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Persistence of rhombomeric organisation in the postsegmental hindbrain

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SUMMARY

Rhombomeres are morphological varicosities of the neural tube that are present between embryonic day (E) 1.5 and E5 and are characterised by compartment organisation, segmentally neuronal organisation and spatially restricted patterns of gene expression. After E5, the segmented origins of the hindbrain become indistinct, while the adult hindbrain has an longitudinal columnar nuclear organisation. In order to assess the impact of the early transverse pattern on later longitudinal organisation, we have used orthotopic quail grafts and in situ hybridisation to investigate the long-term fate of rhombomeres in the embryonic chick hindbrain.

The uniformity of mixing between quail and chick cells was first verified using short-term aggregation cultures. The dispersal of the progeny of individual rhombomeres (r) was then assessed by the unilateral, isochronic and orthotopic transplantation of either r2, r3, r4, r5 or r6 from quail to chick at embryonic day E2. In addition, orthotopic, partial rhombomere grafts, encompassing an inter-rhombomere boundary and adjacent rhombomere bodies were used to assess cell mixing within rhombomeres. Operated embryos were incubated to either E7 or E10 when chimaeric brains were removed. Quail cells were identified in whole mounts or serial sections using the quail-specific antibody QCPN. Subsequently, radial glia morphology was assessed either by immunohistochemistry or DiI labelling. A series of fixed hindbrains between E6 and E9 were probed for transcripts of *Hoxa-2* and *Hoxb-1*.

Fate-mapping reveals that the progeny of individual rhombomeres form stripes of cells running dorsoventrally through the hindbrain. This pattern of dispersal precisely parallels the array of radial glia. Although the postmitotic progeny of adjacent rhombomeres spread to some extent into each others' territory in intermediate and marginal zones, there is little or no mixing between rhombomeres in the ventricular zone, which thus remains compartmentalised long after the rhombomeric morphology disappears. Segmental gene expression within this layer is also maintained after E5. A more detailed analysis of mixing between proliferating cells, using partial rhombomere grafts, reveals that both mixing and growth are non-uniform within the ventricular layer, suggesting, in particular, that longitudinal expansion within this layer is restricted. Together, these observations suggest that rhombomeres do not disappear at E5, as has previously been supposed, rather they persist in the ventricular zone to at least E9, ensuring a continuity in the presumed segmental cues that specify neuroepithelial cells in the hindbrain.

Key words: quail-chick chimaera, fate map, hox gene, rhombomere, hindbrain

INTRODUCTION

The hindbrain undergoes a fundamental change in its organisation during development. The early embryonic hindbrain (at E2-E5) is characterised by transient segmental swellings (Vaage, 1969; Lumsden, 1990), called rhombomeres, which are underlain by equivalent patterns of neuronal organisation (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993) while the later embryonic and adult hindbrain lacks an overtly segmented appearance and is composed of columnar arrays of neuronal nuclei with no obvious traces of earlier segmental organisation (Marín and Puelles, 1995).

The early varicosities and constrictions of the early hindbrain neural tube closely reflect its internal organisation: the motor nuclei of the cranial nerves originate in single rhombomeres or pairs of rhombomeres (Lumsden and Keynes, 1989) and show a transient functional organisation into repeated, autonomous rhythmic units (Fortin et al., 1994, 1995). Segmentation is also reflected in the time course of neurogenesis, where odd-numbered rhombomeres show a delayed maturation compared to even-numbered rhombomeres (Lumsden and Keynes, 1989). Perhaps most significantly, dividing cells do not mix between rhombomeres (Fraser et al., 1990). The interfaces between adjacent rhombomeres become specialised, both in structure (Heyman et al., 1993) and the expression of molecular markers (Heyman et al., 1995; Mahmood et al., 1995). The inter-rhombomere boundaries also correspond with the anterior limits of expression of a number of developmentally significant homeobox-containing genes (McGinnis and Krumlauf, 1992). Each rhombomere can thus be characterised by a specific repertoire of *Hox* gene expression and a clonally restricted population of dividing neuroepithelial cells, arguing that the rhombomeres are specification units.

By contrast, the adult hindbrain acquires a very different organisation and is characterised by columnar neuronal nuclei

that lie orthogonal to the earlier rhombomeric pattern. Overt (i.e. morphological) segmentation disappears at around embryonic stage (Hamburger and Hamilton, 1951) 24 (E5) and is correlated with the expanded expression of radial glia markers, (which earlier characterise inter-rhombomere boundaries alone, Heyman et al., 1995) to all hindbrain regions. By E10, the chick hindbrain has an essentially adult organisation (Marín and Puelles, 1995).

The dramatic difference between early and late patterns of organisation prompts the question of how rhombomeres contribute to and become integrated into adult hindbrain structures. Progeny of individual rhombomeres might form defined sets of nuclei, or related elements within different nuclei, or may simply be irrelevant to later patterning events which are superimposed upon the basic segmental structure. Recent studies suggest the latter is most likely. Cell dispersal is principally by radial (ventricular-to-pial) migration, while axial (anteroposterior) spread is considerably more limited within the intermediate zone of the hindbrain (Tan and Le Douarin, 1991; Hemond and Glover, 1993; Lumsden et al., 1994; Birgbauer and Fraser, 1994; Wingate and Lumsden, 1994; Marín and Puelles, 1995; J. Clarke and A. Lumsden, unpublished observations). Perhaps surprisingly, cell dispersal is largely perpendicular to the layout of adult nuclei.

How are cell division and cell dispersal within the post-rhombomeric hindbrain organised? Are ventricular zone cells free to disperse and mix after rhombomere boundaries disappear? Does the segmental compartmentalisation of cell division become diffuse? Or do the same patterning mechanisms that characterise the rhombomeric hindbrain persist after its overtly segmented structure is lost?

In this study, we have used quail-chick grafting chimaeras (Balaban et al., 1988; Hallonet et al., 1990) to examine the fate of individual rhombomeres. By orthotopic transplantation of a quail rhombomere for a chick rhombomere, the dispersal of grafted neural tissue can be followed through development. Since this study emphasises differential patterns of cell mixing, we have tested the validity of the chimaera technique in vitro by performing short-term aggregation cultures of chick and quail hindbrain cells. Under these conditions, species-related differences in adhesion properties are negligible compared to the species-independent differences that arise according to the rhombomeric origin of cells (Guthrie et al., 1993; A. Wizenmann and A. Lumsden, unpublished observations). Using these observations as a control, we have examined cell mingling between and within rhombomere-derived territories in both the ventricular, proliferative zone and the intermediate, mantle zone of the post-rhombomeric hindbrain. We have also investigated the glial structures that might underlie radial patterns of postmitotic cell dispersal using the fluorescent carbocyanine dye (DiI). Both an asymmetrical restriction on cell mixing and a maintained Hox gene expression in dividing cells suggest that rhombomeres persist within the ventricular zone after overt hindbrain segmentation disappears.

