyngeal arch, and was located in the gyrus cells between r2 and r3 (Fig. 3A,B). Little labeling was observed in the mesenchyme of the head and the pharyngeal arches were negative, indicating that the neuroepithelium in the region of the preotic sulcus does not contribute to the neural crest. The small amount of mesenchymal labeling is likely to be due to some contamination of primary mesodermal mesenchyme at the time of injection, since the injection pipette sometimes penetrated the epithelium. Six embryos were sectioned after photo-oxidation (Fig. 3C). All showed labeling in the r2/3 gyrus, and two additionally showed labeling in the most cranial part of rhombomere 3 (not shown). No labeling was observed in any other rhombomeres.

In order to verify that the absence of labeling in the pharyngeal arches was due to lack of neural crest cell migration from the labeled area and not to the method used, some embryos were

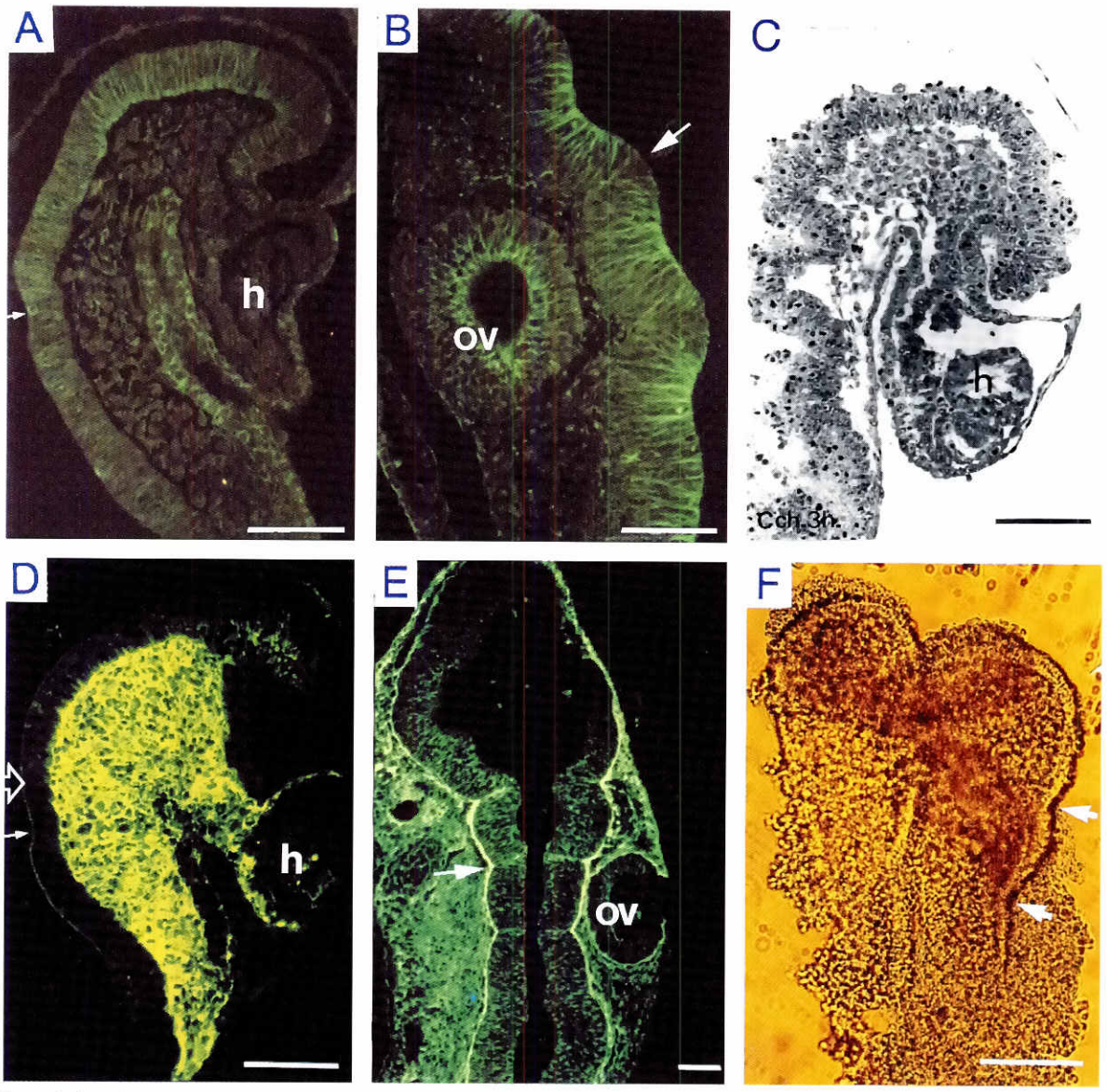

Fig. 4. Tubulin and chondroitin sulphate proteoglycan (CSPG) in the cranial region of 5- somite and 16-somite-stage mouse embryos, and effects of colchicine and cytochalasin D. (A,B) *Immunohistochemical localization of tubulin shows rhombomere-specific* **(B)** *but not prorhombomere-specific features* **(A)**. **(C)** *Breakdown of tubulin by colchicine exposure for 3 h causes neuroepithelial-mesenchymal detachment except at the preotic and otic sulci.* **(D)** *At the 5-somite stage, a low level of CSPG is present in the forebrain and midbrain but not the hindbrain neuroepithelium (the midbrain/hindbrain junction is marked with an arrow), but shows no special relationship to the preotic or otic sulci;* **(E)** *at the 16- somite stage, CSPG is present in the rhombomeric gyri.