

Vital dye analysis of cranial neural crest cell migration in the mouse embryo

GEORGE N. SERBEDZIJA^{1,*}, MARIANNE BRONNER-FRASER² AND SCOTT E. FRASER¹

¹Department of Biology, California Institute of Technology, Pasadena, CA 91125, USA

²Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA 92717, USA

*Author for correspondence

Summary

The spatial and temporal aspects of cranial neural crest cell migration in the mouse are poorly understood because of technical limitations. No reliable cell markers are available and vital staining of embryos in culture has had limited success because they develop normally for only 24 hours. Here, we circumvent these problems by combining vital dye labelling with *ex vivo* embryological techniques. To define better the nature of cranial neural crest cell migration in the mouse embryo, premigratory cranial neural crest cells were labelled by injecting DiI into the amniotic cavity on embryonic day 8. Embryos, allowed to develop an additional 1 to 5 days *ex vivo* in the mother before analysis, showed distinct and characteristic patterns of cranial neural crest cell migration at the different axial levels. Neural crest cells arising at the level of the forebrain migrated ventrally in a contiguous stream through the mesenchyme between the eye and the diencephalon. In the region of the midbrain, the cells migrated ventrolaterally as dispersed cells through the mesenchyme bordered by the lateral surface of the mesencephalon and the ectoderm. At the level of the hindbrain, neural crest cells migrated ventrolaterally in three subectodermal streams that were segmentally distributed. Each stream extended from the dorsal portion of the neural tube into the distal portion of the adjacent branchial arch. The order in which cranial neural crest cells populate their deriva-

tives was determined by labelling embryos at different stages of development. Cranial neural crest cells populated their derivatives in a ventral-to-dorsal order, similar to the pattern observed at trunk levels. In order to confirm and extend the findings obtained with *ex vivo* embryos, DiI (1,1-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate) was applied focally to the neural folds of embryos, which were then cultured for 24 hours. Because the culture technique permitted increased control of the timing and location of the DiI injection, it was possible to determine the duration of cranial neural crest cell emigration from the neural tube. Cranial neural crest cell emigration from the neural folds was completed by the 11-somite stage in the region of the rostral hindbrain, the 14-somite stage in the regions of the midbrain and caudal hindbrain and not until the 16-somite stage in the region of the forebrain. At each level, the time between the earliest and latest neural crest cells to emigrate from the neural tube appeared to be 9 hours. Some aspects of cranial neural crest cell migration were similar to those observed in avian embryos; however, the exact pathways, the timing and the axial levels that contribute to the neural crest cells were distinctly different.

Key words: cell migration, cranial, DiI, mouse, neural crest.

Introduction

In vertebrate embryos, cranial neural crest cells arise from the lateral edges of the neural folds, at the juncture of the neuroectoderm and the ectoderm (Horstadius, 1950; Vermeij-keers and Poelmann, 1980; Nichols, 1981, 1986, 1987; Tosney, 1982). In chicken embryos, ultrastructural studies suggest that emigration of cranial neural crest cells from the neural tube begins shortly after fusion of the neural folds (Tosney, 1982). The pathways of cranial neural crest cell migration have been studied most thoroughly in avian embryos using surgically created chimeric embryos (Johnston, 1966; Le Douarin, 1982; Noden, 1988). These studies

indicate that cranial neural crest cells migrate along a subectodermal pathway that extends ventrolaterally from the dorsal portion of the neural tube between the ectoderm and the underlying mesodermal mesenchyme. After migration, cranial neural crest cells give rise to a variety of craniofacial tissues (Johnston, 1966; Noden, 1975, 1983; Le Douarin, 1982; Tosney, 1982), including the sensory and parasympathetic ganglia, cartilage, bone, muscles and connective tissue of the face (Le Douarin, 1982; Noden, 1983).

