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Late effects of retinoic acid on neural crest and aspects of rhombomere identity

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SUMMARY

We exposed st.10 chicks to retinoic acid (RA), both globally, and locally to individual rhombomeres, to look at its role in specification of various aspects of hindbrain derived morphology. Previous studies have looked at RA exposure at earlier stages, during axial specification. Stage 10 is the time of morphological segmentation of the hindbrain and is just prior to neural crest migration. Rhombomere 4 localised RA injections result in specific alterations of pathways some crest cells that normally migrate to sites of differentiation of neurogenic derivatives. The r4 crest cells that give rise to mesenchymal derivatives are unaffected. In addition, r4 gene expression is also partially altered by RA; within 6 hours of r4 exposure to RA, ectopic expression of *Krox-20* is seen in r4 and *Hoxb-1* expression is lost while *Hoxa-2* expression continues normally. When we examined these RA-treated animals later in development, they showed an anterior displacement of the facial ganglion in addition to a mis-direction of the extensions of its distal axons and a dramatic decrease in the number of contralateral vestibuloacoustic neurons normally seen in r4. Only this r4-specific neuronal type is affected in r4; the motor neuron projections seem normal in experimental animals. The specificity of this result, combined with the loss of *Hoxb-1* expression in r4 and the work by Krumlauf and co-workers showing gain of contralateral neurons co-localised with ectopic *Hoxb-1* expression, indicates a role for *Hoxb-1* and RA in the specification of this cell type in normal development. These results suggest that RA, at st.10, is able to affect some aspects of segment identity while leaving others unchanged.

Key words: chick embryo, retonoic acid, neural crest, rhombomere, *Hoxb-1*

INTRODUCTION

Treatment with retinoic acid at around the time of gastrulation and neurulation causes abnormal development of vertebrate head structures including the brain. This has been documented in mouse (Leonard et al., 1995; Wood et al., 1994), rat (Morriss, 1972), *Xenopus* (Durston et al., 1989; Lopez and Corrasco, 1992; Papalopulu et al., 1991; Sive et al., 1990) and zebrafish (Hill et al., 1995; Holder and Hill, 1991) embryos. Analysis of phenotypes shows that alterations in the organisation of the anterior hindbrain are common to all species. It has been suggested that the spatial organisation of the hindbrain region is regulated by the spatial patterning of the *Hox* genes, as well as other regulatory genes, and since retinoic acid (RA) can affect the expression of these genes it may be that RA is altering hindbrain organisation by altering regulatory gene expression. Recent anatomical studies have thus been complemented by analysis of expression patterns of candidate regulatory genes (Conlon and Rossant, 1992; Marshall et al., 1992; Papalopulu et al., 1991) which are normally expressed in the anterior hindbrain region. These genes include *Hoxb-1*, which is expressed in rhombomere (r)4 (Wilkinson et al., 1989), *Hoxa-2* which in the chick has an anterior expression boundary at the rhombomere 1/2 border (Prince and Lumsden, 1994) and *Krox 20*, which is expressed in r3 and r5 (Wilkinson et al., 1989). The studies in the mouse show that the RA effect on the hindbrain is a complex process involving the expression of regulatory genes and the underlying differentiation of specific groups of cells characteristic to specific rhombomeres.

During late neurula stages the individual hindbrain segments, the rhombomeres (Lumsden and Keynes, 1989; Simon et al., 1995), contain two morphogenetically distinct populations; the neural crest cells and the cells of the neuroepithelium. Later in development, these two cell populations differentiate to form a unique combination of elements either within or derived from each rhombomere. The first group, the neural crest, gives rise to neurogenic and mesenchymal derivatives. Using r4 as an example, the neurogenic derivatives consist of a few neurons and all the support cells of the acoustic and vestibular ganglia, including the Schwann cells which support the axonal extensions of the ganglia (Couly and Le Douarin, 1990; D'Amico-Martel and Noden, 1983; Le Douarin et al., 1986). The mesenchymal derivatives of r4 crest include entoglossal and branchial cartilages and two of the cartilages

in the middle ear (Lumsden et al., 1991; Noden, 1983). The cells of the neuroepithelium will form, among other neuronal groups, part of the facial motor nucleus (Fraser et al., 1990; Lumsden and Keynes, 1989) and the vestibuloacoustic neurons. The latter is a set of specialised sensory efferent neurons, exclusive to r4, some of whose cell bodies migrate across the midline of the neural tube after axonal outgrowth begins (Simon and Lumsden, 1993).

The capacity of cranial neural crest to form both mesenchymal derivatives (dermis, cartilage, bone, connective tissue, and some muscle cells) and neurogenic derivatives (neurons, Schwann cells, and support cells of the cranial ganglia) (Couly et al., 1992; Noden, 1992) has inspired many decades of investigation into the location and timing of cell type specification (Landacre, 1910; Wagner, 1959; for reviews see Anderson, 1989; de Beer, 1937; Le Douarin et al., 1993). However, there is still no clear picture of when cell fate is determined for neural crest, although there are results supporting each of the various hypotheses. It is very possible that some cells are specified before migration while others retain their pluripotentiality until they reach their site of differentiation (Baroffio et al., 1988). The hypothesis of prespecification of regions of hindbrain crest by their axial position is supported by three experiments with premigratory cranial crest (Kuratani and Eichele, 1993; Noden, 1983; Prince and Lumsden, 1994); they demonstrated that heterotopically transplanted crest cells form cartilage elements and express genes appropriate to their original position rather than to their host site. The different question of prespecification of final tissue type of individual premigratory crest cells has been addressed by a number of techniques. Individual cell labelling, culture and transplant experiments in chicks have indicated that, in the trunk, pluripotentiality is not lost until the site of organogenesis is reached (Fraser and Bronner-Fraser, 1991; Le Douarin and Dupin, 1992). However, individual cell labelling in cranial neural crest cells in zebrafish has suggested prespecification of tissue type before migration (Schilling and Kimmel, 1994) while the trunk neural crest has shown similar behaviour to that described in chickens (Riable and Eisen, 1994). In chick cranial neural crest, results have shown some labelled cells giving rise to only a few of the many potential crest-derived tissue types but have given no evidence of specification of an individual final phenotype (Bronner-Fraser and Fraser, 1988; Le Douarin et al., 1993). A third possible prespecification is of a subpopulation of cells within a given segment to restrict their contribution to only one set of crest derivatives. For example, a subset of premigratory cells which would only give rise to neurogenic derivatives of neural crest. This hypothesis is supported currently by two observations. Both distinguish crest that will contribute to neurogenic derivatives, from the rest of the crest, by differential behaviour. The first is its delayed exodus from the neural tube (Hunt et al., 1991; Lumsden et al., 1991) and the second is the retention of *Hoxb-1* expression in some cells that migrate to the position of the cranial ganglia (Hunt et al., 1991; Murphy and Hill, 1991).