MATERIALS AND METHODS

Short-term aggregation cultures

Hindbrains from 20-25 chick embryos were harvested at stage 14, cleaned of adherent mesenchyme cells with dispase (Boehringer, 1 mg/ml), and incubated in red CellTracker dye (CMTMR, Molecular Probes, Oregon, USA). An equivalent number of quail brains were incubated in green CellTracker dye (CMFDA). Quail and chick hindbrains were kept separate while they dissected into individual rhombomeres, which were then pooled according to their axial position (r2,3,4,5). Rhombomeres were incubated in calcium-free medium (HBBS) with 0.002% EDTA for 15 minutes and dissociated cell suspensions were obtained by gently triturating the tissue. Chick and quail cells originating from single rhombomeres were then mixed to produce combinations of like rhombomeres (i.e. chick r2 and quail r2) or combinations of odd and even neighbours (i.e. chick r2 and quail r3). Cell mixtures were allowed to aggregate for 8-24 hours (at 37°C, with gentle agitation) and the patterns of cell segregation or mixing in the resulting spherical cell aggregates was assessed by optical sectioning using a laser scanning confocal microscope equipped with dual wavelength optics (BioRad MRC 600). Previous results show that both in vivo (Guthrie et al., 1993) and in vitro, using identical culture conditions within a single species (A. Wizenmann and A. Lumsden, unpublished observations), cells from odd-numbered rhombomeres only mix freely with other odd cells, while cells from even-numbered rhombomere only mix freely with other even cells. The degree to which this phenomenon is independent of the species from which the rhombomere cell suspension is derived, can be taken as an indication of the validity of observations of quail and chick cell mixing in vivo.

Quail-chick chimaeras

The neural tube of stage 10-11 quail embryos was removed and the dorsal surface of selected rhombomeres marked for orientation purposes by microinjections of DiI C_{12} (Molecular Probes) dissolved in dimethylformamide (6 mg/ml). The isolated neural tube was treated with dispase (1 mg/ml in sterile Howard's Ringer, 30 minutes at room temperature) and then the graft tissue was removed by microdissection from either the left or right side of the rhombencephalon. Three types of unilateral hindbrain graft were made at these stages: the entire extent of an individual rhombomere (r2, r3, r4, r5 or r6); the boundary region between rhombomeres including half of the adjacent, flanking rostral and caudal rhombomere bodies (for example, half r3 plus half r4: 'r3/4') and the basal half of such a 'boundary graft' (Fig. 1). In three embryos, double orthotopic transplants were made of either r2 and r4, or r3 and r5. In six embryos, orthotopic grafts of prospective caudal hindbrain were also made at stage 8-9, before the hindbrain has an overtly segmented structure (Table 1). For all chimaeras, grafts replaced the orthotopic section of the neural tube of stage-matched chicks which was removed cleanly with fine tungsten needles, in ovo. Host eggs were sealed with heavy duty insulation tape ('tesa', Beiersdorf AG, Hamburg, Germany) and placed within an incubator at 37°C for a further 2-8 days. Chimaeric embryos from stage 10-11 were allowed to survive to E7 or E10, while those operated upon at stage 8-9 survived to E4. At these ages, they were decapitated and the brains fixed in 3.5% paraformaldehyde in phosphate buffer (0.1 M). At E10, the hindbrain has an essentially adult complement of hindbrain nuclei (Marín and Puelles, 1995).

Immunohistochemistry

Chimaeric brains at E4 and E7 were processed as whole mounts or as 50 μm parasagittal sections. Brains at E10 were sectioned in either coronal, horizontal or parasagittal planes on a vibrotome at a thickness of 50, 100 or 200 μm. Free-floating sections were stained with the quail-specific perinuclear antibody QCPN (Hybridoma bank, Iowa University, Iowa, USA) and a peroxidase-conjugated secondary antibody, using a whole-mount protocol (Lumsden and Keynes, 1989) to produce a permanent diaminobenzidine reaction product. For a number of sectioned brains, the distribution of labelled cells was logged using a camera lucida and a computer-based morphometry programme (Halasz and Martin, 1984).

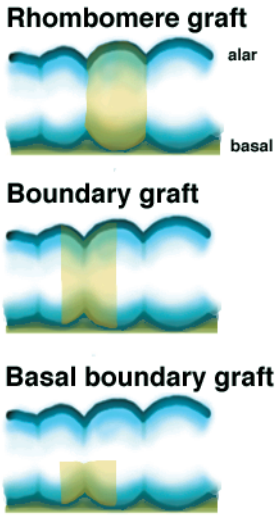


Fig. 1. Schematic diagram of the three types of orthotopic graft employed in this study. (Top) Orthotopic graft of a single rhombomere. (Middle) Orthotopic graft of a boundary region. (Bottom) Orthotopic graft of a basal (ventral) boundary region. All grafts were made unilaterally.

DiI labelling of radial glia

The fourth ventricle of four fixed brains was injected with a suspension of DiI microcrystals following the method of Heyman et al. (1993). After a suitable incubation time, brains were sectioned parasagittally at 100 μm and examined by confocal microscopy.

Whole-mount RNA in situ hybridisation

Chick brains were harvested from embryos at E6, 7, 8 and 9 and rapidly dissected in 3.5% paraformaldehyde in phosphate buffer (0.1 M). Digoxygenin-labelled riboprobes for *Hoxa-2* (Prince et al., 1994) and *Hoxb-1* were used in a whole-mount RNA in situ hybridisation protocol (Wilkinson, 1992: glycine and RNAase treatments were omitted). The *Hoxb-1* probe was a partial cDNA starting at approximately +500 and contains the entire 3′ untranslated region (kindly donated by Dr Vicky Prince). Brains were subsequently sectioned coronally at 100 μm on a vibratome and mounted in glycerol.

