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Rhombomeric origin and rostrocaudal reassortment of neural crest cells revealed by intravital microscopy

Eric Birgbauer[1,†], John Sechrist[2], Marianne Bronner-Fraser[2] and Scott Fraser[1,*]

[1]Division of Biology, California Institute of Technology, Pasadena, California 91125, USA
[2]Developmental Biology Center, University of California, Irvine, California 92717, USA

*Author for correspondence
†Present address: Department of Ophthalmology, University of California, San Francisco, CA 94143, USA

SUMMARY

Neural crest cell migration in the hindbrain is segmental, with prominent streams of migrating cells adjacent to rhombomeres (r) r2, r4 and r6, but not r3 or r5. This migratory pattern cannot be explained by the failure of r3 and r5 to produce neural crest, since focal injections of the lipophilic dye, DiI, into the neural folds clearly demonstrate that all rhombomeres produce neural crest cells. Here, we examine the dynamics of hindbrain neural crest cell emigration and movement by iontophoretically injecting DiI into small numbers of cells. The intensely labeled cells and their progeny were repeatedly imaged using low-light-level epifluorescence microscopy, permitting their movement to be followed in living embryos over time. These intravital images definitively show that neural crest cells move both rostrally and caudally from r3 and r5 to emerge as a part of the streams adjacent to r2, r4, and/or r6. Within the first few hours, cells labeled in r3 move within and/or along the dorsal neural tube surface, either rostrally toward the r2/3 border or caudally toward the r3/4 border. The labeled cells exit the surface of the neural tube near these borders and migrate toward the first or second branchial arches several hours after initial labeling. Focal DiI injections into r5 resulted in neural crest cell contributions to both the second and third branchial arches, again via rostrocaudal movements of the cells before migration into the periphery. These results demonstrate conclusively that all rhombomeres give rise to neural crest cells, and that rostrocaudal rearrangement of the cells contributes to the segmental migration of neural crest cells adjacent to r2, r4, and r6. Furthermore, it appears that there are consistent exit points of neural crest cell emigration; for example, cells arising from r3 emigrate almost exclusively from the rostral or caudal borders of that rhombomere.

Key words: neural crest, cell migration, rhombomere, intravital microscopy, chick

INTRODUCTION

The avian nervous system arises from the neural plate, which closes to form the neural tube, first in the rostral region and then in progressively more caudal portions of the embryo. In the head, the neural tube expands to form the brain, which partitions into the forebrain, midbrain and hindbrain. The hindbrain (rhombencephalon) is subsequently subdivided into seven or eight distinct neuromeres, the rhombomeres. The borders between adjacent rhombomeres restrict cell movement (Fraser et al., 1990; Birgbauer and Fraser, 1994), rendering each rhombomere as a somewhat separate developmental unit. These developmental subdivisions are paralleled by molecular specializations. For example, several antennapedia-class homeodomain (*Hox*) genes show patterns of expression with their rostral and/or caudal extents aligning with rhombomere (r) borders. Their expression pattern shows an overall rostral-to-caudal order that corresponds with their 3′-to-5′ sequence along the chromosome: *Hoxb-2* has its rostral border of expression at the r2/3 border, *Hoxb-3* at the r4/5 border, and *Hoxb-4* at the r6/7 border (Hunt et al., 1991; Keynes and Krumlauf, 1994). Paralogous *Hox* genes of the *a*, *c* and *d* clusters show similar, though not identical, patterns of expression in the hindbrain to that of the *b* cluster.

After neural tube closure in avian embryos, neural crest cells emerge from the neuroepithelium and begin their migration. In the hindbrain, neural crest cell migration is patterned into three broad streams of cells, emanating laterally adjacent to r1/2, r4 and r6; no neural crest cells are apparent adjacent to r3 or r5. Cells within the first stream populate the trigeminal ganglion and maxillomandibular arch; those within the second populate the hyoid arch, as well as the geniculate and vestibulocochlear ganglia; those following the third stream populate the third and fourth branchial arches and peripheral ganglia (the superior, petrosal and nodose; Noden, 1975; D'Amico-Martel and Noden, 1983; Lumsden et al., 1991). *Hox* genes are expressed in the cranial neural crest in a pattern similar to that observed in the neural tube; however, some genes expressed in r3 and r5 do not appear in the associated branchial arches. Although it has been proposed that this mismatch was resolved by r3 and

r5 failing to generate neural crest cells (Lumsden et al., 1991) or by their regional depletion due to apoptosis (Graham et al., 1993), focal DiI injections into individual rhombomeres demonstrate that all rhombomeres contribute to the neural crest. Those cells generated by r3 and r5 appear to deviate rostrally or caudally in their migration to join the streams mentioned above (Sechrist et al., 1993).

To date, the pathways of neural crest cell migration in the hindbrain have been mapped using static techniques that rely on examination of fixed embryos, thus providing no temporal resolution. Quail/chick grafting (Noden, 1983; Couly and LeDouarin, 1990), vital dye labeling (Lumsden et al., 1991; Sechrist et al., 1993) and antibody labeling approaches (Kuratani and Eichele, 1993; Sechrist et al., 1993) each, at best, permits the determination of the site of origin of labeled cells within the dorsal neuroepithelium of the rhombomeres and their final destinations in the branchial arches. Because a careful in vivo analysis of the initial and intermediate stages of migration has not been performed, little is known about the dynamics of cell movement either within the neuroepithelium or in the periphery. Thus, it remains uncertain why two different DiI fate-mapping experiments generated different results (Lumsden et al., 1991; Sechrist et al., 1993). Here, we use a refined dye-labeling method together with low-light-level imaging to follow migrating neural crest cells within living embryos (intravital microscopy). By using very small, focal injections of DiI, we can examine cell behaviors in defined subregions of each rhombomere. The results confirm our previous findings and track the dynamic movements of cells from r3 and r5 that contribute to the neural crest.

MATERIALS AND METHODS

Chick embryos

Fertile White Leghorn chicken eggs were obtained from Lakeview Farms. Eggs were incubated at 38°C on their sides in a rocking incubator for 30-40 hours until they reached the 7-12 somite stage (ss). The embryos were lowered by removing 1.5-2 ml of albumin through an 18-gauge needle, and a 1-2 cm diameter window was cut, with scissors, into the egg shell above the embryo. A 1:10 dilution of Pelikan Fount India Ink (in Howard's Ringer: 0.12 M NaCl, 1.5 mM $CaCl_2$, 5 mM KCl) was injected sub-blastodermally to permit the embryo to be visualized more clearly by epi-illumination. After staging according to the number of somite pairs, the vitelline membrane was gently torn open above the hindbrain with a tungsten needle. After dye injection, the egg was imaged, sealed with tape and allowed to incubate in a humidified, non-rocking incubator at 38°C for up to 24 hours.

Periodically during the incubation, each egg was removed from the incubator, the tape removed, and subjected to intravital microscopy (see below). In one set of embryos (n=11), the embryos were imaged five times, on average at 9ss, 11ss, 14ss, 16ss and 20ss. In all other cases, the embryos were imaged at least three times, on average at 10ss, 13ss and 18ss. The removal of the eggs from the incubator, and the repeated unsealing/sealing procedures did not appear to disrupt embryonic development; the average time required for the addition of new somites was 84±8 minutes, which is well within the normal range.