* **(F)** *4-5-somite-stage embryo after exposure to collagenase and cytochalasin D: on the left side the mesenchyme has been removed and the preotic and otic sulci (arrow on the right side) have disappeared. Bars, 100 μm. h, heart; ov, otic vesicle.*

injected either rostral to or caudal to the preotic sulcus; after culture these showed labeled cells in the first or second pharyngeal arch, the pathways extending from r1/2 and r4, respectively (not shown: similar labeling has been reported elsewhere: Fukiishi and Morriss-Kay, 1992; Morriss-Kay *et al.*, 1993).

Using the same procedure, 5-7-somite stage embryos were labeled in the otic sulcus. After 24 h in culture DiI-labeled cells were present in the rostral half of r5 (not shown).

Immunohistochemistry and cytoskeletal disruption

Immunohistochemical staining for tubulin, actin, chondroitin sulphate proteoglycan (CSPG), heparan sulphate proteoglycan (HSPG) and type IV collagen was compared in sagittally cut 6-7-somite stage mouse embryos (day 8.5) and coronally cut 16-18-somite stage embryos (day 9.0). Anti-tubulin staining showed no relation to the preotic sulcus (Fig. 4A) in contrast to its clear rhombomeric sulcus-specific pattern on day 9 (Fig. 4B). Colchicine treatment affected neuroepithelial structure at day 8.5, but even after 3 h, both the preotic and otic sulcus regions of the epithelium maintained their attachment to the underlying mesenchyme, in contrast to the epithelio-mesenchymal separation observed in non-sulcus regions (Fig. 4C).

Anti-CSPG staining at day 8.5 (Fig. 4D) was strong within the cranial mesenchyme as previously described (Morriss-Kay and

