INTRODUCTION

Segmentation is a mechanism for spatial organisation along the body axis. The vertebrate hindbrain, for example, is subdivided into segmental units called rhombomeres. Cells from each rhombomere are destined to form a precise part of the hindbrain and this is achieved by the almost complete absence of cell mixing between neighbouring compartments (Fraser et al., 1990; Birgbauer and Fraser, 1994). Although each rhombomere will initially generate a similar set of basic neuronal types (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993), they subsequently differentiate along diverse pathways. For example, in the chick, generation of specific branchiomotor nerves, sensory ganglia and fate of neural crest cells are strictly correlated with their rhombomeric origin (Lumsden and Keynes, 1989; Lumsden et al., 1991). Segmentation is characterised by the appearance of a narrow zone of specialized boundary cells that lie at the interface between neighbouring rhombomeres (Lumsden and Keynes, 1989; Heyman et al., 1993, 1995).

Morphological segmentation in the hindbrain is preceded by the expression of two stripes of both the transcription factor Krox-20 (Wilkinson et al., 1989a) and the receptor tyrosine kinase Cek-8 (Nieto et al., 1992; Irving et al., 1996; Cek-8 is the chick orthologue of mouse Sek-1) in the prospective territories of r3 and r5. Cek-8 may be involved in the process of segmentation (Xu et al., 1995) and early phases of Krox-20 expression are required for some aspects of Hox gene expression in these segments (Sham et al., 1993; Nonchev et al., 1996). The expression of a combinatorial code of Hox genes, whose anterior expression limits are coincident with rhombomere boundaries (Wilkinson et al., 1989b; Graham et al., 1989; Duboule and Dolle, 1989), is thought to confer identity to individual rhombomeres. Grafting rhombomeres to more anterior locations suggests that Hox expression is fixed and fate determined just prior to segmentation (Guthrie et al., 1992; Kuratani and Eichele, 1993; Simon et al., 1995), but recent work shows Hox expression can be altered when rhombomeres are grafted to more posterior locations suggesting that Hox expression is not irreversible at these stages (Itasaki et al., 1996).

The generation and maintenance of lineage restrictions at rhombomere borders are thought to be of fundamental importance in the process of segmentation and subsequent regional specification within the hindbrain. The mechanisms that restrict cell mixing at rhombomere borders, however, are not well understood but there are at least two possibilities that could act alone or together. One is that the immiscibility of compartments may be established by alternating surface properties of cells from prospective odd- and even-numbered compartments.
segments. Grafting experiments demonstrate that cell mixing is higher when tissue from two even or two odd rhombomeres are juxtaposed rather than when even tissue is placed next to odd (Guthrie et al., 1993). The second mechanism is that the boundary cells generated at the interface of odd- and even-numbered segments may act as mechanical barriers that prevent mixing (Lumsden, 1990). Boundary cells may form a relatively immobile population as they display reduced levels of interkinetic nuclear migration (Guthrie et al., 1991) and increased levels of a highly adhesive form of NCAM (Lumsden and Keynes, 1989). One approach to understanding the role of boundary cells would be to examine cell movements in hindbrains that have no boundaries. Although there are several mouse mutations in which hindbrain segmentation is disrupted (e.g. McKay et al., 1994; Mark et al., 1993) and exogenous retinoic acid can suppress or alter segmentation in the anterior hindbrain of several species (Papalopulu et al., 1991; Marshall et al., 1992; Wood et al., 1994; Hill et al., 1995), cell movements in these hindbrains has not yet been studied.

Here we report that local application of retinoic acid to chick embryonic hindbrains, just after the initial events of morphological segmentation, leads to loss of boundary cells in the posterior hindbrain. We have analysed Hox, Krox-20 and Cek-8 expression in the boundaryless hindbrain and examined cell movement and neuronal organisation. The results are consistent with the idea that lineage restriction can be maintained by processes other than a mechanical barrier composed of boundary cells. Hox gene expression in the posterior hindbrain is altered but the early spatial organisation of several hindbrain motor nuclei is not affected.

