# In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches

**Paul M. Kulesa** and **Scott E. Fraser**

Division of Biology, Beckman Institute 139-74, California Institute of Technology, Pasadena, CA, 91125, USA

Author for correspondence (e-mail: kulesa@gg.caltech.edu)

## SUMMARY

Hindbrain neural crest cells were labeled with DiI and followed in ovo using a new approach for long-term time-lapse confocal microscopy. In ovo imaging allowed us to visualize neural crest cell migration 2-3 times longer than in whole embryo explant cultures, providing a more complete picture of the dynamics of cell migration from emergence at the dorsal midline to entry into the branchial arches. There were aspects of the in ovo neural crest cell migration patterning which were new and different. Surprisingly, there was contact between neural crest cell migration streams bound for different branchial arches. This cell-cell contact occurred in the region lateral to the otic vesicle, where neural crest cells within the distinct streams diverted from their migration pathways into the branchial arches and instead migrated around the otic vesicle to establish a contact between streams. Some individual neural crest cells did appear to cross between the streams, but there was no widespread mixing. Analysis of individual cell trajectories showed that neural crest cells emerge from all rhombomeres (r) and sort into distinct exiting streams adjacent to the even-numbered rhombomeres. Neural crest cell migration behaviors resembled the wide diversity seen in whole embryo chick explants, including chain-like cell arrangements; however, average in ovo cell speeds are as much as 70% faster. To test to what extent neural crest cells from adjoining rhombomeres mix along migration routes and within the branchial arches, separate groups of premigratory neural crest cells were labeled with DiI or DiD. Results showed that r6 and r7 neural crest cells migrated to the same spatial location within the fourth branchial arch. The diversity of migration behaviors suggests that no single mechanism guides in ovo hindbrain neural crest cell migration into the branchial arches. The cell-cell contact between migration streams and the co-localization of neural crest cells from adjoining rhombomeres within a single branchial arch support the notion that the pattern of hindbrain neural crest cell migration emerges dynamically with cell-cell communication playing an important guidance role.

## INTRODUCTION

During the early development of the vertebrate head in chick, a migratory population of cells called the neural crest cells emerge at the dorsal midline of the neural tube and migrate just below the surface ectoderm to the periphery of the ventral side of the embryo where they populate the branchial arches and give rise to cranial ganglia and bone, cartilage and connective tissue of the face (Le Douarin, 1982; Bronner-Fraser, 1993). In rodents and chick, neural crest cells first appear near the midbrain and continue to emerge along the dorsal midline in a rostral-to-caudal manner over time. As neural crest cells leave the hindbrain and spread out into the surrounding tissue, their migration pathways give rise to a characteristic pattern of densely populated areas separated by neural crest-free zones. The most striking of these is the neural crest streams exiting adjacent to an alternating set of hindbrain segments (rhombomeres; Vaage, 1969; Lumsden and Keynes, 1989). In the rostral part of the hindbrain, adjacent to rhombomere (r1) and r2, neural crest cells disperse over a wide rostrolateral diagonal towards the first branchial arch. The second stream is more narrow and extends laterally from r4 towards the second branchial arch. A third stream extends from r6 on a caudolateral diagonal. Near the branchial arches, this stream forks into two branches which populate the third and fourth branchial arches. The streams of neural crest cells are separated by neural crest-free zones lateral to r3 and r5.

There is evidence to support mechanisms which pre-instruct or prepattern the hindbrain neural crest cells to migrate into distinct branchial arches. In the prepattern hypothesis, it is thought that the neural tube transmits a rostrocaudal identity

and pattern of differentiation to subpopulations of the premigratory neural crest (reviewed by McGinnis and Krumlauf, 1992; Hunt et al., 1991). Specific rhombomere segments are thought to produce neural crest cells which migrate from the even-numbered rhombomeres into distinct branchial arches. A major aspect of this hypothesis are the findings that several Hox genes have specific segmental expression in the hindbrain and in specific exiting streams of neural crest. Other rhombomeres such as r3 and r5 are thought to produce neural crest cells which are inhibited to emigrate due to apoptotic cell death (Graham et al., 1993; Lumsden et al., 1991).

It is also possible that the spatially heterogeneous migration pattern of hindbrain neural crest cells emerges dynamically through the interactions between cells, the environment and with other cell types. In studies of specific extracellular matrix components, there has been no evidence to suggest that these components are expressed along specific migration pathways which would suggest an active role in guiding cells (reviewed in Bronner-Fraser, 1993; Erickson and Perris, 1993). However, the interactions between neural crest cells may be important in guidance. Cell labeling studies show that r3 and r5 neural crest cells mix between adjoining rhombomeres and sort into streams exiting the neural tube (Sechrist et al., 1993; Birgbauer et al., 1995). A typical branchial arch may be populated with neural crest cells which originate from multiple rhombomeres. Analysis of individual cell trajectories in chick explanted embryos show that neural crest cells migrate using a wide variety of individual cell behaviors, ranging from rapid uni-directional movements and backward movements to follow cells in the path of a preceding cell (Kulesa and Fraser, 1998). Neural crest cells also migrate as collectives, extending filopodia to form chain-like cell arrangements, demonstrating opportunities for cell-cell communication during migration. Some neural crest cells from r3 and r5 which emigrate adjacent to their rhombomere of origin collapse filopodia and stop, or abruptly change direction towards a stream exiting adjacent to an even-numbered rhombomere (Kulesa and Fraser, 1998), suggesting repulsive or attractive cues adjacent to the neural tube. Recently, details of specific cues involving members of the Eph receptor tyrosine kinases and their ligands, termed ephrins, in regulating the migration of neural crest cells have been reported (reviewed by Robinson et al., 1997) in trunk neural crest in chick (Wang and Anderson, 1997; Krull et al., 1997) and in branchial neural crest in *Xenopus* (Smith et al., 1997). In order to gain insight into whether similar interactions take place in ovo and to provide a means for testing neural crest cell guidance hypotheses, it is necessary to develop a technique that allows in ovo development to be followed.

Recent advances in imaging technologies have allowed us to take more refined images in ovo, such that we can generate time-lapse movies of chick hindbrain neural crest cell migration. The in ovo imaging represents a more comprehensive picture of the migration pattern of neural crest cells than in explanted embryos, due to the ability to follow cell movements near the neural tube, along the migratory routes and near the branchial arches after embryo rotation. By injecting DiI into the lumen of the neural tube and taking high resolution confocal images as a function of time, in the present study, we have followed the trajectories of individual cells and populations of migrating hindbrain neural crest cells in ovo. Below, we describe some features of in ovo hindbrain neural crest cell migration. Our results support the idea that the in ovo migration pattern of hindbrain neural crest cells arises dynamically and offer time-lapse evidence for cell-cell communication along migration routes.

## MATERIALS AND METHODS

### Embryos

Fertile White Leghorn chick eggs were acquired from a local supplier (Lakeview Farms) and incubated at 38°C until approximately the 6-9 somite stage (SS) of development. Eggs were rinsed with 70% alcohol and 3 ml of albumin was removed prior to cutting a window through the shell. A solution of 10% India ink (Pelican Fount; PL. 51822A143) in Howard Ringer's solution was injected below the blastodisc to visualize the embryos. Embryos were staged according to the stages of Hamburger and Hamilton (1951), denoted as st. 10, for example; in other cases, the embryos were staged by their number of somites, denoted as 10 somite stage (ss).

### In ovo preparation

To label premigratory hindbrain neural crest cells, eggs at the 6-9 ss were injected with an isotonic sucrose solution of DiI (D-282; Molecular Probes; made from a 0.5% stock solution in 100% ethanol, diluted 1:10 in warmed (38°C) 0.3 M sucrose. The dye solution marked a large majority of neural crest cells and cells in the neural tube. After windowing the egg shell, a hole was cut in the vitelline membrane, using a fine tungsten needle, and dye solution was injected into the lumen of the neural tube using a picospritzer, filling the hindbrain region, using a quartz micropipet (Sutter Instrument Co.) positioned just rostral to the first somite pair. A very small amount (approx. 1 mg/ml) of Fast Green FCF (Fisher #42053) was added to the dye solution to make it easier to visualize during injection. Injected eggs were resealed with adhesive tape and incubated at 38°C for 1-2 hours. After this incubation period, we evaluated each embryo in terms of the brightness and uniformity of DiI labeling of the neural tube using an upright epifluorescence microscope (Zeiss) with a 10× long working distance (LWD) objective and selected one embryo for time-lapse imaging.

For two-color injections (*n*=46), DiI (Molecular Probes; D-282) and DiD (D-307), made up separately in 0.5% stock solutions in 100% ethanol, were focally injected in ovo into the dorsal part of the neural tube to label small subpopulations of premigratory neural crest cells. Necrosis results if 100% ethanol injections are too large. The eggs were then resealed with adhesive tape and incubated at 38°C for approximately 24 hours. Embryos were then removed, placed on a glass coverslip and imaged with an inverted confocal microscope (Zeiss 410).