Focal iontophoretic DiI injections

DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate; Molecular Probes, Eugene, OR) was diluted in absolute ethanol to make a 0.5% (w/v) solution. The DiI solution was back-loaded into sharp injection micropipettes (5-10 MΩ resistance if filled with KCl), pulled from thin-walled aluminosilicate glass capillaries (with filament) using a Sutter P-80/PC Micropipette Puller. The micropipettes were held in a pipette holder (E. W. Wright) with a silver wire that was immersed into the DiI solution. After positioning the micropipette into the neural tube, DiI was injected iontophoretically (Myers and Bastiani, 1993) with a maximum of 90 nA for 2-10 seconds using a 9-volt battery through a 100 MΩ resistor. We labeled cells in the dorsal portion of the rhombomere, avoiding the most lateral borders of the neural tube, to circumvent the possible problem that injections performed too laterally may fail to label neural crest precursors. Injections in a variety of mediolateral positions generated neural crest (see Fig. 8, filled circles).

Intravital image acquisition and processing

Immediately after the injection and at indicated timepoints thereafter, the embryos were examined by intravital microscopy with a SIT camera (Hamamatsu) mounted on an epi-fluorescence microscope (Zeiss UEM). The output of the SIT camera was averaged (8 or 16 frames) to minimize noise with the assistance of an Imaging Technologies 151 processor and the VidIm software package (Belford, Stollberg and Fraser, unpublished). Images were stored on a laser disc recorder (OMDR, Panasonic). Simultaneous exposures were obtained for bright and fluorescence images. The overall anatomy of the embryo was observed with white light epi-illumination from a fiber optic illuminator, concentrated on the embryo with a small auxiliary lens placed 1-2 cm from the embryo. The DiI-labeled cells were visualized using an epi-illumination filter set designed for rhodamine. The intensity of the mercury light source was attenuated with neutral density filters and an iris diaphragm to a few percent of the burner's usual output. This attenuation and the use of electronic shutters (Uniblitz) on the epi-illumination light path kept the dose of light to a minimum, thereby avoiding possible phototoxic effects of viewing the labeled cells.

The resolution of the intravital images collected with a SIT camera was sufficient to resolve single labeled cells on the surface of the neural tube or in the early phases of neural crest migration. Light scattering by the intervening tissues sometimes made it impossible to image single cells in the later phases of migration, as they moved deeper into the embryo, without the use of confocal microscopy or cryostat sectioning. Light scattering from intensely labeled cells deep within the neural tube sometimes made the injection sites appear artificially enlarged. Such light scattering lacked cellular detail and, therefore, could not be confused with labeled neural crest cells.

After fixation, embryos were examined as whole-mounts on a BioRad MRC-600 laser-scanning confocal attachment mounted on a Zeiss Axiovert microscope. Filters and laser lines appropriate for rhodamine fluorescence were employed. The confocal microscope was used to collect a z-series of epifluorescence images at 5 μm intervals; this stack of images was projected computationally into a single plane using the CoMOS software package. Transmitted-light images showing the overall anatomy of the embryo were collected using either a bright-field or dark-field arrangement of the condenser.

For display and analysis, the bright-light images and the epi-illumination images were transferred to Adobe Photoshop for the Macintosh computer. The images were superimposed, scaled to the appropriate aspect ratio, and output using either Nikon or Kodak electronic image printers.

Fixation and cryostat sectioning

DiI-labeled embryos were fixed 12-24 hours with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). For cryostat sectioning, embryos were washed in 0.1 M PB for 1 hour and soaked in 5% and 15% sucrose solutions for 4-12 hours each at 4°C. Next, they were infiltrated with a solution of 15% sucrose and 7.5% gelatin (Sigma, 300 Bloom) for 4 hours at 37°C, and then embedded in fresh gelatin.

After the gelatin set, the embryos were kept in the refrigerator for up to 4 days before cryostat sectioning. Immediately before sectioning, embryos were quick-frozen in liquid nitrogen and allowed to equilibrate to −25°C prior to cutting transverse 10 μm sections on a Zeiss Microm cryostat. Sections of DiI-labeled embryos were viewed without a coverslip shortly after sectioning, but also could be stored with little degradation for several months in the refrigerator in a light-tight slide box.

RESULTS

Iontophoretic application of DiI to small numbers of dorsal neural tube cells

In previous studies (Sechrist et al., 1993), we used a pressure injection technique to apply DiI to the dorsal neural tube, and demonstrated that all rhombomeres produce neural crest cells. Those cells emerging from rhombomere (r) 3 and r5 appeared to deviate rostrally and caudally in their migration, whereas neural crest cells from r2, r4 and r6 appeared to move directly laterally. Because relatively large numbers of cells were labeled, it was difficult to deduce the trajectory of individual cells; furthermore, after incubation of the injected embryo, many of the cells that remained within the neural tube appeared to abut one of the rhombomere borders, leading to the criticism that the injections may not have been sufficiently refined to accurately map the contribution from individual rhombomeres.

In the present study, we circumvent these limitations by applying a more-refined DiI application technique, and examine the dynamics of neural crest cell movement, es-

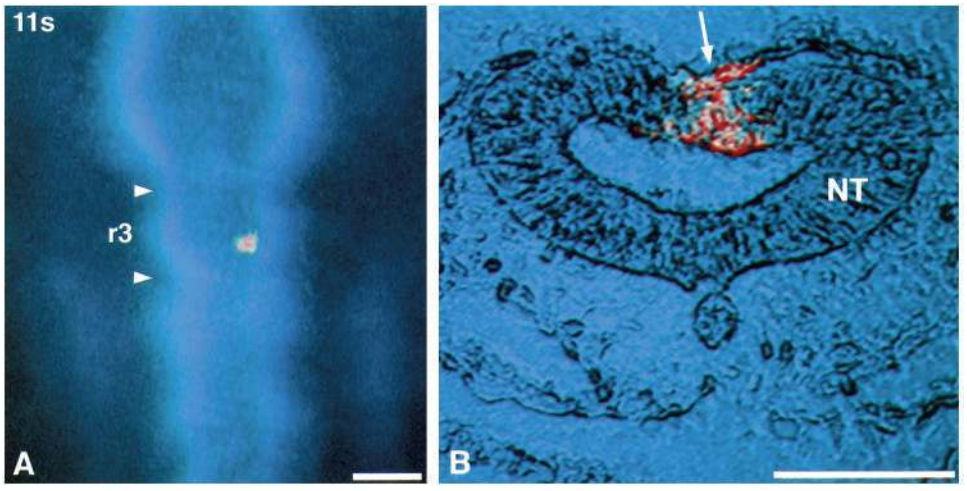

Fig. 1. An embryo injected into r3 at the 11 somite stage and fixed shortly thereafter. (A) A dorsal view of a living 11 somite (11s) stage embryo immediately after DiI injection into mid to caudal r3. The arrowheads indicate the borders of r3, and the embryo is oriented with rostral toward the top of the page. (B) A transverse section at the level of the injection site in the same embryo, fixed within 45 minutes of injection, illustrates that DiI-labeled cells (arrow) are localized in the dorsal midline of the neural tube (NT) as well as in the overlying ectoderm. In this and all subsequent figures, the stage of the embryo is given by its number of somites in the upper left corner of the panel, and the time after injection is shown in the upper right corner. Bars: 100 μm.