Although alteration of neural crest migration has been proposed as an explanation for RA induced craniofacial defects, there has been little work examining crest behaviour after exposure to RA. Some of this work has reported inhibition of migration (Pratt et al., 1987; Thorogood et al., 1982; Webster et al., 1986) while the work of Lee and co-workers (Lee et al., 1995) has shown a specific mis-migration of crest. In the latter experiment, animals were exposed globally to RA and no correlation between altered migration, early gene expression, and subsequent alteration in morphology was made. In contrast, our study examines how crest is affected by local exposure to RA at the time of the initiation of crest migration, while also examining alterations in regulatory gene expression and later phenotype resulting from the same experimental treatment.

Just prior to crest migration the hindbrain undergoes many changes. At the 9 somite stage (ss), in chicks, rhombomeres are forming morphological boundaries and certain homeobox genes are developing precise borders of expression which coincide with those boundaries. *Krox-20* expression is localised to the regions of the two stripes of cells forming r3 and r5 (Wilkinson et al., 1989), *Hoxa-2* is developing its anterior border of expression at the r1/r2 border (Prince and Lumsden, 1994) and *Hoxb-1* is expressed in the entire posterior hindbrain up to the developing r3/r4 border (Kuratani and Eichele, 1993). In the next few hours, as the rhombomeres develop morphological boundaries and crest migration begins, *Krox-20* and *Hoxa-2* expression is sharpened to coincide with those boundaries, while *Hoxb-1* expression is blocked in r3 and r5 to leave a precisely defined band of expression in r4.

Given the variety of developmental events occurring at this stage it would be useful to know the effect of RA exposure on these processes. Despite many detailed studies (Conlon and Rossant, 1992; Cunningham et al., 1994; Holder and Hill, 1991; Kessel, 1993; Marshall et al., 1992; Wood et al., 1994) recording RA effects, it is still unclear if RA acts endogenously to control the normal expression of *Hox* genes. However, a number of recent lines of evidence suggests that it does; RA is present endogenously in Henson's node (Hogan et al., 1992) and the *Xenopus* dorsal lip during gastrulation (Chen et al., 1994), which is the time of first expression of the *Hox* genes which later acquire anterior expression boundaries in the hindbrain (e.g. *Hoxa-1* and *Hoxb-1*; Kolm and Sive, 1995; Wilkinson et al., 1989). Recent work has also shown that retinoic acid response elements (RAREs) are present in the regulatory regions of the *Hoxa-1* (Langston and Gudas, 1992) and *Hoxb-1* genes (Studer et al., 1994; Marshall et al., 1994). It is also important to consider the specific stages at which RA exerts its action on the regulation of *Hox* gene expression in the embryo; different stages of development show a differential sensitivity to RA (Conlon and Rossant, 1992; Cunningham et al., 1994; Wood et al., 1994). Presence of RA from the onset of gastrulation suggests that it is active then, whereas the activity of RAREs in controlling an early and a late pattern of *Hoxb-1* expression indicates an additional later role for RA.

The accessibility of the chick embryo during development allows RA treatment to be done at a stage precisely defined by somite number and, uniquely, allows isolated exposure of a specific area of the body. By localising exposure, we have, for the first time, examined the specific response of the hindbrain to RA in isolation from any other tissue response. We have chosen a stage of exposure which is later than in previous work to avoid confusion with the early effects of RA in axial specification. The current study focuses on individual aspects of segment identity; gene expression, differentiation of the neuroepithelium, neural crest migration and neurogenic and mesenchymal crest derivatives. The changes in rhombomere identity we see must neces-

sarily derive from changes in the microenvironment of the rhombomeric cells as opposed to respecification of a gastrula stage ancestral population which could result from early RA exposure. This could explain the apparent contradiction with the results of other published works showing a gain of *Hoxb-1* expression after RA exposure in contrast to the loss of *Hoxb-1* expression we report. We have directed our analysis to examine r4 and its derivatives because of the differential sensitivity to RA seen in that segment at this stage of development. We describe the immediate effect of RA on *Hoxb-1*, *Krox-20* and *Hoxa-2* expression and on neural crest migration as well as the subsequent effect on the later development of the cranial ganglia, cartilages and neuroepithelium. The results are discussed in terms of the possible influence of altered gene expression on other aspects of segment identity as well as an endogenous role for RA in their specification.

MATERIALS AND METHODS

Chicken eggs from a mixed flock (Needle's Farm, Enfield) were incubated to between the 7- and 14-somite stage (ss) at 37°C then windowed for experimental manipulation. Whole embryos were exposed to RA by implantation, adjacent to the embryo, of a cube of approximately 0.5 mm^3 of Silastic-medical elastomer (Dow Corning) impregnated with 10 mg/ml or 100 mg/ml all *trans*-retinoic acid (RA) (Sigma) or injection of RA into individual rhombomeres (see below). Silastin cubes without RA were implanted in control animals. The embryos were incubated for an additional 24-72 hours before preservation in perfix (4% paraformaldehyde, 2% trichloracetic acid, and 20% 2-propanol in H_2O) or 3.5% paraformaldehyde then processed for analysis as described below.

HNK-1, tubulin and SC1/DM-GRASP labelling

Whole-mount immunocytochemistry using HNK-1 mouse IgM (Developmental Studies Hybridoma Bank, Baltimore, Maryland) or anti-acetylated tubulin (Sigma) was carried out by blocking of endogenous peroxidase activity with 0.05% hydrogen peroxide in phosphate buffer with 1% Triton (PBStriton), incubating with 1:10 HNK-1 IgM in PBStriton or 1:20 anti-acetylated tubulin with 10% normal goat serum for 2 days at 4°C, reacting the bound antibody with 1:200 anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma) in PBStriton with 1% normal goat serum, then visualising the bound secondary antibody with 3,3′-diaminobenzidene tetra hydrochloride (DAB-Sigma D-5637) and clearing with 50% glycerol in phosphate buffer. Whole-mount immunocytochemistry using the SC1/DM-GRASP antibody was carried out as described by Guthrie and Lumsden (1992).

DiI injections

Individual rhombomeres of 7-12ss chicks were injected with the lipophilic dye DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) in N,N-dimethylformamide (DMF) at 3 mg/ml using micro-injection techniques. The site of injection was confirmed by observation both after injection and at the time of preservation. All injections were clearly contained within the boundaries of a given rhombomere and were placed dorsally in the rhombomere keeping dye exposure of surface epithelial cells to a minimum. For RA treatment, RA powder was added at 5 mg/ml to the DiI solution immediately before injection. The experimental animals were incubated for a further 6-48 hours, then observed under episcopic-fluorescence using a rhodamine B excitation filter (546 nm wavelength) before preservation in perfix or fresh 3.5% paraformaldehyde in PBS. Some animals were injected with RA in DMF into the lateral mesoderm of the 10ss chick, incubated for an additional 48 hours then preserved in perfix. Specimens preserved in perfix were processed for whole-mount immunocytochemistry using HNK-1 antibody. Those fixed in paraformaldehyde were washed in PBS with 0.1% Tween-20 (PBST) then dehydrated in 100% methanol where they were stored at −20°C for in situ hybridisation.