RESULTS

Quail and chick cells have similar aggregation properties

To ensure that the patterns of dispersal of cells from quail embryo rhombomeres did not reflect species-specific cell surface properties, short-term chimaeric aggregation cultures were made from hindbrains at stage 14. Various combinations of cell suspensions from chick and quail embryos were used to assess whether species differences affect cell-cell adhesion. The confocal micrograph in Fig. 2A shows quail r6 (green) and chick r6 (red) cells that had reaggregated in culture over a period of 24 hours to form a number of clusters of evenly mixed cells. By comparison, a heterotypic culture of chick r6 (red) and quail r5 (green) produces a majority of segregated aggregates such as that shown in Fig. 2B. The aggregates from a number of such homotypic and heterotypic combinations of dissociated rhombomere cells were scored as either ‘mixed’ or ‘segregated’. ‘Pure’ aggregates consisting entirely of either chick or quail cells formed less than 1% of the experimental population. The average percentage of aggregates that were mixed is contrasted with that which were segregated in the bar charts in Fig. 2A and B. The frequency of mixed aggregates was significantly higher in homotypic combinations (Fig. 2A, five experimental combinations; number of aggregates, n=326) and significantly lower in heterotypic combinations (Fig. 2B, four experimental combinations, n=797). Therefore, differences in axial level were more significant than species differences in determining cell mixing and/or sorting-out properties. This allows confidence that, in subsequent quail-chick chimaera grafting experiments, species-specific cell cohesion properties are not a significant factor in determining cell dispersal.

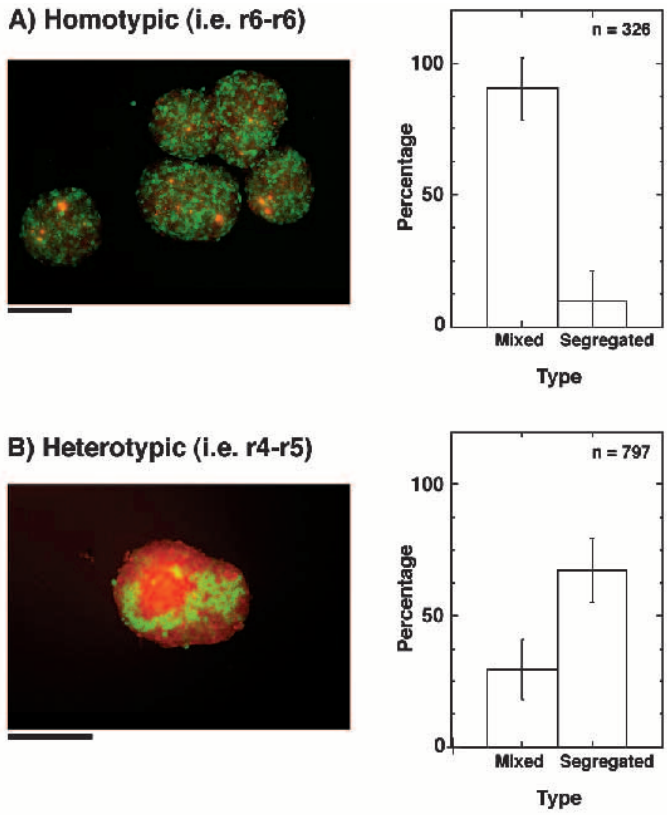


Fig. 2. Mixing properties of chick (red) and quail (green) hindbrain cells were assessed in short-term aggregation cultures. (A) When quail and chick rhombomeres from the same axial level are combined, cells mix together evenly. The confocal photomicrograph shows five ‘mixed’ aggregates formed by cells from a quail r6 and a chick r6. The bar chart (right) shows the mean number of mixed and segregated aggregates, with standard deviations, from five co-culture experiments in which cells from odd-odd or even-even pairs of rhombomeres were combined (r2-r2, r3-r3, r4-r4, r5-r5 and r6-r6: n=number of aggregates assessed). (B) An example of a ‘segregated’ aggregate from a co-culture quail r4 and chick r5. The bar chart shows pooled results from four heterotopic combinations of rhombomeres (r2-r3, r3-r4, r4-r5 and r5-r6). Scale bars, 100 μm.

Cell dispersal parallels radial glia organisation

Single rhombomere grafts were used to assess the fate of segments after E5, when boundaries in the hindbrain disappear. A total of 27 chimaeric brains (Table 1) were analysed from a larger number of experimental embryos. Brains were rejected (n=9) if they showed signs of asymmetrical development (all grafts were unilateral). In each case, the pattern of quail cell dispersal was consistent and the accompanying figures illustrate representative chimaeric brains.

Table 1. Summary of grafts

	Number of grafts analysed
(a) Double rhombomeres	
r3 & r5	2
r2 & r4	1
(b) Single rhombomeres	
r2	1
r3	7
r4	7
r5	4
r6	5
(c) Boundary grafts	
Whole	5
Basal half	4
(d) Unsegmented neural tube grafts (stage 9)	
Presumptive *r7*	6

The progeny of single rhombomeres formed stripes of cells in the inside-outside (ventricular-pial) axis of the hindbrain. Fig. 3A shows a parasagittal section through an E10 hindbrain where r3 and r5 were replaced unilaterally by orthotopic quail rhombomeres. Stripes of quail cells run from the ventricular to pial surfaces where they are at their broadest. The progeny of r3 and r5 have maintained their original segmental disposition although there is also a degree of mixing between neighbouring rhombomere territories. Reconstructions of single rhombomere grafts shows that cells move only a limited distance along the rostrocaudal axis: Fig. 3B shows a camera lucida drawing of a parasagittal E10 hindbrain section in which quail cells derived from r6 have been plotted over the outline of slice.

The dispersal of the progeny of each rhombomere is parallel to the orientation of radial glia fibres through the hindbrain. Fig. 3C shows a section through a brain in the same plane and at the same stage as Fig. 3B, in which the radial glia have been labelled with DiI. An array of radial cell bodies spans the ventricular-pial axis of the entire region with their basal endfeet at the pial surface.

Boundaries between territories remain sharp at the ventricular surface

By comparison to the indistinct, overlapping boundaries between the progeny of neighbouring rhombomeres in the intermediate zone of the E7-10 hindbrain, the borders between clonal progeny in the ventricular zone remain exquisitely sharp. Fig. 4A shows a dorsal view of the ventricular surface of hindbrain at E7. Labelled cells were derived from a orthotopic transplant of a quail left r3 and form a geometrical strip of approximately 150 μm width at the ventricular surface. Cells are tightly packed and there is no leakage out of r3 territory. The same tight boundaries are apparent in a horizontal section through a unilateral r5 graft examined at E10 (Fig. 4B). In contrast to the E7 hindbrain, the ventricular surface is convoluted and the scatter of labelled cells outside the densely labelled stripe of cells are at a deeper level with respect to the ventricular surface. Both photomicrographs are at the same magnification and demonstrate that, although the hindbrain has grown greatly in size between E7 and E10, the width of rhombomere-derived territory at the ventricular surface remains similar between the two ages. Mixing appears to be limited to postmitotic cells which have migrated out of the ventricular zone. The proliferative cells of each rhombomere remain segregated after rhombomere boundaries have disappeared as morphological entities.

Hox gene expression is segmental at the ventricular surface at E6

Probes for *Hoxa-2* and *Hoxb-1* were used to examine the pattern of expression of these genes at E6. Fig. 5A and B show in situ hybridisation patterns of riboprobes in dorsal views of the ventricular surface of whole mounts, and in coronal section (Fig. 5C,D) at the level of the VIIth/VIIIth nerve ganglion complex.