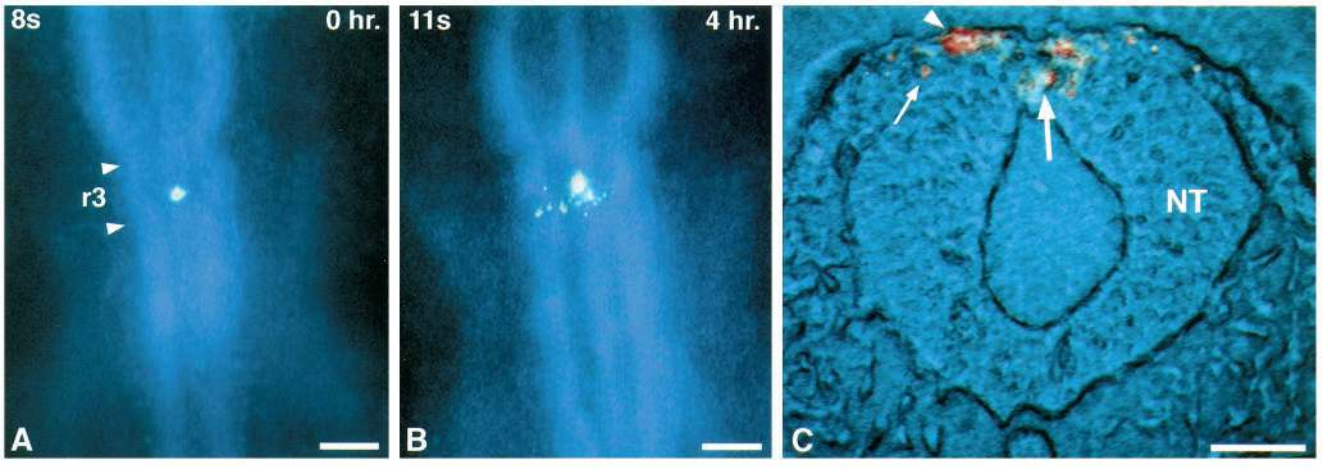

Fig. 2. An embryo injected into mid r3 at the 8 somite stage, imaged immediately and after 4 hours of incubation. (A) A dorsal view immediately after a mid r3 injection into an 8ss embryo (no. 29 in Table 1). Arrowheads indicate the borders of r3; rostral is oriented to the top. (B) The same embryo imaged 4 hours later at the 11ss shows labeled cells moving caudally and laterally close to the r3/4 border; the initial injection spot appears to be only a little more caudal than in A. (C) A transverse section through the same embryo, at the level indicated by the thin line in B, illustrates DiI-labeled cells in dorsal neuroepithelial cells (large arrow), a few migratory neural crest cells (small arrow) that have not yet moved beyond the lateral edge of the hindbrain, and some labeled cells in the ectoderm (arrowhead). NT, neural tube. Bars: A,B, 100 μm; C, 40 μm.

Table 1. Injection site, injection stage and final destination of labeled neural crest cells

Embryo no.	Position of injection	Injection stage (ss)	Stage at fixation (ss)	Neural crest migration (arch)†
	rhombomere 2			
1	caudal	10	20	1st
2	caudal	10	20	1st
3	caudal	9	16	1st
4	caudal	9	18	1st
5	caudal	9	17	1st
6	r2/r3 border	9	17	1st
	rhombomere 3			
7*	rostral	9	19	1st & 2nd
8	rostral	9	17	1st
9	rostral	10	21	1st & 2nd
10	rostral	10	16	1st
11	rostral	9	15	1st
12	rostral	9	19	1st
13	rostral	9	15	1st & 2nd
14	rostral	8	15	1st & 2nd
15	mid-rostral	8	16	2nd
16	mid-rostral	8	19	2nd
17	mid	9	14	2nd
18*	mid	8	17	2nd
19*	mid	11	18	2nd
20	mid	9	16	1st & 2nd
21	mid	9	18	2nd
22	mid	8	22	2nd
23	mid	8	20	2nd
24	mid	10	18	2nd
25	mid	9	16	none
26*	mid	9	18	2nd
27	mid	10	21	2nd
28	mid	8	18	2nd
29	mid	8	12	2nd
30*	mid	9	18	2nd
31*	mid	11	18	none
32*	mid-caudal	9	18	2nd
33	caudal	8	13	2nd
34	caudal	10	18	2nd
35*	caudal	9	18	2nd
36*	caudal	9	17	2nd
37	caudal	8	11	2nd
38	caudal	9	19	2nd
39*	caudal	8	18	2nd
	rhombomere 4			
40	mid	10	15	2nd
41	mid	11	18	2nd
42	mid	11	18	2nd
43	mid	9	16	2nd
44	caudal	10	18	2nd
45	caudal	11	24	2nd
	rhombomere 5			
46	rostral	11	21	2nd & 3rd
47	rostral	11	22	2nd & 3rd
48	rostral	10	18	2nd
49	mid-rostral	11	16	2nd
50	mid	10	21	2nd & 3rd
51	mid	11	18	2nd & 3rd
52	mid	11	17	2nd
53	mid	11	15	2nd
54	mid	10	18	3rd
55	mid	11	19	2nd
56	mid	8	17	none
57	mid	11	19	3rd
58	mid	11	18	2nd & 3rd
59	mid	11	17	3rd
60	mid	11	18	2nd & 3rd
61	mid	12	19	none
62	mid	10	20	2nd
63	mid	10	25	none
64	mid-caudal	11	22	2nd & 3rd
65	r5/r6 border	9	22	2nd & 3rd
66	r5/r6 border	10	16	2nd
67	r5/r6 border	10	16	none
	rhombomere 6			
68	rostral	11	18	3rd
69	rostral	12	23	3rd
70	mid	12	23	3rd
71	mid	10	20	3rd & 4th

*Embryos in which both dorsal and ventral dye injections were performed.
†Neural crest migration away from the neural tube is categorized by the arch or the associated ganglia forming region in which the neural crest cells were observed. Those cases in which no labeled neural crest cells were observed are marked 'none'.

pecially from r3 and r5. DiI was applied by focal iontophoretic injections to small numbers of cells (fewer than 10-20 cells in an area approx. 20-40 μm in diameter). Intravital, low-light-level video microscopy was used to visualize the fluorescent dye in living embryos three to five times as development proceeded, establishing not only the size and exact site of origin of the labeled population, but also their migratory trajectories. The distribution of the labeled cells immediately after injection was confirmed in some of the embryos using laser-scanning confocal microscopy and/or histological sections. Such examinations show clearly that injections could be made that were contained entirely within individual rhombomeres; in the images taken immediately post-injection, no overlap between labeled cells and the rhombomere borders was observed. Examples of embryos imaged as living whole-mounts and then fixed 0.5 and 4 hours after injection are illustrated in Figs 1 and 2, respectively. Transverse sections (Figs 1B, 2C) through these embryos reveal the presence of DiI in dorsal neural tube cells and neural crest cells. In some cases, label is visible within the ectodermal cells through which the injection pipette passed. These are easily distinguishable from the other cell types because of their characteristic position and morphology.

The dynamic nature of neural crest cell movement was analyzed by taking three to five images of the same living embryo as it developed. In every embryo, images were obtained immediately after injection to verify the location of labeling within the rhombomere. Any embryo in which the labeled cells were not initially restricted to the confines of a single rhombomere was discarded. On average, images were acquired at the 9 somite stage (ss), 11 ss, 14 ss, 16 ss and 20 ss (for embryos imaged five times) or 10 ss, 13 ss and 18 ss (for embryos imaged three times). The final image was obtained after fixation 14-24 hours post-injection on the confocal microscope. The data from all labeled embryos are summarized in Table 1 and Fig. 8.

Neural crest cell movement after focal DiI injections into r3

DiI injections into r3 (n=33; Table 1) were performed between the 8-11 somite stage (ss); our previous results demonstrate

that r3 no longer produces neural crest by the 12 ss (Sechrist et al., 1993). As injections in the rostral and caudal portions of r3 tended to give different patterns of cell movements, they will be presented separately.