In situ hybridisation

In situ hybridisation was performed as previously described (Prince and Lumsden, 1994). Expression patterns of the *Hoxa-2* (Prince and Lumsden, 1994), *Krox-20* (Wilkinson et al., 1989) and *Hoxb-1* genes were analysed. We obtained a 240 bp cDNA of chick *Hoxb-1* (previously *Ghox-lab*; Sundin et al., 1990) corresponding to nucleotides 656-895 by RT-PCR using primers: (1) 5′ ATTCGCACCAACTTCACC; (2) 5′ AGGTGCAGTTGGACTGATCC; (3) 5′ ACTTCACCACCAAGCAGC. The 240 bp cDNA was used to probe a chick stage 12-16 cDNA library in λZAPII (generously provided by Angela Nieto and David Wilkinson) and a 2 kb cDNA (900 bp of coding region plus 3′ utr) obtained and subcloned into pSK (Stratagene). This plasmid was linearised at *Xba*I and antisense digoxigenin *Hoxb-1* riboprobe synthesised.

Cartilage staining

To examine the effects of RA on the cartilaginous elements of the head, injected animals were incubated for an additional 5-8 days before preservation in formalin. They were then stained with 0.02% alcian blue (w/v) in 30% glacial acetic acid in absolute ethanol, rinsed in absolute ethanol, hydrated and cleared in 0.5% KOH.

Vestibuloacoustic backfills

Embryos were incubated for an additional 48-72 hours after r4 injections to examine the development of the neurons within the neural tube. They were preserved in 3.5% paraformaldehyde and the mesenchyme was dissected away to expose the proximal root of the seventh and eighth cranial ganglia. The exposed root was injected with 3 mg/ml DiI in DMF and left overnight. The neural tube was dissected clean then coverslipped in glycerol and examined using a fluorescent microscope.

RESULTS

Retinoic acid effects on neural crest migration

Firstly, we treated embryos globally with RA in silastin and examined its effects on neural crest migration by HNK-1 antibody labelling. Secondly, to reveal the specific axial level of the affected crest, we localised the RA exposure to the neuroepithelium and examined migration of regions of crest by injecting DiI with RA into individual rhombomeres.

HNK-1

Embryos exposed to low levels of retinoic acid (RA) before or at early stages of crest migration show a very precise alteration of a normal neural crest pathway. We used the HNK-1 antibody to reveal hindbrain neural crest distribution (Tucker et al., 1984). In normal development, the neural crest cells migrate along discrete pathways from the hindbrain into the branchial arches (ba). There is no crest migration between pathways. This is particularly obvious in the crest-free area of the lateral mesoderm between the neural crest migrating into ba 1 and the stream migrating into ba 2. The boundaries of this 'crest free zone' are sharply defined (Fig. 1A) and respected by all migrating neural crest cells. The pathways of migration into ba 2 and into ba 3 are separated by the otic vesicle and the strip of lateral mesoderm ventral to the otic vesicle and dorsal

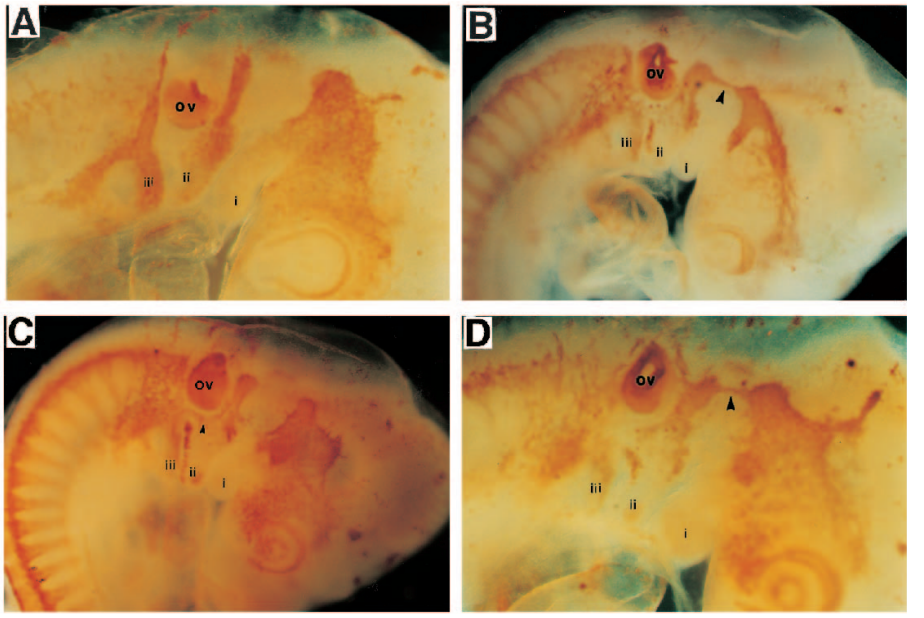

Fig. 1. HNK-1 labelling of migrating neural crest in st.15 chick embryos. (A) Control. (B-D) RA-treated. B and D show the band of crest cells between the r2 and r4 streams in the 'crest-free zone' (large arrowhead). C shows the mis-migration of crest ventral to the otic vesicle (small arrowhead). All photos reveal normal migration of crest into the branchial arches. ov, otic vesicle; i, ii and iii, branchial arches 1, 2 and 3 respectively.

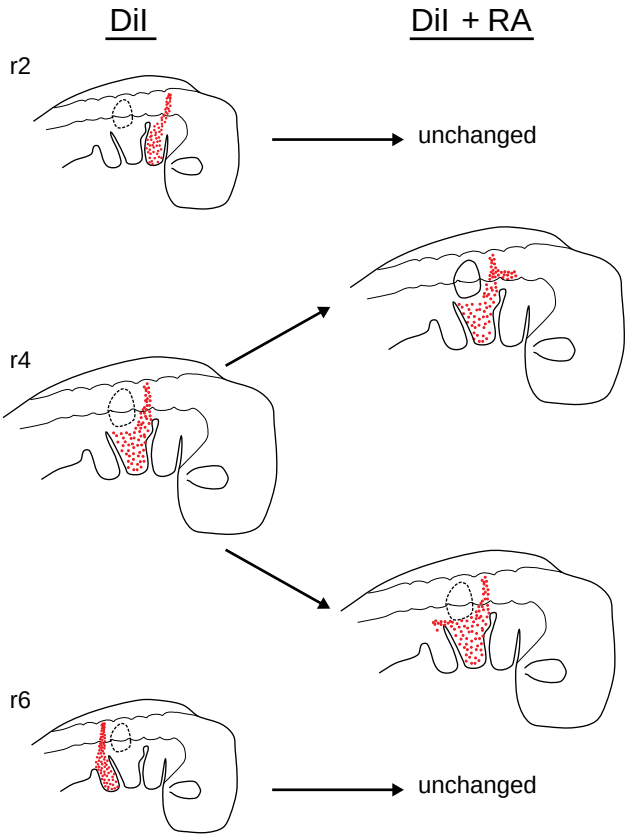

Fig. 2. Drawings of migrating DiI-labelled neural crest cells in st.14 embryos. Injections of DiI alone at stage 10, into crest cells in r2, r4 and r6, are depicted on the left, showing the normal migration pattern. The addition of RA to the DiI injections had no effect on migration from r2 or r6. On the right are drawings of the two abnormal migration patterns resulting from injections of DiI with RA into r4. Upper right; rostral mis-migration of r4 crest cells at the level of the otic vesicle. lower right; caudal mis-migration of cells from r4 ventral to the otic vesicle.

