Development 129, 433-442 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV3568

Signalling between the hindbrain and paraxial tissues dictates neural crest migration pathways

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Accepted 24 October 2001

SUMMARY

Cranial neural crest cells are a pluripotent population of cells derived from the neural tube that migrate into the branchial arches to generate the distinctive bone, connective tissue and peripheral nervous system components characteristic of the vertebrate head. The highly conserved segmental organisation of the vertebrate hindbrain plays an important role in pattering the pathways of neural crest cell migration and in generating the distinct or separate streams of crest cells that form unique structures in each arch. We have used focal injections of DiI into the developing mouse hindbrain in combination with in vitro whole embryo culture to map the patterns of cranial neural crest cell migration into the developing branchial arches. Our results show that mouse hindbrain-derived neural crest cells migrate in three segregated streams adjacent to the even-numbered rhombomeres into the branchial arches, and each stream contains contributions of cells from three rhombomeres in a pattern very similar to that observed in the chick embryo. There are clear neural crest-free zones adjacent to r3 and r5. Furthermore, using grafting and lineage-tracing techniques in cultured mouse embryos to investigate the differential ability of odd and even-numbered segments to generate neural crest cells, we find that odd and even segments have an intrinsic ability to produce equivalent numbers of neural crest cells. This implies that inter-rhombomeric signalling is less important than combinatorial interactions between the hindbrain and the adjacent arch environment in specific regions, in the process of restricting the generation and migration of neural crest cells. This creates crest-free territories and suggests that tissue interactions established during development and patterning of the branchial arches may set up signals that the neural plate is primed to interpret during the progressive events leading to the delamination and migration of neural crest cells. Using interspecies grafting experiments between mouse and chick embryos, we have shown that this process forms part of a conserved mechanism for generating neural crest-free zones and contributing to the separation of migrating crest populations with distinct *Hox* expression during vertebrate head development.

Key words: Neural crest, Hindbrain, Segmentation, Cell migration, Cell death, Head patterning, Mouse, Chick, Transplantation

INTRODUCTION

The cranial neural crest is a pluripotent mesenchymal population that plays a critical role in construction of the vertebrate head. Arising at the junction between the neural plate and surface ectoderm (Selleck and Bronner-Fraser, 1995) cranial neural crest forms connective tissue, nerves, ganglia, cartilage and skull bones (Le Douarin, 1983; Noden, 1982). Many craniofacial malformations, therefore, are largely attributable to defects in the patterning, proliferation, migration or differentiation of this cell population (Sulik et al., 1988). The cranial neural crest is derived primarily from the hindbrain, which is transiently subdivided into seven rhombomeres (r), and these form lineage-restricted compartments (Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000a; Vaage, 1969). This segmental organisation is conserved and critical for patterning the pathways of neural crest cell migration. In chick and other vertebrate embryos, hindbrain-derived neural crest cells generally migrate as three distinct streams adjacent to r2, r4 and r6, contributing to the first, second and third branchial arches (ba), respectively (Kulesa et al., 2000; Kulesa and Fraser, 2000; Lumsden et al., 1991; Osumi-Yamashita et al., 1994; Sadaghiani and Theibaud, 1987; Schilling and Kimmel, 1994; Sechrist et al., 1993; Serbedzija et al., 1992; Trainor and Tam, 1995). In contrast, there are clear neural crest-free zones adjacent to r3 and r5, generating a two-segment periodicity in the patterns of neural crest migration. Since neural crest arising at distinct axial levels helps to establish the unique anterior-posterior (A-P) identity of structures in its adjacent branchial arch (Hunt et al., 1991; Noden, 1983; Trainor and Krumlauf, 2000a), it is important to understand the mechanisms that segregate these migrating populations. In this respect these crest-free zones could be important for branchial arch patterning by preventing

the intermixing of neural crest populations with different positional identities.

The function and the mechanism by which the r3 and r5 crest-free zones are established are contentious. On one hand, analyses in avian embryos reported elevated levels of cell death in the premigratory neural crest populations of r3 and r5 (Graham et al., 1993), mediated by *Bmp4* signalling from the even rhombomeres. This induces *Msx2* in r3 and r5, leading to the elimination of neural crest before migration (Ellies et al., 2000; Graham et al., 1994; Graham et al., 1993). Hence, inter-rhombomeric signalling would be the key factor in modulating the generation of crest cells from odd rhombomeres. In contrast, lineage and time-lapse analyses in avians clearly show that r3 and r5 do generate neural crest cells, but they move anteriorly and posteriorly, joining the even rhombomere streams (Kulesa, 1998; Kulesa et al., 2000; Kulesa and Fraser, 2000; Sechrist et al., 1993). Furthermore, in frog and fish embryos, r5 produces equivalent amounts of crest cells that migrate laterally, compared to the even rhombomeres (Smith et al., 1997). The variation between species in the amount and migratory pathways of neural crest cells generated by r5, implies that the crest-free zones are not an intrinsic property of odd-numbered rhombomeres themselves. This suggests that environmental influences adjacent to r3 and r5 may be important in controlling the pathways of hindbrain neural crest migration (Farlie et al., 1999), and distinct mechanisms may be utilised by different species to pattern these events. Therefore it is important to understand the mechanisms responsible for segregating the branchial arch streams in mouse embryos and determine if they are functional in other vertebrates.

In this study, we used cell grafting and lineage-tracing techniques in cultured mouse embryos to investigate the interactions between the hindbrain and the environment with respect to patterning the pathways of mouse cranial neural crest cells. We demonstrate that the patterns of cell death in the hindbrain do not correlate with the generation or migration of neural crest. Furthermore, odd-numbered rhombomeres have the same capacity to generate neural crest cells as the even segments. Reciprocal transpositions between even and odd rhombomeres show that specific regions are inhibitory to neural crest cell migration. Our findings demonstrate that the pathways of hindbrain neural crest migration and crest-free zones adjacent to r3 in the mouse are generated by combinatorial signalling events between the hindbrain and the adjacent environment, and this mechanism appears to be conserved in the chick.

MATERIALS AND METHODS

Donor and host embryo isolation and culture

Embryos were obtained from timed-pregnant matings of CBA×C57/BL6 or from transgenic line (*EphA4-GFP; Hoxb1-lacZ*) (Pöpperl et al., 1995) crosses with F_1 females. Host embryos used in culture experiments, 8.5-9.5 days post coitum (d.p.c.), were dissected from the uterus with an intact visceral yolk sac, amnion and ectoplacental cone (Trainor et al., 1994). Previous work has shown that the first migrating population of neural crest cells in the mouse leaves the caudal midbrain-rostral hindbrain neuroepithelium at the 5-6 somite stage (Chan and Tam, 1988). Therefore to catch the earliest waves of migrating neural crest cells in rhombomere grafting experiments, only donor and host embryos having 5 or less pairs of somites were used. After grafting, host embryos were cultured in vitro for 24-48 hours as previously described (Sturm and Tam, 1993; Trainor and Krumlauf, 2000b).

Isolation and labelling of rhombomeric, mesoderm and ectoderm tissue

Consistent neuromeric landmarks, described by Trainor and Tam (Trainor and Tam, 1995), were used to identify the axial level of tissue to be grafted and the correct site of transplantation. Finely polished alloy and glass needles were used to separate the neuroectoderm from adjacent tissues. Tissue fragments that could not be cleanly separated from adjacent tissues were incubated in 0.5% trypsin, 0.25% pancreatin, 0.2% glucose and 0.1% polyvinylpyrolidone in phosphate-buffered saline (PBS) for 5 minutes at 37°C or in Dispase for 5 minutes at 37°C to ensure a pure population. Isolated tissue fragments were then washed in DMEM (Dulbecco's modified Eagles medium), before being labelled with DiI or DiO by soaking in a 1:1 mix of DiI:DR50 or DiO:DR50 (Manzanares et al., 1999), for 2 minutes. Tissue fragments were then washed in DMEM and dissected in DR50 (Sturm and Tam, 1993) using glass needles into smaller fragments consisting of approximately 15-30 cells.