Iontophoretic injections into the caudal or middle portions of r3 resulted in a small dye spot consisting of 10-20 labeled cells (Figs 1A, 2A, 3A) that increased in diameter over the next 4.5 hours (Figs 2B, 3B), reflecting cell dispersal and possibly some cell division. In addition, the population of labeled cells within the neural tube typically moved caudally, with some of the labeled cells encroaching on the r3/4 border (Fig. 2B). Few, if any, neural crest cells were seen any distance from the neural tube surface at this time. By 8.5 hours, the caudal shift in the labeled cells was still evident; labeled cells within and/or on the neural tube were primarily within the caudal portion of r3 as well as abutting the r3/4 border. In addition, there was a progressive increase in the number and extent of migration of DiI-labeled cells away from the neural tube (Fig. 3C). Many neural crest cells had emigrated from the r3/4 border of the neural tube and coursed laterally, rostral to the otic vesicle, apparently *en route* to the second branchial arch. After 13-14.5 hours, the pattern of labeled cells was quite similar to that at 8.5 hours, although cells had moved further ventrally into the region of the second branchial arch and the associated ganglia (Fig. 3D-G). Thus, labeled cells course caudally from injection sites in the mid or caudal regions of r3, leaving the surface of the neural tube, preferentially at the caudal border or r3, within hours after initial labeling.

A less dramatic, but somewhat reversed, movement of ectodermal and neural crest cells was observed after injections into the rostral portion of r3. Focal injections (n=8) into the rostral third of the r3 produced well-defined groups of labeled cells; Fig. 4A shows one group of labeled cells just caudal to the r2/3 border. After 6 hours, the dye-labeled cells had spread rostrally to encompass the r2/3 border, and, often, caudally to reach or pass the midpoint of r3. In the example shown (Fig. 4B), a small trail of DiI-labeled cells was visible leaving the neural tube and migrating rostrally. By the time

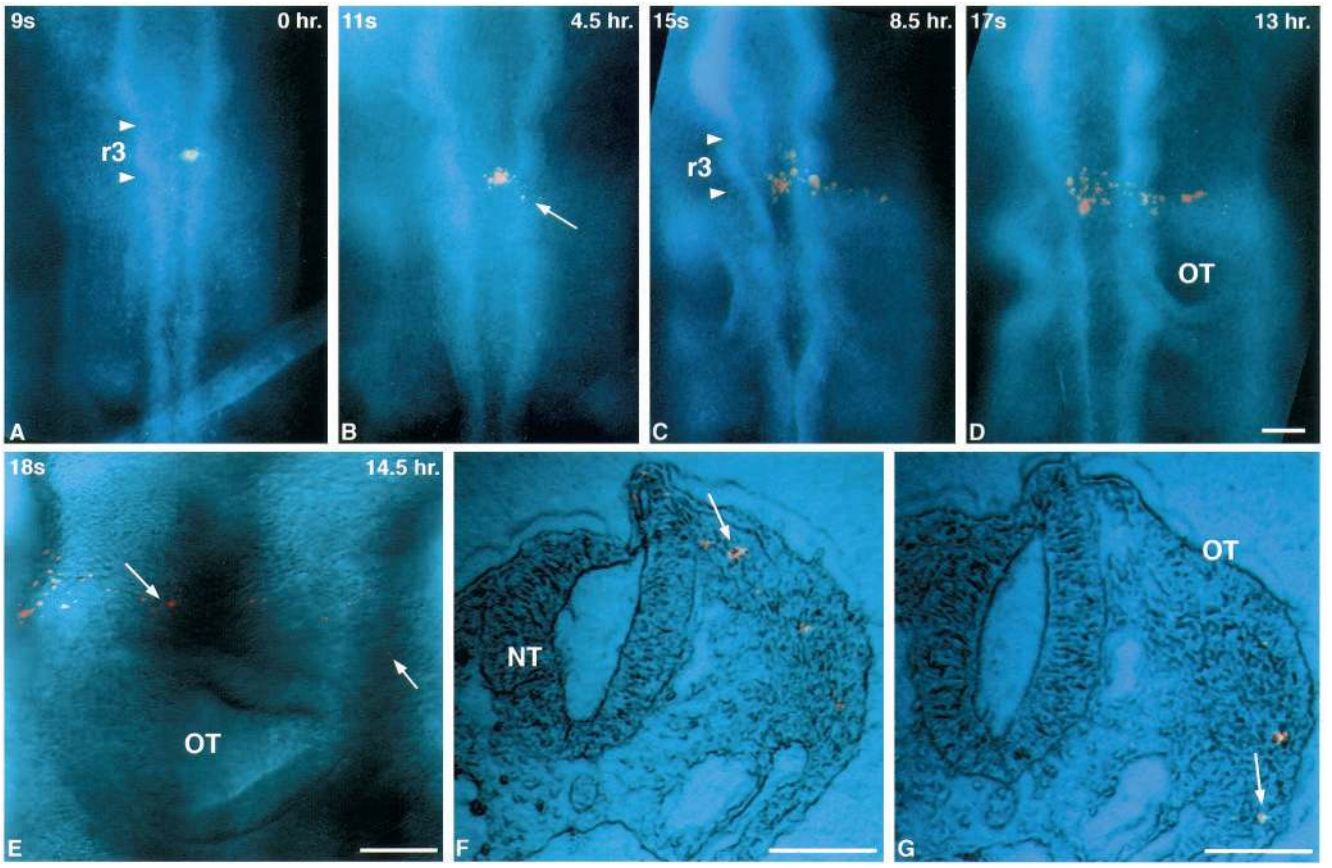

Fig. 3. An embryo injected into mid r3 showing caudal movement of the injection spot and of neural crest. (A) In this embryo (no. 21 in Table 1), injected at the 9ss, the initial injection site is clearly visible within the confines of r3. (B) By 4.5 hours after injection (11 ss), the injection spot has begun to shift caudally and appears more dispersed; a few individual cells (arrow) are visible near the r3/4 border. (C) By 8.5 hours after injection (15 ss), labeled cells have moved beyond the surface of the neural tube and toward the second branchial arch; the main dye spot is now level with the r3/4 border. (D) By 13 hours (17 ss), more labeled neural crest cells have dispersed and appear to penetrate further ventrally in front of the otic vesicle (OT). (E) Confocal image showing a lateral view of the embryo 14.5 hours after injection (18 ss), with numerous labeled neural crest cells (arrows) rostral to the otic vesicle and moving ventrally toward the second branchial arch. (F) Transverse section through the embryo 14.5 hours after injection, caudal to the original injection site, revealing DiI-labeling in migrating neural crest cells (arrow). (G) Another section more caudal (at the pre-otic level; note the edge of the OT in this section) through the embryo illustrates that labeled neural crest cells (arrow) have migrated ventrally and are entering the second branchial arch. NT, neural tube; OT, otic vesicle. Bars: 100 μm.