MATERIALS AND METHODS

Retinoic acid application

Eggs were purchased from Poyndon Farm, Hertfordshire, England, incubated at 38°C and staged according to Hamburger and Hamilton (1951). All-trans-retinoic acid (Sigma, UK) was dissolved in dimethylsulphoxide (DMSO) and loaded on AG1-X2 beads of approximately 100 μm diameter as described by Tickle et al. (1985). Control beads were soaked in DMSO only. Beads were placed into the fourth ventricle of the hindbrains through a small slit in the roof plate of stage 10-11 chick embryos. Eggs were resealed, returned to the incubator and harvested at different time points. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline.

Whole-mount in situ hybridisation

Preparation of digoxigenin-labelled RNA probes and the protocol for whole-mount in situ hybridisation were as described by Nieto et al. (1996). A 420 bp antisense RNA probe corresponding to nucleotides 394-813 was used to detect Cek-8 transcripts (Sajjadi and Pasquale, 1993). Follistatin transcripts were detected using antisense RNA for the whole cDNA (1.1 kb, Conolly et al., 1995). The Hoxb-4 antisense riboprobe (1.3 kb) is complementary to the homeobox and sequences 5’ and 3’ to it (Burke et al., 1995). The riboprobe to detect Krox-20 transcripts spanned 0.23 kb of the Krox-20 cDNA. The Hoxa-2 probe was generated as described in Prince and Lumsden (1994) and Hoxb-1 expression was screened using an antisense riboprobe stretching over 2 kb.

3A10 antibody staining

Whole-mount staining followed the protocol of Lumsden and Keynes (1989).

Iontophoretic application of Dil and DiA

Small deposits of the lipophilic membrane dye Dil (Molecular Probes D-282) were applied in ovo to the developing hindbrain by iontophoresis. Microelectrodes with a tip diameter of approximately 2 μm were filled at their tips with a small quantity of Dil (3 mg/ml in dimethyl formamide) and then backfilled with 1 M lithium chloride. These were connected to the positive pole of a 9 volt battery. The electrode was micromanipulated into position and the dye driven out of the electrode by completing the circuit with a second silver wire electrode placed in the egg albumen and attached to the battery’s negative terminal. Completing the circuit for about 2-3 seconds was sufficient to label a small patch of cells. The success and position of the labelled cells was checked on an epifluorescence microscope fitted with a extra long working distance ×20 objective, and imaged and measured using a cooled CCD camera and Biovision software. For double-label experiments, the membrane dye DiA (Molecular Probes D-3883) was applied in the same way. The embryos were allowed to develop for 48 hours and flat-mounted hindbrains examined using a Nikon fluorescence microscope or Leica confocal microscope.

Retrograde labelling

Trigeminal, facial and glossopharyngeal nerve roots of control and experimental embryos fixed in 4% paraformaldehyde at stages 18-20 were exposed by dissection. The roots were injected with 3 mg/ml DiI or DiA in dimethylformamide and left overnight in fixative at room temperature. Hindbrains were dissected free of mesenchyme, prepared as flat mounts and examined on a Nikon fluorescence microscope.

RESULTS

Retinoic acid leads to loss of hindbrain boundaries

We implanted AG1X2 beads (100 μm diameter) soaked in 10 μg/ml retinoic acid into the 4th ventricle at the level of r4 in stage 10-11 chick embryos. Beads of 200 μm diameter release retinoic acid continuously over at least 20 hours but this period is probably reduced for beads with half the diameter as used here (Eichele et al., 1984). Embryos were allowed to develop for 24 or 48 hours, up to stages 15/16 or 19/20, respectively, and their hindbrains dissected and flat mounted. Phase-contrast optics revealed that this local retinoic acid application caused loss of boundaries between posterior rhombomeres. In 60% of cases, only rhombomeres 1 to 3 (r1 to r3) were distinguishable and boundaries posterior to r3 were missing (Fig. 1A,B). In 26% of cases, one or two rhombomeres were visible and boundaries posterior to r1 or r2 missing. In the remaining cases (14%), either 4 or 5 rhombomeres were visible. The identity of rhombomeres was deduced from their relationship with the cranial nerve roots.

Morphological loss of posterior boundaries was associated with the absence of follistatin transcripts, which mark rhombomere boundaries in normal embryos at stages 18-20 (Conolly et al., 1995). Normally five stripes of follistatin expression co-localise with morphological boundaries from the r2/3 boundary down to the r6/7 boundary, whereas a maximum of two stripes could be seen in retinoic-acid-treated embryos (n=4/4, Fig. 1C,D). Parasagittal sections of the hindbrain revealed that the characteristic fan-shaped array of boundary cells and the increased extracellular spaces normally associated with boundaries (Heyman et al., 1993, 1995) were also lost (Fig. 1E,F).