For in ovo imaging, a teflon membrane was assembled on a cylindrical acrylic ring (2.2 cm inner diameter × 2.6 cm outer diameter × 0.5 cm high) to provide a window into the eggshell. The acrylic ring, held by forceps, was dipped into warmed white beeswax (Eastman Kodak Co. #1126762) for approximately 10 seconds. The ring was then laid on a rectangular piece of high-sensitivity, oxygen permeable teflon membrane (Fisher #13-298-83; 3.8 cm × 7.5 cm × 15 µm) and before the beeswax could harden, a rubber o-ring (2.4 cm id × 3.1 cm od) was placed around the circumference of the acrylic ring, pulling the teflon membrane taut. After 5 minutes, the rubber o-ring was removed and the excess teflon cut away. The ring was then placed into the hole cut in the chicken eggshell. Melted beeswax was used to seal the circumference of the ring to the eggshell. Because hindbrain neural crest cells begin to emerge at the 6 somite stage in

chick, we did not attempt to image embryos with this technique earlier than the 5 somite stage.

### Time-lapse confocal microscopy

DiI-labeled embryos were visualized using an upright confocal microscope (modified Biorad MRC-500). The aperture of the confocal microscope was fully opened such that with a 10× Neofluar objective, numerical aperture (NA) 0.30, each optical section represented about 30-40 μm in *z*-height. Although this large aperture sacrificed some resolution, it increased the sensitivity of the microscope and allowed for a maximum optical section thickness so that we could capture a majority of cell trajectories for longer periods of time. The microscope (Zeiss) was surrounded by cardboard (4 mm thick) covered with thermal insulation (Reflectix Co.; 8 mm thick) which enclosed a warming heater (Lyon Electric Co. Inc. #115-20) that maintained the environment at 38C for the duration of time-lapse filming, with only mild temperature fluctuations. The fluorescent dye, DiI, was excited with the 568 nm laser line using the YHS filter set intended for rhodamine. During a typical time-lapse imaging session (*n*=36), we took 4-dimensional data sets by collecting up to 11 *z*-sections at 10 μm increments. This 100 μm image cube allows us to follow more complete trajectories of individual and populations of neural crest cells. The sections were projected into one image and recorded every 2 minutes onto either a video optical memory disk recorder (OMDR; Panasonic TQ3038F) or digitally to magneto-optical disk (3M #15175; 590 MB) using the COMOS software package. Images were analyzed and played back as a movie after conversion (using macros from H. Karten) using the image processing and analysis program, NIH Image 1.62.

### Time-lapse data analysis

Time-lapse data were analyzed with an automated 2-D cell tracking program (XVtrack; developed by Drs J. Solomon and S. Speicher/Caltech), which could identify and follow even cells that were challenging to follow by eye. The program acquired cell targets from an initial image of the time series and used several criteria, including cell shape and brightness, to track cells frame by frame throughout the time-lapse sequence. If the time interval is short between frames of the movie, cell shape, intensity and position changes little between frames. In such settings, XVtrack is extremely reliable. A majority of individual cells were tracked throughout the entire time-lapse sequence, with the program continuously calculating cell position and velocity. Some targets which were lost or suddenly appeared along the way were indexed by the program; many of these may have been the result of cells moving in or out of the focal plane.

## RESULTS

### Visualizing hindbrain neural crest cell migration in ovo

To characterize the movements of

neural crest cells migrating from the hindbrain to the branchial arches, we labeled potential neural crest cells with DiI and followed their trajectories in ovo using a new windowing technique. Intact embryos that have a teflon window placed in the eggshell maintain normal morphology and growth rates for 3 to 5 days, compared with that of windowed, but not imaged embryos of the same initial stage. This technique allowed us to visualize neural crest cell migration 2-3 times longer than in whole embryo explant cultures, providing a more complete picture of cell migration from emergence at the dorsal midline to entry into the branchial arches (Fig. 1). Below, we describe the migration behaviors of individual neural crest cells and outline several fascinating characteristics of the spatial patterning of the branchial arches on the cellular level using individual frames
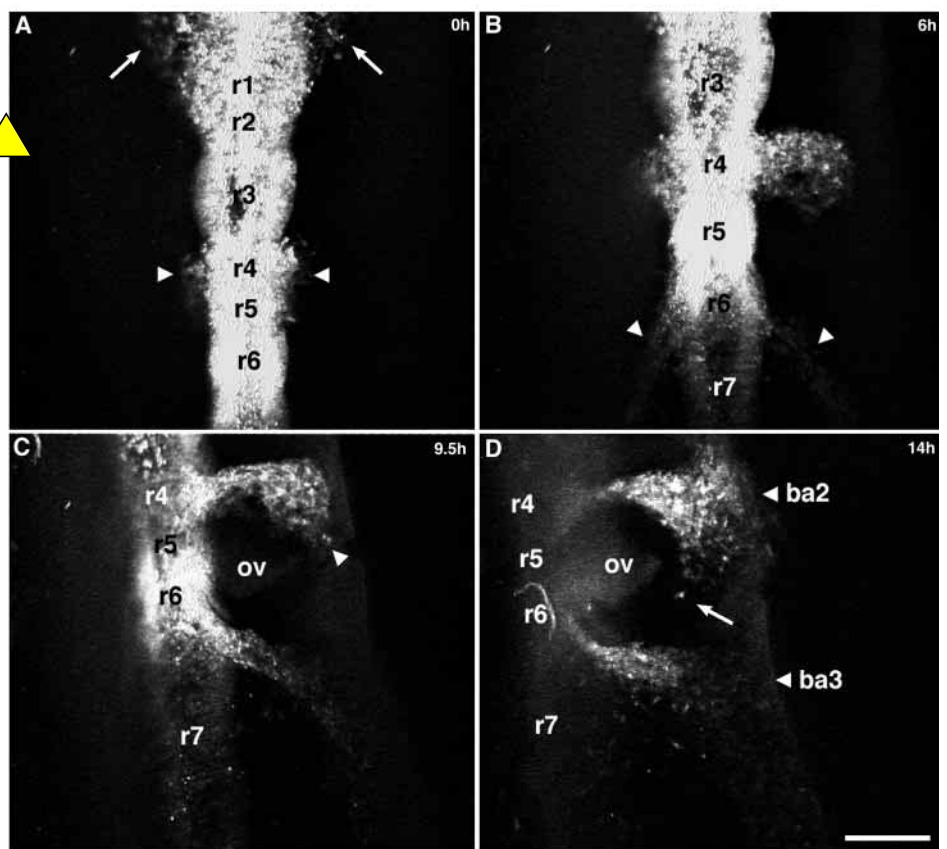