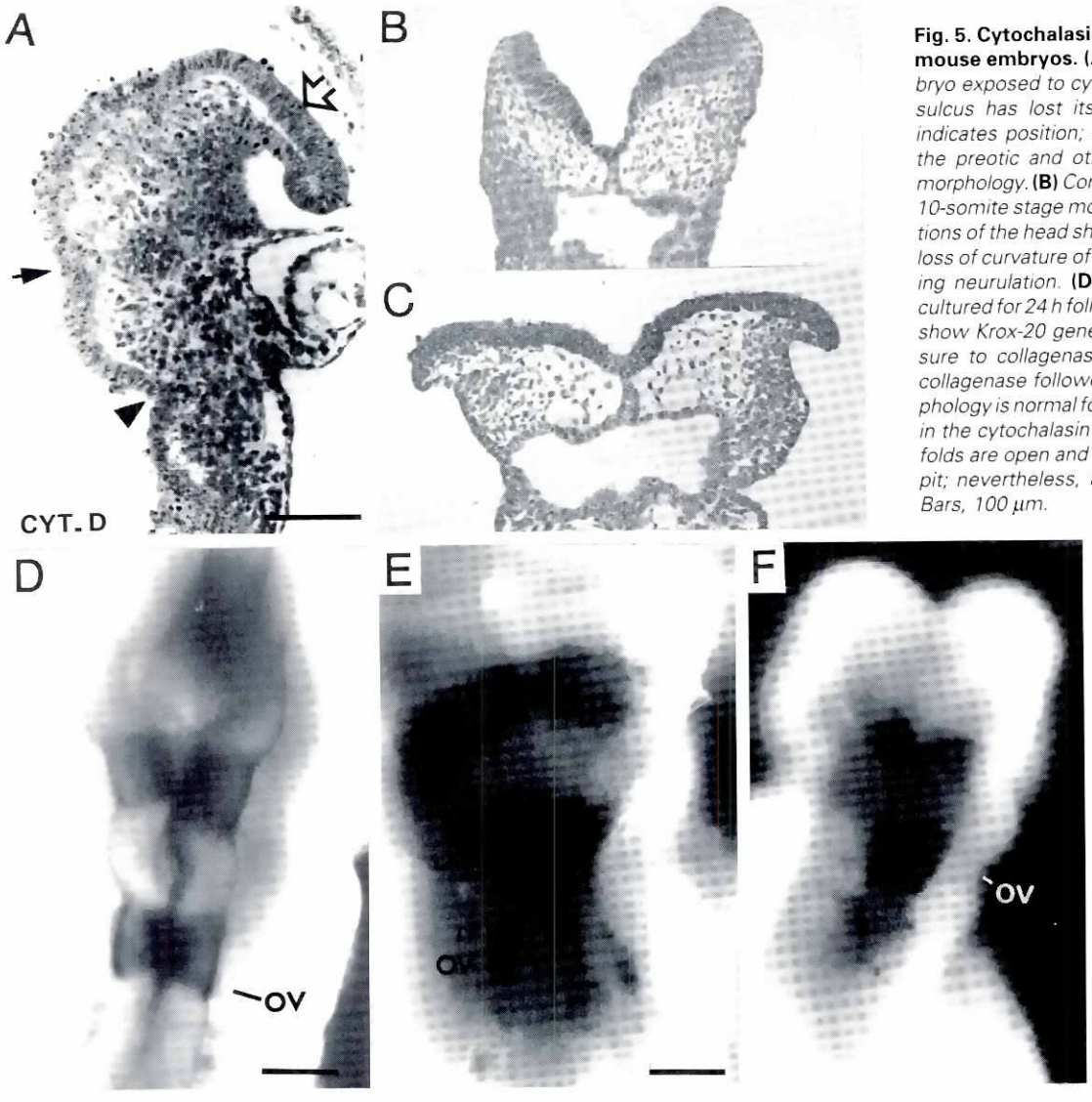

Fig. 5. Cytochalasin and collagen treatment of mouse embryos. (A) *7-somite stage mouse embryo exposed to cytochalasin D for 1 h: the optic sulcus has lost its concave curvature (bracket indicates position; compare with Figure 1D) but the preotic and otic sulci (arrow) show normal morphology.* **(B)** *Control;* **(C)** *cytochalasin D-treated 10-somite stage mouse embryos: transverse sections of the head showing cytochalasin D-induced loss of curvature of the neuroepithelium undergoing neurulation.* **(D-F)** *16-somite-stage embryos cultured for 24 h followed by in situ hybridization to show Krox-20 gene expression after a 1 h exposure to collagenase* **(D)**, *cytochalasin D* **(E)**, *or collagenase followed by cytochalasin D* **(F).** *Morphology is normal following collagenase alone, but in the cytochalasin D-treated embryos the neural folds are open and the otic vesicle (ov) is an open pit; nevertheless, Krox-20 expression is normal. Bars, 100 μm.*

Tuckett, 1989). It was also observed within the neuroepithelium of the forebrain and midbrain and their basement membranes; this distribution appeared to coincide with the region in which apical cytoplasmic bridges were observed by TEM (Fig. 2A). CSPG staining was not observed in the hindbrain neuroepithelium in day 8.5 embryos (Fig. 4D), but on day 9.0 it was present throughout the neuroepithelial basement membrane and in the rhombomeric gyri (Fig. 4E). Type IV collagen and HSPG immunostaining were observed throughout the neuroepithelial basement membranes at day 8.5 and day 9.0, with no regional specializations (not shown).