In normal embryos, staining with the monoclonal antibody
3A10 revealed that neuronal differentiation in r2 and r4 is advanced ahead of r3 and r5, and that boundaries contain more circumferential axons than non-boundary regions (Fig. 1G, and Lumsden and Keynes, 1989). In retinoic-acid-treated hindbrains, at stage 15 (i.e. 24 hours after treatment), there was virtually no sign of the usual alternating levels of neuronal differentiation (Fig. 1H) and the periodic axon accumulations normally associated with boundaries was lost at these and subsequent stages (Fig. 1I).

Changes in Cek-8 and Krox-20 expression precede morphological changes

Cek-8 and Krox-20 are first expressed in a region that will become r3 at stages 8− and 8, respectively (Irving et al., 1995) and in both r3 and r5 from stage 10 onwards (Fig. 2A,B). Cek-8 expression lasts until at least stage 22 (Fig. 2C) whereas Krox-20 is only present until stage 18. Retinoic acid beads placed anterior to r1 or posterior to r6 did not affect Cek-8 expression (n=3). At 4 to 6 hours after placing a retinoic acid bead at any position between r1 and r6, Cek-8 expression in r3 was unaffected but abolished in r5 in all treated embryos (n=10/10, Fig. 2D). Complete loss of Cek-8 expression in r5 was observed 4 hours after treatment (n=2/2). At this time, all boundaries were still visible. Cek-8 remained absent from r5 but present in r3 throughout the following 48 hours (to stage 20, n=9/9, Fig. 2E). In stage 20 embryos with three or more rhombomeres, the presence of morphological boundaries was correlated with sharp expression boundaries of Cek-8 in r3 (n=9/9, Fig. 2E).

In specimens where only two rhombomeres were clearly distinguishable, Cek-8 had a sharp expression boundary at the anterior part of r3, where a morphological boundary was still visible, but not at the posterior part, where Cek-8 expression was downregulated and the morphological boundary abolished (n=2/2 Fig. 2F). In two specimens, a r4/5 boundary was also present and, in both cases, this correlated with residual Cek-8 expression in r5, although this expression was not immediately adjacent to the boundary.

The effect on Krox-20 expression varied with the position of the bead. At 6 hours, a bead placed at r4 level abolished Krox-20 expression in both r3 and r5 (n=1, Fig. 2G), and a bead placed at r5 or r6 abolished Krox-20 transcripts at r5 level only (n=2). Surprisingly beads placed at r2 or r3 level, had no affect on r3 expression but complete downregulation in r5 (n=2). Beads anterior to r2 or posterior to r6 did not affect Krox-20 expression. With beads placed at r4, Krox-20 in both r3 and r5 remains completely downregulated 24 hours after retinoic acid treatment when the embryos have reached stage 15/16 (Fig. 2H, n=4/5, the fifth embryo had weak expression in r3). Despite the loss of Krox-20, the morphological boundaries of r3 were maintained.

In general, Cek-8 expression was maintained in r3 and abolished in r5 after retinoic acid application, whereas Krox-20 could be abolished in both r3 and r5. Furthermore, at this stage of development, expression of both Cek-8 and Krox-20 in r5 was more sensitive to retinoic acid than expression in their r3 domains. The expression of Cek-8 in r3 was maintained despite the loss of Krox-20 transcripts from stage 11/12 onwards, i.e. 6 hours after implanting the bead. Boundary maintenance was strictly correlated with Cek-8 expression.
**Hoxb-4, Hoxb-1 and Hoxa-2**

*Hox* genes are thought to confer segment identity and are known to be responsive to retinoic acid. *Hoxb-4* is normally expressed in the neural tube up to the r6/r7 boundary (Fig. 3D). At 16 hours after retinoic acid implants when *Cek-8* and *Krox-20* expression are already affected, *Hoxb-4* expression remained unchanged (*n*=3). But, by 24 hours, the domain was shifted anteriorly to the last visible boundary at the posterior limit of r3 (*n*=3, Fig. 3E).