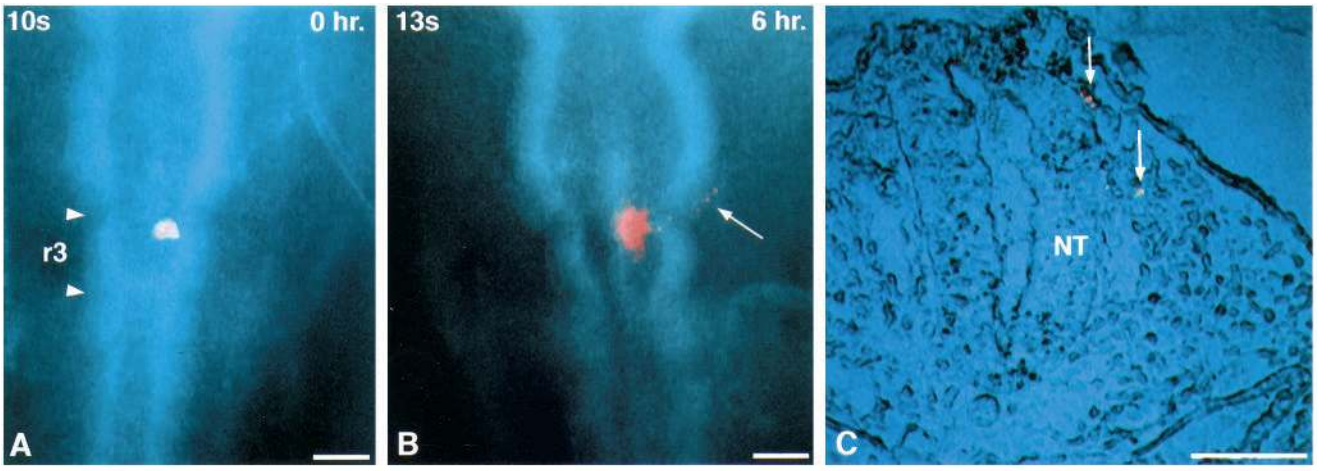

Fig. 4. An embryo injected into rostral r3 showing neural crest migration rostrally. (A) In this embryo (no. 10) injected at the 10ss, the spot of DiI is confined to rostral r3. (B) After 6 hours at the 13ss, the injection spot appears to have dispersed both rostrally and caudally. A single stream of neural crest cells (arrow) has moved laterally beyond the r2/3 border. (C) A transverse section through the level of caudal r2 in the same embryo later at the 16 ss confirms that some DiI-labeled neural crest cells (arrows) have migrated lateral to the neural tube (NT). Bars: 100 μm.

of fixation of this example (16 ss), this stream of cells was no longer visible in the whole-mount because it was too deep within the interior of the embryo; however, histological sections of the embryos clearly illustrated the distribution of the labeled neural crest (Fig. 4C). In other embryos (Fig. 5), rostrally moving cells were more obvious, and individual DiI-labeled cells, presumably neural crest cells, were observed spread over the surface of r2 and r3. Cumulatively, injections into rostral r3 produce neural crest cells that either leave the neural tube at the r2/3 border and migrate toward the first branchial arch (n=4), or move caudally as well, contributing labeled cells to both the first and second branchial arches (n=4; Table 1; Fig. 8).

Caudal shift of dorsal relative to the ventral neural tube cells in r3

The majority of injections into r3 labeled cells that later shifted caudally from the injection site (Table 1; Fig. 8). For example, 17 of the 18 embryos with injections in the middle region of r3 contained labeled cells that underwent a caudal shift, with many exiting the neural tube at the r3/4 border and migrating toward the second branchial arch. Even injections into the rostral region of r3 gave rise to some cells that coursed caudally (n=4 of 8). Interestingly, the group of cells that moved caudally from a rostral r3 injection included not only labeled neural crest cells but also labeled cells that remained within the neural tube (Fig. 5D); in contrast, the group of cells that moved rostrally consisted of only neural crest cells and ectodermal cells (Fig. 5B). There are two possible explanations for the apparent shift of the labeled cells and the preponderance of cells moving caudally; there may be: (1) a general rearrangement of all neural tube cells with respect to the rhombomere borders; or (2) a selective caudal-shifting of migrating neural crest cells and dorsal neuroepithelial cells toward the r3/4 border.

To test between these possibilities, we double-labeled some embryos, injecting DiI into both the ventral neural tube on the left side of the embryo and the dorsal neural tube, at the same rostrocaudal position, on the right side (Fig. 6A). As with dorsal injections, the ventral labeled region became larger and dimmer as the cells dispersed and divided; however, there was little, if any, net shift in the rostrocaudal position of the labeled region with respect to the rhombomere boundaries when examined at 6.5 (Fig. 6B) and 14 hours (arrow, Fig. 6C) after injection. In the same embryos, the labeled cells in the dorsal neural tube underwent a significant caudal shift relative to both the rhombomere borders and the labeled ventral cells. This shift was clearly evident after 6.5 hours (Fig. 6B), at which time the dorsal spot appeared to have moved approximately 50 μm caudally. At 14 hours, neural crest cells had dispersed over much of the surface of mid-r3 to rostral-r4. The labeled cells on the left side of caudal r3 (Fig. 6C) were not within the neural tube; instead, as confirmed in cryostat sections, they were more superficially positioned neural crest cells that had migrated across the midline from the dorsal injection on the right side. Of the 10 embryos with double DiI injections into r3 (denoted by * in Table 1), a distinct, systematic caudal shift of the cells that remained within the neural tube was noted in all but two. These were embryo 31, which gave rise to no labeled neural crest cells, and embryo 35, which gave rise to neural crest cells that subsequently moved caudally to the r3/4 border and migrated into the second arch, leaving the labeled cells within the neural tube in place.

To determine if a caudal shift is unique to r3, we performed similar DiI injections into r2, which is aligned with and contributes neural crest cells to the first branchial arch (n=6). In all cases, we observed ample migration of labeled cells into the first arch. In none of these embryos did the remnants of the injection site shift in position within r2, even in the one case in which there was a rostral dispersion of some labeled neural crest cells or ectodermal cells. Because this shift is restricted to dorsal neuroepithelial cells in r3, it cannot result from a general rearrangement or growth of the entire rhombomere or the remnants of the convergent-extension movements of neurulation (Schoenwolf and Sheard, 1990); instead, it may reflect a developmental program unique to r3.