Anti-actin immunostaining was present at a low level within the day 8.5 neuroepithelium, with no regional differences (not shown). Disruption of the microfilament structure with cytochalasin D for one hour abolished the optic sulcus, but had no effect on the structure of the preotic or otic sulci, in 3-6-somite stage embryos (Fig. 5A); it was sufficient to reverse the concave curvature of the neural folds in 10-somite-stage embryos (Fig. 5B,C). Embryos cultured for 24 h after this treatment failed to close their neural tubes and developed rhombomeres with reversed curvature (see also Tuckett and Morriss-Kay, 1985a), but showed the normal two bands of *Krox-20* expression and *Krox-20*-labeled neural crest cells extending caudally from the lateral edge of the r5 domain (Fig. 5E).

Exposure of embryos to collagenase had no effect on the preotic sulcus either alone or when followed by colchicine or cytochalasin D treatment (Fig. 5D,F); however, embryos in which the neuroepithelium and mesenchyme were separated after collagenase treatment and then exposed to cytochalasin D showed loss of morphology of both the preotic sulcus and the otic sulcus (Fig. 4F).

Effect of retinoic acid on prorhombomeric segmentation and 3' HoxB gene expression

Embryos exposed to RA at presomite stages (day 7.75 to day 8.0) failed to form a preotic or otic sulcus, and *Hoxb-1* and *Hoxb-2* genes were ectopically expressed at abnormally rostral levels (Fig. 6). Exposure to RA at day 8.0 to day 8.25 (2- to 5-somite-stages) had no effect on prorhombomere morphology, which was already present.

Discussion

Limitations of the structural basis of the prorhombomere concept

The preotic and otic sulci are clear morphological landmarks in the neuroepithelium of human and other mammalian embryos during very early somite stages of development. Our morphological study found that the preotic sulcus forms during segmentation of the first two pairs of somites, and the otic sulcus is present by the 3- somite stage. The two sulci divide the hindbrain into prorhombomeres (pr) A, B and C. The junctions between prA and the midbrain, rostrally, and prC and the occipital hindbrain, caudally, are not morphologically distinct by light microscopic observation until formation of the rhombomeres. In this study we observed two features that revealed the putative position of the midbrain/prA junction: cytoplasmic bridges were present between neuroepithelial cells of the forebrain and midbrain but not the hindbrain, and immunohistochemical staining for CSPG was present in the forebrain and midbrain but not the hindbrain neuroepithelium. The site of the junction between prC and the occipital hindbrain (termed rhombomere D by Müller and O'Rahilly, 1983) is only distinguishable by the position of the somites; no distinguishing features were observed in the neural epithelium by the methods used here.