**Fig. 1.** Selected images from a time-lapse sequence showing the progression of hindbrain neural crest cell migration from the dorsal neural tube into the surrounding unlabeled tissue and into the branchial arches. (A) The field of view shows six of the rhombomeres (r), r1-r6. Hindbrain neural crest cell migration begins from the axial level near rhombomere 1 (r1), with the first cells becoming visually distinct as they migrate into the surrounding unlabeled tissue (arrows point to individual neural crest cells), and proceeds in a caudal manner. Neural crest cells are just beginning to emigrate lateral to r4 (arrowheads). (B) As neural crest cells continue to stream out adjacent to r4, they form densely populated streams. Notice how neural crest-free zones arise adjacent to r3 and r5. Some neural crest cells are starting to emigrate adjacent to r6 and are heading in a caudolateral direction (arrowheads). r7 is now in the field of view. (C) As the embryo rotates, the migration pattern of neural crest cells into the branchial arches is revealed on one side of the embryo. Notice how neural crest cells have migrated around a portion of the otic vesicle (OV), with some individual cells near the front of the stream (arrowhead). (D) As neural crest cells fill the second (ba2) and third (ba3) branchial arches, some individual cells from the ba2 migration stream begin to migrate caudally into the region between the second and third branchial arches (arrow). Scale bar, 100 μm.
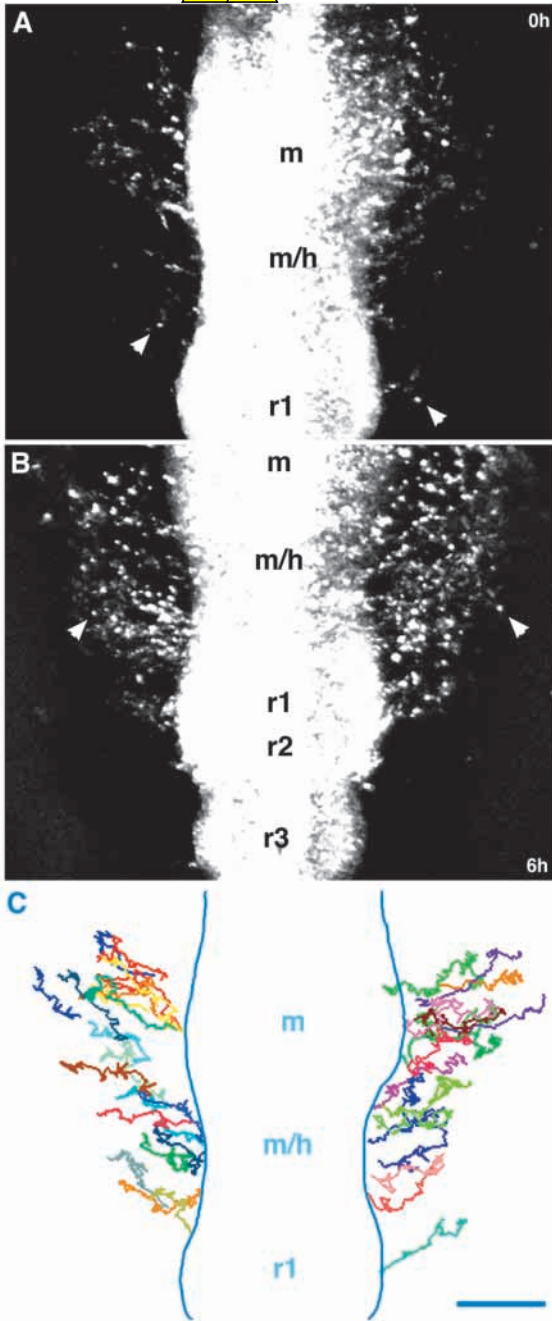
**Fig. 2.** Selected frames from a confocal time-lapse imaging session show DiI-labeled neural crest cells leaving the neural tube and migrating into the periphery from the axial level of the midbrain (m), midbrain/hindbrain boundary (m/h) and rhombomere 1 (r1). (A) Neural crest cells have emigrated from the midbrain and are just starting to appear adjacent to r1 (arrowheads). (B) After 6 hours (6h), neural crest cells have dispersed into the surrounding tissue and spread out in a wide distribution from the midbrain (m) to rhombomere 2 (r2). The arrowheads point to individual migrating neural crest cells. Notice how the distribution of neural crest cells does not cover the region adjacent to r3, rather the distribution of cells seems to be spread out rostral to an imaginary border drawn from the caudal part of r2 extending on a rostrolateral diagonal from r2. (C) Analysis of the time-lapse data reveals that the individual cell trajectories tend to follow a rostrolateral diagonal from the lateral edge of the neural tube (for clarity, only a subset of all the trajectories are shown). Scale bar, 50 µm.
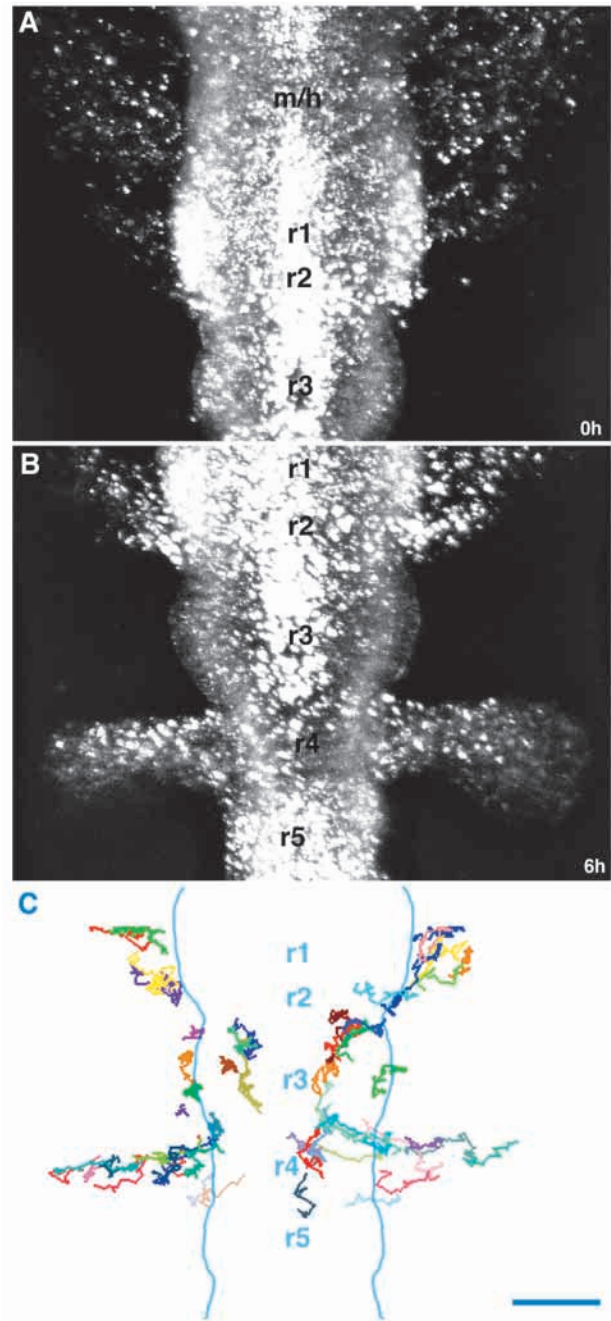


**Fig. 3.** Selected frames from a confocal time-lapse imaging session showing DiI-labeled neural crest cells leaving the neural tube and migrating into the periphery from the axial level of r3 to r5. (A) Neural crest cells have spread out into the surrounding unlabeled tissue adjacent to the midbrain/hindbrain boundary (m/h), r1 and r2. (B) After 6 hours (6h), neural crest cells have started to emigrate in densely populated streams adjacent to r4. Neural crest-free zones appear adjacent to r3 and r5. (C) Some individual cell trajectories are shown. Notice how some individual cell trajectories of r3 neural crest cells near the midline follow a rostrolateral diagonal towards r2 and the first branchial arch. Other cell trajectories from the caudal part of r3 extend into r4 and join the cells migrating adjacent to r4. Notice how the individual r3 neural crest cell trajectories immediately adjacent to r3 show that the cells do not continue to migrate lateral to r3, but instead loiter near r3 or stop. Neural crest cells adjacent to r4 migrate with mostly lateral trajectories to the second branchial arch. Scale bar, 50 µm.

of the time-lapse movies. These movies are available at the website: http://www.its.caltech.edu/~fraslab.

## Neural crest cells migration into branchial arch 1

The first hindbrain neural crest cells emerge along the dorsal midline from the midbrain and caudal to rhombomere (r) r1 in a rostro-to-caudal manner. Fig. 2A shows many DiI-labeled neural crest cells emigrating into the surrounding unlabeled tissue adjacent to the midbrain (m) with a few neural crest cells beginning to migrate away adjacent to r1 (arrowheads). The migrating cells spread out to cover a broad region of the surrounding tissue, but do not appear to cross an imaginary boundary which extends on a rostrolateral diagonal from the border between r2 and r3 (Fig. 2B). Individual cells which migrate near this region often extend and retract filopodia in the caudal direction, but continue movement rostrolaterally.

Time-lapse analysis reveals that the neural crest cells emerging from the midbrain and r1 display a wide variety of migration behaviors, including chain-like cell arrangements. Chains of cells form near the dorsal midline and extend to the lateral extent of the neural tube, where they appear to disassemble and migrate as individuals. Individual cell trajectories appear to maintain a rostrolateral or lateral direction extending away from the neural tube (Fig. 2C). These cells actively extend filopodia and migrate at an average speed of 158±2 µm/hour.

## Neural crest cells migration into branchial arch 2

The migration pattern of neural crest cells into the second branchial arch is different from the first branchial arch. As neural crest cells continue to emerge along the dorsal midline in a rostro-to-caudal manner from r3 to r5, their migration pathways set up densely populated exiting streams lateral to r4 interdigitated by neural crest-free zones adjacent to r3 and r5 (Fig. 3). Neural crest cells first appear to leave the neural tube adjacent to r4 in a densely populated stream. Cells at the front of the stream spread out to form a fan shaped front which tapers back towards the neural tube (Fig. 3B). Time-lapse analysis reveals that a substantial number of r3 neural crest cells contribute to the first and second branchial arches. Some r3 neural crest cells migrate to join cells emigrating adjacent to r2 and moving towards the first branchial arch (Fig. 3C). Notice in Fig. 3C that the trajectories of these cells are along a rostrolateral diagonal direction. Other r3 neural crest cells move to join cells leaving adjacent to r4 (Fig. 3C). Many of these cell trajectories are along a caudolateral diagonal from near the midline (Fig. 3C). A few cells, which migrate lateral to r3, were seen to collapse filopodia and stop or change direction to migrate towards neural crest streams adjacent to the even-numbered rhombomeres. Cell trajectories show these cells adjacent to r3, as well as other r3 neural crest cells which remain near the midline (Fig. 3C). Typical cell trajectories of r5 neural crest cells which contribute to the second branchial arch resemble those of r3 neural crest cells. The cell trajectories run from the dorsal midline to the lateral edge of the neural tube, moving close to the boundary with r4 before exiting the neural tube. Neural crest cells which migrate towards the second branchial arch move away from the neural tube at an average speed of 143±2 µm/hr.