Hoxa-2 expression has a sharp anterior boundary. Different levels of staining define four distinct regions (Fig. 5A). A band of weak expression is followed, caudally, by high expression over two band widths. Weaker staining defines a further band and then more caudally, expression is uniform to the spinal cord. By comparing this expression to brains carrying quail rhombomeres, it is evident that each band corresponds to the territory derived from successive rhombomeres. Thus the anterior boundary is at the r1/r2 boundary, the more darkly stained stripe is in the territory of r3 and r4, the posteriorly adjacent stripe of weaker expression has a posterior boundary at r5/r6. An intensely stained longitudinal stripe of r3 and r4 cells runs close to the lateral edge of the hindbrain, between the Vth and VIIth/VIIIth (VIIth) ganglia.

Similarly, *Hoxb-1* expression (Fig. 5B) is restricted at E6 to two lateral (dorsal/alar in the early embryo) patches at the level of the r4-derived stripe. Within these lateral patches, the more medial part stains most strongly. Between this patch and the lateral edge of the ventricular surface there is a lightly staining stripe of cells. The width and position of these partial stripes are equivalent to the r4 half of corresponding stripe defined by the probe for *Hoxa-2*.

Fig. 5C and D show coronal sections of the same brains as in Fig. 5A and B, at the level of r4-derived territory and just anterior to the VIIth nerve ganglion. With a specific exception, transcripts of both *Hoxa-2* and *Hoxb-1* are concentrated in the ventricular zone: the dark lateral, longitudinal band of *Hoxa-2* expression in (r2), r3 and r4 lies in the lateral, pial region between the Vth and VIIth nerve ganglia. This lateral region probably overlies, or corresponds with, neurons associated with the vestibulo-ocular complex (Deiter's nucleus: Labandeira-Garcia et al., 1989; Pétursdóttir, 1990; Marín and Puelles, 1995).

Hox gene expression in the ventricular zone is down regulated between E6 and E9

At E7, *Hoxa-2* still shows distinct bands of expression (Fig. 6A) while, for *Hoxb-1*, the weaker ventricular expression at the lateral edge of the dorsal surface is lost. This leaves two weak mid-lateral bands of staining (Fig. 6B). At E8, the ventricular surface becomes corrugated into a more complex three-dimensional configuration. The segmental *Hoxa-2* expression in the ventricular zone becomes less distinct (Fig. 6C), and expression of *Hoxb-1* can no longer be detected (Fig. 6D). As at E6 (Fig. 5C and D), gene expression is restricted to the ventricular zone.

At E9, the relatively strong expression of *Hoxa-2* in the ventricular zone has all but disappeared. Fig. 7A shows two brains

at E9 that were probed for *Hoxa-2* and then developed for different periods. In the relatively lighter stained hindbrain (left), the two dark lateral patches correspond with the extraventricular, possibly vestibulo-ocular, areas which labelled strongly at E6. In the more developed brain (right), it is still possible to see some relatively stronger ventricular expression in the r3- and r4-derived territories. Expression of *Hoxa-2* is essentially uniform throughout the radial thickness of the hindbrain from at least the level of r2 and more caudally. The cerebellum does not express *Hoxa-2* (Fig. 7B).

Cell movement both within and between rhombomeres is restricted along the longitudinal axis of the ventricular layer

From the observations above, it is apparent that segmental gene expression and restricted cell movement within the ventricular layer are maintained after obvious rhombomere boundaries have disappeared. Is cell movement restricted at the boundaries of rhombomere-derived territories? Or is there a general restriction of longitudinal cell migration in the ventricular layer? Previous studies have shown that cells within rhombomeres are able to mix freely (Fraser et al., 1990; Lumsden et al., 1994), but also that tangential spread and mixing may be quite limited within the ventricular zone (Balaban et al., 1988; Hallonet et al, 1990; Tan and Le Douarin, 1991; Guthrie et al., 1993).

To assess the mixing between proliferating cells, unilateral orthotopic grafts of boundary regions and the surrounding half rhombomere bodies were made from quail to chick at E2 (Fig. 1; Table 1). For example, grafts were made of regions encompassing the caudal half of r3 and the rostral half of r4 (r3/4). Fig. 7A shows the distribution of quail cells in the ventricular zone of E7 chimaeric hindbrains following the orthotopic transplant of a r4/5 boundary graft. There is no spread of cells out of the grafted ventricular zone and, moreover, the width of this band (c.150 μm) is equivalent to that derived from the graft of a single rhombomere (cf. Fig. 4A). This suggests that territorial boundaries are not required to restrict the movement of dividing cells and, moreover, that mixing within the ventricular layer along the longitudinal axis is severely restricted.

Ventricular zone cells are free to mix in the mediolateral axis

To investigate whether cell movement per se is restricted for ventricular cells, orthotopic boundary grafts were made of just the basal (ventral) half of the neural tube (Fig. 1). Fig. 7B-D show examples of the spread of quail-derived ventral ventricular zone cells in E7 chimaeric brains. Cells are free to spread and mix along the mediolateral axis. There was considerable variability in the degree of spread of cells from medial (originally basal) to lateral (originally alar). In Fig. 8B, a strip of quail cells spreads almost to the lateral edge of the hindbrain. The graft in Fig. 8C gives rise to far fewer ventricular cells, although small clusters of cells have spread a considerable distance from the floor plate towards the lateral edge of the brain. Fig. 8D shows an intermediate state between these extremes, at a higher magnification. By contrast to the rostrocaudal boundary, the interface between basal and alar territories is broken and uneven. Quail cells form tongues of extending clones that intercalate with unlabelled proliferating cells from the (chick-derived) dorsal region. Small groups and, more rarely, single quail cells have broken away from these tongues. Similarly, unlabelled (chick) cells form lacunae within the ventral, quail-derived region.

The extent and variability of mediolateral cell mingling contrasts markedly with the lack of spread along the longitudinal axis within the ventricular layer. The variability in the distribution of proliferating quail cells may reflect the size of the graft but also reflects the underlying lack of uniformity in cell division across the dorsoventral axis of the hindbrain (J. Clarke and A. Lumsden, unpublished observations). The patterns of mixing of basal boundary grafts with host tissue shows that the interface between graft and host cells is not in itself a restriction on cell movement.