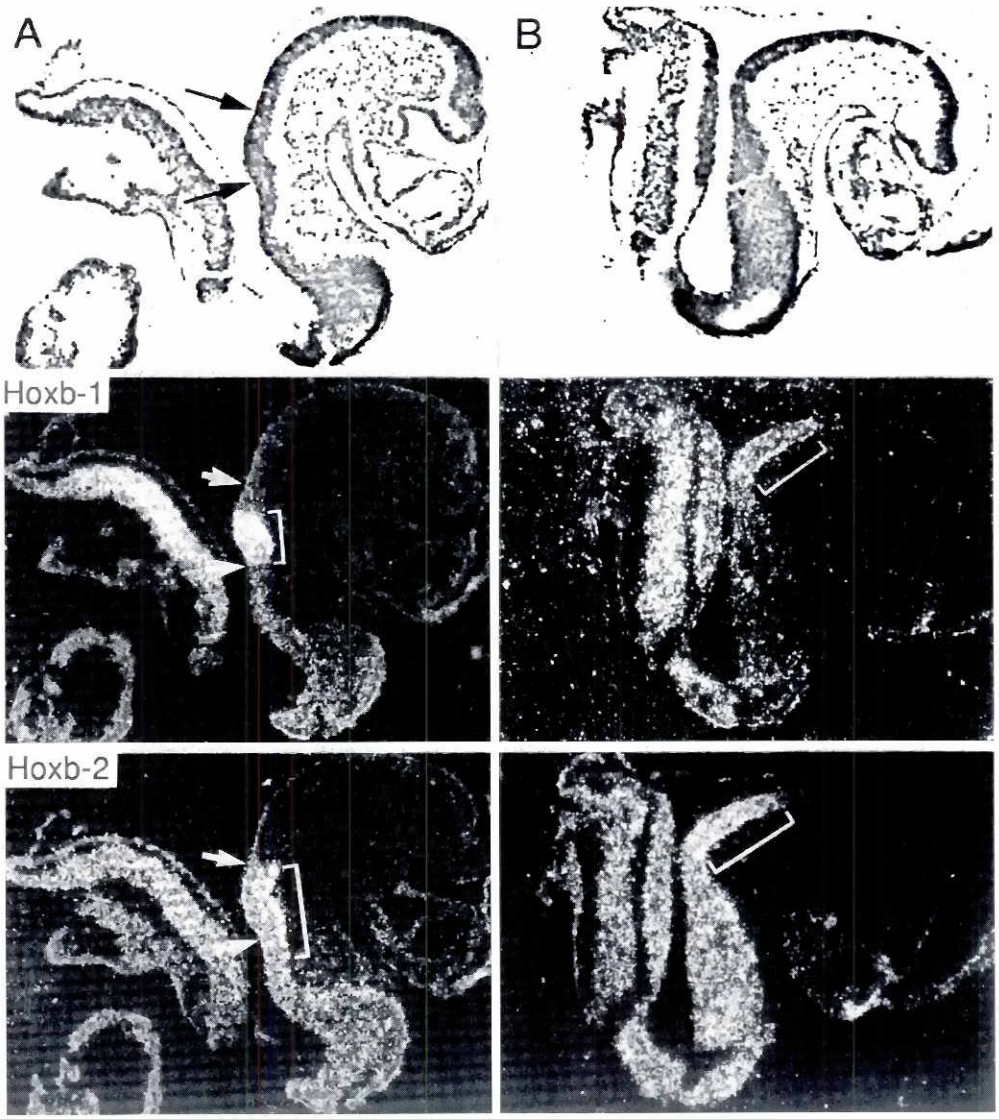

Fig. 6. Parasagittal sections of 6-somite-stage mouse embryos: (A) control and (B) exposed to retinoic acid at the late presomite stage. *Top: bright field micrograph showing preotic and otic sulci in the control but not in the RA-exposed embryo. Middle: the highest level of expression of Hoxb-1 (bracket) is from the middle of prB to the cranial border of the otic sulcus in* **(A)**, *but more rostral in* **(B)**. *Bottom: the highest level of expression of Hoxb-2 (bracket) is from the preotic sulcus to the caudal border of the otic sulcus in* **(A)** *but more rostral in* **(B)**. *Arrows indicate the preotic and otic sulci on all control micrographs.*

Maintenance of sulcus structure

The preotic sulcus is a discrete, deeply recessed structure, whereas the otic sulcus is a gentle concavity of the neuroepithelium. Nevertheless, both are firmly anchored to the underlying cranial mesenchyme, as shown by the ability of colchicine to cause separation of the neuroepithelium and mesenchyme elsewhere, but not in the two sulci. Our experiments did not reveal what mechanisms maintain the structure of the two sulci. They were not disrupted by exposure to cytochalasin D, although the optic sulci were lost in the same specimens, and the same treatment of later stage embryos reversed the concave curvatures of neurulation and of the rhombomeric sulci. In previous studies we have cultured embryos in the presence of heparitinase, which degrades the basement membrane component heparan sulphate proteoglycan (Tuckett and Morriss-Kay, 1989), and chondroitinase ABC, which degrades chondroitin sulphate proteoglycan (Morriss-Kay and Tuckett, 1989). These enzymes inhibited either cranial neurulation (heparitinase) or neural crest cell emigration (chondroitinase), but neither of them affected the formation or maintenance of the preotic and otic sulci. Exposure of early somite stage mouse embryos (day $8^{1/4}$) to raised retinoic acid levels does not result in loss of the already formed preotic and otic sulci (our unpublished observations). We induced loss of preotic and otic sulcus structure only by separation of the neuroepithelium from its underlying mesenchyme, and suggest that the morphological integrity of the two sulci depends on an epithelial- mesenchymal interaction, but our results do not provide any information about the nature of that interaction.