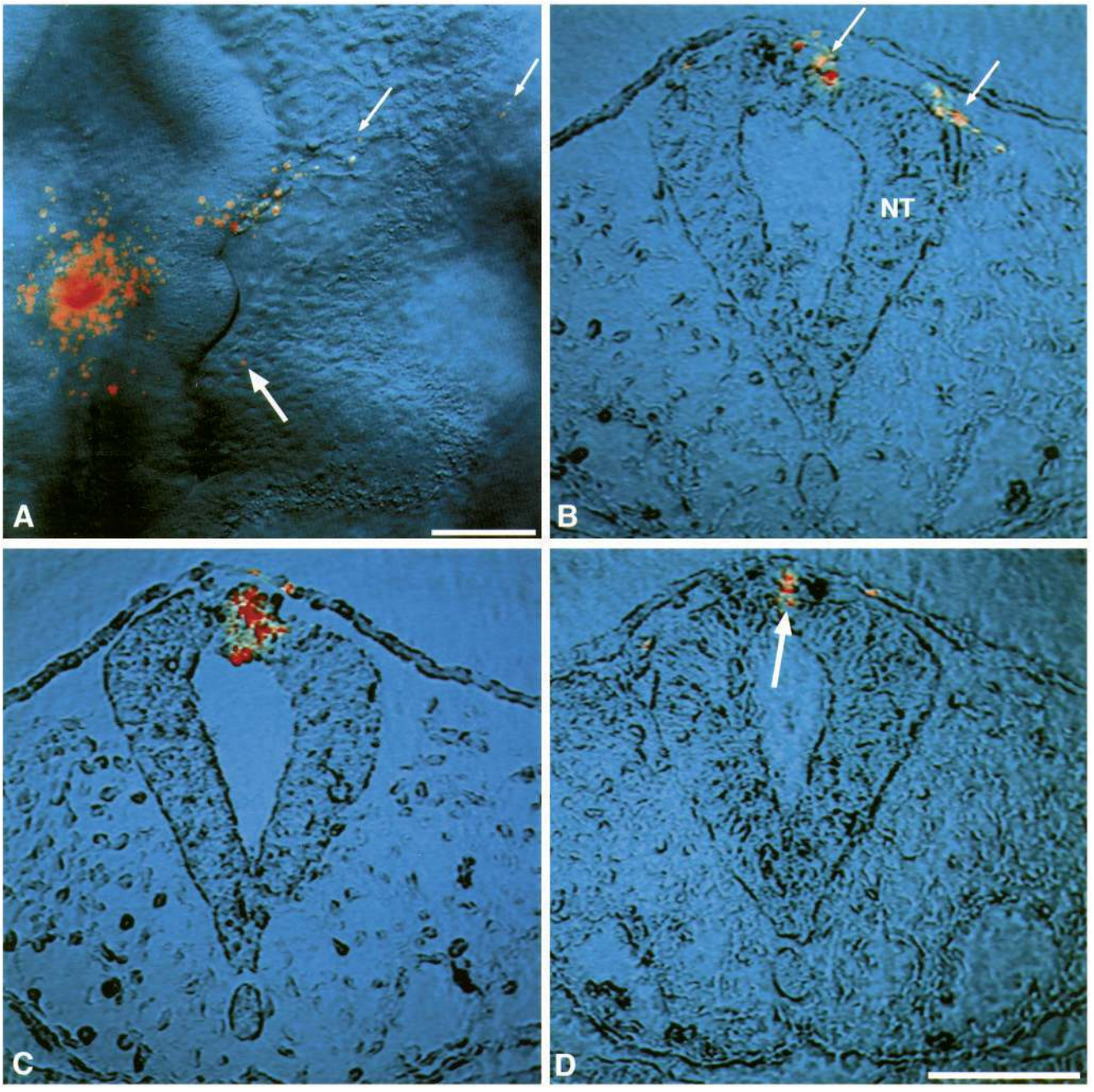

Fig. 5. Sections of an embryo injected into rostral r3 showing types of labeled cells at different rostrocaudal levels. (A) A confocal image of embryo no. 14 at the 15 ss (10 hours after injection at the 8ss) shows numerous DiI-labeled cells that have dispersed over r3. More neural crest cells have moved rostrally than in the embryo illustrated in Fig. 4, exiting the neural tube at the r2/3 border and moving toward and into the first branchial arch (small arrows). Note that a neural crest cell (large arrow) also appears to have exited the neural tube at the r3/4 border. (B) Transverse section shows that labeled cell types rostral to the injection site appear to be restricted to migrating neural crest cells (arrows) and ectodermal cells. (C) Transverse section at the injection site shows labeled neuroepithelial and ectodermal cells. (D) A transverse section caudal to the injection site shows DiI-labeled dorsal neuroepithelial cells (arrow) as well as neural crest cells and ectodermal cells. Bars: 100 μm.

Neural crest cell movement after focal DiI injections into r5

Our previous analyses showed that DiI-labeled cells emerging from both r3 and r5 migrated rostrally and caudally to contribute to the neural crest. To examine the behavior of neural crest cells derived from r5 in more detail, we performed focal iontophoretic injections of DiI (n=19). A representative embryo is illustrated in Fig. 7. Initially, a small group of labeled cells was observed within the confines of the rhombomere (Fig. 7A). After 4.5 hours, the injection spot had enlarged, suggesting some cell dispersion and/or proliferation (Fig. 7B). By this time point, several individual neural crest cells were evident migrating both rostrally and caudally away from the initial injection site. By 8.5 hours, a prominent stream

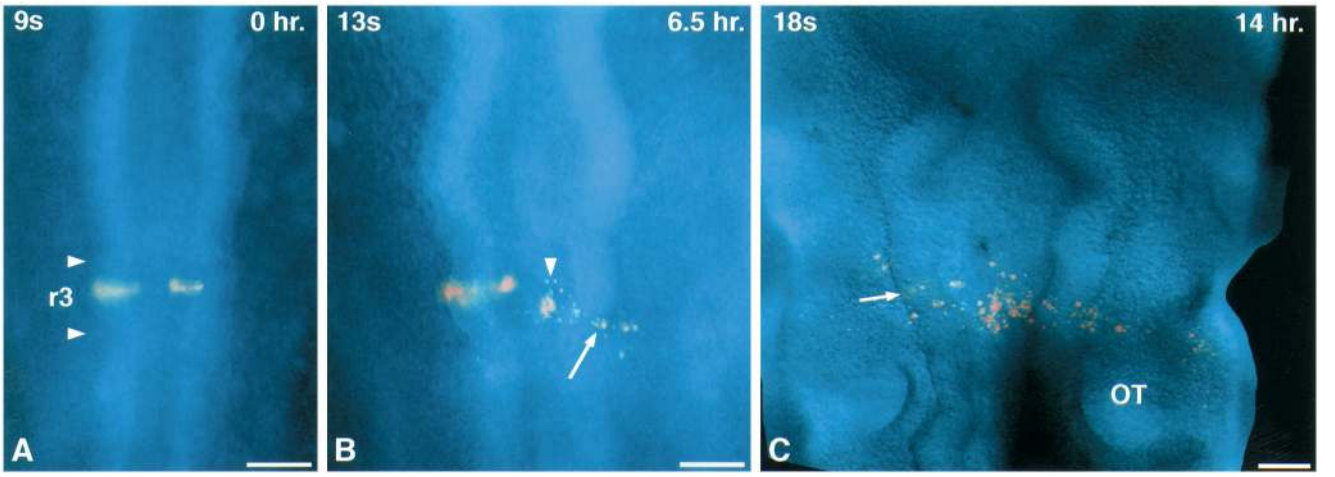

Fig. 6. An embryo that was double-labeled with DiI in both the ventral neural tube (left side) and dorsal neural folds (right side) showing caudal movement of dorsal cells. (A) In this embryo (no. 26) injected at the 9 ss, both dye spots appear to be in the middle of r3. (B) By 6.5 hours after injection (13 ss), the ventrally labeled cells remain at the same rostrocaudal position with respect to the rhombomere boundaries. In contrast, the dorsal injection spot has shifted caudally, toward the r3/4 border, and labeled neural crest cells (arrow) are seen, even more caudally, migrating away from the r3/4 border. A few scattered dorsal cells (probably ectodermal cells) have not shifted caudally (arrowhead). (C) By 14 hours after injection (18 ss), the dorsal dye spot appears to have dispersed and the labeled cells overlap the r3/4 border; numerous labeled cells have exited the neural tube surface and are migrating toward or are already in the second branchial arch. The ventrally labeled cells (arrow) are partially obscured by the migrating dorsal cells, but their position has been confirmed in transverse sections through this embryo. OT, otic vesicle. Bars. 100 μm.

of labeled cells can be could be moving rostrally (Fig. 7C) into the second branchial arch, and by 12.5 hours, a large caudally directed stream could be visualized immediately caudal to the otic vesicle and continuing toward the third branchial arch (Fig. 7D). When viewed by confocal microscopy (Fig. 7E) or in transverse sections (Fig. 7F,G) after 24 hours, this embryo had some DiI-labeled cells in the second branchial arch but many more in the third. There was no apparent shift in the position of the initial injection site in r5 as there was in r3.

In order to compare the pattern of neural crest migration in r3 and r5 with other rhombomeres, a few embryos (n=19 and previous studies; Sechrist et al., 1993) were injected with DiI at the level of r2, r4, the r5/6 border or r6. In all cases, the labeled neural crest cells exited from the neural tube and migrated laterally into the first, second, third and/or fourth branchial arches, respectively (Table 1; Fig. 8); there was no systematic shift in the position of the labeled cells that remained in the neuroepithelium near the original injection site.

Taking the data from all injections together, it seems clear that all rostrocaudal levels in the hindbrain can give rise to neural crest cells (Fig. 8). All but six injections gave rise to neural crest cells that migrated into the periphery. The two cases in r3 and the four in r5 (open symbols; Fig. 8) that failed to give rise to migratory cells cannot be interpreted with certainty. They may reflect injections that either missed the dorsal region of the neural tube that gives rise to the neural crest or labeled only the superficial ectoderm, injections that killed the labeled cells due to damage or over-labeling, or authentic regional variations in the origins of the neural crest.