Mixing between subventricular cells is constrained at boundaries

Some E7 chimaeric brains were sectioned parasagittally to contrast the distribution of postmitotic cells in the intermediate zone with the spread of proliferating cells in the ventricular zone. Fig. 9A and B are photomicrographs showing typical grafts of a single rhombomere (r3) with a boundary graft of the similar size (r4/5). The characteristic differences in cell behaviour were consistent for all the chimaeras analysed. For both types of graft, there was no significant anteroposterior mixing of proliferating cells within the ventricular zone. However, the spread of postmitotic cells in the mantle layer was strikingly dependent on whether the interface between grafted and host neural tissue fell at a rhombomere boundary (Fig. 9A) or half-way along a segment (Fig. 9B). While the progeny of r3 (Fig. 9A) form a stripe with discrete and approximately parallel edges, the progeny of the boundary graft (Fig. 9B) fan out to fill a territory that ventrally is more than twice the width of the stripe from an intact rhombomere. This is consistent with rhombomere boundaries acting as barriers to the movement of the majority of postmitotic cells. When the edge of a graft falls in the middle of a rhombomere, cells outside the ventricular layer may move along the anteroposterior axis. This movement is constrained if the grafted cells abut territory derived from an adjacent rhombomere.

In Fig. 9C and D, dorsal views of the distribution of cells across 500 μm of the hindbrain have been reconstructed from a rhombomere (r3) and a basal boundary graft (basal r3/4). Chimaeric brains were sectioned parasagittally. For each section, cell positions relative to the tissue outline were logged onto a computer using a camera lucida. A projected pseudo-dorsal view of cell distribution, through the depth of the hindbrain, was then computed from the stack of aligned, digitised sections. Examples of sections from each reconstruction, from the levels marked by an asterisk, are shown in Fig. 9E and F. Ventricular cells are shown in red and subventricular cells are marked in green. The degree of longitudinal spread of post-mitotic cells is far greater across the entire width of the hindbrain in the boundary graft (Fig. 9D).

These observations show that there are different constraints on the mixing of ventricular and subventricular cells. Proliferating quail cells at the ventricular surface form a discrete cluster with very limited longitudinal spread regardless of graft type. Cells in the mantle layer, which we presume are postmitotic, show patterns of inter- and intra-rhombomeric spread that are consistent with in vitro cell mixing experiments (Fig. 1).

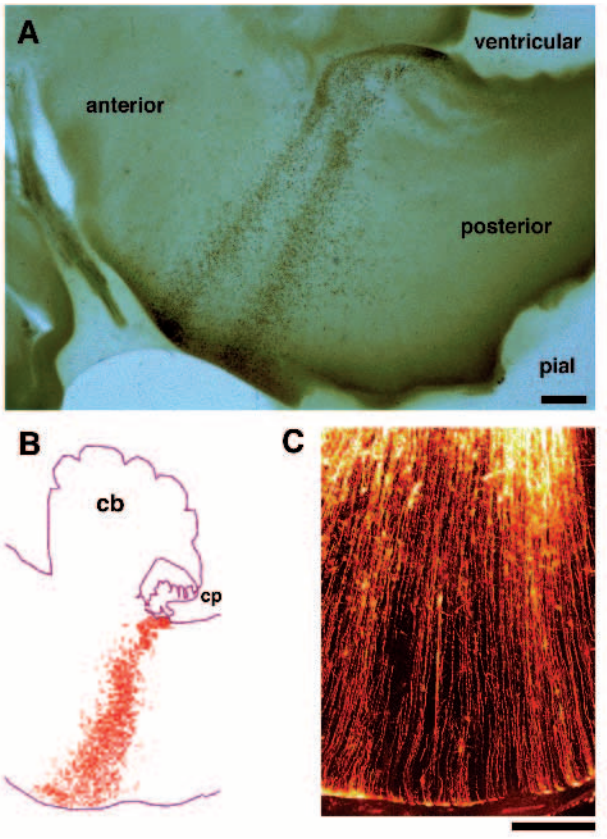


Fig. 3. The distribution of quail cells at E10 following orthotopic rhombomere transplants at E2. (A) r3 and r5 were unilaterally replaced with orthotopic grafts from an age-matched quail embryo. The chimaeric brain was sectioned parasagittally. Quail cells were labelled by the QCPN anti-quail antibody and stained by peroxidase immunohistochemistry. (B) Camera lucida drawing of a parasagittal section through an E10 chimaeric hindbrain showing the distribution of quail cells from a unilateral, orthotopic r6 graft. (C) Confocal micrograph of a parasagittal section through a hindbrain where radial glia fibres had been labelled by DiI. Scale bars, 250 μm. cb, cerebellum; cp, choroid plexus.

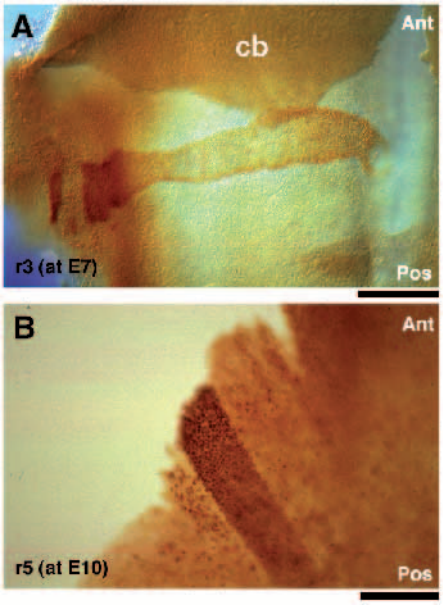


Fig. 4. The progeny of rhombomeres remain segregated at the ventricular surface. (A) The dorsal surface of an E7 chimaeric hindbrain where the left side of r3 had been replaced with a orthotopic quail graft at E2. Overlying roofplate and choroid plexus were removed. (B) Horizontal section through the ventricular surface of an E11 brain which received a unilateral (right), orthotopic r5 graft of quail neural tissue at E2. Although the orientation is the same as in A, the folding of the ventricular surface changes the apparent orientation of the stripe of ventricular quail cells when viewed from above. Scale bars, 250 μm.

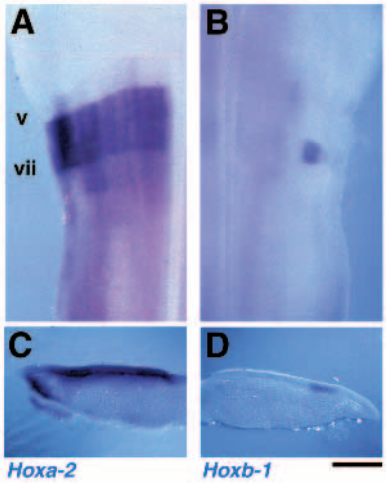


Fig. 5. Hox gene expression is segmental within the ventricular layer of the hindbrain at E6, after rhombomeres have disappeared. (A) Dorsal view of the left side of a whole-mount hindbrain stained with a digoxygenin-labelled riboprobe for *Hoxa-2* by in situ hybridisation. The roofplate was removed in order to visualise the ventricular surface. (B) Dorsal view of the right side of hindbrain stained for *Hoxb-1*. (C) Coronal section (100 μm) through a *Hoxa-2*-labelled hindbrain at the level of r4-derived cells, just anterior to the VIIth nerve ganglion. (D) Coronal section at the same level through a *Hoxb-1* probed hindbrain. Scale bar, 250 μm. v, level of Vth nerve ganglion; vii, level of VIIth/VIIIth nerve ganglion.