Prorhombomeres and rhombomeres

The main aim of the present study was to correlate morphology of the neural epithelium at prorhombomere stages with the rhombomeric segmentation of the later hindbrain. DiI labeling and

embryo culture showed that the cells of the preotic sulcus are later found at the r2/3 boundary, and that the otic sulcus cells were located within r5, mainly close to its rostral border. These fates show a clear correlation with *Krox-20* expression. The earliest cells containing *Krox-20* transcripts appear to be those of the preotic sulcus itself; the domain of expression then spreads caudally to define the extent of the future r3. Rostral-to-caudal spread of *Krox-20* expression also occurs within the otic sulcus, the lateral edge of the domain extending caudally ahead of the medial part. Formation of r3 and r4 involves reversal of the convex curvature of prB, except at its rostro-caudal mid-point, to form two sulci divided by the r3/4 gyrus.

In the original reports localizing *Krox-20* expression to r3 and r5, it was proposed that this gene plays a role in hindbrain segmentation and in the regulation of *Hox* gene expression (Wilkinson *et al.*, 1989a; Chavrier *et al.*, 1990; Wilkinson and Krumlauf, 1990; Gilardi *et al.*, 1991). More recently it has been shown to play a role in enhancing *Hoxb-2* gene expression in r3-r5 (Sham *et al.*, 1993). A role in preotic sulcus formation is ruled out by the observations of Schneider-Maunoury *et al.* (1993), who disrupted the *Krox-20* gene so that it would make a truncated protein with no zinc fingers and therefore no DNA binding activity. The disrupted gene was expressed in the normal two domains at early somite stages, but was downregulated early, and r3 and r5 failed to form. The illustrations in their report show that preotic and otic sulcus morphology is normal in *Krox-20*- disrupted embryos. Thus although a role for Krox-20 in rhombomeric segmentation is well established, it clearly plays no part in prorhombomere formation. However it is interesting to note that in the chick, which does not have prorhombomeres, both the r3 and the r5 *Krox-20* stripes appear much later than in the mouse (at the 6-somite and 8-somite stages, respectively), and also differ from the gradually extending pattern of expression shown in the mouse (and *Xenopus* and zebrafish) in being expressed throughout their full domains from the outset (Irving *et al.*, 1996, and references therein).

Prorhombomeres and gene expression

Inhibition of prorhombomeric segmentation by exposure to retinoic acid at late presomite stages, as shown here and in a previous study (Mahmood *et al.*, 1996), indicates that the molecular mechanisms underlying preotic and otic sulcus formation (but not maintenance: see above) are retinoic acid-sensitive. In normal embryos, the rostral boundaries of *Hoxb-2* and *Hoxb-1* are the preotic sulcus and mid-prB respectively; they are ectopically expressed rostral to their normal domains in RA-exposed embryos lacking prorhombomeric segmentation. On morphological grounds, *Hoxb-2* is the most serious contender for involvement in the mechanism of prorhombomere formation, since its rostral boundary is established at the presomite stage (Hunt *et al.*, 1991), i.e. before *Krox-20* expression or preotic sulcus formation. Upregulation of its expression in prB and the otic sulcus occurs as the otic sulcus deepens and prB becomes more distinct as a convexity of the neuroepithelium. Many aspects of 3' *Hox* gene regulation involve retinoic acid response elements (e.g. Studer *et al.*, 1994), suggesting that their sensitivity to RA may be directly correlated with the RA sensitivity of prorhombomere formation.

Sek-1 is another candidate gene for involvement in prorhombomeric segmentation, since it is co-expressed from pre- and early somite stages with *Krox-20* in the preotic and otic sulci of mouse embryos (Irving *et al.*, 1996), but little is known about the function of the *Sek-1* gene product.