## Neural crest cells migration into branchial arch 3

Neural crest cells from r6 contribute to the third branchial arch population. Time-lapse analysis reveals that r6 neural crest
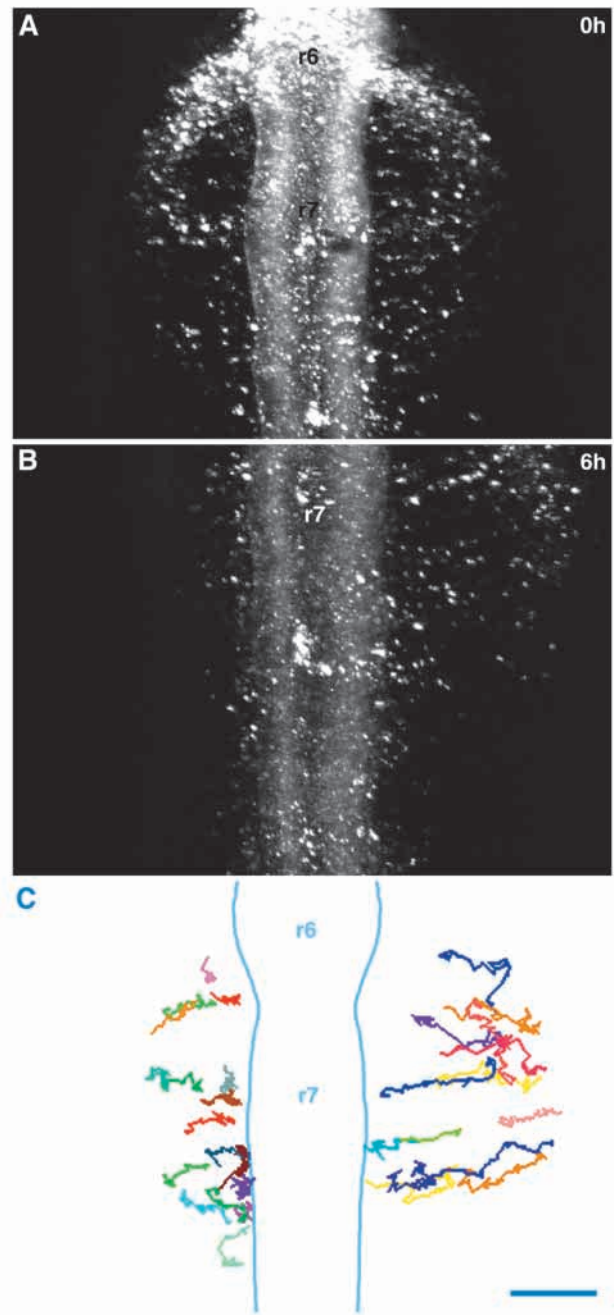


**Fig. 4.** Selected frames from a confocal time-lapse imaging session showing DiI-labeled neural crest cells leaving the neural tube and migrating into the periphery from the axial level of r6 and r7. (A) Neural crest cells have spread out into the surrounding unlabeled tissue adjacent to either side of r6 in densely populated streams, while cells which have migrated adjacent to r7 have a more sparse distribution. (B) After 6 hours (6h), the embryo has rotated to display r7 neural crest cells which continue to emigrate laterally from r7. (C) A sample of some individual cell trajectories is shown. Notice how individual cell trajectories stretch laterally into the surrounding tissue. Scale bar, 50 µm.

cells migrate into the surrounding tissue and maintain a densely populated exiting stream which extends in a caudolateral diagonal direction from r6 towards the third branchial arch (Fig. 4A). As cells reach the lateral side of the otic vesicle, they spread out and cover a larger region caudally. With the exception of a few neural crest cells, cell trajectories maintain an imaginary border extending laterally out from the caudal side of the otic vesicle. Near the third branchial arch, the population of neural crest begins to split into two distinct branches of a fork. The rostral branch populates the third branchial arch and the caudal part populates the fourth branchial arch. Neural crest cells from r5 also migrate into the third branchial arch. The neural crest cells from r5 emerge at the dorsal midline and migrate caudally into r6, or directly into the third branchial arch, exiting the neural tube region near the boundary (b) between r5 and r6, b5/6. Neural crest cells which migrate into the third branchial arch move at an average speed of 166±4 µm/hr.

In contrast to migration in a densely populated stream, neural crest cells from r7 leave the dorsal midline of r7 and migrate as individuals and collectively in chain-like cell arrangements. Individual cells may cross between the chain-like cell arrangements, which tend to be 1 cell wide and may include 5-10 cells. This migration pattern gives rise to a sparse distribution of cells between the neural tube and the fourth branchial arch (Fig. 4B). Time-lapse analysis reveals that individual cell trajectories and chains extend in a lateral direction from the neural tube (Fig. 4C).

## Cell-cell contact near the otic vesicle and between branchial arches 2 and 3

The ability to watch neural crest cell migration as the embryo rotates onto its side reveals a particularly interesting characteristic of the migration pattern, namely cell-cell contact between distinct migration streams. Below, we first describe the time evolution of the neural crest cell
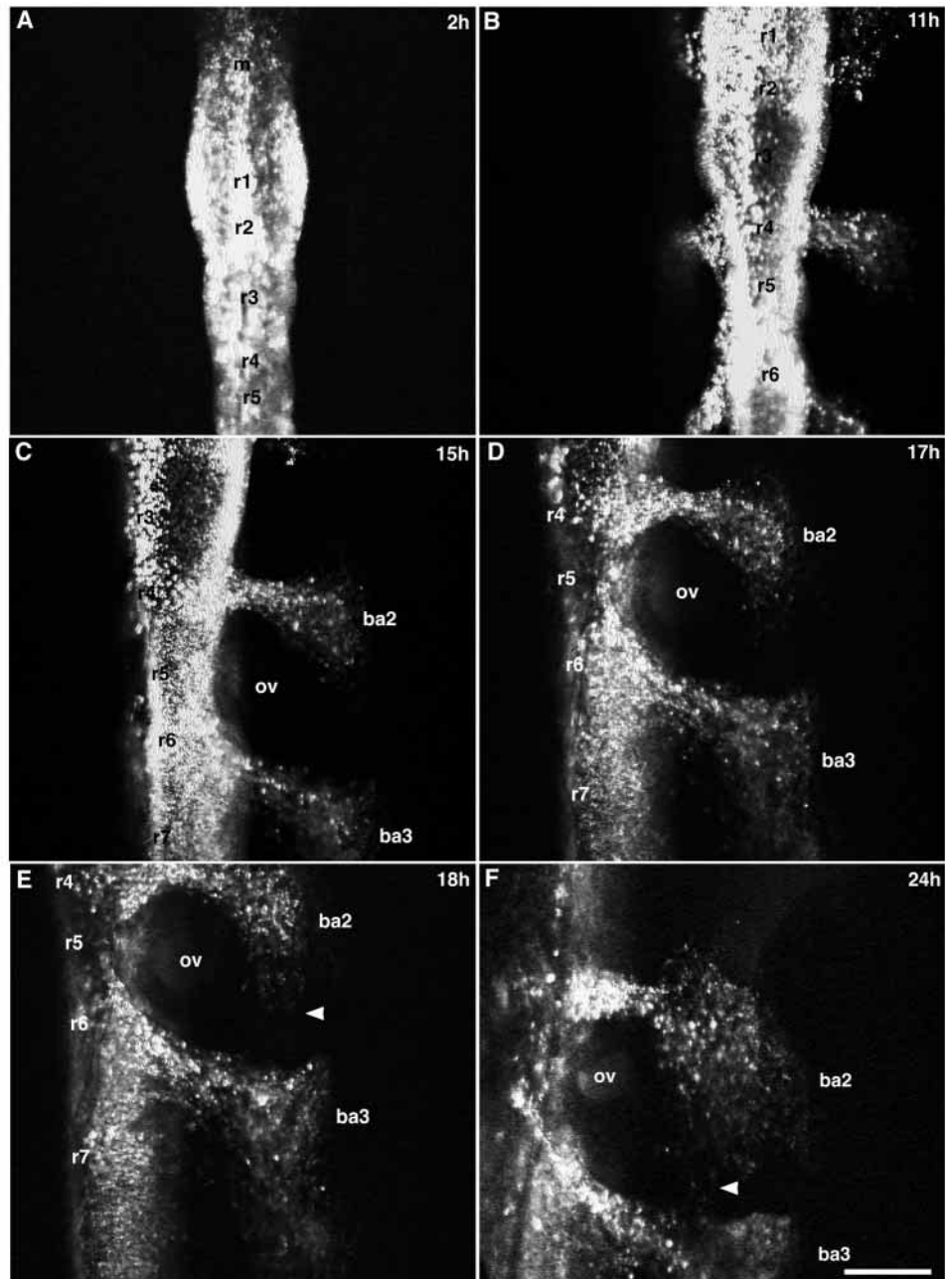