DISCUSSION

The present study uses intravital microscopy to follow the migratory patterns and destinations of small numbers of labeled cells and their progeny in living chick embryos. This approach offers direct evidence for some interesting aspects of hindbrain neural crest development. First, our findings confirm and extend our previous conclusions that all hindbrain rhombomeres produce neural crest cells (Sechrist et al., 1993); although there appears to be no neural crest migration lateral to r3 and r5, the intravital microscopy performed here shows that neural crest cells arise from r3 and r5, only to deviate rostrally or caudally to contribute to the major migratory streams adjacent to r2, r4 and r6. As described previously (Sechrist et al., 1993), there were somewhat fewer neural crest cells arising from r3 than other rhombomeres; however, r5 produced numbers of cells comparable to those produced by the even-numbered rhombomeres. The positions of the labeled cells and their migratory directions are summarized in schematic form in Fig. 8. Second, the more refined injections used here document a sub-segmental organization to r3: the rostral third of r3 gives rise to neural crest cells that move predominantly rostrally, whereas the middle and caudal portions of r3 produce neural crest cells that move primarily caudally. In contrast, injections at various rostrocaudal levels of r5 labeled neural crest cells that migrate both rostrally and caudally. Another property, unique to rhombomere 3, is the significant shift of neuroepithelial cells in the dorsal neural tube caudally toward the r3/4 border.

The series of images collected for each embryo in this study by intravital microscopy offers a direct explanation for the differences between our previous analysis (Sechrist et al., 1993) and another performed using seemingly similar techniques (Lumsden et al., 1991). Lumsden and his colleagues (Lumsden et al., 1991) found little if any contribution to the neural crest after a focal injection of DiI into r3 or r5, and therefore proposed that the segmental pattern of migration in the

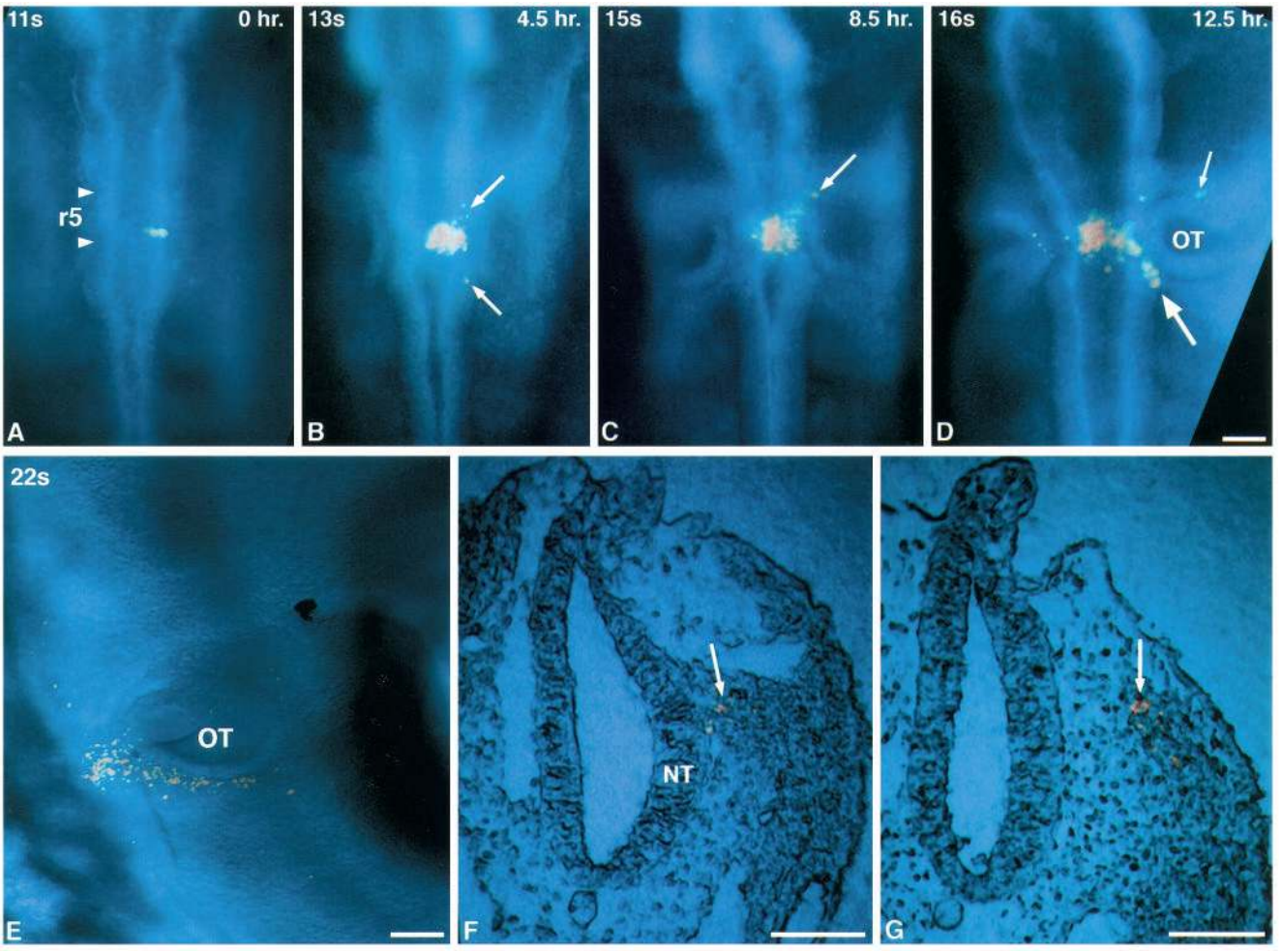

Fig. 7. An embryo injected into caudal r5 showing both rostral and caudal migration of neural crest. (A) In this embryo (no. 64) injected at the 11ss, the DiI is restricted to a small spot within the confines of r5 (arrowheads). (B) After 4.5 hours (13 ss), the spot has dispersed both rostrally and caudally. A few individual cells (arrows), presumably labeled neural crest cells, are visible both rostral and caudal to r5. (C) By 8.5 hours after injection (15 ss), a few more labeled cells (arrow) have moved beyond the surface of the neural tube and are migrating rostral to the otic vesicle. (D) By 12.5 hours after injection (16 ss), a prominent group of labeled neural crest cells (large arrow) is seen migrating caudal to the otic vesicle (OT). The rostrally migrating cells (short arrow) have moved into the second branchial arch. (E) Confocal image of the embryo at the time of fixation (22 ss), showing numerous DiI-labeled cells caudal to the OT that are approaching the third branchial arch; barely seen in this image are a few cells rostral to the otic vesicle. (F) Transverse section through the embryo fixed at the 22 ss, at a level just rostral to the otic vesicle, shows a few DiI-labeled cells (arrow) in the region of the forming auditory ganglion lateral to the neural tube (NT). About 12 cells were observed in this location in 4-5 sections. (G) Transverse section at a level caudal to the otic vesicle shows labeled neural crest cells (arrow) migrating toward the third branchial arch. Bars: 100 μm.

hindbrain must result from a regional absence of neural crest progenitors at these levels. In support of this proposal, they found zones of cell death that preferentially but not exclusively involve r3 and r5 (Graham et al., 1993). We find that the majority of neural crest emigration occurs prior to the period of cell death (i.e., before 11-12 ss), making it unlikely that cell death is the major determinant of the segmental pattern of hindbrain neural crest migration. The early redistributions of the labeled neural crest cells within the first few hours after injection demonstrated in the images presented here suggest an alternate interpretation of the data from Lumsden and colleagues (1991). In their study, the original site of DiI labeling was inferred in large part from the positions of the labeled cells at the termination of the experiment; those in which the labeled zone remained definitively within a single rhombomere were taken as successful injections. Our images show that small, focal injections, clearly positioned in the middle of r3 or r5, give rise to labeled progeny that move within and/or along the neural tube toward the boundary with the immediately rostral or immediately caudal rhombomere within the first few hours. This rapid redistribution was observed in all but the six injections from which no neural crest arose. In other words, the migratory pathways taken by the progeny of any successfully labeled neural crest progenitors in r3 and r5 moved them to or across the boundaries of the injected rhombomere. Therefore, virtually all of the cases in which neural crest progenitors were labeled in this study would have been misjudged to be 'bad' injections by the criteria of Lumsden et al. (1991).