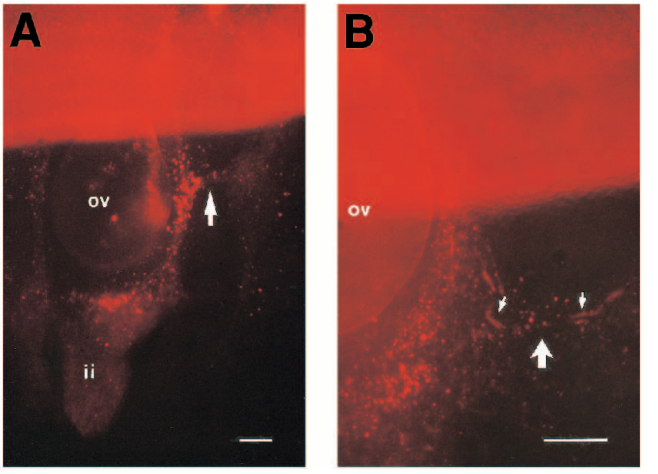

Fig. 3. DiI-labelled neural crest cells migrating from r4 in two experimental st. 14 animals. Large arrows in A and B point out a subpopulation of r4 crest cells mis-migrating rostrally through the lateral mesoderm. (Normal neural crest migration is depicted in Fig. 2.) (A) Mis-migrating cells make a rostral turn at mid-otic vesicle (ov) level. Many r4 crest cells of RA-treated animals continue to migrate normally into branchial arch 2 (ii). (B) Individual DiI labelled cells (small arrows) can be seen turning anteriorly. Scale bar, 50 μm.

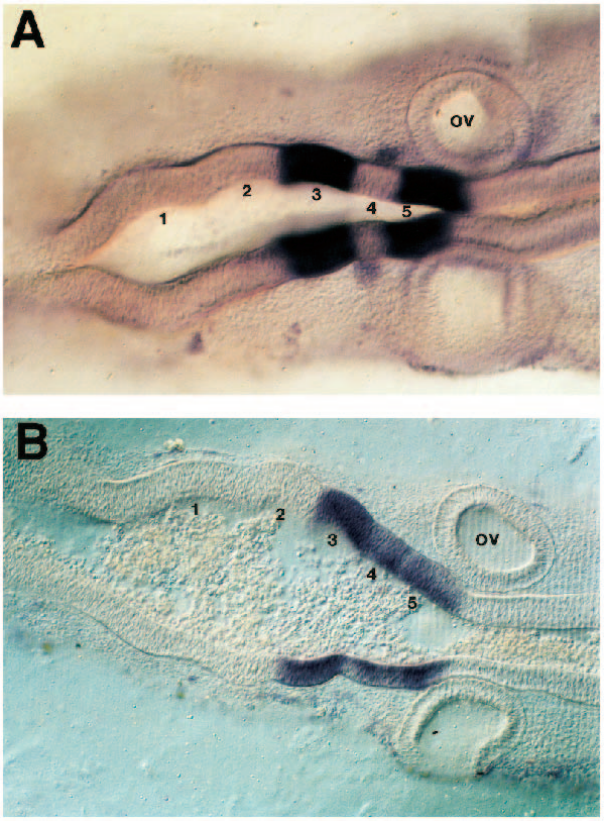

Fig. 4. *Krox-20* expression in coronal sections of st.15 chick. (A) Control displaying normal expression in r3 and r5. (B) r4 RA-injected specimen showing abnormal expression in r4. Note the boundary between r4 and r5 which is morphologically less distinct in treated animals. (The areas of expression lateral to the hindbrain mark the axon exit points of r4; Heyman et al., 1995). 1-5, rhombomeres; ov, otic vesicle.

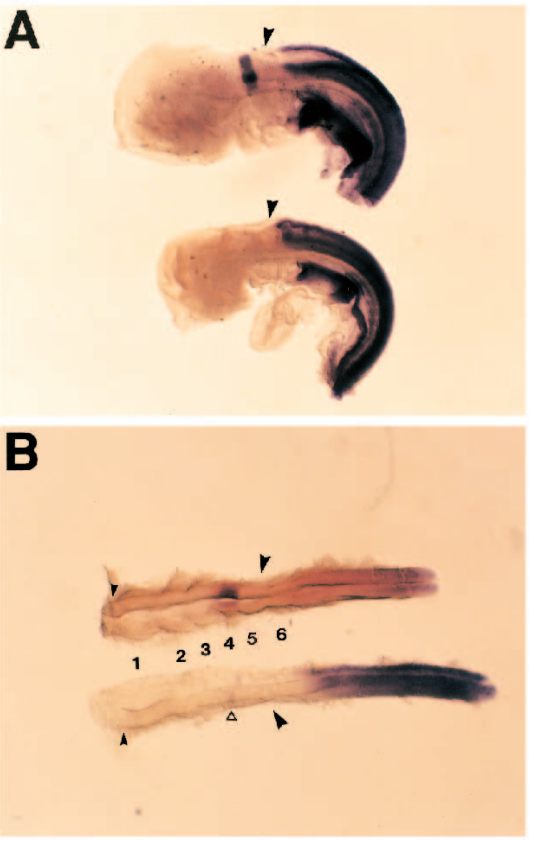

Fig. 5. *Hoxb-1* expression in whole-mount st.15 chick embryos. In both photos the control is positioned above the r4 RA-injected animal. (A) Control shows normal *Hoxb-1* stripe in r4 and general expression posterior from r6. The treated animal has lost the r4 stripe but displays normal posterior expression. (B) Dissected hindbrains of control (top) and injected (bottom) animals. The treated animal has a pale thin band of r4 expression (open arrowhead). Note that the hindbrains are the same length and while the morphological boundaries of the rhombomeres of the experimental animal are less obvious, the same number of rhombomeres are visible. Large arrowheads mark the position of the otic vesicle, small arrowheads mark the midbrain-hindbrain border. 1-6, rhombomeres.