Tissue transplantations

Odd rhombomere to even rhombomere grafts

Small groups of approximately 15-30 cells from r3 or r5 of 8.5 d.p.c. embryos were orthotopically transplanted back to the same sites and heterotopically transplanted into r2 or r4 of isochronic embryos.

Even rhombomere to odd rhombomere grafts

Small groups of approximately 15-30 cells from r4 of 8.5 d.p.c. embryos were orthotopically transplanted back to the same site and heterotopically transplanted into r2 and r3 of isochronic embryos.

Mesoderm and ectoderm grafts

Small fragments of mesoderm or ectoderm were isolated from adjacent to r3 and transplanted next to r4 in isochronic embryos.

Embryo analysis

Grafted embryos were analysed for DiI and green fluorescent protein (GFP) labelling by fluorescence microscopy and with a Leica TCS NT confocal microscope (567 nm excitation) before being assayed for cell death.

Detection of cell death

8.5-9.5 d.p.c. embryos were assessed for rhombomeric cell death via Nile Blue, Acridine Orange or TUNEL staining. For Nile Blue and Acridine Orange staining, embryos were cultured in DR50 (containing a 1:400 dilution of 1.5% Nile Blue in water or 5 μg/ml of Acridine Orange) for 30-40 minutes. Apoptotic cells stain intensely blue when incubated in Nile Blue and are epifluorescent (rhodamine) after incubation with Acridine Orange. TUNEL staining labels apoptotic cells fluorescently (fluorescein) and was performed according to the manufacturer's instructions (Boehringer Mannheim).

Whole-mount in situ hybridisation

Bmp4 and *Msx2* digoxigenin-labelled riboprobes were synthesised (Boehringer Mannheim) and whole-mount in situ hybridisation was performed as previously described (Wilkinson, 1992).

RESULTS

Fate-mapping neural crest migration

In order to understand the mechanisms regulating the pathways of neural crest migration in mouse embryos it was necessary

to precisely map the neural crest contributions made by each rhombomere to the branchial arches. We therefore performed focal injections of DiI into the developing hindbrain of 8.25 d.p.c. mouse embryos to mark small groups of cells in specific pre-rhombomeric territories (r1-r6), preceding the migration of cranial neural crest cells. Care was taken to label only very small groups of cells to ensure that any labelled neural crest cells were derived from only one rhombomere. These embryos were cultured in vitro for 24-36 hours and assayed for the relative contribution and patterns of migration of neural crest cells from each marked group of cells (Fig. 1A-F). Labelling in r1 and r2 produce neural crest cells, which migrate laterally and extensively populate the entire proximo-distal extent of the ba1 (Fig. 1A,B). By comparison, r3 produces relatively few neural crest cells, but some migrate anteriorly to join the r2 stream and contribute to the most proximal region of the ba1 (Fig. 1C, arrowhead). Hence, the first branchial arch (ba1) is populated by neural crest cells derived from the midbrain and r1-3. The remainder of the r3-derived neural crest cells migrate posteriorly and make a minor contribution to the proximal region of ba2 (Fig. 1C, arrow). A lineage tag in future r4 reveals that neural crest cells derived from this segment primarily populate the second arch and they colonise the entire proximo-distal extent of ba2 (Fig. 1D). r5-derived neural crest migrates anteriorly and posteriorly to join the even-numbered neural crest streams from r4 and r6 (Fig. 1E) in a manner similar to cells derived from r3. However, r5 generates more neural crest cells than r3 and contributes less neural crest to ba2 than r4, but significantly more than r3. As a consequence of these patterns, ba2 is composed of neural crest derived from three rhombomere segments, r3-r5 (Fig. 1C-E,G). Lineage tracing r5 and r6 reveal that the third arch (ba3) is populated primarily by r6-derived neural crest cells (Fig. 1F) and also, to a lesser extent, by the posteriorly migrating neural crest derived from r5 (Fig. 1E, arrow, and data not shown). Together, these results show that mouse hindbrain-derived neural crest cells migrate in three segregated streams adjacent to the even-numbered rhombomeres into the branchial arches (Fig. 1G), and that this pattern is very similar to that observed in the chick embryo. Each rhombomere in the mouse produces neural crest cells, with the even-numbered segments the most, followed by r5 and then r3. There are also clear neural crest-free zones adjacent to r3 and r5.

Absence of segmental patterns of cell death in r3 and r5

Since the patterns of neural crest migration from r3 and r5 were similar between the mouse and chick, it was important to examine whether there was also a conservation in the rhombomeric patterns of apoptosis (Graham et al., 1994; Graham et al., 1993) that could account for the diminished production of neural crest cells relative to the other rhombomeres.

To score for general patterns of apoptosis, we used the vital dyes Nile Blue Sulphate and Acridine Orange, which stain the highly chromatin-rich apoptotic bodies. This enabled the detection of apoptotic cells in embryos cultured in vitro, so that a picture of cell death can be accumulated over a number of hours during development rather than at a single time point. Using mouse embryos from 8.5-9.5 d.p.c., vital staining demonstrated that, during the period of neural crest migration, cell death occurs in a temporally and spatially dynamic manner (Fig. 2A-D). Although we observed consistent and reproducible cell death in the hindbrain, there was no specific pattern that could be attributed to either odd or even rhombomeres. Even within a single embryo, the unfused neural folds often display completely different patterns of cell death, highlighting the dynamic nature of events (Fig. 2A, arrowheads). The elevated