In normal stage 15 embryos, *Hoxb-1* expression is detected at low levels up to the r6/r7 boundary and at high level in r4 (Fig. 3A). As with *Hoxb-4*, *Hoxb-1* expression remains unchanged 5-6 hours after retinoic acid treatment (*n*=3). At 24 hours after treatment, *Hoxb-1* was still expressed to a variable level at the r4 level in a width equivalent to one rhombomere while its caudal domain shifted anteriorly to the region of the r5/6 boundary (*n*=5, Fig. 3B). At 48 hours, *Hoxb-1* at the r4 level was further down-regulated and the caudal domain again shifted anteriorly to lie close to the r3/4 boundary (Fig. 3C). All hindbrain expression of *Hoxb-1* was reduced to a very low level.

From stage 11, the normal anterior limit of *Hoxa-2* expression is at the r1/2 border and remains until stage 24 (Prince and Lumsden, 1994 and Fig. 3F). *Hoxa-2* is also expressed in the crest that emanates from r4 to populate the second branchial arch. In retinoic-acid-treated hindbrains, the anterior limit of *Hoxa-2* expression was unchanged at the r1/2 border (Fig. 3G). There was uniform expression from the caudal r3 boundary backwards, thus the high levels of expression immediately adjacent to the floor plate in r4 of control brains (Fig. 3F) was lost while those adjacent to the floor plate in r2 and r3 were maintained. *Hoxa-2* continued to be expressed in the second branchial arch of retinoic-acid-treated embryos (*n*=4).

In summary, expression of *Hoxb-4* and the caudal domain of *Hoxb-1* shifted anteriorly in retinoic-acid-treated embryos whereas the anterior expression limit of *Hoxa-2* was unaltered. In contrast to *Cek-8* and *Krox-20*, the changes in *Hoxb-4* and *Hoxb-1* expression domains occur after the loss of posterior boundaries, 24 hours after retinoic acid treatment.

**Precursor dispersal**

We investigated whether loss of boundaries and the molecular changes that follow retinoic acid treatment lead to increased cell dispersal and cell mixing between former neighbouring rhombomeres. We first established the anteroposterior dispersal of labelled cells in individual rhombomeres. Cells in rhombomeres 3, 4 and 5 were labelled with DiI or DiA at stage 10/11 and some embryos treated with retinoic acid and others used as controls. Labelling was performed as close as possible to a boundary (Fig. 4A,B) and, initially, each application usually labelled 5-10 neighbouring cells. 48 hours later labelled cell clusters of various shapes and sizes were seen, ranging from elongated stripes of cells to small oval patches of cells in both control and retinoic-acid-treated specimens. Fig. 5 summarises the results and illustrates that the mean antero-posterior dispersal of neural precursor cells (78±6 μm, range 16-155 μm in control brains) was not changed significantly in retinoic-acid-treated embryos (85±5 μm, range 16-181 μm) and amounted to 40% of the width of a rhombomere at stage 19. This was true regardless of how many rhombomeres remained after retinoic acid application.

We next tested whether cells from former rhombomeres retained their relative positions by injecting DiI into r3, close to the r3/r4 boundary, and into r5, close to the r4/r5 boundary in the same embryo and then treating with retinoic acid. After 48 hours, the labelled patches were on average 220±10 μm apart (range 155-310 μm, *n*=15). This distance spans at least the normal width of rhombomere 4 at stage 19 (145-224 μm depending on the dorsoventral level) as measured on 11 control embryos. Labelled cells thus remained within their original territory despite the absence of morphological boundaries and there was no significant tissue loss from r4.

To assess cell mixing between r4 and r5, DiA and DiI were
Fig. 3. Hox gene expression shown in flat-mounted hindbrains from stage 15-20 chick embryos. (A) Hoxb-1 is expressed up to r6 and also in r4 in normal stage 15 hindbrain. Bidirectional arrow indicates gap between two expression domains. (B) Hoxb-1 is still expressed in r4 24 hours after retinoic acid treatment in hindbrain with 4 rhombomeres remaining, but gap to more posterior domain is reduced (bidirectional arrow). (C) By 48 hours, Hoxb-1 expression in r4 is lost and low level expression in caudal domain has shifted anteriorly to lie close to r3/4 boundary (arrow). (D) Hoxb-4 is expressed up to 6/7 boundary (arrow) in normal stage 15 hindbrain. (E) After 24 hours of retinoic acid treatment anterior limit of Hoxb-4 expression is shifted anteriorly to r3/4 boundary (arrows). (F) Normal anterior expression limit of Hoxa-2 lies at the r1/r2 boundary. (G) Anterior expression limit is unchanged 48 hours after retinoic acid treatment, but the high level of expression normally present next to floor plate in r4 is lost. Bar is 200 μm in A, B, C, F and G, and 400 μm in D and E.