**Fig. 5.** Typical time course of the neural crest cell migration pattern leading up to a connection between the second and third branchial arch migration streams. These selected images from a time-lapse sequence show the progression of DiI-labeled hindbrain neural crest cell migration from the dorsal neural tube into the surrounding unlabeled tissue and into the branchial arches. (A) Premigratory hindbrain neural crest cells and neural tube cells have been labeled with DiI. The midbrain (m) and caudal regions to rhombomere 5 (r5) are in the field of view. (B) As neural crest cells start to emigrate from the neural tube, the characteristic pattern of densely populated neural crest areas separated by neural crest-free zones adjacent to r3 and r5 begins to emerge. (C) As the embryo rotates, neural crest cells which have migrated around the rostral and caudal sides of the otic vesicle (OV) towards the second (ba2) and third (ba3) branchial arches can be seen. (D) Notice that as the embryo turns, more of the branchial arch sites are visible and the large numbers of neural crest cells are present. (E) Some neural crest cells from the second branchial arch stream begin to migrate into the neural crest-free zone adjacent to the lateral side of the otic vesicle (an arrowhead points to the front of the migrating cells) and (F) form a connection (arrowhead) with neural crest cells that migrate rostrally from the third branchial arch stream. Time is in hours (h). Scale bar, 100 µm.
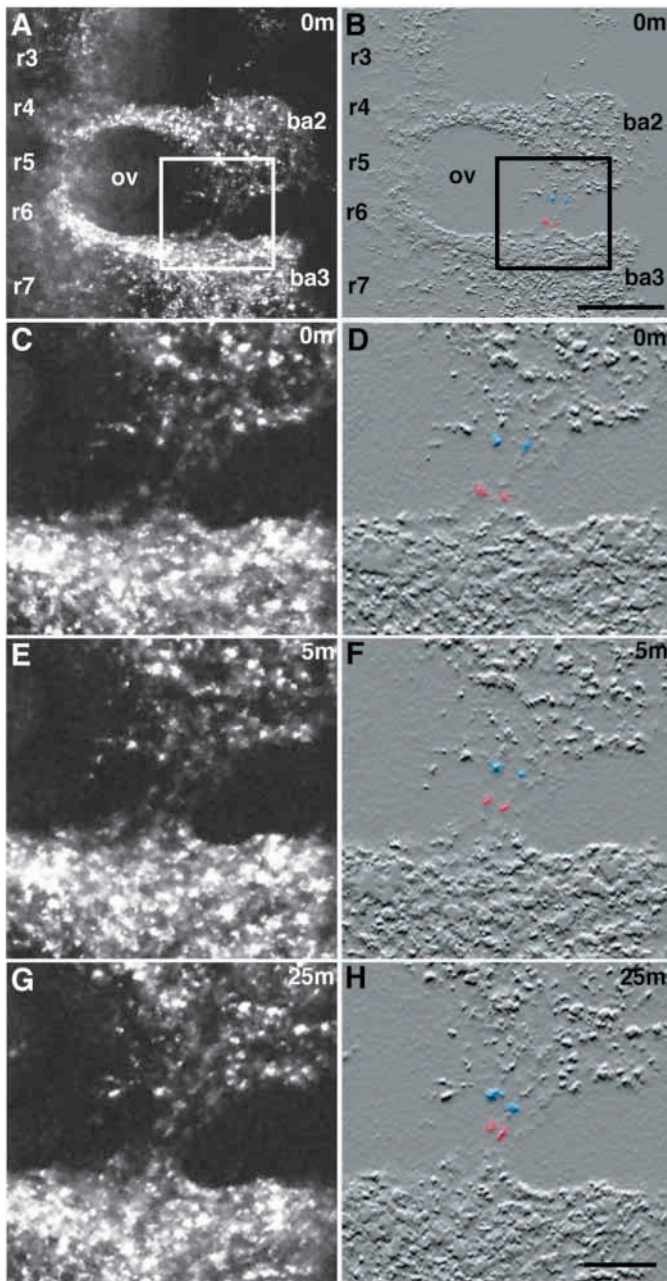
**Fig. 6.** This figure and Figs 7 and 9 present selected frames from confocal time-lapse imaging sessions showing DiI-labeled neural crest cells migrating to the second (ba2) and third (ba3) branchial arches, in separate streams, interacting near the lateral side of the otic vesicle (OV). In these images, the embryo has rotated to reveal a more complete picture of the migration pathways of neural crest cells into the branchial arches. In all three figures the left column of frames represent the raw data and the right column of frames are the raw data images which have been processed using the emboss filter in Adobe Photoshop 4.0 to visually enhance the outline of cells. Time is in minutes (m). (A,B) The embryo has rotated to reveal the rhombomeres (r), r3-r7. Notice how the neural crest cell streams adjacent to r4 and r6 have extended laterally and cells have spread out into ba2 and ba3. The box surrounds particularly interesting cell interactions between the migrating streams of neural crest cells, on the lateral side of the otic vesicle (OV). In B, two blue-colored cells migrating within the second branchial stream and two red-colored cells in the third branchial arch stream are labeled. (C-H) The region enclosed within the box (in frames A and B) has been magnified to show the interaction between cells from the separate exiting neural crest streams. Notice how the individual colored cells move closer together (E,F) and come to within a cell diameter of each other (G,H). Scale bars, 100 μm (B) and 50 μm (H).

region (Fig. 5E; arrowhead). Neural crest cells at the caudal border of the second branchial arch stream actively extend filopodia into the caudal region of unlabeled tissue while neural crest cells at the rostral edge of the third branchial arch stream extend filopodia in the rostral direction. As more neural crest cells fill the streams and branchial arches, the gap between the two populations narrows in the area just lateral to the otic vesicle and a few neural crest cells stretch out and contact cells in the other exiting stream (Fig. 5F; arrowhead). Individual filopodia will often extend and establish a contact between another cell in the other exiting stream, while maintaining a connection back to their stream. The connection between streams (which occurred in 10 of 18 (56%) time-lapse sessions) takes place after initial neural crest cells have reached the second and third branchial arches and in a region presumed to be a neural crest-free zone.

Neural crest cells from distinct exiting streams which come in contact form a connection between the streams, but there does not appear to be widespread mixing of cells. In Fig. 6, we show a typical formation of this contact between cell populations. The region of cell interaction between cells of the second and third branchial arch streams is shown in Fig. 6A,B (boxed) and magnified in the remainder of Fig. 6. Notice that the blue-colored cells are in the second arch stream and the red-colored cells are in the third arch stream (Fig. 6B). These cells migrate to within about 1 cell diameter of each other (Fig. 6C-H) but do not mix into opposite populations. The cells eventually return to the branchial arch population from which they migrated: it was common to see neural crest cells from a separate population migrate and form a connection to the other population, but then return to their original exiting stream. In Fig. 7, notice how the blue-colored cell starts in the third branchial arch stream (Fig. 7B), migrates in a rostral direction (Fig. 7C,D) and then divides (Fig. 7E-H). The cells eventually move back towards the third branchial arch stream. Tracking of individual neural crest cells shows the trajectories by which cells from the second and third branchial arch streams move

migration pattern which leads to the contact between streams. As the DiI-labeled hindbrain neural crest cells begin to emerge from the neural tube, they can be seen migrating into the surrounding unlabeled tissue (Fig. 5A,B). Streams of exiting neural crest cells form adjacent to r4 and r6, separated by neural crest-free zones lateral to r3 and r5 (Fig. 5B,C). Notice how the neural crest-free zone adjacent to r3 appears to be maintained laterally all the way to the branchial arches (Fig. 5C). As more of the periphery of the embryo is revealed, the second (ba2) and third (ba3) branchial arch destination sites appear densely populated with neural crest cells (Fig. 5D). The region lateral to the otic vesicle (OV) which lies between the second and third branchial arches is compromised. Surprisingly, time-lapse analysis reveals that a few neural crest cells from separate exiting streams migrate into this unlabeled

into the region between the streams (Fig. 8A) and interact (Fig. 8B). Individual cells within the second branchial arch stream may migrate from the middle of the streams (Fig. 8A, arrow) and from the front of the streams (Fig. 8A, asterisk, arrowhead) into the region between the streams. Individual cells that come from the separate branchial arch streams (Fig. 8B, white arrowhead) then interact in between the streams (Fig. 8B, arrow).