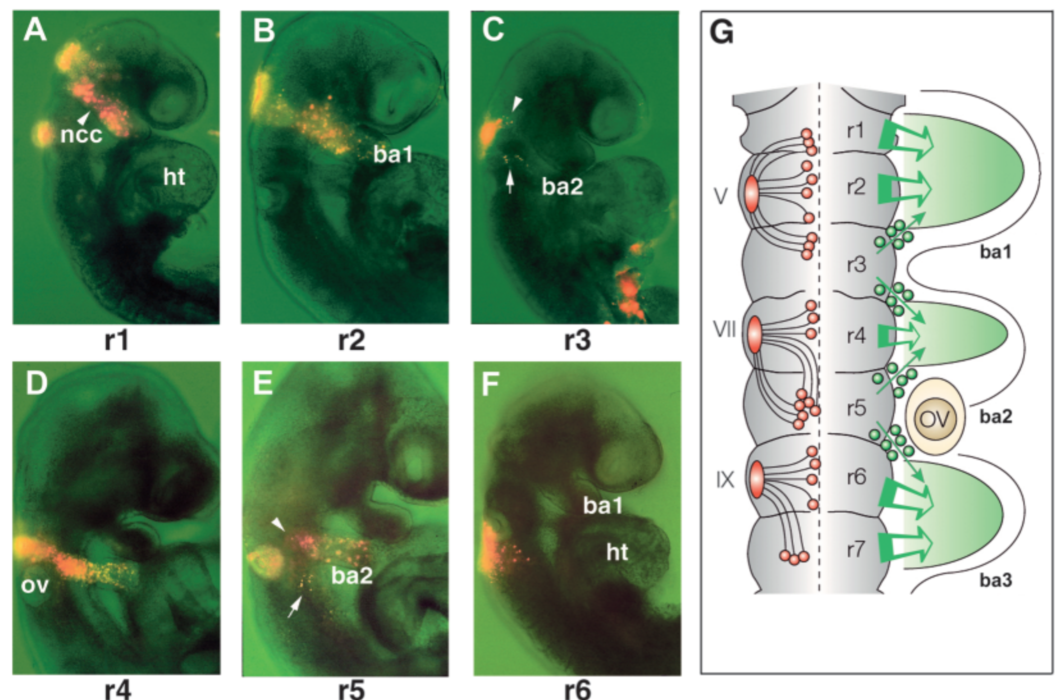

Fig. 1. Migration patterns of mouse cranial neural crest cells (ncc). (A,B) The entire proximodistal extent of the first branchial arch (ba1) is extensively populated by laterally migrating DiI-labelled, neural crest cells derived from r1 (A) and r2 (B). (C) r3 produces relatively few ncc, which do not migrate laterally. Rather they move anteriorly (arrowhead) and posteriorly (arrow) to contribute to the most proximal regions only of ba1 and ba2, respectively. (D) The second branchial arch (ba2) is composed primarily of laterally migrating r4-derived ncc. (E) r5 produces more ncc than r3, which contribute to ba2 and also ba3. r5 ncc migrate anteriorly (arrowhead) and posteriorly (arrow) around the developing otic vesicle. (F) The third branchial arch (ba3) is composed primarily of ncc derived from r6. (G) Summary of ncc migration patterns [adapted from fig. 1a in Trainor and Krumlauf (Trainor and Krumlauf, 2000a)]. ht, heart; ov, otic vesicle; V, trigeminal; VII, facial; IX, glossopharyngeal motor nerves.

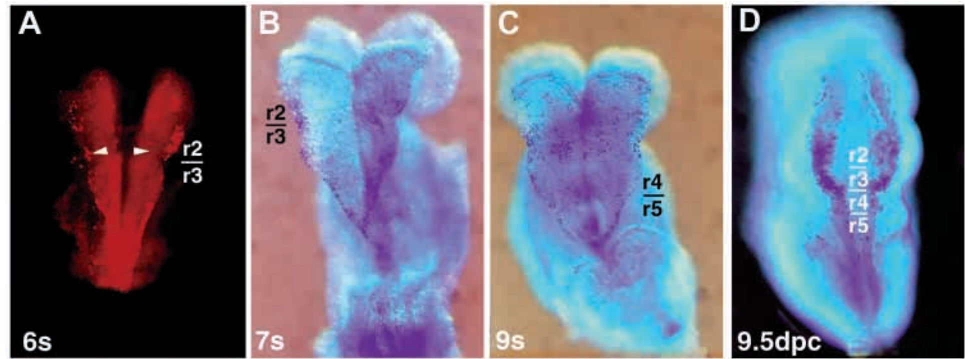

Fig. 2. The patterns of cell death during ncc migration. (A) Acridine Orange stain of a 6-somite stage embryo showing different patterns of cell death in adjacent neural folds (arrowheads). (B-D) Nile Blue staining of 7s (B), 9s (C) and 9.5 d.p.c. (D) embryos showing temporally and spatially dynamic patterns of cell death and the absence of rhombomere-specific death.

levels of cell death detected between 8.5-9.5 d.p.c. were associated with the normal caudal-to-rostral progression of neural tube closure and subsequent formation of the roof plate in the hindbrain (Fig. 2A-D). Similar results were obtained independently by TUNEL staining of fixed embryos (data not shown).

The absence of localised cell death in r3 and r5 prompted us to examine the patterns of *Bmp4* and *Msx2* gene expression during mouse hindbrain development and neural crest migration (Fig. 3). In contrast to the chick, mouse *Bmp4* is not expressed in r3 or r5 during this period. At 8.5 d.p.c., mouse *Bmp4* is completely absent from the dorsal neural tube but is expressed in more ventral tissues, such as the heart (Fig. 3A). By 9.5 d.p.c., *Bmp4* is expressed in Rathke's pouch (arrow) and along the anterior epithelium of the first branchial arch, but is still absent from the hindbrain (Fig. 3B,C). Similarly, *Msx2* expression, which is activated by BMP signalling in apoptosis, also differs markedly from the patterns of its chick homologue, which are confined to r3 and r5 during hindbrain development (Graham et al., 1994; Graham et al., 1993). In the mouse *Msx2* is expressed uniformly in the dorsal edges of the neural tube along the entire A-P axis (Fig. 3D,E). By 9.5 d.p.c., mouse *Msx2* expression is reduced in the hindbrain but is elevated in the roof plate and the distal halves of the branchial arches (Fig. 3E,F). Therefore, *Bmp4/Msx2* mediated apoptosis of odd-rhombomere neural crest cells co-ordinated by signalling between even- and odd-numbered segments is unlikely to be responsible for patterning the pathways of neural crest migration in the mouse.

The ability of r3 to generate neural crest cells depends upon the environment

The absence of rhombomere-specific cell death raised the possibility that the reduced capacity of r3 to generate neural crest is an intrinsic property of this rhombomere. To address this issue we isolated and transposed cells from r3 to different rhombomeres to test their ability to generate neural crest cells in novel environments. In homotopic control grafts, r3 cells (labelled with DiI) transplanted back to r3 generated very few neural crest cells (Fig. 4A). In agreement with the lineage studies described above, these cells migrated anteriorly or posteriorly to populate the proximalmost region of the first and second branchial arches (Fig. 4A, and data not shown). Surprisingly, r3 cells transplanted heterotopically to r2 or r4 gave rise to large numbers of neural crest cells, which populated the entire proximodistal extent of the first and second branchial arches, respectively (Fig. 4B,C). Similarly r5 also has the capacity to generate large amounts of neural crest when transplanted into r2 and r4 (Fig. 4D, and data not shown). Hence r3 and r5 have the intrinsic capability to generate neural crest cells in a manner indistinguishable from even-numbered rhombomeres. However, this ability appears to be restricted during normal development.

One explanation for the ability of r3 to generate elevated levels of neural crest in r2 or r4 is that these cells have changed their identity. To address this issue we utilised an *EphA4-GFP* transgenic line expressing the reporter specifically in r3 and r5 under the control of *Krox20* as a marker for segmental identity (Theil et

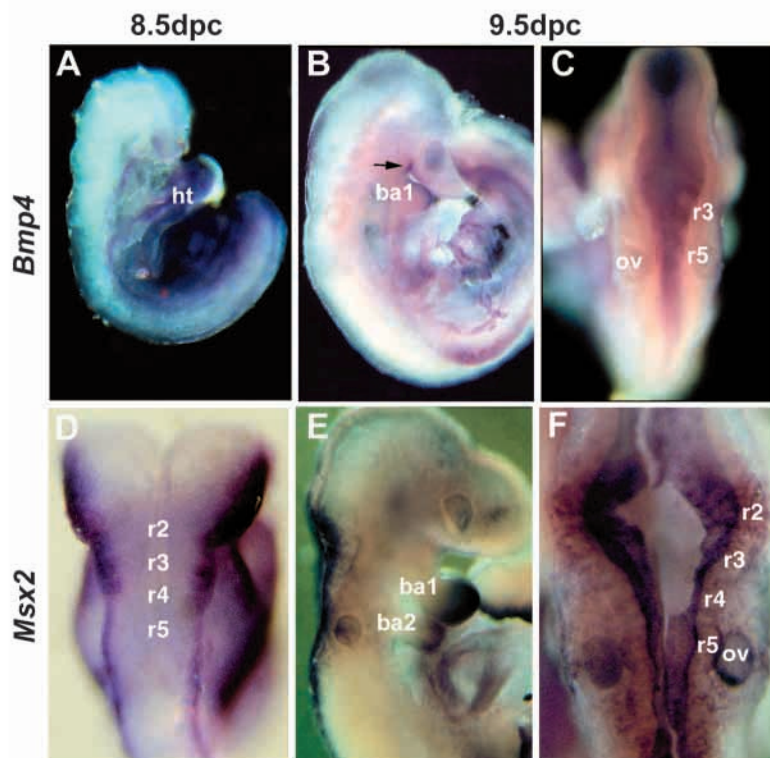

Fig. 3. *Bmp4* and *Msx2* are not segmentally expressed. (A-C) *Bmp4* expression is confined to ventral tissues such as the heart (ht) at 8.5 d.p.c. (A) and to Rathke's pouch and the anterior ectoderm of ba1 in 9.5 d.p.c. embryos (B, arrow). *Bmp4* is not expressed in the dorsal neural tube in 8.5-9.5 d.p.c. embryos (A-C). (D-F) *Msx2* is initially expressed uniformly in the dorsal neural plate of 8.5 d.p.c. embryos (D), but by 9.5 d.p.c. becomes restricted to the distal halves of ba1 and ba2 (E) and also to the roof plate (F).