Injected on either side of the r4/5 boundary (Fig. 4B). We measured the distance between the injections using a cooled CCD camera and Biovision software and embryos were treated with retinoic acid and examined 48 hours later. The descendants of cells that are initially closer than the average dispersal of 40% of a rhombomere’s width should have the possibility to mix. Since 40% of a stage 10/11 rhombomere is 56 μm, we analysed the results from 14 pairs of injections that were less than 56 μm apart in hindbrains in which the r4/5 boundary was abolished. In 12/14 embryos, despite the absence of a morphological boundary, no cell mixing was seen. In eight embryos, the two labelled clusters were separated by a thin stripe of unlabelled cells (Fig. 4D). In four cases, the two labelled clusters abut one another at a fuzzy interface but no intermingling could be seen (Fig. 4E). Intermingling of labelled cells over the whole of the interface of the two populations was only apparent in one embryo (Fig. 4G), where the maximum overlap was 20 μm, and in one further embryo a few intermingling cells were seen at the interface (Fig. 4F).

To ensure that our injections were close enough not only to each other but also to the boundary, we made injections close to the r4/r5 boundary in untreated embryos and analysed how many labelled cells spread up to the boundary over the subsequent 48 hours. In 13/23 cases fluorescent cells had spread at least up to the boundary. Furthermore cell mixing within a normal rhombomere was tested by injecting DiI towards the anterior and DiA towards the posterior boundary of r4. In this test, cell mixing within a rhombomere occurred in 50% (4/8) of cases, although it was not extensive (Fig. 4C). In the cases where labelled clusters did not mix, the original injections were more than 56 μm (40% of a rhombomere width) apart.

Organisation of branchiomotor nuclei
The anterior shifts in Hoxb-4 and Hoxb-1 expression suggest that the affected rhombomeres may have been posteriorised and the lack of boundary cells raises the possibility that the organisation of branchiomotor nuclei normally segregated at a boundary may be disrupted. To address these questions, we performed retrograde labelling studies from cranial nerves at 48 hours (stage 19/20) and 3A10 antibody staining at 48 and 72 hours (stage 23-25) after retinoic acid treatment. At these times, cells at former r4, r5 and r6 levels have been expressing Hoxb-4 for 24 and 48 hours, respectively. In normal embryos, the VIIth (facial) nerve root is located at r4 level and recruits its motor neurons from r4 and r5, whilst cell bodies of motor neurons contributing to the IXth (glossopharyngeal) nerve are found in r6 and r7. There is normally little or no spatial overlap between these two branchiomotor nuclei (Fig. 6A,B). In retinoid-treated hindbrains, there was only a slight increase in the spatial overlap of the facial and the glossopharyngeal nuclei (Fig. 6C-E). The maximum overlap that we found (Fig. 6D) was equivalent to 10% of the total anteroposterior extent of the facial nucleus and usually restricted to less than 10 overlapping cells from each nucleus.

In agreement with Gale et al. (1996), the number of contralateral vestibulo-acoustic (CVA) neurons located in the floor plate at the level of r4 was markedly reduced in retinoic-acid-treated embryos (Fig. 6C). Somewhat surprisingly, we found the motor neurons in r3 were altered by retinoic acid treatment. Normally all r3 motor neurons would contribute to the trigeminal nerve root on r2 but, in retinoic-acid-treated embryos, the number of r3 neurons labelled from the trigeminal was reduced and a significant number of r3 motor neurons were labelled by DiI application to the facial root on r4 (Fig. 6F). Combined facial and trigeminal fills demonstrated a relative lack of motor neurons in r3 (data not shown). The contribution of r3 neurons to the facial root was despite the presence of a r3/4 boundary (Fig. 6F).
Normally the exit point of the facial nerve is restricted to a tightly defined region on r4 (Fig. 6A) but, in retinoic-acid-treated embryos, retrograde labelling demonstrated that the facial nerve exits from a line stretching from r4 down into r5 (Fig. 6G). The facial exit point was transformed to an ‘exit line’ in 7/8 cases and, in one case, even fused with the trigeminal exit point. The transformation of the r4 exit point to an exit line stretching into r5 that resembles the exit line on r6 and r7 for the glossopharyngeal motor axons. It is possible that, since exit points are marked by local accumulations of neural crest which may be instructive for the patterning of motor axons (Niederlander et al., 1996), this change in exit points may be caused by the alterations in neural crest migration previously demonstrated in RA-treated hindbrains (Lee et al., 1995; Gale et al., 1996).