Prorhombomeres as a specifically mammalian feature: functional significance

Why should mammalian embryos, but not embryos of other vertebrate classes, show prorhombomeric subdivision of the hindbrain before the development of rhombomeric segmentation? Clearly it is not a prerequisite for rhombomere formation, and has a significance that is specifically mammalian. We have previously suggested a function for the preotic sulcus as a barrier to cell movement between the region caudal to it, in which cell division leads to intrinsic growth of the neuroepithelial and neural crest cell populations, and the region rostral to it, in which the neuroepithelium is a fluid sheet of cells flowing towards and into the rapidly expanding forebrain (Tuckett and Morriss-Kay, 1985b; Morriss-Kay and Tuckett, 1987). Rapid growth of the forebrain during neurulation is a specifically mammalian feature, and lays the developmental foundations for the specifically mammalian 6-layered neocortex. Forebrain growth and forebrain- specific gene expression domains are reduced in embryos exposed to RA prior to prorhombomere formation (Morriss-Kay *et al.*, 1991; Simeone *et al.*, 1995). Invention of the preotic sulcus may have been a key event in evolution of the mammalian forebrain.

The otic sulcus is different in kind from the preotic sulcus, showing no specialized ultrastructural features, and remaining as a sulcus to become r5. Its functional significance may simply be caudal delineation of the prB segment. PrB is the source of a discrete population of neural crest cells. These cells emigrate from the neural folds as a separate group, populating the second pharyngeal arch and acoustico-facial ganglion (Tan and Morriss-Kay, 1985, 1986); they have a unique pattern of *Hox* gene expression, and an exceptionally high level of CRABP I expression (Hunt *et al.*, 1991; Ruberte *et al.*, 1991, 1992). The prB segment lies between two structures, the preotic and otic sulci, from which (except for the most caudal part of the otic sulcus) neural crest cells do not arise. Before emigration of its neural crest cells, prB shows a high level of expression of *Fgf-3*, the domain of which extends laterally to the nascent second arch; by early rhombomere stages of development, *Fgf-3* expression extends further caudally, to r5 and r6, and the neuroepithelial origin of the surface ectodermal domain has moved caudally with it (Mahmood *et al.*, 1996). This surface ectodermal domain includes the area of the otic placode/pit, and its caudal shift may explain the variability in position of the otic ectoderm observed in human embryo specimens (Müller and O'Rahilly, 1983).

Conclusions

Morphologically, there is only one fully delineated area within the hindbrain prior to rhombomere formation, and that is prorhombomere B: there are no distinctive features intrinsic to the neural epithelium delineating prC from the occipital region, and only subtle cellular and extracellular features delineating prA from the midbrain. The concept of prorhombomeric segmentation may therefore be somewhat artificial, based on (a) the early formation of a structure, the preotic sulcus, that is involved in control of cell movement rostral to it, (b) the absence of neural crest cell-forming tissue within the neuroepithelium of the preotic and otic sulci, and (c) the firm anchorage of these two sulci to the underlying mesoderm.

Materials and Methods

Embryos

C57Bl/6 mouse embryos at days 8 (2-7-somite stages) and 9 (12-16-somite stages) of gestation, and AHA rat embryos at days 9 (2-7-somite stages) and 10 (12-16-somite stages) of gestation were used for the experimental studies. For scanning electron microscopy (SEM) a complete series of 2-16-somite stage embryos was used.

Light and electron microscopy

After removal of the membranes the embryos were fixed in 2.5% glutaraldehyde fixative in 0.1% sodium cacodylate buffer, post-fixed in 1% cacodylate-buffered osmium tetroxide, rinsed and dehydrated with graded alcohols before embedding in Spurr resin. Semi-thin sections 0.5-1 μm thick were mounted on glass slides and stained with methylene blue in borax solution. When the desired sections were obtained, thin sections of 80-90 nm thickness were collected, mounted on copper grids and double stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM). For SEM, the embryos were fixed as for TEM, but not post-fixed, and dehydrated in graded acetones. The embryos were then critical point dried, mounted on aluminium stubs and coated with gold (60 nm thick).