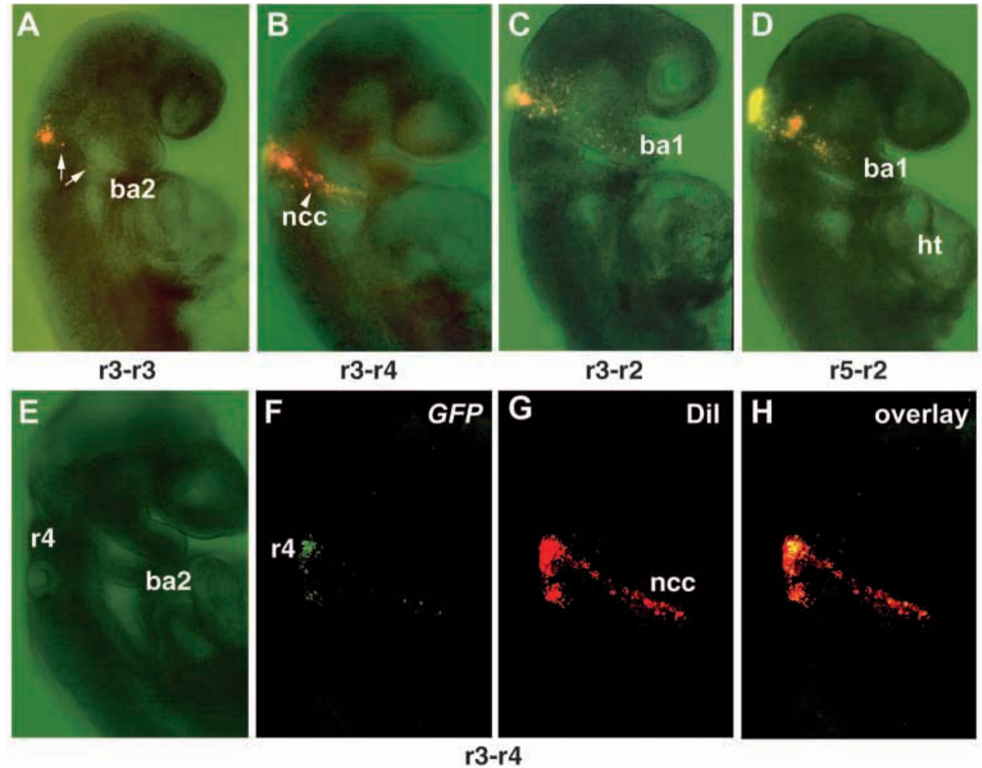

Fig. 4. Odd rhombomeres have the capacity to generate ncc. (A) DiI-labelled r3 cells transplanted back into r3 generate small numbers of ncc, which migrate primarily into the proximal region of ba2 (arrows). (B) r3 cells transplanted into r4 generate elevated numbers of ncc, which colonise the entire length of ba2. (C) r3 cells transplanted into r2 also generate increased amounts of ncc, which colonise the entire length of ba1. (D) r5 cells produce ncc, which extensively populate ba1 when grafted into r2. Ht, heart. (E) Bright-field image of 9.5 d.p.c. embryo post grafting of r3 cells into r4 and 24 hours culture. (F) Confocal image showing that r3 cells, taken from an *EphA4-GFP* transgenic embryo and transposed into r4, maintain their identity in the hindbrain environment. In contrast, graft-derived neural crest cells have reduced reporter expression (*GFP*), showing that they do alter their identity. (G) Confocal image of DiI-labelled r3 cells grafted into r4, showing increased levels of ncc generation that populate ba2. (H) Confocal overlay of F and G, highlighting the maintenance of identity of the grafted rhombomeric tissue and plasticity of the graft-derived ncc.

al., 1998). We isolated genetically marked r3 cells, labelled them with DiI and then transplanted cells homotopically to the same site or heterotopically to other rhombomeres in host embryos (Fig. 4E-H). This enabled us to test for the ability of r3 to generate neural crest cells in novel environments and to monitor the regional identity of the grafted tissue. As expected from the results above, r3 tissue grafted into r4, generates increased amounts of neural crest cells (Fig. 4G,H). In the hindbrain the transposed r3 cells retain their original identity, confirmed by *GFP* expression in the ectopic r4 environment (Fig. 4E,F). In contrast, nearly all the graft-derived crest cells downregulated the reporter, consistent with the idea of plasticity in dispersed migrating cranial neural crest populations, as described in previous studies (Kulesa et al., 2000; Kulesa and Fraser, 2000; Schilling, 2001; Trainor and Krumlauf, 2000a; Trainor and Krumlauf, 2000b). Therefore the ability of r3 to generate increased amounts of neural crest in the r2 or r4 environment cannot be attributed to a change in identity of the transplanted rhombomere cells.

The ability of r4 to generate neural crest cells also depends upon the environment

These experiments suggest that the environment adjacent to r3 plays an important role in restricting the delamination, migration and/or proliferation of neural crest cells in ba2. Hence, an important issue is whether even-numbered rhombomeres retain their ability to generate substantial amounts of neural crest cells in ectopic r3 environments. To test this, we used a *Hoxb1-lacZ* transgenic line expressing the reporter specifically in r4, to isolate, DiI-label and graft small groups of r4 cells into host embryos (Fig. 5). In control transplantations, r4 cells grafted back into r4 gave rise to numerous neural crest cells, which extensively populated ba2 (Fig. 5A). Similarly, when r4 cells were transposed into r2 they retained the ability to generate numerous neural crest cells, which migrate into ba1 (Fig. 5B). In contrast, when r4 cells were transplanted into r3, their capacity to produce migrating neural crest cells was repressed (Fig. 5C,D). We confirmed that this repression is not due to a change in their segmental identity, as indicated by continued *lacZ* expression in the grafted hindbrain tissue (data not shown). To exclude the possibility that the absence of migrating neural crest cells was due to cell death of the grafted tissue, we performed TUNEL staining (Fig. 5E-G). TUNEL and staining for β-gal show that the grafted cells are retained in r3, express the appropriate marker and are hence not eliminated by cell death. These results imply that the local environment adjacent to r3 prohibits the formation and migration of neural crest cells equally well from odd- and even-numbered rhombomeres.