DISCUSSION

Precursor movements in hindbrains lacking boundary cells
With only a few exceptions (Birgbauer and Fraser, 1994), cells of adjacent rhombomeres do not mix in normal embryos (Fraser et al., 1990; Guthrie et al., 1993). We have explored whether loss of boundary cells and segmental gene expression in retinoic-acid-treated embryos leads to increased mixing between cells that derive from adjacent rhombomeres. This question has not been addressed in previous reports of mor-
phologically unsegmented hindbrains. In the absence of boundaries, we detect no change in the overall anteroposterior dispersal of precursor cells and, in most cases, no substantial mixing between cells of former rhombomeric units. While we cannot rule out the possibility that a small increase in mixing occurs precisely at the position of the former boundaries, our observations seem to rule out a substantive role for boundary cells in the restriction of anteroposterior cell dispersal and mixing. This supports the original suggestion of Fraser et al. (1990) that clones of cells spread up to the middle of rhombomere boundaries rather than only up to the zone of boundary cells, and is consistent with the idea that lineage restriction can be maintained by processes other than a mechanical barrier composed of boundary cells (Guthrie and Lumsden, 1991; Guthrie et al., 1993).

Our data lend support to the proposal that the principal mechanism of lineage restriction at boundaries is the expression of alternating surface properties on cells from odd- and even-numbered rhombomeres (Guthrie et al., 1993; Wingate and Lumsden, 1996). The nature of these surface properties remains largely unknown but may be similar to the calcium-dependent mechanisms that in vitro can segregate cells taken from distinct regions of the forebrain (Götz et al., 1996). Either repulsive interactions between odd and even cells and/or preferential adhesion between like cells could be involved. The expression domains of both Krox-20 and Cek-8 prior to morphological segmentation suggests they could play a role in establishing the alternating cellular properties responsible for lineage restriction. The segmental defects that follow dominant negative inhibition of the zebrafish ortholog of Cek-8 could be explained by increased cell movement (Xu et al., 1995), but this has not been demonstrated directly. Our finding that down-regulation of Cek-8 and Krox-20 in r5 does not lead to an increase in cell movement suggests that other factors can maintain the restrictions to cell movement. Cek-8, for example, could normally work in conjunction with other segmentally expressed members of the Eph-like receptor tyrosine kinase family (Becker et al., 1994; Bergeman et al., 1995; Flenniken et al., 1996) which may not be affected by retinoic acid treatment.

**Loss of boundary cells**

Under experimental conditions, boundaries develop at the interface of rhombomeres with divergent properties (Guthrie and Lumsden, 1991). In general, no boundaries form when rhombomeres of the same kind are juxtaposed. One interpretation of the loss of boundaries in the retinoic-acid-treated hindbrain is that the divergent properties necessary for boundary formation and maintenance have been lost. We find that boundary loss is closely correlated with and preceded by down-regulation of Cek-8 suggesting that signalling through this receptor tyrosine kinase may be important for boundary initiation and maintenance. However, since cell mixing between former rhombomeric units is still restricted in retinoic acid hindbrains, the cell properties that prevent mixing must still be present and this suggests that these properties differ from those required for