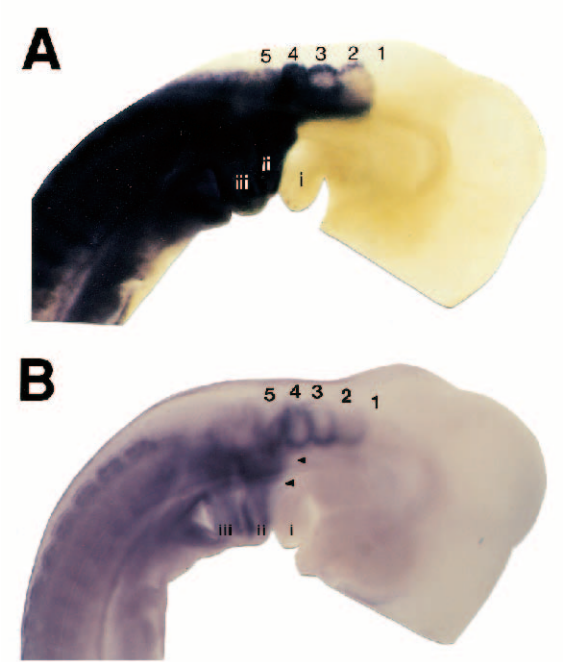

Fig. 6. Expression of *Hoxa-2* in the (A) control and (B) r4 RA-injected chick. All RA-injected embryos show a normal pattern of expression. Note the sharp line of expression at the anterior edge of the ventrally migrating r4 crest (small arrowheads) indicating that r4 crest cells migrating normally into branchial arch 2 and 3 express *Hoxa-2* while anteriorly mis-migrating r4 cells do not. 1-5, rhombomeres; i, ii, and iii, branchial arches 1, 2, and 3 respectively.

to ba 2 and 3. After global exposure to 10 mg/ml RA, 55% of the embryos (35 of 63) display HNK-1 labelling within this 'crest-free zone' (band 1-2, Fig. 1B,D) and 65% of the embryos (41 of 63) display a continuous band between the ba 2 stream and the ba 3 stream (band 2-3) (Fig. 1C). These pathway alterations occur at remarkably consistent dorsoventral positions. Band 1-2 runs between the site of the developing vestibuloacoustic ganglia and the site of the developing trigeminal ganglion. Band 2-3 passes immediately ventral to the otic vesicle along the dorsal edge of ba 2 and ba 3. There were also 4 cases in which HNK-1 labelling indicated crest in inappropriate arches. All animals treated with silastin with no RA were normal.

Embryos treated with low levels of RA (10 mg/ml), with the exception of a few cases, are superficially normal in appearance. In contrast, embryos exposed to high levels of RA (100 mg/ml) either died or showed the drastic deformations of the cranial morphology described in many previous studies on RA effects (see (Maden and Holder, 1992 for review). The surviving embryos, about half of those treated, display apparent reduction of frontonasal mesenchyme, small branchial arches and shortening of the hindbrain concurrent with the anterior shift of the otic vesicle with respect to the eye. The HNK-1 labelling in these animals often indicated completely chaotic crest migration.

DiI injections

Premigratory crest DiI injections localised to individual rhombomeres reveal the normal pathways of migration of neural crest cells of each rhombomere, confirming the precision of the pathways seen with HNK-1 labelling, and identify which segment of crest showed alteration of pathway with exposure to RA. DiI is a lipophilic dye rapidly taken up by cells adjacent to the injection site and, in the case of neural crest cells, is carried in the cells and their progeny during migration. Injections of DiI alone, localised to r4, resulted in labelled cells uniquely in the second branchial arch and adjacent to the otic vesicle at the future site of the facial ganglion (Fig. 2). When 5 mg/ml RA was added to the injection, there continued to be labelled cells in ba 2, but in 31 of 57 injected animals there was an additional stream of labelled cells migrating anteriorly at the level of the otic vesicle (Fig. 3). (While we have ensured the localisation of the injection within the rhombomere boundaries we have not attempted to label all migrating cells, therefore it is possible that more injections resulted in altered migration pathways which were not visible because the cells were not labelled.) The dorsoventral position of the altered pathway was similar for all mis-migrating individual crest cells (Fig. 3B) in an affected embryo and for all affected embryos. The dorsoventral level is also the same as that of the band 1-2 seen in HNK-1 labelled embryos (Fig. 1B,D). A few animals injected in r4 with RA showed an abnormal stream of crest cells migrating posteriorly, ventral to the otic vesicle (Fig. 2) at the same level as the band 2-3 seen in HNK-1 labelled embryos (Fig. 1C). The migration of these neural crest cells from r4 has been respecified or the pathway changed. In contrast to r4-injected animals, there was no difference between the pattern of DiI-labelled cells in those animals injected with DiI and RA and those with DiI alone from r2 (DiI+RA n=38 DiI n=10) or r6 (DiI+RA n=17 DiI n=4). The r2 localised injections, with or without RA, showed cells migrating exclusively into ba 1 and the region where the trigeminal ganglion will form. All r6 localised injections result in labelled cells in ba 3 and in the region where the glossopharyngeal ganglion will form. In summary, the neural crest migrations from r2 and r6 are unaffected by RA but subpopulations of crest from r4 migrate abnormally but in a defined pathway.

To examine whether the RA injected into individual rhombomeres was influencing crest pathways by affecting the mesoderm through which the crest migrates, ten embryos were injected with RA into the mesoderm lateral to r2, r3, and r4 of the 10ss chick. The migration of neural crest as shown by HNK-1 staining was completely normal in all ten embryos.

Animals injected with DMF then labelled with HNK-1 showed no alteration in neural crest migration from normal and appeared morphologically unaffected.

RA effects on gene expression

Using specimens injected with RA and DiI, we examined the effects of RA on the expression patterns of three genes whose expression becomes localised to individual rhombomeres of the anterior hindbrain at the time of neural crest migration: *Krox-20*, *Hoxb-1*, and *Hoxa-2*. In normal development, *Krox-20* is expressed in two precisely defined bands of cells which correspond to r3 and r5 with no expression in r4 (Fig. 4A). RA exposure results in a single broad band of expression extending from the rostral edge of r3 caudally through r4 and r5 (Fig. 4B) or two broad stripes divided by a very thin band of cells not expressing *Krox-20*. While the anterior boundary of expression of r3 and the posterior boundary of expression of r5 seem unchanged, the cells of r4 express *Krox-20* after RA exposure. These alterations in expression pattern occur with the highest frequency after injection into r4 (Table 1) but some changes in *Krox-20* expression are seen from RA treatment of each rhombomere. This may indicate a bleeding of RA into adjacent rhombomeres occurring after the injections even though embryos showing DiI spread were discarded from analysis.

Hoxb-1 expression in r4 is diminished in 14 of 17 r4 RA-injected animals while the normal *Hoxb-1* expression in r6, r7, and r8 was unaffected (Fig. 5A,B). Of those 14, 9 had lost all *Hoxb-1* expression in r4 (Fig. 5A) the remaining 5 had a very narrow or pale band in the r4 position (Fig. 5B). Again injections into rhombomeres other than r4 resulted in alteration of *Hoxb-1* expression. This prevents us from being fully confident of exclusive localisation of RA to the injection site. Even if some spreading of RA occurs, the effective dosage of RA seems to only be present very close to the injection site as is shown by the low numbers of RA-affected animals from r2 or r6 injections (Table 1).

In contrast to *Krox-20* and *Hoxb-1*, *Hoxa-2* expression appears unchanged after exposure to RA (Fig. 6). The normal expression from the r1/r2 border caudal throughout the hindbrain (Fig. 6A) (Prince and Lumsden, 1994) was observed in all experimental animals. In addition, as is present in normal animals, a very well defined anterior edge of expression was seen in the r4 crest stream in RA-injected animals (Fig. 6B). Interestingly, the rostrally mis-migrating cells from r4 in the RA-treated animals do not express *Hoxa-2*.