The mesoderm and ectoderm are not sufficient to repress neural crest migration

The inability of r3-derived neural crest cells to migrate laterally, and the existence of a neural crest-free territory adjacent to r3, suggest the existence of environmental zones with combinatorial signals that prohibit neural crest formation and migration. To explore the source of the inhibitory signal(s), we examined whether paraxial tissues, such as the cranial mesoderm or surface ectoderm, were responsible for patterning the migration pathways of neural crest cells. To test if these tissues are sufficient to block the lateral migration of neural crest cells, we isolated mesoderm or surface ectoderm adjacent to r3 and transplanted small amounts of each tissue next to r4 (Fig. 6). The grafted DiO-labelled mesoderm and ectoderm

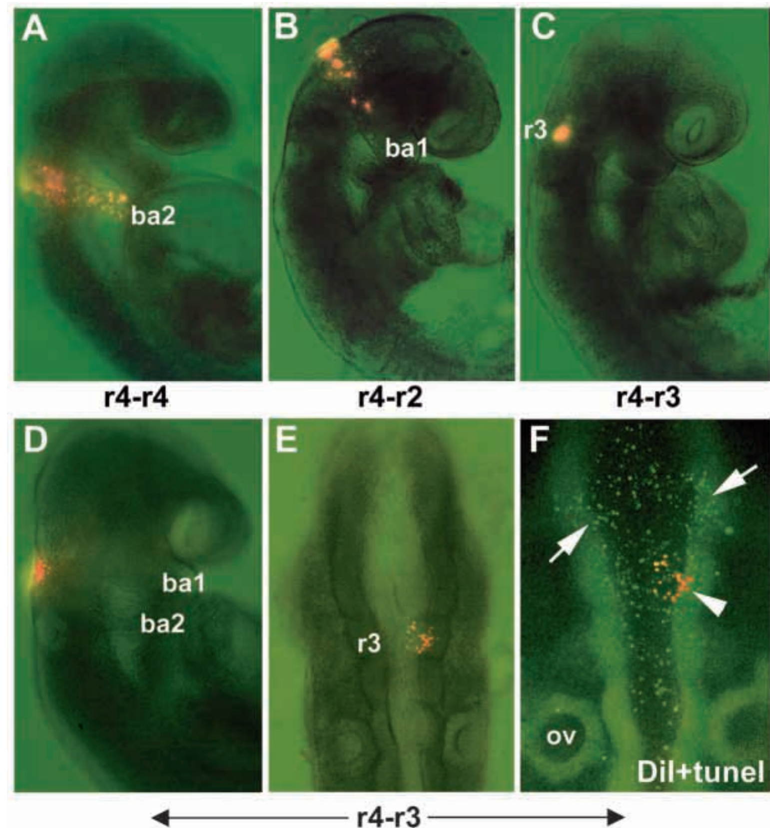

Fig. 5. The capacity of r4 to generate ncc is governed by the environment. (A) Control graft of DiI-labelled r4 cells back into r4, showing that r4 generates ncc, which colonise ba2. (B) Heterotopic graft of r4 cells into r2, showing that r4 can generate ncc in this ectopic environment, and populate ba1. (C) Heterotopic graft of r4 cells into r3, showing that the capacity of r4 to generate ncc is repressed in this ectopic environment. (D,E) Lateral (D) and dorsal (E) views of an embryo in which r4 cells transposed into r3 fail to generate crest. (F) Overlay of TUNEL (green) and DiI (red) staining in the same embryo showing that the grafted rhombomeric cells are not being eliminated by cell death.

tissue colonised the host, proliferated and contributed to the second branchial arch, as expected for tissues placed in this location (Fig. 6B,D). In all cases, irrespective of the proximo-distal location of the transplanted tissue, mesoderm and ectoderm did not inhibit migration of r4-derived neural crest cells (DiI labelled) into ba2 (Fig. 6A,C,D). These results imply that neither the cranial mesoderm nor the cranial ectoderm alone is sufficient for dictating the pathways of neural crest migration in the mouse. It may be possible that the cranial mesoderm and surface ectoderm act together to pattern the neural crest pathways. However, this seems unlikely given that in the chick, 180° rotations of the cranial ectoderm and mesoderm adjacent to r3-r4 do not alter the pathways of neural crest migration (Sechrist et al., 1994). This implies that mesoderm and surface ectoderm do not appear to secrete a broadly diffusible signal that dominantly inhibits neural crest migration.

One caveat to these experiments is that it is difficult to control or maintain the relative position of the grafted mesoderm and surface ectoderm. If these tissues need to be in direct contact with the neural crest cells as they are beginning to delaminate or migrate from the neural epithelium, we might not have been able to assay for short-range signals or interactions. Hence we cannot formally exclude the possibility that surface ectoderm or mesoderm alone are critical in these events, as the proper balance of inhibitory signals might not have been established due to improper positioning or time of exposure. Nonetheless, together these analyses argue that signalling between the hindbrain and these tissues in the developing arch environment is required to mediate the inhibition of neural crest formation and migration. This is consistent with the fact that neural crest is generated at the junction between the surface ectoderm, mesoderm and neural plate and a series of interactions may be involved in setting up and maintaining the inhibitory influences adjacent to r3.

Conservation of signalling interactions between mouse and chick

Our rhombomere transpositions in mouse showed that the relatively low production of r3-derived neural crest cells is not intrinsic to the rhombomere itself. Rather, combinatorial interactions between r3 and the surrounding arch environment as it develops, restrict the generation and migration of cranial neural crest cells. In contrast, in chick embryos it has been postulated that inter-rhombomeric signalling between even- and odd-numbered rhombomeres is responsible for limiting the production of neural crest (Ellies et al., 2000; Graham et al.,

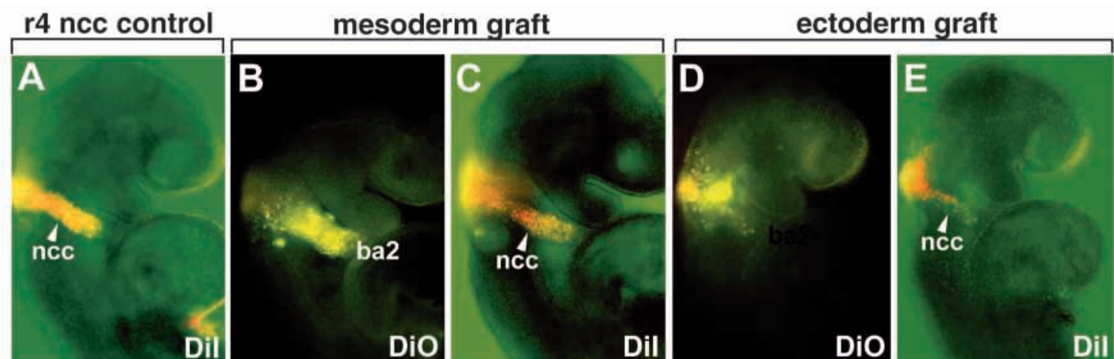

Fig. 6. Environmental influence of mesoderm and surface ectoderm on ncc migration. (A) Control of r4 DiI-labelled ncc migrating into ba2. (B-E) DiO-labelled mesoderm (B) and surface ectoderm (D) isolated from adjacent to r3 and transplanted next to r4 were unable to block the migration of r4-derived ncc (labelled with DiI), which populated ba2 (C and E, respectively).

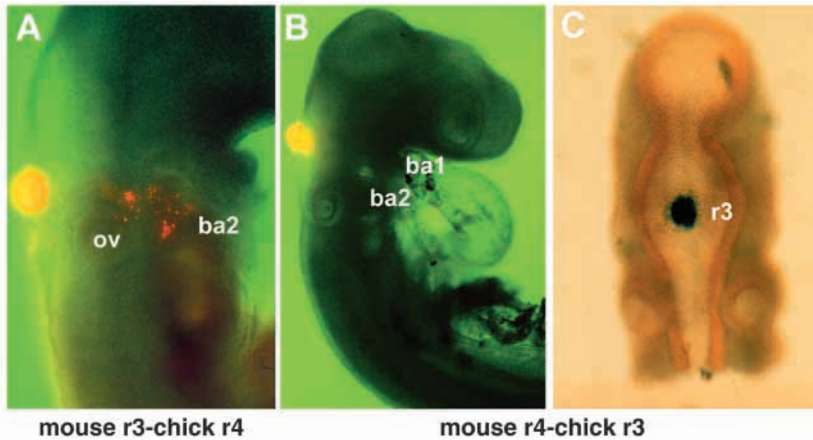

Fig. 7. Conserved repression of crest migration in mouse/chick grafts. (A) DiI-labelled mouse r3 cells transplanted into r4 of the chick generate numerous ncc, which colonise ba2. Ov, otic vesicle. (B) DiI-labelled r4 cells derived from the *Hoxb1-lacZ* transgenic line and transplanted into r3 of the chick are unable to generate ncc. (C) This is not due to a change in their A-P identity or cell death, as grafted mouse cells continue to strongly express the reporter gene.