Our analysis of the dynamics of neural crest migration indicates that the hindbrain neural crest emigrates in two

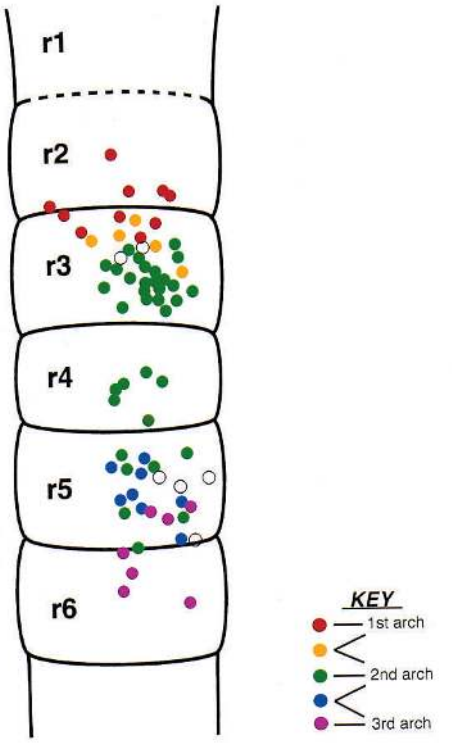

Fig. 8. Schematic diagram summarizing the locations of all injections and their trajectories toward the branchial arches. The positions are plotted as they would appear from above, and are positioned on the cartoon of the hindbrain using the percentage of the rhombomere length and width measured at the time of injection. The size of the dots is somewhat smaller than the actual size of the injection spot. The colors of the dots indicate the eventual distribution of the cells that arose from the injection: Red indicates contribution to the first arch only; yellow indicates contribution to both first and second arch; green indicates second arch only; blue indicates both second and third arch; purple indicates cells contributing to the third and/or fourth arch. Open circles indicate the 6 injections that failed to produce neural crest cells.

distinct manners. In the even-numbered rhombomeres, neural crest cells migrate predominantly laterally from the dorsal aspect of the neural tube. In r3 and r5, the cells first shift toward a neighboring rhombomere, where they move from the neural tube into the periphery. This initial migration either within or along the neural tube surface, rather than a direct entry into the cranial mesenchyme, could result from a variety of physical or chemical differences in the hindbrain. For example, the dorsolateral neural tube surface might be excessively adhesive in r3 and r5, favoring rostrocaudal motions along the dorsal margin. Another possibility is that the odd rhombomeres or adjacent ectoderm/mesenchyme might present an inhibitory substrate for neural crest cell movement. Such specializations might favor the neural crest cells remaining in contact with the neural tube surface instead of emigrating into the periphery until they reach the boundary with the next rhombomere. Evidence for some adhesive or cell surface differences has been noted between r3 and r5 and other rhombomeres. For example, grafting of r3 into other rhombomeres results in the formation of a physically distinct boundary zone, suggesting an adhesive mismatch (Guthrie et al., 1993); in addition, cell surface markers such as HNK-1 (Kuratani, 1991) and butyrylcholinesterase (Layer et al., 1988) are expressed in r3 and r5 but not in even-numbered rhombomeres. An alternative explanation is that physical interactions with surrounding tissues may contribute to the pattern of emigration. For example, the otic vesicle has been proposed to act as a mechanical barrier for migrating neural crest cells (Anderson and Meier, 1981), trapping those from r5 so that they must deviate around it rostrally and caudally (Sechrist et al., 1993, 1994). A combination of experimental manipulation of the ectoderm adjacent to the neural tube together with intravital microscopy of labeled neural crest cells should permit these and other suggestions to be addressed directly.

The migration patterns demonstrated in this study present a major challenge for understanding the significant differences between the complement of *Hox* genes expressed in the neural tube and the adjacent neural crest. *Hox* gene expression in r2, r4 and r6 is, in general, identical to that in neural crest derivatives within branchial arches 1, 2 and 3, respectively (Hunt and Krumlauf, 1991; Hunt et al., 1991). This one-to-one correspondence might be taken as evidence that axial identity is set within the neural tube and that neural crest cells arising from particular rhombomeres carry the appropriate *Hox* genes to their final sites. A complication to this hypothesis is provided by r3 and r5: *hoxb2* is expressed within r3, but does not appear to be expressed by the neural crest cells migrating to or present within the first branchial arch; similarly, r5 cells express *hoxb3,* but neural crest cells migrating into the second arch do not. Given that all rhombomeres contribute to the neural crest (data presented here and by Sechrist et al., 1993), the previous proposal that r3 and r5 fail to produce neural crest cells (Lumsden et al., 1991) cannot explain the differences in the gene expression patterns. There remain two possible explanations for the mismatch in the gene expression patterns between the odd-numbered rhombomeres and the branchial arches to which they contribute neural crest cells: First, there may be heterogeneity in gene expression within premigratory neural crest cells; for example, premigratory neural crest cells in rostral r3 might be *hoxb-2* negative. Second, there may be rapid gene regulation upon departure from the neural tube or during the early stages of migration; for example, all premigratory neural crest cells in rostral r3 might express *hoxb-2*, but it may be rapidly down-regulated upon emigration or migration into the r2 stream. Such rapid down-regulation of gene expression has been observed for *hoxa-2,* which is present in r2 but not in the newly emigrated neural crest cells from this rhombomere (Prince and Lumsden, 1994). It will be necessary to perform high-resolution, in situ hybridization in combination with dye-labeling to follow the rhombomeric origin of the neural crest cells to distinguish between these two possibilities.

Due to their superficial migration, particularly at early stages, it has been possible to visualize hindbrain neural crest migration in avian embryos despite the fact that the embryos are not fully transparent. By using a refined dye-labeling method, together with low-light-level imaging, we have followed migrating neural crest cells within living embryos to examine cell behavior in defined sub-regions of selected rhombomeres. Our results reveal that there is dynamic rearrangement of both neuroepithelial cells and neural crest cells in the hindbrain, particularly in r3, persisting until well after formation of rhombomere borders. The findings show clear inhomogeneities in the regions of the neural tube from which

neural crest cells emigrate, since cells arising from r3 emigrate almost exclusively from the rostral or caudal borders of that rhombomere. Therefore, performing a dynamic analysis of neural crest cell migration in the chick hindbrain has revealed interesting new properties of both neural crest cells and the rhombomeres from which they emerge. Previously, this type of study was only possible in more accessible lower vertebrates such as *Xenopus laevis* (Collazo et al., 1993) or zebrafish (Collazo, et al., 1994; Raible et al., 1994; Schilling et al., 1994). Based on these studies, it is clear that many aspects of neural crest migration differ markedly between lower and higher vertebrates, including the numbers of neural crest cells, their exact pathways of migration, and their extent of rostrocaudal dispersion. Therefore, it seems critical to apply dynamic analyses of cell migration to higher vertebrate embryos in order to determine which, if any, of these differences play critical roles in neural crest development.

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