The limits on mixing between dividing cells revealed by grafting after stage 10 are not typical of earlier hindbrain development. When orthotopic grafts are made before rhombomeres form, in a caudal stage 8-9 hindbrain, cells in the ventricular zone are free to mix along the anteroposterior axis. Figure 10A shows the distribution of quail cells from such a graft in a parasagittal section through an E4 chimaeric brain. Radial clusters of quail cells have moved anteriorly away from the edge of the graft and include cells within the ventricular layer. These clusters are reminiscent of clones derived from single cells (Hemond and Glover, 1993). Inset, at a higher magnification, a single cluster from an adjacent section contains a single rounded mitotic cell and a number of elongated cell profiles within the ventricular zone. By comparison, in a boundary graft at stage 11^{-} (Fig. 10B), anteroposterior spread takes place only outside the ventricular layer. Whether, this later longitudinal migration occurs at the edge of the ventricular zone, or deeper within the mantle, cannot be discerned by our methods.

DISCUSSION

We have shown that the adult hindbrain is composed of a segmental array of lineages derived from individual rhom-

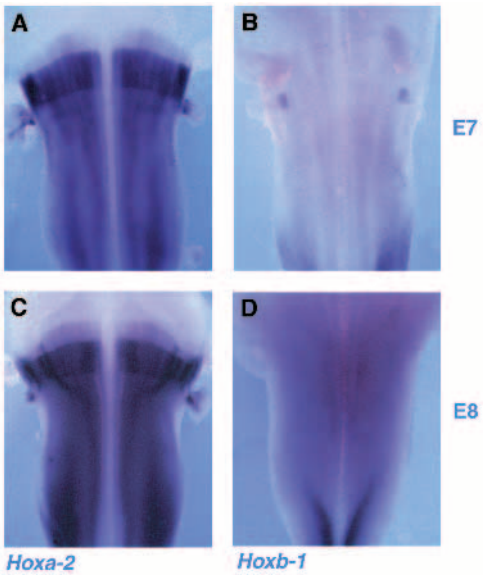


Fig. 6. After E6, the levels of *Hoxa-2* and *Hoxb-1* maintain different levels of segmental expression. (A) At E7, *Hoxa-2* is clearly segmentally expressed. Staining is also visible in the VIIth cranial ganglion although neither in the Vth ganglion nor in the developing cerebellum. (B) *Hoxb-1* staining is weaker than at E6. The faint bands of label that were visible extending from the strong patches of *Hoxb-1* to the lateral edge of the ventricular surface have disappeared. (C) By E8, the ventricular surface has become significantly more convoluted but segmental expression of *Hoxa-2* remains distinct. Relative levels of *Hoxa-2* in the mantle of the hindbrain have increased. (D) *Hoxb-1* segmental expression in rostral hindbrain can no longer be detected at E8 by whole-mount in situ.

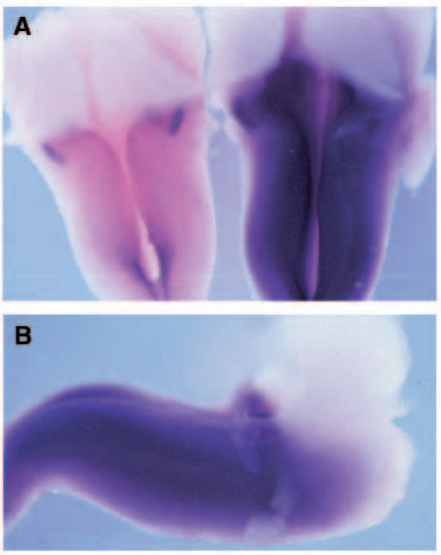


Fig. 7. At E9, *Hoxa-2* expression is virtually uniform throughout the hindbrain. (A) Two brains that were stained for different periods of time. The relatively stronger labelling in lateral extraventricular, vestibulo-ocular regions is more easily seen in the lighter stained hindbrain (left). Segmental labelling of the ventricular surface is still detectable in a more deeply stained hindbrain (right). (B) A side view of a stained brain shows the extent of *Hoxa-2* message in the mantle of the hindbrain.

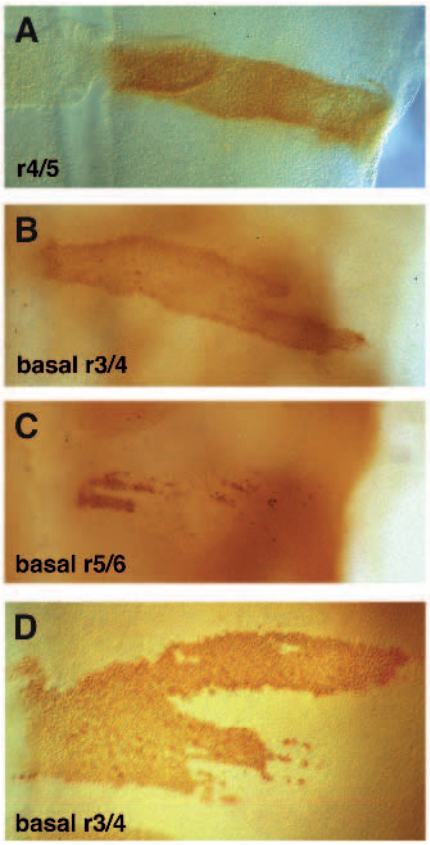


Fig. 8. Quail cells at the ventricular surface of E7 hindbrains following orthotopic boundary grafts. Anterior (rostral) is top, posterior (caudal) bottom. At E2, the rhombomere boundary and the adjacent halves of the neighbouring rhombomeres in a chick host were replaced by equivalent quail tissue. (A) Ventricular distribution of quail cells following a boundary graft (r4/5) across the entire mediolateral extent of one half of the neural tube. When just the basal quarter of the neural tube was fate mapped, the spread of quail cells from medial to lateral ventricular surface was variable. (B) Two tongues of cells extend laterally in a chimaeric brain that received a basal R/5 quail graft. The anterior and posterior boundaries of the quail-derived territory are unbroken. (C) A basal r5/6 graft, which has produced relatively fewer proliferative cells. The spread of cells is again medial to lateral. (D) A basal r3/4-derived population of proliferative quail cells shown to a slightly higher magnification.

bomeres. The boundaries between these polyclonal units are not sharp, except at the ventricular surface where both sharp boundaries and segmental gene expression are maintained until at least half-way through embryonic life. Mixing between cells at the ventricular surface is constrained along the anteroposterior axis. This contrasts with mixing behaviour of proliferating cells along the mediolateral axis and patterns of dispersal of postmitotic cells within rhombomeres. The organisation of the ventricular zone of the postsegmental embryonic hindbrain is considered below.