1994; Graham et al., 1993). In this model the ability of r3 to generate neural crest cells is repressed by signals received from its interaction with the adjacent r2 and r4 territories.

To distinguish between these mechanisms we performed transpositions from mouse to chick. Mouse r3 tissue grafted into r4 in the chick, becomes incorporated and proliferates, generating neural crest cells, which populate ba2 (Fig. 7A). This is further evidence that r3 has the intrinsic capacity to generate neural crest cells. Conversely the ability of r4 to generate neural crest is lost when mouse r4 tissue is transplanted into r3 in the chick (Fig. 7B). This is not due to cell death or changes in identity as the grafted cells continue to express the *lacZ* reporter (Fig. 7C). This illustrates that the environment adjacent to r3 in the chick also restricts the ability of r4 to generate migratory neural crest and that mouse rhombomeric cells have conserved the ability to respond to these signalling interactions. Since in both of these types of transpositions, r3 and r4 cells are juxtaposed and capable of interacting with each other, our experiments show that interactions between the arch environment and rhombomeres are a critical determinant in controlling the production of neural crest. Together with the neural crest-free zones adjacent to r3 in other vertebrates, these results suggest that there is a common mechanism involving interactions between r3 and arch tissues used to govern the patterning of neural crest derived from r3.

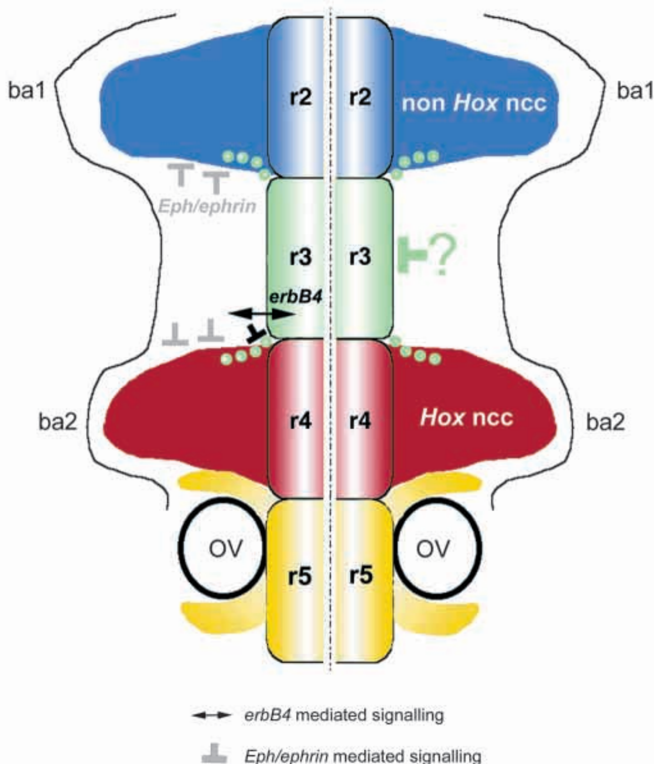

Fig. 8. Model of the mechanisms patterning the pathways of mouse ncc migration. Each rhombomere has the capacity to generate ncc. From r2 and r4, ncc migrate laterally into ba1 and ba2, respectively. r3 and r5 both generate neural crest that migrates anteriorly and posteriorly to join the laterally migrating even-rhombomere ncc streams. Note that r3 produces fewer ncc (small green circles) than r5 (yellow curves). This creates crest-free zones adjacent to the odd-numbered rhombomeres. Neural crest generation from r3 is regulated by inhibitory signals (?) in the adjacent environment (green bar). This crest-free zone adjacent to r3 is maintained by multiple mechanisms. *ErbB4* signalling (black arrows and bar) from the hindbrain to the adjacent mesenchyme keeps even-rhombomere ncc streams segregated dorsally, and Eph/ephrin signalling (grey bars) maintains the segregation ventrally. This prevents the infilling of neural crest from flanking regions. In the case of the neural crest-free zone adjacent to r5 this pattern is generated by the physical inhibition created by induction and formation of the otic vesicle. Adapted from fig. 6F in Golding et al. (Golding et al., 2000).

DISCUSSION

Environmental influences on the migration and generation of cranial neural crest

In this study we demonstrate that environmental signals adjacent to r3 in the mouse, established by combinatorial tissue interactions, inhibit the lateral migration of neural crest cells. This is consistent with time-lapse analyses in the chick showing that crest cells arising in r3 extend filopodia into the adjacent territory but retract these projections and fail to migrate laterally (Kulesa, 1998). Recently, evidence has been obtained for a neurite growth inhibitor in the r3-adjacent mesenchyme that patterns central projections of cranial sensory axons, and this axon-growth inhibitory activity may be part of the mechanism that restricts neural crest migration from r3 into the adjacent mesenchyme (Golding et al., 1999). As a consequence of this lateral inhibition, r3-derived neural crest cells migrate anteriorly and posteriorly to join the neural crest streams emigrating from r2 and r4, and this is a common feature of all vertebrates (Fig. 8).

Our lineage analyses in the mouse show that r3 has a reduced capacity to generate neural crest cells, and reciprocal transpositions experiments between r3 and r4 revealed that this is not an intrinsic property of the rhombomere. Since the interfaces between r3 and r4 and their respective axial identities in these transposition experiments were maintained, this implies that inter-rhombomeric signalling is not responsible for the reduced levels of neural crest cells generated from r3. Furthermore, lineage analyses in the chick have shown that the reduced level of neural crest cell delamination from r3 occurs throughout the entire segment and not just at the borders with even rhombomeres (Kulesa, 1998; Sechrist et al., 1993). This implies that environmental signals are not only limiting the lateral migration of neural crest but also, directly or indirectly, influencing the ability of r3 to generate neural crest. It may be that the inhibition of lateral neural crest migration feeds back on r3 itself to regulate the amounts of neural crest cells that can be made. Alternatively, signals in the adjacent environment could act directly on r3 to modulate the induction of neural crest cells.

The transposition of cranial mesoderm and surface ectoderm suggest that the signals influencing the generation and migration of neural crest are established by interactions between the hindbrain and these tissues. This suggests several possibilities. Positive, emigration-provoking signals, potentially in concert with extracellular matrix differences, may be present in some mesodermal populations either intrinsically or through interactions with the endoderm. Furthermore, neural crest survival, emigration and migration into the arches may result from the sequential interactions initiated in the hindbrain during neural plate stages. Hence, in a manner similar to what we have found in studying cranial neural crest plasticity in A-P patterning, the proper program of events governing the migration of crest may need first to be established in the hindbrain, to allow migratory crest cells to interpret and respond to environmental signals set up through a series of tissue interactions. This contrasts with the trunk, where the segmental migratory pattern of neural crest through the rostral sclerotome is directly imposed by the rostrocaudal character of the mesoderm (Bronner-Fraser and Stern, 1991; Keynes and Stern, 1984). The exclusion of neural crest adjacent to r3, and the rostral and caudal patterns of migration of r3-derived neural crest observed in the mouse, create a neural crest-free zone. This is a common feature in vertebrates (Farlie et al., 1999) and suggests that the signalling interactions we have observed in the mouse reflect a conserved mechanism for patterning the distribution of neural crest cells from r3. This raises several interesting issues with respect to neural crest and craniofacial patterning.