There were a few instances where cells did cross between neighboring streams (Fig. 9). Notice that the blue-colored cell (Fig. 9B) which starts near the stream adjacent to r4 migrates in the caudal direction around the lateral side of the otic vesicle (Fig. 9C,D) and then divides (Fig. 9E,F). One progeny (Fig. 9F; red-colored cell) migrates to join the stream of cells heading towards the third branchial arch and the other daughter cell (blue colored) moves back towards the original site from where it separated from the migrating population (Fig. 9G-J).

## Focal 2-color injections of premigratory neural crest cells in adjoining rhombomeres

To test to what extent neural crest cells from adjoining rhombomeres mix along migration routes to the branchial arches, we used two different fluorescent dyes, DiI and DiD to label premigratory neural crest cells in adjacent rhombomeres. After injecting groups of cells in ovo, we resealed and reincubated the eggs and harvested the embryos for imaging after 24 hours. Fig. 10 shows a typical embryo 24 hours after injection of DiI (green) and DiD (red) to label premigratory neural crest cells in r6 and r7, respectively. Notice in Fig. 10A,B that r6 neural crest cells form distinct and densely populated migration streams. One stream extends laterally and appears to terminate at the medial side of the otic vesicle (Fig. 10A), while a separate stream wraps around the caudal side of the OV and splits into two distinct branches (Fig. 10A,B). The rostral branch populates the third branchial arch and the caudal branch (Fig. 10B; arrow) populates the fourth branchial arch. Neural crest cells from r7 spread out laterally and densely populate the fourth branchial arch (Fig. 10C; asterisk). Notice in Fig. 10C that very few r7 neural crest cells wind up mixed within the r6 neural crest cell migrating population (Fig. 10C,D; one particular r7-derived cell is circled) and there are no r7-derived neural crest cells in the third brachial arch. r6 and r7 neural crest cells intermingle in a common area within the fourth branchial arch (Fig. 10D; arrow).
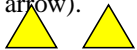
## DISCUSSION

The experiments presented here use a new in ovo imaging technique to follow the trajectories and migratory behavior of individual DiI labeled neural crest cells in the developing hindbrain. We followed

hindbrain neural crest cells throughout their migration, as they mix near the dorsal midline, exit the neural tube into the branchial arch streams, and populate the branchial arches. The trajectories of individual cells are much less stereotyped than may have been assumed, and demonstrate that the distinct streams of cells moving towards the branchial arches do not
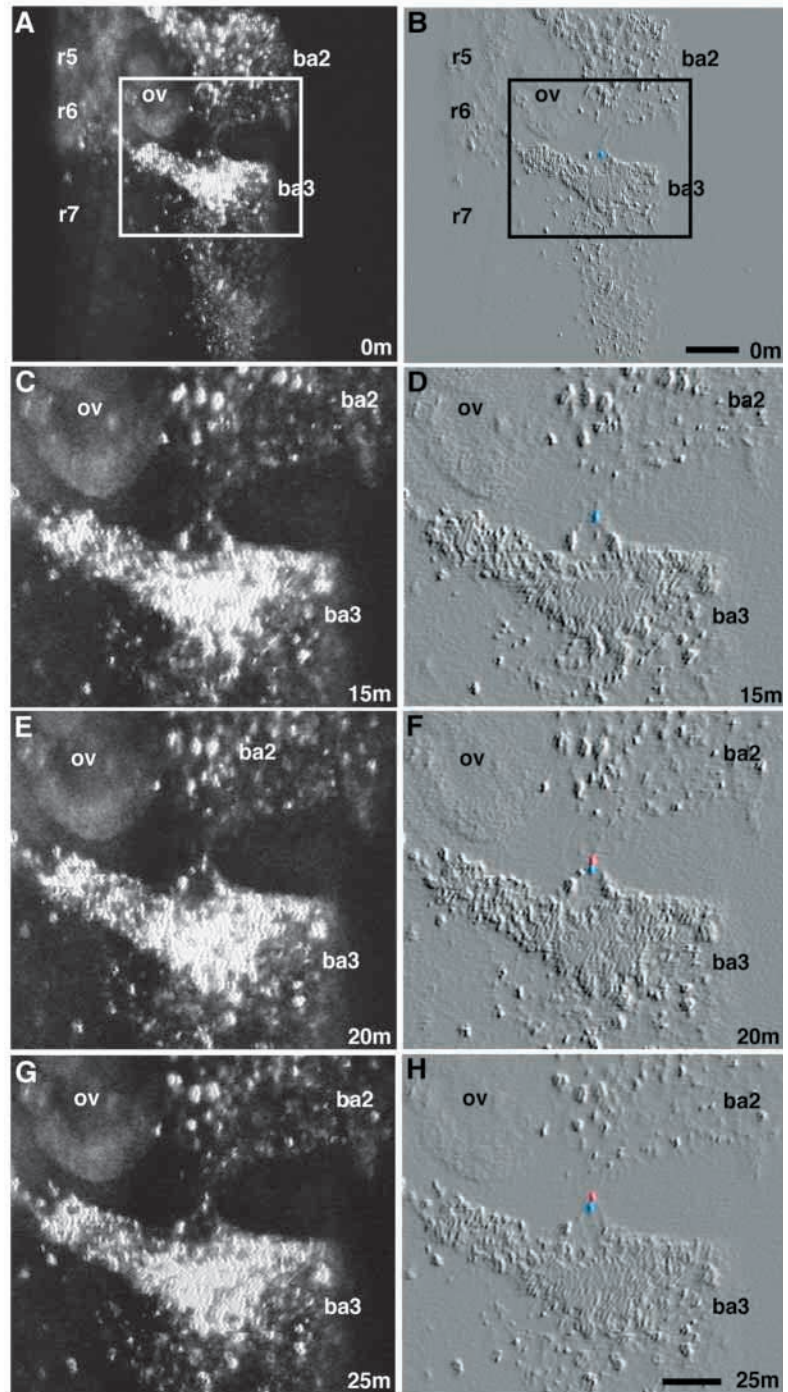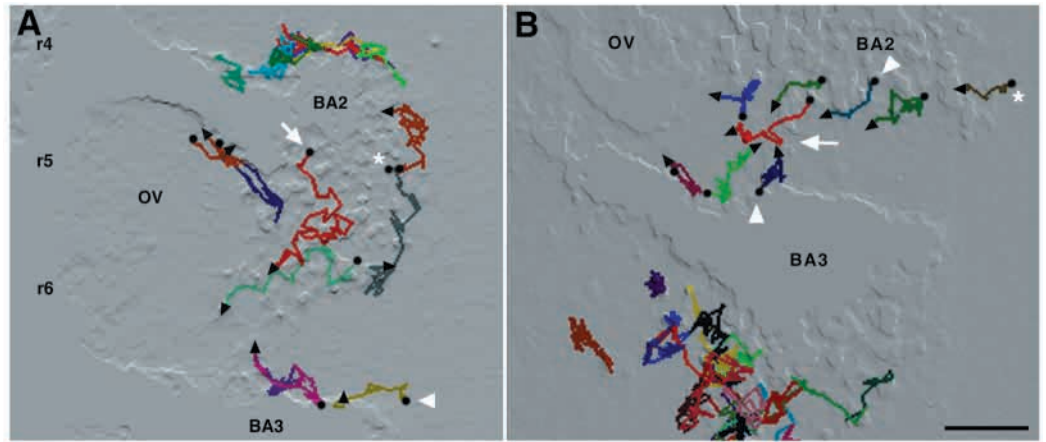


**Fig. 7.** (A,B) The box surrounds the region where hindbrain neural crest cells from neighboring migration streams are interacting. In B, we highlight a typical individual cell (blue colored) within the third branchial arch stream. The blue-colored cell migrates in the rostral direction to midway between the streams (C,D) and then divides (E-H) to produce a daughter cell (red colored). The scale bars are 100 µm (B) and 50 µm (H).

**Fig. 8.** Individual neural crest cell trajectories are plotted from cell movements during time-lapse imaging sessions. Cell tracks outline the pathways of individual neural crest cells in the region of the second and third branchial streams. Each colored cell trajectory is marked at the start (black circle) and end (black arrowhead) of the time-lapse sequence. The background image is the raw data of the fluorescently labeled neural crest cells, which has been embossed in Photoshop, at the end of the individual time-lapse sessions. (A) Individual neural crest cells migrate from the middle (arrow) and front (asterisk) of the second branchial arch stream to the region between the second and third branchial arch streams. Notice that neighboring cells may move in opposite directions (asterisk). Cells from the third branchial arch stream may also migrate backwards from the front of the stream (white arrowhead). The trajectories represent 3.5 hrs of time-lapse imaging. (B) The interaction between branchial arch streams takes place when individual neural crest cells from the second and third branchial arch streams (white arrowheads) move further into the region between the two streams (arrow). Cells outside of this region continue to migrate towards the site of interaction (asterisk). The trajectories represent 3 hours of time-lapse imaging. Scale bar, 50 μm.



result from strictly segregated behavior of the cells. Cells within a given stream come in contact with cells in other streams, and sometimes mix or intermingle with cells in other streams. These findings suggest that the patterned nature of hindbrain neural crest cell migration arises dynamically, and is shaped through interactions with other cells and with the environment surrounding the neural tube and along the migration routes.