Progeny of rhombomeres are radially organised in the mantle zone

While we did not attempt a detailed fate-map of individual rhombomeres with respect to nuclear identity (see Marín and

Puelles, 1995), it is clear from orthotopic rhombomere grafts that there is a characteristic distribution of the progeny of individual rhombomeres orthogonal to the columnar nuclear organisation of the maturing hindbrain. The array of segmental cell lineages is underlain by a radial glia structure, throughout the hindbrain, that parallels the dispersal of cells.

Rhombomeres persist in the ventricular zone

Although after E5 no boundary structures are identifiable by either characteristic morphological or glial markers (Heyman et al., 1993, 1995; Mahmood, 1995), cells do not mix between rhombomere territories. In addition, we find that the transcripts of developmentally important homeobox-containing genes retain a similar segmental distribution in the ventricular zone to the pattern found when rhombomeres were present. Ventricular cells hence show similar patterns of lineage restriction and gene expression to those of the early embryonic, overtly segmented hindbrain. It seems reasonable to conclude that this proliferating zone is still organised into rhombomeres.

If rhombomeres persist in the ventricular zone after E5, then a continuity of segmental positional cues will be maintained for neurons born over subsequent days. The dynamics of mRNA expression in the ventricular zone may directly reflect the period over which new cells are being contributed to specific nuclei. *Hoxb-1* message, for example, has a restricted mediolateral distribution and cannot be detected after E8. Do progenitors expressing *Hoxb-1* mRNA give rise to a specific subset of neurons at different times of development?

Surprisingly, the rostrocaudal extent of each ventricular rhombomere is only c.150 μm. Thus, proliferative cells of the embryonic hindbrain are not only organised into rhombomeres after overt hindbrain segmentation is lost, they are also of a fixed anteroposterior length. The length of the ventricular surface of the hindbrain is hence constant as the mantle layer expands, producing a gross morphogenic asymmetry, which accentuates the pontine flexure.

Cell mixing within the ventricular zone is asymmetric

The asymmetry in the growth of the segmentally organised ventricular surface is reflected in an asymmetry of cell mixing within the ventricular layer. While cells mix along the mediolateral axis, there is no mixing of ventricular cells across any graft boundaries along the longitudinal axis when grafts were made at stage 10-11. Since proliferating cells can spread along the axis at stage 8-9 (Fig. 10; and Fraser et al., 1990), this suggests that this asymmetry is present soon after, or at the time that, rhombomeres form. Asymmetric growth appears not to be a result of oriented cell division (Guthrie at al., 1991). By contrast to the ventricular zone, subventricular (and hence presumably postmitotic) cells are free to move within rhombomeres and restricted only at the boundaries between rhombomeres (Fig. 9B). This restriction on precursor cell mixing is in distinct contrast to the developing chick spinal cord (Leber and Sanes, 1995).

How do these observations compare to analyses of clonal dispersal within the brainstem? Fraser et al. (1990) showed that proliferating cells are free to mix along the axis prior to neural tube segmentation. At this stage (stage 8-9), a detailed analysis of the composition of clones derived from single cells, which were intracellularly dye-labelled, shows that both neuroepithelial cells and postmitotic neurons mix freely with their neighbours producing a ‘salt and pepper’ pattern of dispersal (Lumsden et al., 1994; J. Clarke and A. Lumsden, unpublished observations). By contrast, Hemond and Glover (1993) have shown that, when retrovirally labelled at stage 12-16, clones analysed at E6-9 include a tightly clustered group of neuroepithelial cells within the ventricular zone and more widely dispersed cells outside the ventricular zone. The results of our study show that this restriction on the mixing of precursor cells appears as early as stage 10, shortly after, or at the same time as, rhombomere boundaries themselves are formed.

However, an alternative explanation for our observations is that the asymmetry of cell mixing both within the ventricular zone and between the ventricular and subventricular layers is an experimental artefact. We have tried to control for species differences in cell adhesion by performing short-term aggregation cultures and orthotopic grafting prior to rhombomere formation. In vitro aggregation studies demonstrate that rhombomere-specific adhesion properties are more important in determining cell sorting than species-specific qualities (Fig. 1). Orthotopic grafts at stage 8-9 demonstrate that the graft interface per se is not a restriction on the mixing of quail and chick cells within the ventricular zone (Fig. 10).

Significance of a segmentally organised hindbrain ventricular zone

The persistence of rhombomeres after overt segmentation is lost suggests that the maintenance of early embryonic patterning cues are important for subsequent cell proliferation. However, relatively little is known about late neuronal production in the chick brainstem except for the birthdates of cells within a few, well-characterised nuclei (see Rubel et al., 1976). Despite this, the observed asymmetry in the pattern of growth and cell mixing allows broader speculation on the role of hindbrain segmentation.

Firstly, the asymmetry in the pattern of ventricular cell mixing suggests that rhombomere boundaries, per se, are not barriers to the movement of proliferating cells. Rather, the formation of rhombomeres is either concurrent with, or precedes, a reduced capacity for dispersal of precursor cells along the longitudinal axis. If the transverse segmentation of the neural tube is, in fact, facilitated by a freeze on the anteroposterior movement of proliferating cells, then boundary regions clearly become a secondary feature of an emergent segmental organisation. This is consistent with the interfaces between rhombomeres being the regions of induction of a third cell state: boundary cells are the first cell types in the hindbrain to express radial glia markers (Heyman et al., 1995). Outside the ventricular zone, cells move freely within rhombomeres while dispersal is restricted at segmental boundaries (Fig. 9). Cells that ‘violate’ rhombomere boundaries at stage 17 (Birgbauer et al., 1994) appear to leave no progeny in the ventricular zone, suggesting that they are most likely either migratory neuroblasts or young neurons. Our observations from grafting experiments are thus entirely consistent with the interpretation that rhombomere boundaries maintain polyclonal lineage restriction compartments (Fraser et al., 1990).