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**Fig. 6.** Organisation of cranial nerves. (A-G) Retrograde labelling from VII/VIII nerve root with DiI (red) and from IX root with DiA (yellow). (A) Control fills to show lack of overlap between red and yellow motor neurons. Arrow marks r5/6 boundary. Asterisk marks compact exit point of VII/VIII nerve. (B) Higher power of another control that shows minimal spatial overlap of red and yellow cells at r5/6 boundary. (C) Low-power micrograph to show overall organisation of facial and glossopharyngeal neurons on both left and right sides of retinoic-acid-treated embryo. There is little or no overlap between red and yellow cells. (D, E) High-power view of two retinoic-acid-treated hindbrains both of which show limited spatial overlap of red and yellow cells. (F) Motor neurons filled in r3 from VII/VIII root in retinoic-acid-treated hindbrain. Same specimen as in C. Arrow marks r3/4 boundary. (G) Compact exit point of trigeminal root (->) and exit ‘line’ of facial root to right in retinoic-acid-treated hindbrain. Compare exit line to VII/VIII exit point asterisked in A. (H) 3A10 staining reveals peripheral organisation of VII, IX and X nerves in control embryo. Compact exit point of VII nerve is asterisked. (I) Retinoic-acid-treated embryo reveals VII exit point (asterisk) now resembles exit line of IX/X (white arrow) for VII nerve and anastomoses between IX and X nerves (black arrow). (J) Low-power view of retinoic-acid-treated embryo showing normal position of abducens nerve roots, which are shown at higher power in K. V, trigeminal; VI, abducens; VII, facial; IX, glossopharyngeal; X, vagus; OV, otic vesicle. Bar is 100 μm for A and B, 40 μm for D and E.
boundary formation. Retinoic acid treatment may therefore alter the cell surface differences that normally maintain boundaries but not those that prevent cell mixing.

In contrast to Cek-8, loss of Krox-20 expression did not correlate with loss of segmentation. We observed a complete loss of Krox-20 expression in both r3 and r5 but the r2/r3 and usually the r3/r4 boundary remained in retinoic-acid-treated hindbrains. Analysis of Krox-20 null mutant mice demonstrates its requirement for the correct development of r3 and r5 (Schneider-Manourí et al., 1993, 1997); it also controls some aspects of the expression of Hoxa-2 (Nonchev et al., 1996) and Hoxb-2 (Sham et al., 1993) and the early phases of Cek-8 expression (Thiel, T., Gilardi, P., Charnay, P. and Wilkinson, D. G., unpublished). Our results are consistent with the view that Krox-20 plays a role in establishing the identity of r3 (see later), but that its continued expression is not required for the maintenance of segmentation.

Plasticity of Hox gene expression and neuronal organisation in hindbrains lacking boundary cells

A summary of the retinoic-acid-induced changes to neuronal organisation and Hox, Krox-20 and Cek-8 expression is given in Fig. 7. Hox genes are thought to impart segment identity to individual rhombomeres. In our experiments, the domains of Hoxb-1 and Hoxb-4, in contrast to those of Cek-8 and Krox-20, are not altered until after morphological segmentation is lost. Then Hoxb-4 shifts anteriorly up to the posterior boundary of r3 and an anterior shift in the caudal domain of Hoxb-1 also occurs together with the down-regulation of its r4 domain. The relatively delayed shift in Hox expression suggests that this response may not be a direct one. A more speculative suggestion is that the ectopic, anterior shift in expression occurs as a wave spreading forward in the plane of the neuroepithelium from its original domain and that the progress of this spreading expression is initially obstructed by the presence of rhombomere boundaries. Boundary cells have reduced junctional communication (Martínez et al., 1992) and this has been proposed to limit the spread of small inductive signalling molecules (Martínez et al., 1995). Perhaps in this way boundaries normally help maintain the long-term stability of Hox expression borders.

At stage 10, rhombomeric Hox expression and the phenotype of r4 efferent neurons appears to be irreversibly fixed when grafted to ectopic more anterior locations (Guthrie et al., 1992; Kuratani and Eichele, 1993; Simon et al., 1995; Grapin-Botton et al., 1995). However, Hox expression can be altered at this stage by grafting somites next to the hindbrain and within grafts moved from anterior to posterior locations (Itasaki et al., 1996), but whether these late changes in Hox expression are accompanied by alterations to neuronal organisation is uncertain. Our results demonstrate that the spatial regulation of at least two Hox genes, Hoxb-1 and Hoxb-4, can be altered by the exogenous application of retinoic acid to the hindbrain at stages 10-11. Here the late changes in Hox expression do not appear to have altered the early organisation of facial (r4/5) and glossopharyngeal (r6/7) efferent neurons nor the appearance of the abducens motor nucleus (r5/6). If these aspects of regional identity are controlled by Hox genes, they must have been determined before stage 15, as this is the time that Hox expression is altered (20 hours after retinoic acid treatment) and maintained despite changes in expression after this time. In contrast to this, the CVA neurons characteristic of r4 are lost (our data and previously shown by Gale et al., 1996) suggesting their identity may be more plastic. Mutations of the Hoxb-1 gene have shown that the contralateral migration of CVA neurons is dependent on expression of this gene (Studer et al., 1996).