Immunohistochemistry

Mouse embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1-2 h at 4°C, embedded in Paraplast and cut at 7 μm. The sections were dewaxed, rinsed with PBS and incubated with appropriate dilutions of one of the following antibodies: mouse monoclonal anti-α-tubulin (ICN); anti-chondroitin sulfate proteoglycan (CSPG) antibody CS-56 (Sigma); rabbit polyclonal anti-actin (ICN); anti-basement membrane heparan sulfate proteoglycan (HSPG) (a gift from John Hassell); anti-collagen IV (ICN). After rinsing with PBS the sections were incubated with a fluorescein-labeled goat anti-mouse or anti-rabbit IgG antibody (ICN) diluted to 1:10 for 30 min at room temperature.

Embryo culture

AHA rats and C57/Bl6 mice were used. Early somite-stage embryos were dissected from the decidual swellings in Tyrode's saline. Reichert's membrane was removed leaving the yolk sac and ectoplacental cone intact. The embryos were cultured at 38°C in 50 ml glass bottles or plastic tubes containing equal volumes of heat-inactivated rat serum and Tyrode's saline, and 10 μl penicillin/streptomycin (5000 iu/μl and 5000 μg/μl). Prior to sealing, the vessels were gassed with 5% CO_2: 5% O_2: 90% N_2 for 3-6-somite stage embryos, and regassed with 5% CO_2: 95 % air from the 10 somite-stage onwards. They were continuously rotated at 30 r.p.m.

DiI injections

Micropipettes were made using 50 μl disposable capillary tubes (Clark electromedical instruments, UK), pulled with an Ealing Vertical microelectrode puller. Fifteen rat and fifteen mouse embryos (3- to 6-somite-stage) were used. Each embryo was placed in a drop of culture medium and held by gentle suction of the yolk sac with a holding pipette. The micropipette was filled with a solution of 0.05% DiI in 0.3 M sucrose and manipulated by hand; it was pushed between the yolk sac and the amniotic membranes and then into the neuroepithelium of the preotic or otic sulcus. A drawing recording the precise position of the injection was made for each embryo. All successfully injected embryos were transferred into culture bottles, gassed and placed in a roller culture apparatus. The bottles were wrapped in foil to avoid photodegradation of the fluorescent dye. After 24 h (mouse) or 36 h (rat) the embryos were rinsed in Tyrode's solution and fixed in 4% formaldehyde/0.25% glutaraldehyde in phosphate buffer at 4°C. The embryos were examined and photographed by epifluorescence (rhodamine filter set). Five rat and five mouse embryos were processed for photo-oxidation of 3,3'-diaminobenzidine (DAB) (Selleck and Stern, 1991). They were then conventionally processed for embedding in Paraplast, sectioned at 7 μm and stained with Alcian blue at pH 2.5.

Colchicine, cytochalasin D and collagenase treatment of embryos

Twelve 2-7 somite-stage embryos were treated for either 1 or 3 h with colchicine (Sigma) diluted in Tyrode's saline which had been added to the culture medium at a final concentration of 0.2 μg/ml. Four 3-6 somite-stage embryos and two 8-10-somite stage embryos were treated for 1 h in medium containing cytochalasin D in DMSO at a final concentration of 0.15 μg/ml. Eight 2-7-somite-stage embryos were treated in Hank's balanced salt solution containing 0.04% collagenase at 37°C for 1 h prior to the colchicine treatment (4 embryos) or cytochalasin D (4 embryos) and the mesenchyme was partially or completely separated from the neuroepithelium in two of each group. Four embryos of each stage-group were cultured in the presence of DMSO as controls. After treatment the embryos were fixed in Bouin's fluid, embedded in Paraplast and cut at 5 μm. Sections were stained with hematoxylin and eosin. Twelve embryos treated for 1 h with collagenase, 17 treated for 1 h with cytochalasin D, 16 with collagenase followed by cytochalasin D, and 6 untreated control embryos were washed in Tyrode saline and cultured for a further 24 h in addition-free medium, followed by *in situ* hybridization for detection of *Krox-20* gene expression.

Retinoic acid treatment and whole-mount in situ hybridization

Retinoic acid treatment was as described previously (Wood *et al.*, 1994). The *in situ* hybridization protocol was as described by Wilkinson and Green (1990), using *Krox-20* riboprobes donated by David Wilkinson and *HoxB* probes donated by Robb Krumlauf.

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