Secondly, asymmetric mixing between dividing cells results in a proliferative ventricular surface of a constant length to at least E9. Is there any reason why a constant rhombomere length might be important? Studies by Graham and colleagues

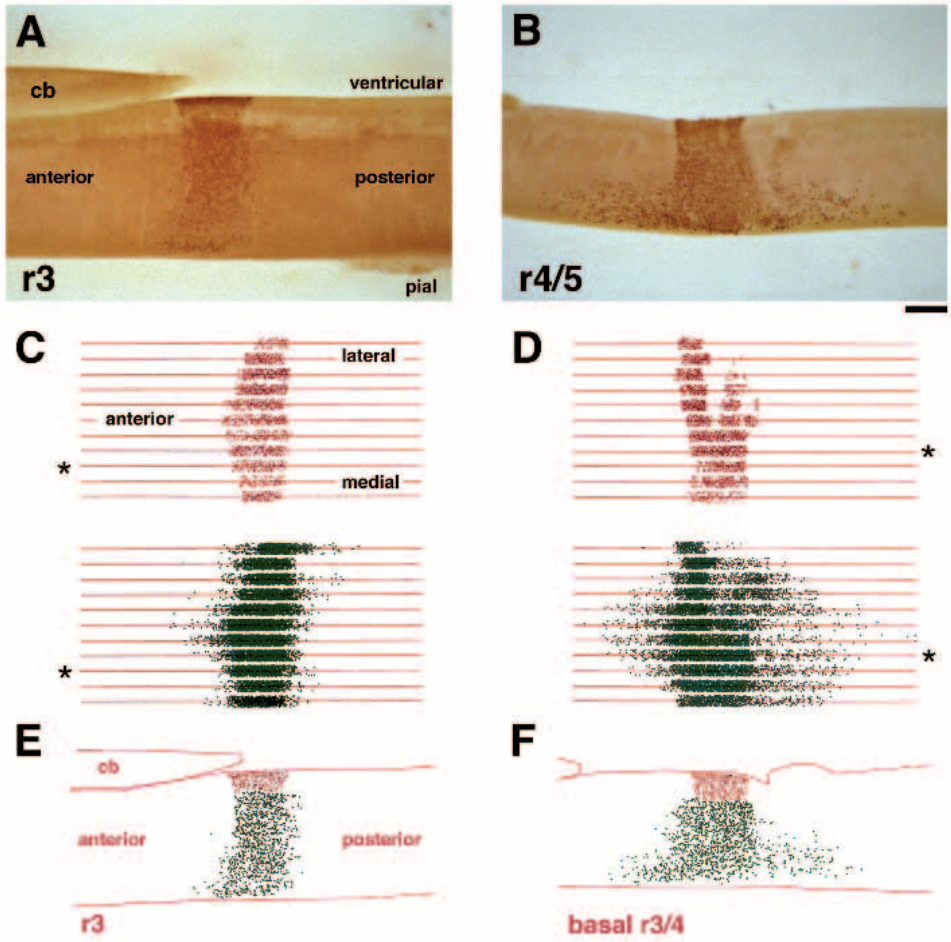
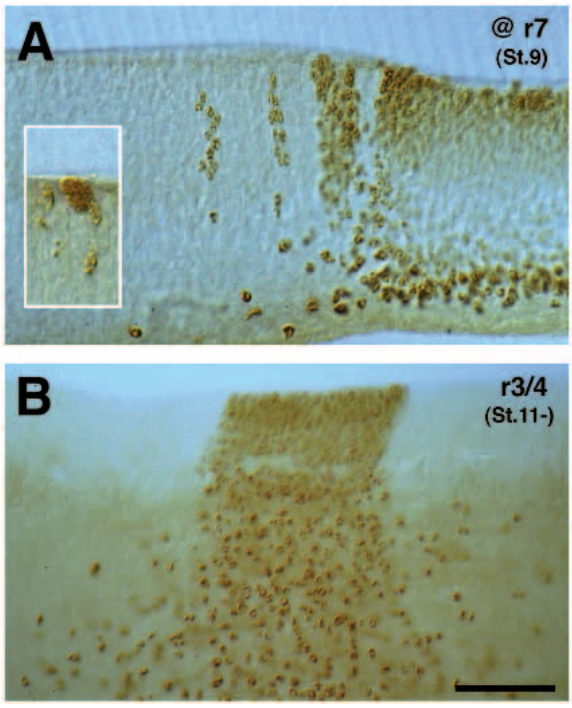


Fig. 9. A comparison of cell mixing between ventricular and subventricular cells for single rhombomere and 'boundary' grafts. (A) Parasagittal section through an r3-derived stripe in an E7 hindbrain which shows the relatively limited degree of spread of quail cells in the mantle with respect to the dorsal, ventricular cells. (B) By contrast, an r4/5 boundary graft in parasagittal section shows that the spread of cells in the ventral mantle is quite considerable, and much greater than for a single rhombomere graft. Scale bar, 100 μm. (C) Comparison of the spread of ventricular (red) and subventricular (green) cells derived from a grafted r3 (shown in A). A camera lucida linked to a computer-aided morphometry programme (Halasz and Martin, 1984) was used to digitise cell positions relative to the boundaries of the sectioned brain. The reconstructed brain is viewed as if from the ventricular surface. Each line represents the centre of a section. Red dots (top) represent the position of ventricular quail cells. Green dots (middle) contrast the relative displacement of postmitotic cells in the mantle. So as to view cell position the cells have been 'jittered' mediolaterally relative to the plane of section. Sections were at 50 μm and the reconstruction is to scale. (D) Equivalent reconstruction of the basal boundary graft (r3/4) shown in Fig. 8D. (E,F) Examples of one of the sections in the reconstructions (the level of section is marked by an asterisk). In all figure elements, anteroposterior axis runs left to right.



(Graham et al., 1994; Graham and Lumsden, 1996) show that various aspects of characteristic segmental gene expression are maintained by interactions between rhombomeres. Such interactions establish segmental identity. If these planar interactions are mediated by diffusible molecules, then the length of a rhombomere limits the potential for communication between segments. We hypothesise that ventricular rhombomeres remain compressed along the anteroposterior axis to maintain such interactions and hence to prevent the breakdown of rhombomeric identity within the ventricular zone of the postsegmental embryonic hindbrain.

Fig. 10. A comparison of cell mixing along the anteroposterior axis (left to right) following grafts at different ages. (A) Parasagittal section through a chimaeric brain at E4 following an orthotopic graft of prospective quail r7 at stage 9, prior to segmentation of the hindbrain. Clusters of radially dispersed quail cells within the ventricular layer have broken away from the anterior edge of the graft. Inset, a single cluster of quail cells from an adjacent section shown to a higher magnification. (B) Parasagittal section showing the dispersal of quail cells in an E7 chimaeric hindbrain following an r3/4 boundary graft at stage 11⁻. Anteroposterior mixing of quail cells can be seen in the mantle but not with the ventricular layer. Scale bar, 40 μm.

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