We also find that some of the motor neurons in r3 are altered such that their axons exit via the facial nerve rather than the trigeminal. Similar alterations in r3 motor neurons are seen in mouse embryos exposed to retinoic acid during gastrulation and early neurulation (Marshall et al., 1992; Kessel, 1993) but, in these cases, this was accompanied by ectopic expression of Hoxb-1 in r2 and/or r3. In contrast, by studying the expression of Hoxa-2, Hoxb-1 and Hoxb-4, we did not detect any changes in Hox expression in r3. This alteration in motor neuron phenotype is however anticipated by the early loss of Krox-20 expression from r3 and is consistent with the view that Krox-20 plays a role in establishing the identity of this rhombomere (Schneider-Manourí et al., 1993, 1997). Loss of Krox-20 may be accompanied by the loss of ‘odd-ness’ or ‘three-ness’ from r3 and its motor neurons may therefore be attracted equally to the two ‘even’ exit points on either side.

Since our analysis has been carried out only 24 hours after the alterations in Hox gene expression and before the migrations and condensations of motor nuclei are complete, it is possible that other changes in neuronal organisation would become apparent in retinoic-acid-treated brains analysed at more mature stages of development. However, the loss of CVA neurons and alterations to r3 motor neurons demonstrate that some phenotypic changes can occur within the time scale of our observations. The use of specific markers for motor neuron subsets might also reveal patterns of respecification not apparent with the retrograde labelling analysis.

The effect of retinoic acid is variable, depending on dose, time and method of application

Non-localised applications of retinoic acid during gastrulation...
and early neurulation commonly result in a dose-dependent disruption and/or resegmentation of segmentation specifically in the anterior hindbrain of several species (see for example Durston et al., 1989; Papalopulu et al., 1991; Marshall et al., 1992; Wood et al., 1994; Hill et al., 1995). Our results suggest that after hindbrain segmentation is established the sensitivity to retinoic acid shifts to the posterior hindbrain and the anterior hindbrain is largely unaffected.

The method of delivery of retinoic acid to the hindbrain appears to be influential for the resulting phenotype as our results differ to those previously reported by Gale et al. (1996). In both studies the treatment was performed at the same time (stage 10) and at the same location (r4). Whilst Gale et al. (1996) injected a pulse of retinoic acid, we applied retinoic acid from slow-release beads which provide a continuous steady state release for a prolonged period. In their retinoic-acid-treated embryos, segmentation was unaffected and Krox-20 is expressed in r3, r4 and r5 and Hoxb-1 expression is diminished in r4 but normal posteriorly. However, in agreement with our results, they also see a reduction of CVA neurons at r4 level and suggest that r4 is neither lost nor respecified, but rather that there are some alterations of its individual characteristics. The differences in phenotype could be accounted by a later response to retinoic acid mediated by the longer release from the beads.

Lack of segmentation
Lack of morphological segmentation in the posterior hindbrain described here is similar to the unsegmented hindbrains observed in kreisler and Hoxa-1 mutant mice (McKay et al., 1994; Mark et al., 1993) and also in vitamin A-deficient quail embryos (Maden et al., 1996). The partly unsegmented hindbrains of kreisler and Hoxal mice as well as vitamin A-deficient quail embryos are due to the lack of development of some or all posterior rhombomeres. In contrast, it seems that, in our study, loss of morphological segmentation is a consequence of the loss of only boundary cells and not whole rhombomeres.

So what do boundary cells do?
Boundary cells do not appear to play a substantial role in the restriction of cell movement at the borders between rhombomeres. They are the site of local accumulations of circumferential axons in the hindbrain (Lumsden and Keynes, 1989; Heyman et al., 1993, 1995) and these accumulations are lost when boundaries are lost; however, the significance of this role in axonal organisation is not yet understood. Possible roles for boundary cells are either that they form a structural scaffold of specialised glial cells, which may be important for axon and neuronal patterning or that they constitute centres for the generation of particular cell types (Wilson et al., 1993; Heyman et al., 1995). The fate of boundary cells is not known.

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