Model for signals influencing neural crest migration

An important question is why is it necessary to establish exclusion zones and restrict r3 neural crest migration? One major reason may be a need to prevent mixing between non-*Hox* and *Hox* expressing neural crest cells, which populate ba1 and ba2, respectively (see Fig. 8). A variety of gain- and loss-of-function studies have shown that *Hoxa2* is primarily responsible for specifying second branchial arch fate and can also inhibit the lower jaw skeleton (Couly et al., 1998; Gendron-Maguire et al., 1993; Grammatopoulos et al., 2000; Kanzler et al., 1998; Pasqualetti et al., 2000; Rijli et al., 1998; Rijli et al., 1993). Therefore *Hoxa2*-expressing populations must be excluded from the first branchial arch neural crest. This is achieved in part by restricting the generation of neural crest from r3 and also by changes in identity of the small number of anteriorly migrating cells, due to plasticity and cell community effects (Schilling, 2001; Trainor and Krumlauf, 2000b). Furthermore, the tight contacts and communication between migrating neural crest cells derived from a similar location may help to feed back the influences of inhibitory signals in the arch environment (Kulesa et al., 2000; Kulesa and Fraser, 2000).

While this mechanism excludes the lateral migration of r3-derived neural crest, other mechanisms must operate to prevent in-filling and mixing of neural crest from adjacent territories to maintain an exclusion zone. For example, in *ErbB4* mutants a population of r4-derived neural crest cells acquires the ability to migrate through the dorsal mesenchyme adjacent to r3 (Golding et al., 2000). This phenotype arises due to changes in the mesenchyme and is not autonomous to the neural crest (Golding et al., 2000). Since *ErbB4* is expressed only in r3 and r5, this phenotype reflects defects in signalling between r3 and its adjacent environment (Golding et al., 2000). There is also evidence that *Eph/ephrin* signalling is another mechanism that contributes to restrictions in the mixing of branchial arch neural crest populations (Fig. 8). In *Xenopus* embryos, the neural crest delaminates as a contiguous A-P band and separation is achieved only during the later phases of migration through the differential expression of the *Eph* receptor and *ephrin* ligand families of genes (Sadaghiani and Theibaud, 1987; Smith et al., 1997). Hence these mechanisms keep neural crest streams segregated and prevent infilling from adjacent territories, working in concert with restrictions in the lateral migration of r3 neural crest to establish crest-free zones.

Differences between the r3 and r5 environments

There are important differences in the mechanisms governing neural crest migration from r3 and r5. Firstly, in mouse, fish and frog embryos r5 generates considerably more neural crest than r3. Secondly, the migration patterns of r5 neural crest are not as well conserved in vertebrates as they are for r3. In frog and fish embryos r5 generates equivalent amounts of neural crest compared to the even rhombomeres, which also migrate laterally (Smith et al., 1997). In contrast, in mouse (Fig. 1) and chick embryos (Kulesa, 1998; Kulesa et al., 2000; Kulesa and Fraser, 2000; Sechrist et al., 1993), crest from r5 migrates rostrally and caudally to join adjacent even-rhombomere streams (Fig. 8). Whilst the general migration pattern of r5-derived cells is similar to r3, it does not appear to arise by the same mechanism. The otic vesicle is positioned immediately adjacent to r5, which provides a physical barrier rather than an exclusion zone that inhibits lateral migration from r5 (Fig. 8). This is consistent with the fact that in fish and frog embryos, the otic vesicle is positioned more laterally, thus allowing the unimpeded lateral migration of neural crest cells from r5 that is seen in these species (Sadaghiani and Theibaud, 1987; Schilling and Kimmel, 1994; Smith et al., 1997; Snape et al., 1991). Furthermore, in mouse and chick transplantations, moving r5 from the proximity of the otic vesicle results in the lateral migration of r5-derived neural crest cells (Fig. 4D) (Saldivar et al., 1996). Thus there are differences in the

ability of the environments adjacent to the odd-numbered rhombomeres to influence neural crest migration. Hence the interactions between r3 and the environment, which generate signals that establish a neural crest-free zone, appear to be unique to that rhombomere.

Similarities and differences between the mouse and chick patterning of neural crest

Our interspecies grafting experiments, in which mouse r3 was transposed into chick r4, confirms that r3 has the capacity to generate neural crest in a different species (Fig. 7). Conversely transplants of mouse r4 into chick r3 display a repressed ability to generate neural crest cells. This suggests that the environmental signals adjacent to r3, which restrict the initial generation and migration of neural crest cells, are conserved between the species. In contrast, other mechanisms contributing to neural crest patterning appear to have diverged. In the chick, interactions between odd and even rhombomeres generate segmental patterns of apoptosis mediated by a *Bmp4-Msx2* signalling loop that contributes to the reduction of neural crest from r3 (Ellies et al., 2000; Graham et al., 1994; Graham et al., 1993). However, in the mouse hindbrain the patterns of *Bmp4* and *Msx2* expression are not segmentally restricted in comparison to the chick. *Bmp4* is not expressed in the mouse hindbrain and *Msx2* is expressed uniformly along the dorsal edge of the neural tube. While it is possible that other Bmps may be involved, neither Bmp7 or Bmp2 are segmentally expressed in odd rhombomeres. Furthermore, this is consistent with our findings in the mouse that while cell death occurs in a temporally and spatially dynamic manner, there is no evidence for cell death specifically in odd rhombomeres during the period of neural crest migration (Fig. 2).

Since cell death in odd-numbered rhombomeres does not contribute to the patterning of mouse cranial neural crest, this raises the issue of why there are differences between the species. One explanation may be related to fundamental differences in the timing and manner in which neural crest cells are generated in each species. Neural crest migration in the mouse occurs during neural fold elevation prior to neural tube closure, whereas in the chick, neural crest migration occurs well after neural tube closure (Le Douarin, 1983; Le Douarin and Kalcheim, 1999). As a consequence the conserved interactions adjacent to r3 that inhibit crest migration may cause crest cells to build up between the neural tube and surface ectoderm in the chick and not the mouse. Hence, in chick, cell death may be required to clear cells in r3 and r5 that are unable to migrate.

In conclusion, our grafting experiments, together with existing studies in the chick (Farlie et al., 1999; Saldivar et al., 1996), suggest that signalling interactions between r3 and the surrounding environment restrict the generation and migration of cranial neural crest. To date few molecules that influence the pathfinding of cranial neural crest cells have been identified, but examples include the *ephrins* and their *Eph* receptors (Helbling et al., 1998; Smith et al., 1997) and evidence is emerging to indicate that *collapsin-1/semaphorin-III* might also be involved (Eickholt et al., 1999). Future analyses in mouse embryos will be aimed at identifying the molecular networks that interact to generate and pattern the migration pathways of cranial neural crest cells.

We thank P. Mealyer for help with animal husbandry, and N. Itasaki, L. Ariza-McNaughton and members of the Krumlauf laboratory for valuable discussions. P. T. was supported by EMBO and HFSP postdoctoral fellowships. This work was funded by Core MRC Programme support, The Stowers Institute for Medical Research and an HFSP International Network Research Grant (RG0146/2000-B) with R. K. as Principal Investigator.

REFERENCES

Bronner-Fraser, M. and Stern, C. (1991). Effects of mesodermal tissues on avian neural crest cell migration. *Dev. Biol.* **143**, 213-217.

Chan, W. Y. and Tam, P. P. L. (1988). A morphological and experimental study of the mesencephalic neural crest cells in the mouse embryo using wheat-germ agglutinin gold conjugate as the cell marker. *Development* **102**, 427-442.