Previous analyses of the final destinations of neural crest cells have led to the presumption that branchial arch streams are set up as distinct populations that remain segregated on their way to the branchial arches after being specified within the neural tube. In support of such a hypothesis, some cell heterotopic grafting studies appear to indicate that premigratory neural crest cells are determined in the neural tube to give rise to certain cell types and structures (Noden, 1983). The strong correlation between rhombomere boundaries and *Hox* gene expression patterns has been taken to indicate that axial level is specified by the stable expression of a combination of specific Hox genes (Hunt et al., 1991). The thought that the cells are pre-specified before they leave the neural tube is consistent with some early studies of the neural crest fate map, which suggested that a subset of rhombomeres give rise to the streams and that cells from r3 and r5 are eliminated by cell death (Lumsden et al., 1991; Graham et al., 1993). By imaging the labeled cells as they proliferate and exit the neural tube, more recent DiI-labeling studies show that the segmental pattern is not sculpted by cell death alone, but primarily by a deflection of the neural crest cells from r3 and r5 towards an adjoining rhombomere or exiting stream (Sechrist et al., 1993; Birgbauer et al., 1995). Time-lapse observations of neural crest cell trajectories both in explanted embryos (Kulesa and Fraser, 1998) and in ovo (present data) demonstrate conclusively that neural crest cells arise from all rhombomeres. Both time-lapse studies show that these neural crest-free zones result largely from a redirection of r3 and r5 neural crest cells to join neighboring exit streams.

The trajectories of the cells exiting the neural tube and coursing into the segmental stream offer some insights into the interactions that shape them. As shown in Fig. 3C, r3 neural crest cell trajectories become directional only after the cells enter the exiting streams. Within r3, neural crest cells meander and sometimes reach the lateral edge of the rhombomere. Cells that continue laterally either stop and collapse filopodia, or rapidly change direction towards a neighboring exit stream. These behaviors suggest the local action of a repulsant, reminiscent of the mechanism proposed to pattern trunk neural crest migration. Neural crest migration in the trunk is segmental (Rickmann et al., 1985; Bronner-Fraser, 1986), in which cells avoid migrating through the caudal halves of somites. The presence of Eph receptors and their ephrin ligands on neural crest cells and cells within the somites play a dominant role in directing trunk neural crest cells (Wang and Anderson, 1996; Krull et al., 1997) and may be involved in shaping distinct cranial neural crest cells streams. Indeed, complementary expression of Eph receptors and ephrins mediate cell sorting and restrict cell intermingling (Xu et al., 1999; Mellitzer et al., 1999); altering their function leads to a mixing of the second and third branchial arch streams in *Xenopus* (Smith et al., 1997).

The long-term nature of our in ovo neural crest cell trajectories allow us to catch transient events, and show that the neural crest cell free zones are not absolute; instead, they can be violated as the cells move laterally away from the neural tube. For example, neural crest cells from the second and third branchial arch streams show significant interactions after they migrate lateral to the otic vesicle (see Figs 6-9). More than half of the time-lapse recordings show cells interacting or crossing between the BA2 and BA3 streams. Even though the distance between the first and second branchial arch streams is larger, cells from both streams migrate into the intervening region suggesting similar processes take place in the r3 crest-free zone. Because the cells can migrate across the region between the streams and establish contacts, these "crest free" areas are

best viewed as being dynamically maintained. The most extreme case of streams being dynamic in nature comes from the separation of the third (BA3) and fourth (BA4) branchial arches. Distinct BA3 and BA4 streams are sculpted from a broad mass of migrating cells, and become apparent only after the neural crest cells migrate lateral to the otic vesicle (Fig. 10).

In light of previous hypotheses suggesting that neural crest cells are prespecified to populate a particular branchial arch, it is notable that cells cross between the branchial arch streams. Although the number of cells mixing between the branchial arch streams is small, any number of crossings is significant, as it shows that the streams are not absolute. It seems unlikely that this mixing is merely an error correction device, as some neural crest cells were seen to migrate into the region between the streams and divide; the daughter cells often moved into separate branchial arch streams (Figs 6-9). The cell-cell interactions and mixing of cells between streams raise the question of whether individual cells express a stable combination of genes related to their level of hindbrain origin. The evidence of mixing between branchial arch streams and the uniformity of gene expression patterns reported by others implies that the cells must alter their gene expression to conform with their branchial arch destination. In agreement with this, other studies imply that migrating neural crest cells have the ability to alter gene expression after leaving the neural tube (Prince and Lumsden, 1994; Nieto et al., 1995; Saldivar et al., 1996).

The present results do not argue against any role for the rhombomere of origin. Instead there may be a refined choreography that guides neural crest cells from neighboring rhombomeres into distinct subpopulations within a branchial arch. As shown in Fig. 9, neural crest cells from r6 contribute to the third and fourth branchial arches; whereas r7 neural crest cells migrate only to the fourth branchial arch. These distinct domains of mutual exclusion and of co-mingling raise the question of whether interactions between r6 and r7 neural crest cells are both competitive and synergistic. Cell ablation studies will be required to determine if the interactions are positive, negative or both, in a context-dependent
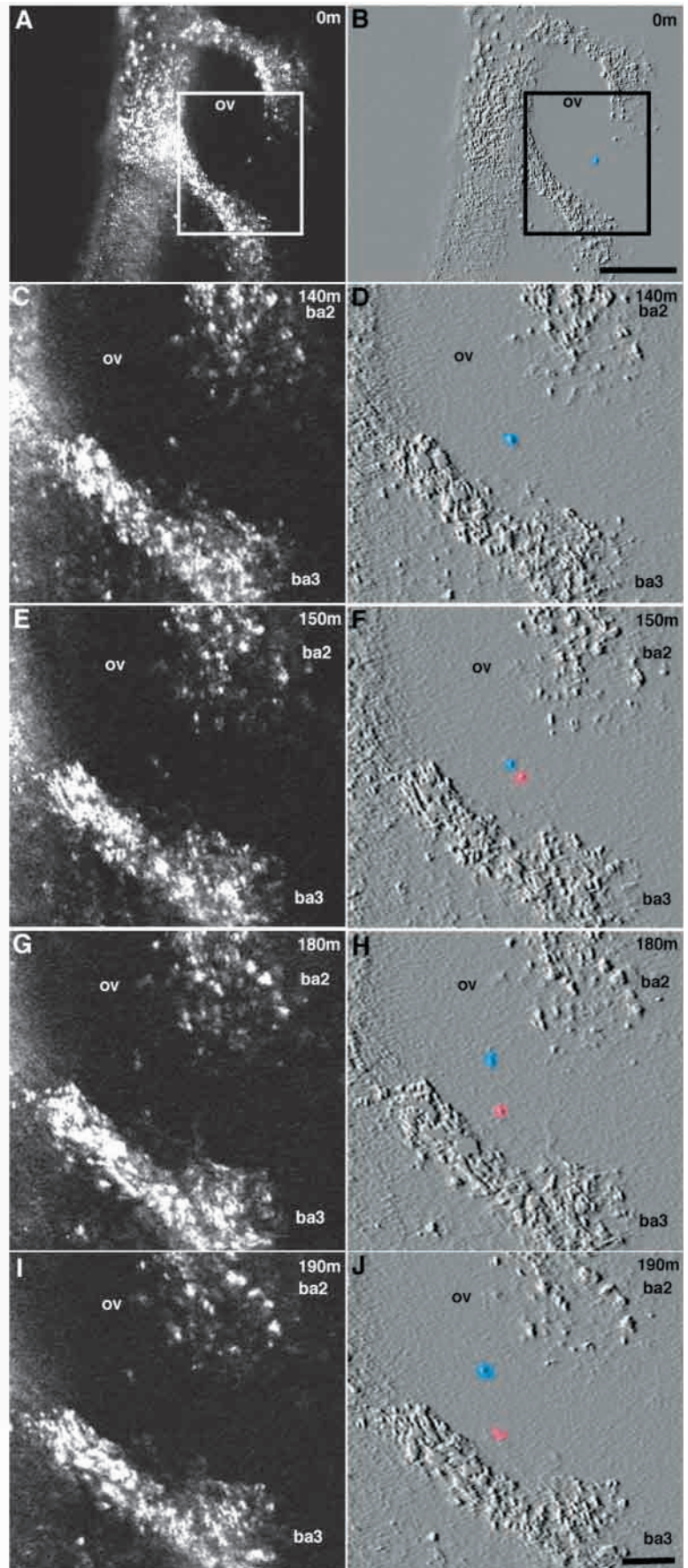
**Fig. 9.** Some individual neural crest cells mix between migrating streams. (A,B) Notice that the cell that originates from near to the second branchial arch stream has migrated into the region inbetween the two exiting streams. In (B), the cell has been highlighted in blue. The region within the box in A and B has been magnified in C-J. The blue-colored cell migrates closer to the third branchial arch stream (C,D) and then divides (E,F) to give rise to a daughter cell (red colored in F). The red-colored cell migrates in the caudal direction (G,H) and comes close to the third branchial arch stream while the blue-colored cell remains closer to the second branchial arch stream (I,J). Scale bars, 100 μm (B) and 50 μm (J).