In situ analysis was generally performed on animals preserved between 24 and 48 hours after injection. To test the

Table 1. Effects of RA on gene expression

			Krox-20 expression		
	n	inj. site	One band	*	Normal
	8	r2	2	2	4
	6	r3	5	1	
	4	r4	3		1
	2	r5	1		1
	1	r6		1	
Total	21		11	4	6
	8 control				8

*Specimens with thick bands showed two very broad bands of expression separated by a very thin band of non-expressing cells

			Hoxb-1 r4 expression		
	n	inj. site	Absent	Thin band	Normal
	8	r2		1	7
	3	r3	2	1	
	17	r4	9	5	3
	1	r5	1		
	3	r6			3
Total	32		12	7	13
	6 control				6

		Hoxa-2 expression
		Normal
	15	15
	3 control	3

n, number of embryos injected.

rapidity of the RA effect, a few specimens were fixed 6 hours after injection. This short exposure to RA also resulted in the described alteration of *Hoxb-1* and *Krox-20* expression. In situ controls were injected with DiI in DMF alone and displayed normal patterns of expression of all three genes.

RA effects on phenotype

The cranial ganglia of embryos treated with 10 mg/ml RA were analysed by labelling with anti-acetylated tubulin antibody. Anterior displacement of the facial ganglion (Fig. 7B) is indicated by the axons extending posteriorly as opposed to their normal ventral projection to the second branchial arch. The space between the facial ganglion and the otic vesicle demonstrates that it is only the ganglion and not the otic vesicle that is displaced in the RA exposed animals. In normal development, the distal axons from the facial ganglion project exclusively into the second branchial arch (D'Amico-Martel and Noden, 1983) (Fig. 7A). The mis-direction resulting from RA treatment is of two types: from the facial ganglion anteriorly connecting with the trigeminal ganglion (Fig. 7B,D) or posteriorly, ventral to the otic vesicle, often connecting with the axons of the glossopharyngeal ganglion (Fig. 7B,C).

Comparison of backfills of the proximal root of the seventh and eighth cranial ganglia of 3 control (Fig. 8A) and 9 RA-treated (Fig. 8B) animals reveals normal organisation of the motor neurons in r4 and r5 with axons exiting from r4 (Fig. 8A,B). The effect of RA is seen only in the complete absence or reduction (Fig. 8B) of contralateral cell bodies in the treated r4 as compared to the controls (Fig. 8A). In st. 21 normal chicks, we counted a mean of 58 (4.8 standard deviation) contralateral cell bodies, as defined by their presence in the floor plate or on the contralateral side of the neural tube, per animal. Ten RA-injected chicks from st. 21 to st. 25 were examined; two have no contralateral cell bodies and together the treated animals have a mean of 5.2 (5.1 standard deviation) contralateral cell bodies each. As with neural crest migration, we saw no effect of RA on efferent neurons in r2.

The general normality of the motor neurons in experimental animals was confirmed by use of the SC1/DM-GRASP antibody. SC1/DM-GRASP antibody recognises a glycoprotein expressed by motorneurons (Burns et. al., 1991) but the motorneurons of r2 and r3 have a much lower level of immunoreactivity than those of r4 and r5 (Guthrie and Lumsden, 1992). This different level of staining intensity provides a distinguishing marker for r4 and r5 motor neurons (Fig. 9A). Six whole-mount preparations with the antibody showed no change in immunoreactivity of the r4 motor neurons in stage 17-18 chick embryos injected in r4 with RA at stage 10 (Fig. 9B).

Cartilaginous elements of the head were stained with alcian blue to visualise their morphology. Only animals injected in r4 were analysed for branchial arch cartilage alterations. Both animals injected with RA and DiI (n=6) and those injected with DiI alone (n=2) had completely normal cartilage formation (Fig. 10). Specifically, the cartilages of the otic region, the lower jaw and the hyobranchial apparatus, which are all derived from hindbrain neural crest (Le Lievre and Le Douarin, 1975; Noden, 1983) were normal in size and shape after RA injections.

DISCUSSION

Previous works describing the effects of RA on the central nervous system report phenotypes arising from pulsed and continuous and global exposure to RA from gastrulation through neurulation and, in some cases, to later developmental events. This has made it impossible to distinguish the early from the later effects of RA and the specific tissue on which RA is acting. Our experiments show that at the 10ss, r4 is sensitive to RA exposure in the hindbrain. Within 6 hours of r4 exposure to RA, ectopic *Krox-20* expression is seen in r4, a decrease in *Hoxb-1* expression is seen while *Hoxa-2* expression continues normally. Only a subpopulation of premigratory r4 crest is affected by RA treatment, and looking later in development, individual aspects of the r4 phenotype are altered. These results are summarised in Fig. 9 which highlights the colocalisation of altered gene expression and affected crest. RA influences the specification of r4 in some but not all of its of characteristics at the time of rhombomere boundary formation. Contralateral vestibuloacoustic neurons, the pathways of a subpopulation of crest and the pathways of facial ganglia extensions are influenced by RA while other elements of the r4 phenotype, mesenchymal crest migration, cartilaginous elements and motor neuron extensions, are not.

The isolated band of *Hoxb-1* expression in the hindbrain along with the flanking bands of *Krox-20* expression have been described as molecular markers for r4 (Krumlauf, 1993; Wilkinson, 1993). The loss of *Hoxb-1* and gain of *Krox-20* expression at the axial level of r4 in our experiments might be interpreted as a deletion of this hindbrain segment. We feel this is unlikely because of the normality of hindbrain length, of r4