Couly, G., Grapin-Botton, A., Coltey, P., Ruhin, B. and Le Douarin, N. M. (1998). Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between *Hox* gene expression and lower jaw development. *Development* **128**, 3445-3459.

Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S. and Doherty, P. (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-2189.

Ellies, D. L., Church, V., Francis-West, P. and Lumsden, A. (2000). The WNT antagonist cSFRP2 modulates programmed cell death in the developing hindbrain. *Development* **127**, 5285-5295.

Farlie, P. G., Kerr, R., Thomas, P., Symes, T., Minichiello, J., Hearn, C. J. and Newgreen, D. (1999). A paraxial exclusion zone creates patterned cranial neural crest cell outgrowth adjacent to rhombomeres 3 and 5. *Dev. Biol.* **213**, 70-84.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Golding, J., Trainor, P., Krumlauf, R. and Gassman, M. (2000). Defects in pathfinding by cranial neural crest cells in mice lacking the Neuregulin receptor ErbB4. *Nature Cell Biol.* **2**, 103-109.

Golding, J. P., Tidcombe, H., Tsoni, S. and Gassmann, M. (1999). Chondroitin sulphate-binding molecules may pattern central projections of sensory axons within the cranial mesenchyme of the developing mouse. *Dev. Biol.* **216**, 85-97.

Graham, A., Francis-West, P., Brickell, P. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-686.

Graham, A., Heyman, I. and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.

Grammatopoulos, G. A., Bell, E., Toole, L., Lumsden, A. and Tucker, A. S. (2000). Homeotic transformation of branchial arch identity after Hoxa2 overexpression. *Development* **127**, 5355-5365.

Helbling, P. M., Tran, C. T. and Brandli, A. W. (1998). Requirement for EphA receptor signaling in the segregation of *Xenopus* third and fourth arch neural crest cells. *Mech. Dev.* **78**, 63-79.

Hunt, P., Gulisano, M., Cook, M., Sham, M., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991). A distinct *Hox* code for the branchial region of the head. *Nature* **353**, 861-864.

Kanzler, B., Kuschert, S. J., Liu, Y.-H. and Mallo, M. (1998). *Hoxa2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. *Development* **125**, 2587-2597.

Keynes, R. and Stern, C. (1984). Segmentation in the vertebrate nervous system. *Nature* **310**, 786-789.

Kulesa, P. (1998). Neural crest cell dynamics revealed by time-lapse video microscopy of whole chick explant cultures. *Dev. Biol.* **204**, 327-344.

Kulesa, P., Bronner-Fraser, M. and Fraser, S. (2000). In ovo time-lapse analysis after dorsal neural tube ablation shows rerouting of chick hindbrain neural crest. *Development* **127**, 2843-2852.

Kulesa, P. M. and Fraser, S. E. (2000). In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches. *Development* **127**, 1161-1172.

Le Douarin, N. (1983). *The Neural Crest.* Cambridge: Cambridge University Press.

Le Douarin, N. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge Univesity Press.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Manzanares, M., Cordes, S., Ariza-McNaughton, L., Sadl, V., Maruthainar, K., Barsh, G. and Krumlauf, R. (1999). Conserved and distinct roles of *kreisler* in regulation of the paralogous *Hoxa3* and *Hoxb3* genes. *Development* **126**, 759-769.

Noden, D. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev. Biol.* **96**, 144-165.

Noden, D. M. (1982). Patterns and organization of craniofacial skeletogenic and myogenic mesenchyme: a perspective. *Prog. Clin. Biol. Res.* **101**, 167-203.

Osumi-Yamashita, N., Ninomiya, Y., Doi, H. and Eto, K. (1994). The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. *Dev. Biol.* **164**, 409-419.

Pasqualetti, M., Ori, M., Nardi, I. and Rijli, F. M. (2000). Ectopic *Hoxa2* induction after neural crest migration results in homeosis of jaw elements in *Xenopus*. *Development* **127**, 5367-5378.

Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., Brenner, S., Mann, R. and Krumlauf, R. (1995). Segmental expression of *Hoxb1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/Pbx*. *Cell* **81**, 1031-1042.

Rijli, F., Gavalas, A. and Chambon, P. (1998). Segmentation and specification in the branchial region of the head: The role of *Hox* selector genes. *Int. J. Dev. Biol.* **42**, 393-401.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-1349.

Sadaghiani, B. and Theibaud, C. H. (1987). Neural crest development in the *Xenopus laevis* embryo, studies by interspecific transplantation and scanning electron microscopy. *Dev. Biol.* **124**, 91-110.

Saldivar, J., Krull, C., Krumlauf, R., Ariza-McNaughton, L. and Bronner-Fraser, M. (1996). Rhombomere of origin determines autonomous versus environmentally regulated expression of *Hoxa3* in the avian embryo. *Development* **122**, 895-904.

Schilling, T. (2001). Plasticity of zebrafish Hox expression in the hindbrain and cranial neural crest hindbrain. *Dev. Biol.* **231**, 201-216.

Schilling, T. F. and Kimmel, C. B. (1994). Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. *Development* **120**, 483-494.

Sechrist, J., Scherson, T. and Bronner-Fraser, M. (1994). Rhombomere rotation reveals that multiple mechanisms contribute to segmental pattern of hindbrain neural crest migration. *Development* **120**, 1777-1790.

Sechrist, J., Serbedzija, G. N., Scherson, T., Fraser, S. E. and Bronner-Fraser, M. (1993). Segmental migration of the hindbrain neural crest does not arise from its segmental generation. *Development* **118**, 691-703.

Selleck, M. A. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-538.

Serbedzija, G., Fraser, S. and Bronner-Fraser, M. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.

Smith, A., Robinson, V., Patel, K. and Wilkinson, D. G. (1997). The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr. Biol.* **7**, 561-570.

Snape, A. M., Winning, R. S. and Sargent, T. D. (1991). Transcription factor *AP-2* is tissue specific in *Xenopus* and is closely related or identical to keratin transcription factor 1 (*KTF-1*). *Development* **113**, 283-293.

Sturm, K. and Tam, P. P. L. (1993). Isolation and culture of whole postimplantation embryos and germ layer derivatives. *Meth. Enzymol.* **225**, 164-190.

Sulik, K., Cook, C. and Webster, W. (1988). Teratogens and craniofacial malformations: relationships to cell death. *Development* **103**, 213-232.

Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P. and Wilkinson, D. (1998). Segmental expression of the *EphA4 (Sek-1)* receptor tyrosine kinase in the hindbrain is under the direct transcriptional control of Krox20. *Development* **125**, 443-452.

Trainor, P. and Krumlauf, R. (2000a). Patterning the cranial neural crest: Hindbrain segmentation and *Hox* gene pasticity. *Nature Rev. Neurosci.* **1**, 116-124.

Trainor, P. and Krumlauf, R. (2000b). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nature Cell Biol.* **2**, 96-102.

Trainor, P. A. and Tam, P. P. L. (1995). Cranial paraxial mesoderm and neural crest of the mouse embryo-codistribution in the craniofacial mesenchyme but distinct segregation in the branchial arches. *Development* **121**, 2569-2582.

Trainor, P. A., Tan, S. S. and Tam, P. P. L. (1994). Cranial paraxial mesoderm-regionalization of cell fate and impact on craniofacila development in mouse embryos. *Development* **120**, 2925-2932.

Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (*Gallus domesticus*). *Adv. Anat. Embryol. Cell Biol.* **41**, 1-88.

Wilkinson, D. G. (1992). Whole mount in situ hybridisation of vertebrate embryos. In *In Situ Hybridisation, A Practical Approach* (ed. D. G. Wilkinson), pp. 75-83. Oxford: IRL Press at Oxford University Press.