**Fig. 10.** Selected time-lapse frames from a typical embryo injected with DiI (green) and DiD (red) to label premigratory hindbrain neural crest cells in rhombomeres (r) r6 and r7 are shown 24 hours after reincubation. The embryo has rotated and the dorsal neural tube is to the left. (A) The fluorescence images (DiI in B and DiD in C) have been overlaid onto a bright-field image to show the location of the injection sites and the distribution of labeled neural crest cells into the surrounding tissue and third (ba3) and fourth (ba4) branchial arches. Notice in A and B how the r6-derived neural crest cells are distributed laterally from the dorsal neural tube to the otic vesicle (OV) and around the caudal side of the OV. The migration stream of r6 neural crest cells splits into two distinct branches to contribute to the third and fourth (B, arrow) branchial arches. (C) r7-derived neural crest cells have migrated laterally to populate the fourth branchial arch (asterisk). Notice that only a few individual cells appear to be mixed in with the r6-derived neural crest cells prior to entering the arches (circled cell), and there are no r7-derived neural crest cells in the third branchial arch. (D) An overlay of the fluorescence images (B and C) points out the striking feature that the r7-labeled neural crest cells are co-localized with the r6-derived neural crest cells in the fourth branchial arch (arrow). An individual r7-derived neural crest cell (circled) lies mixed in with the r6-derived neural crest cells. Scale bar, 50 µm.
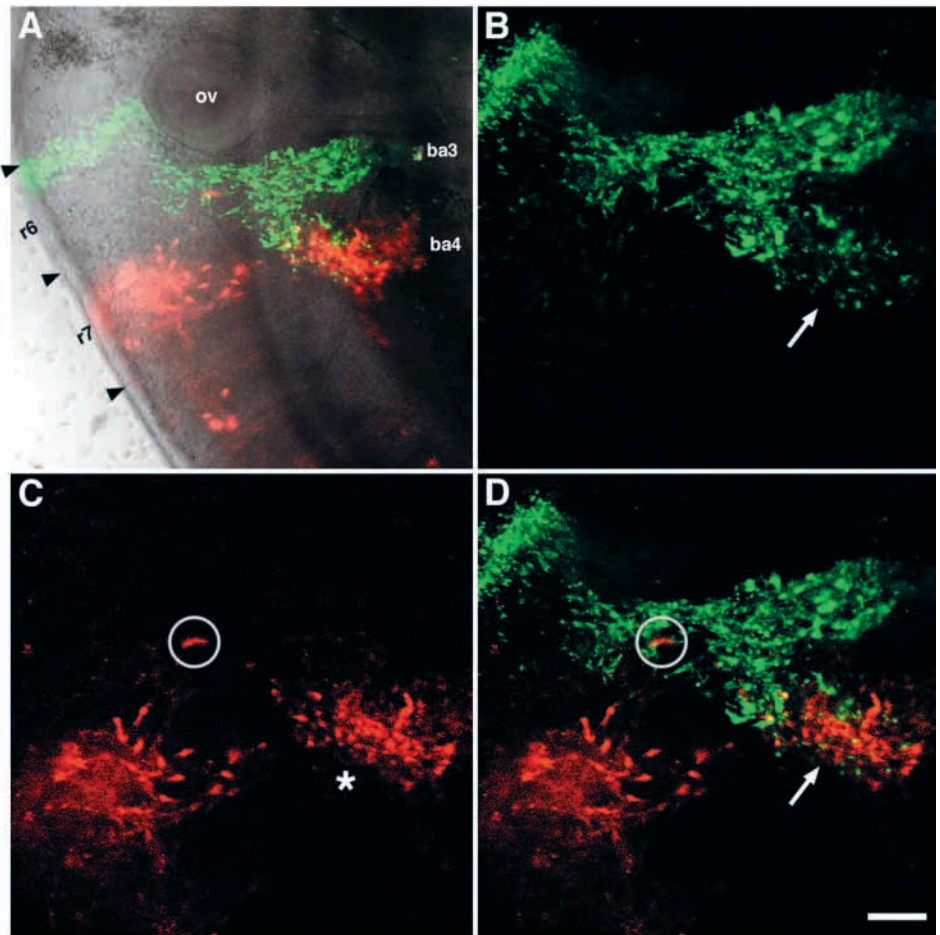


fashion (negative in BA3, positive in BA4). Our result, that the third arch is mostly void of r7-derived neural crest cells may have implications to long-term craniofacial patterning. During head patterning, hindbrain neural crest cells contribute to the visceral skeleton and connective tissue of the tongue and jaw. Long-term fate mapping studies suggested a reliable contribution pattern (Köntges and Lumsden, 1996), but considered the third and fourth branchial arch neural crest as a single population. Given our data on the distinct mixture of cells in the two arches, there may be further segregations involved in head patterning.

Our analyses of individual in ovo cranial neural crest cell trajectories and migration behaviors provide key information on the formation and maintenance of the apparently orderly migration pattern from the neural tube to the branchial arches. Near the neural tube, cranial neural crest cells mix and sort into segmental streams that extend laterally from the neural tube, suggesting that positive guidance cues influence the emigration of cells from the neural tube. Each segmental stream has contributions from more than one rhombomere, implying that the streams are not set up as distinct subpopulations. Individual cell trajectory data show that neural crest-free zones form after cells enter the tissue surrounding the neural tube, suggesting that repulsive cues act adjacent to r3 and r5 to segregate the streams. Enroute to the branchial arches, neural crest cells maintain a directed movement towards the branchial arches, but are free to migrate across regions between the streams and may even cross into other streams. Thus, branchial arch stream formation and migration are not deterministic, solely dictated by early events within the neural tube; instead, it seems wiser to consider it an ongoing process, formed and continually shaped through cell-cell and cell-environment interactions.

## REFERENCES

**Birgbauer, E., Sechrist, J., Bronner-Fraser, M. and Fraser, S. E.** (1995). Rhombomeric origin and rostrocaudal reassortment of neural crest cells revealed by intravital microscopy. *Development* **121**, 935-945.
**Bronner-Fraser, M.** (1986). Analysis of early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115**, 44-55.
**Bronner-Fraser, M.** (1993). Cell interactions in neural crest cell migration. *Adv. Dev. Biol.* **2**, 119-152.
**Erickson, C. and Perris, R.** (1993). The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. *Dev. Biol.* **159**, 60-74.

**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.

**Hamburger, V. and Hamilton, H. L.** (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.

**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 vertebrate head. *Nature* **353**, 861-864.

**Köntges, G. and Lumsden, A.** (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

**Krull, C., Lansford, R., Gale, N.W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E. and Bronner-Fraser, M.** (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7**, 571-580.

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

**LeDouarin, N. M.** (1982). *The Neural Crest.* Cambridge.: Cambridge University Press.

**Lumsden, A. and Keynes, R. J.** (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

**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.

**McGinnis, W. and Krumlauf, R.** (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

**Mellitzer, G., Xu, Q. and Wilkinson, D. G.** (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

**Nieto, M. A., Sechrist, J., Wilkinson, D. G. and Bronner-Fraser, M.** (1995). Relationship between spatially restricted Krox-20 gene expression in branchial arch neural crest and segmentation in the chick embryo hindbrain. *EMBO J.* **14**, 1697-1710.

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

**Prince, V. and Lumsden, A.** (1994). Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.

**Rickmann, M., Fawcett, J. and Keynes, R. J.** (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Embryol. exp. Morph.* **90**, 437-455.

**Robinson, V., Smith, A., Flenniken, A. M. and Wilkinson, D. G.** (1997). Roles of Eph receptors and ephrins in neural crest pathfinding. *Cell Tissue Res.* **290**, 265-274.

**Saldivar, J. R., Krull, C. E., Krumlauf, R., Ariza-McNaughton, L. and Bronner-Fraser, M.** (1996). Rhombomere of origin determines autonomous versus environmentally regulated expression of Hoxa-3 in the avian embryo. *Development* **122**, 895-904.

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

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

**Vaage, S.** (1969). The segmentation of the primitive neural tube in chick embryos (Gallus domesticus). A morphological, histochemical and autoradiographic investigation. *Ergbn. Anat. EntwGesch* **41**, 3-87.

**Wang, H. U. and Anderson, D. J.** (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-396.

**Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D.G.** (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-271.