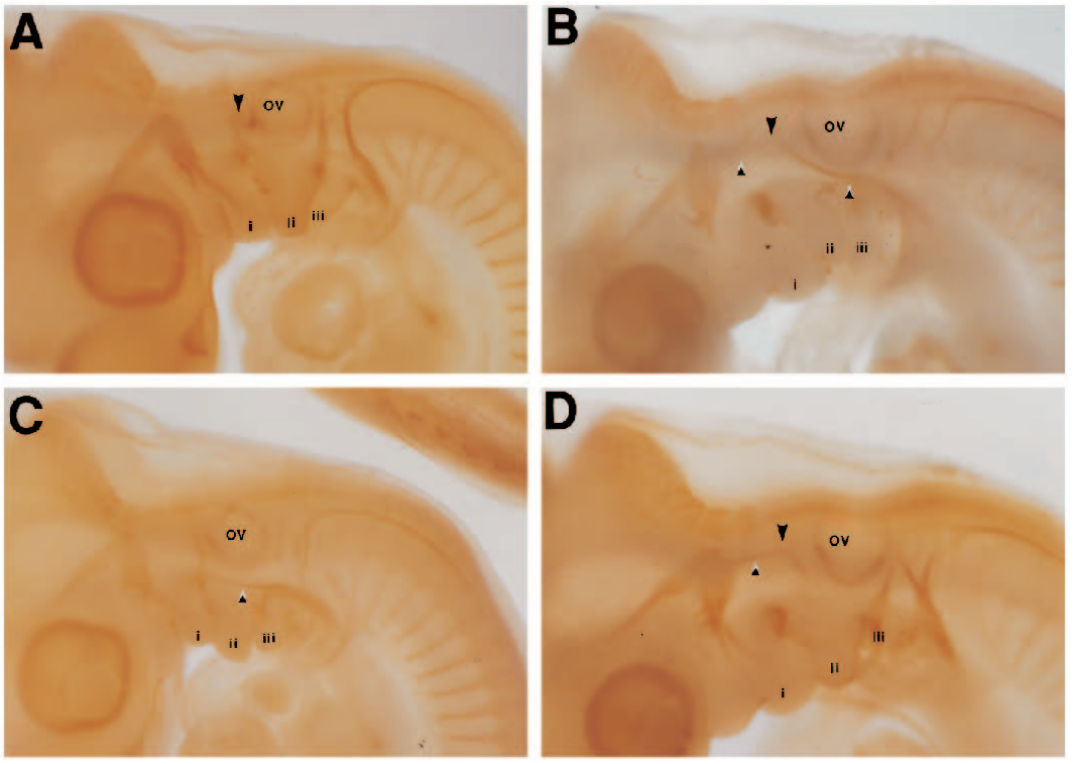

Fig. 7. Acetylated-tubulin labelling of whole-mount st.18 chick embryos. (A) control. (B-D) RA-treated embryos. Black arrowheads point to site of facial ganglion. White and black arrowheads point to inappropriate projections of facial ganglion. A shows normal facial ganglion extension: into branchial arch 2. B shows a rostrally displaced facial ganglion as well as extensions from the facial ganglion to the trigeminal ganglion and ventral to the otic vesicle (ov). C shows an extension ventral to the otic vesicle. D shows the connection between trigeminal and facial ganglia. i, ii, and iii, branchial arches 1, 2 and 3.

and r5 motorneurons, of cranial cartilage development and of migration of neural crest into the branchial arches. Backfills (Fig. 8), antibody staining (Fig. 9), DiI labelling (Fig. 2) and morphological analysis (Fig. 10) demonstrate that the motorneurons and neural crest cells which give rise to the cartilage elements are unaffected by RA despite the changes in molecular identity of the rhombomere from which they are derived. This suggests that RA exposure does not result in a loss of r4 or even a respecification of the entire segment identity but instead results in an alteration of individual characteristics of the r4 phenotype. This finding is consistent with that seen in zebrafish exposed to RA (Hill et al., 1995).

Neither gene expression nor crest migration from r2 and r6 is affected by RA while a subset of r4 crest cells shows precise respecification of migration pathway. Crest cells normally migrates within very sharply defined boundaries (Lumsden et al., 1991). Although r4 crest continues to migrate within these boundaries after RA exposure, two consistent exceptions occur. An abnormal stream of crest cells is seen in the area between the normal position of the facial ganglion and the trigeminal ganglion (Fig. 3) or, sometimes additionally, a stream moves posteriorly from the normal stream just ventral to the otic vesicle (Fig. 2). Looking 48 hours later, the RA exposure results in the anterior displacement of the facial ganglion with respect to the otic vesicle and the distal axons of the facial ganglion making inappropriate extensions through the same region of lateral mesoderm as the abnormally migrating crest. Mesenchymal derivatives, such as the epi-

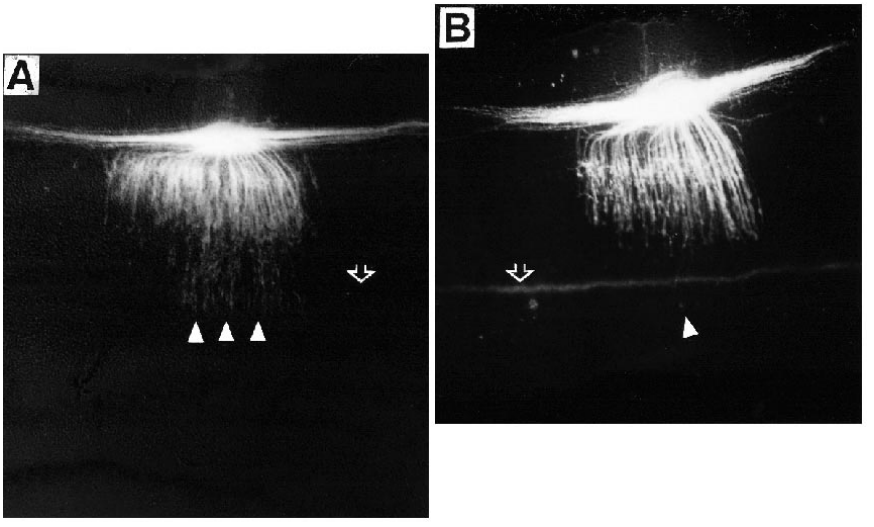

Fig. 8. DiI backfills of VIIth and VIIIth cranial ganglia. (A) Control whole-mount hindbrain. (B) Hindbrain which had an r4 RA injection 3 days previously. Very few cell bodies of contralateral neurons have migrated into the floor plate in the treated animal as opposed to the control (arrowheads). Open arrowheads, midline of floor plate.

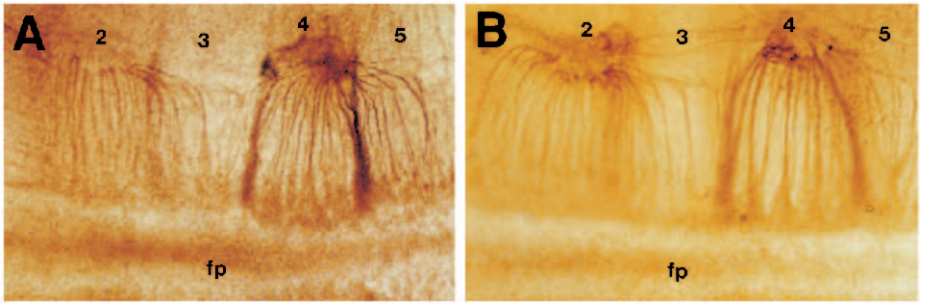

Fig. 9. SC1/DM-GRASP labelling of st.18 chick rhombomeres. (A) Control whole-mount hindbrain (B) Hindbrain which had an r4 RA injection at stage 10. The normal r4 characteristic of increased immunoreactivity of r4 motorneurons as compared to r2 motorneurons appears unaffected by RA exposure. 2-5, rhombomeres; fp, floor plate.

branchial elements, are normal as is the migration of the crest into branchial arch 2, as shown by DiI labelling, HNK-1 staining, and *Hoxa-2* expression. Our results show that only a subpopulation of r4 crest, which does not include crest that gives rise to mesenchymal derivatives, is affected by RA exposure.

Crest cells which migrate to the sites of their neurogenic derivatives are selectively affected in our experiments suggesting a differential sensitivity of a subpopulation of premigratory neural crest. While the injections expose all the crest in the neural tube to RA equally, only some crest shows an altered pathway of migration. Injections in the lateral mesoderm at the same stages have no effect on migration, ruling out the possibility of RA altering either the mesenchyme through which the crest migrates or the final site of differentiation. This differential response within a morphologically homogeneous population of cells supports the hypothesis of some prespecification of crest before migration begins. It is interesting to note that *Hoxa-2* null mutants have implicated that gene in determination of mesenchymal crest derivatives (Gendron-Maguire et al., 1993) which is consistent with our finding that RA affected neither Hoxa-2 nor mesenchymal crest.

A variety of different types of perturbation experiments have demonstrated alterations of exclusively or principally neurogenic derivatives of neural crest. Global RA exposure, as well as the localised exposure described in this paper, has repeatedly been shown to affect neurogenic derivatives. The anterior shift of the facial ganglion and disruption of the 9th and 10th nerve have been reported by Kessel (1993), Morriss-Kay (1992), Papalopulu et al.(1991) and Holder and Hill (1991). In some cases, the reported shift of the facial ganglion might be explained by an anterior shift in the position of the otic vesicle but we see no such displacement in our work nor is it visible in the results presented by Kessel (1993). In the light of our results from RA localised to premigratory crest, we suggest these malformations in neurogenic derivatives result from a change in migration specified in a subpopulation of the r4 crest. As well as altering cranial ganglia, RA exposure has been shown, by a number of researchers in addition to ourselves, to alter *Krox-20* expression to a single broad band (Conlon and Rossant, 1992; Marshall et al., 1992; Morriss-Kay et al., 1991). Altering *Krox-20* expression pattern, through any of a number of experimental methods, results in malformation of neurogenic derivatives of crest. *Krox-20* expression disrupted by direct mutation (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) or by mutation of *Hoxa-1* (Chisaka et al., 1992;

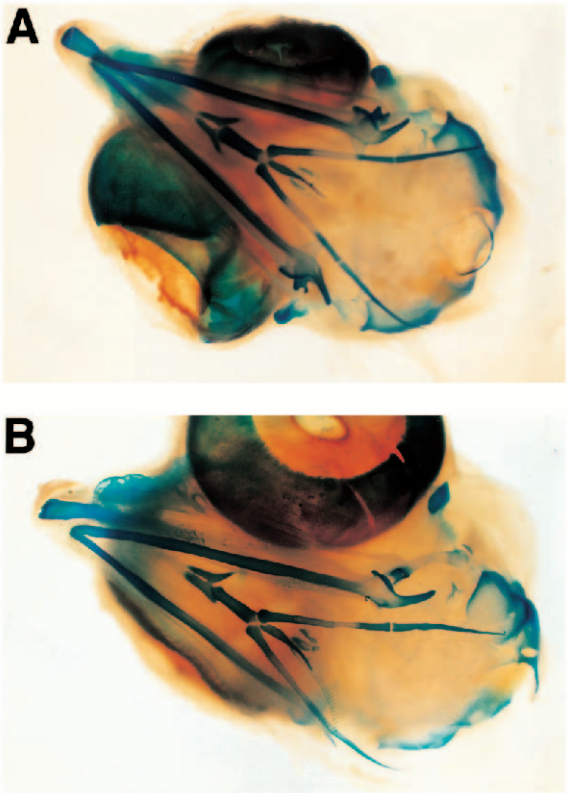

Fig. 10. E8 chick embryos cleared and stained with alcian blue; skull ventral view. (A) Control embryo. (B) Embryo 6 days after r4 RA-injection. As can be seen cartilage (stained blue) development appears unaffected by neural crest exposure to RA.

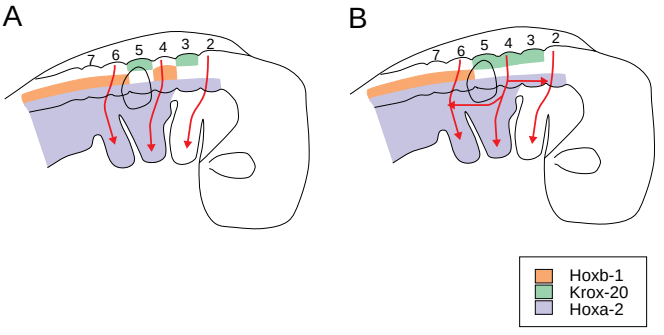

Fig. 11. Summary drawings of gene expression and crest cell migration pathways. (A) Normal st. 14 embryo. (B) r4 RA-injected embryo. Green, *Krox-20* expression; orange, *Hoxb-1* expression; purple, *Hoxa-2* expression; red indicates the neural crest cell migration pathways.

Dolle et al., 1993; Lufkin et al., 1991) resulted in changes in crest-derived cranial ganglia and not in placodally derived ganglia and, with the exception of one reported alteration of a mesenchymally derived element, there is no alteration seen in any other crest derivatives. On the strength of this repeated coincidence, it is attractive to postulate that disruption of *Krox-20* expression affects crest migration and subsequent malformation of cranial ganglia but that has yet to be proved.

A population of cells in the neuroectoderm, the contralateral vestibuloacoustic efferent neurons, is a unique aspect of r4 identity (Simon et al., 1995). Fig. 8 shows that RA exposure 3 days previously effected a decrease or loss of these neurons. *Hoxb-1* involvement in specification of this cell population is suggested by the work of Krumlauf and colleagues (Marshall et al., 1992; Zhang et al., 1994). They showed that ectopic expression of *Hoxb-1* in the hindbrain, resulting either from pre-neurula exposure to RA or over expression of *Hoxa-1*, coincided with ectopic contralateral neurons in those segments having additional expression. Even though we see a loss, instead of a gain, both of our groups see a colocalisation of affected *Hoxb-1* expression and affected contralateral neurons; this argues for a role for *Hoxb-1* in specification of these neurons.

A direct mechanism by which endogenous RA plays a role in normal hindbrain development has recently been shown. Marshal et al. (1994) and Studer et al. (1994) have reported the requirement of two retinoic acid response elements (RAREs) for normal expression of *Hoxb-1*, each responsible for the distinct expression patterns visible at different stages of hindbrain development. One element is necessary for *Hoxb-1* expression in the hindbrain before the rhombomere boundaries form when expression is continuous to the preotic sulcus (Marshall et al., 1994). The second is responsible for the repression of *Hoxb-1* expression in r3 and r5 at the 12-14ss (Kuratani and Eichele, 1993) leaving a sharply defined stripe of expression in r4 (Studer et al., 1994). It seems reasonable to postulate that availability of RA to the RARE controls the repression of *Hoxb-1* expression in r3 and r5 while the unavailability of RA to this RARE in r4 at the 12ss leads to Hoxb-1 expression in r4. This endogenous RA mechanism for localisation of *Hoxb-1* in r4 could explain the specificity of exogenous RA effects on r4. The additional RA we injected at the 10ss, before *Hoxb-1* is localised to r4, makes RA accessible to the RARE triggering the repression mechanism normally only affecting r3 and r5, resulting in the loss of *Hoxb-1* in r4. The possible role of *Hoxb-1* in specifying at least one aspect of the r4 phenotype, the contralateral vestibuloacoustic neurons, suggests an endogenous role for RA at this stage in creating the phenotype as well as localising the gene expression pattern.

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