In the mouse, a variety of genetic mutations exist that appear to affect neural crest cell migration and differentiation (Gruneberg and Truslove, 1960; Auerbach, 1954; see review by Morrison-Graham and Weston, 1989). By com-

paring neural crest cell migration and differentiation in these mutant embryos with that in normal embryos, it may be possible to learn much about the mechanisms involved in both processes. Unfortunately, we have only a limited understanding of cranial neural crest cell migration in the mouse, owing to the relative inaccessibility of the embryos to microsurgical and cell marking techniques. Our present knowledge of the pathways of migration derives from ultrastructural studies using either cellular morphology (Verwoerd and van Oostrom, 1979) or toluidine blue staining (Nichols, 1981, 1986) to identify neural crest cells. These studies suggest that cranial neural crest cells complete their emigration from the neural folds prior to neural tube closure in the head. Cranial neural crest cells are thought to migrate ventrolaterally along a subectodermal pathway, similar to the pathways observed in chick embryos (Johnston, 1966; Noden, 1975; Le Douarin, 1982). As migration continues, neural crest cells become indistinguishable, both morphologically and histologically, from cells of mesodermal origin; therefore, some method for marking neural crest cells is required in order to study the subsequent stages of cranial neural crest cell migration.

Cell marking techniques have been used to follow some aspects of cranial neural crest cell migration. The microinjection of gold-conjugated wheat germ agglutinin into the amniotic cavity of mouse embryos in culture has permitted the analysis of the early stages of migration (Smits-van Prooijie *et al.*, 1986; Chan and Tam, 1988). Wheat germ agglutinin adheres to cell surface glycoconjugates (Gesink *et al.*, 1983), thus labelling neural crest cells while they are contiguous with the ectoderm prior to neural tube fusion (Smits-van Prooijie *et al.*, 1986). Current culturing techniques permitted approximately 24 hours of normal development; therefore, this technique was useful for studying only the first day of cranial neural crest cell migration. Because of the nature of the label, one cannot rule out the possibility that it passed unequally to daughter cells or passed to unlabelled cells (Smits-van Prooijie *et al.*, 1986; Chan and Tam, 1988). Despite these potential limitations, these experiments clearly suggested that in the mouse, as in the chicken, cranial neural crest cells from the rostral hindbrain region migrate ventrolaterally along a subectodermal pathway and populate the trigeminal ganglia and the first branchial arch (Chan and Tam, 1988).

Previously, we labelled premigratory trunk neural crest cells within the neural tube of the mouse embryo by intraluminal injection of the fluorescent carbocyanine dye, DiI (1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Serbedzija *et al.*, 1990, 1991). Because DiI is lipid-soluble and hydrophobic, it incorporates nearly irreversibly into the plasma membranes of all cells that it contacts (Sims *et al.*, 1974). This dye does not spread from labelled to unlabelled cells, nor does it appear to have any adverse effects on neuronal or neural crest cells (Honig and Hume, 1986; Serbedzija *et al.*, 1989). However, mouse cranial neural crest cells begin migrating prior to the fusion of the neural folds (Verwoerd and van Oostrom, 1979; Nichols, 1981, 1986). This makes injection of DiI into the neural tube inappropriate for labelling this cell population.

Here, we report experiments performed using a modification of the DiI-labelling technique that permits identifi-

cation of premigratory cranial neural crest cells. DiI was injected into the amniotic cavity of a mouse embryo from which it labelled all cells in contact with the amniotic fluid, including the entire ectoderm, the neural plate and the neural folds. With further development, only the cells of neural crest and placodal origin become internalized. The embryos, maintained within the mother, remained attached to the uterus throughout the experimental period, utilizing the *ex vivo* technique first developed for work on the limb (Muneoka *et al.*, 1986). This avoided the limited developmental times over which whole embryo culture gives normal development and permits the analysis of cranial neural crest cell migration over extended periods. To confirm and refine some aspects of the migration observed in *ex vivo* embryos, premigratory neural crest cells were labelled by injecting small amounts of DiI directly into the forming neural crest of embryos maintained in culture. By using both the *ex vivo* and the *in vitro* labelling techniques, it was possible to determine the pathways of neural crest cell migration, the temporal pattern of neural crest cell emigration from the neural folds and the order in which neural crest cells populate their derivatives. While the general pattern of cranial neural crest cell migration was similar to that observed in avians, important differences were observed in the origin and the trajectories of the migrating neural crest cells.

Materials and methods

Animal preparation

Embryos were obtained by mating CD-1 females with BDF-1 males (Charles Rivers). In the evening, male and female mice were placed in the same cage, but kept separate by a gated partition. The gate was opened at 6:00 a.m. the following morning and the male and female mice were allowed to mate for three hours. The presence of a vaginal plug at the end of the three hour period was taken to indicate pregnancy, and the time and date that the gate was opened was designated embryonic day 0 (E0). This mating scheme reduced the normal variability in the actual time of mating as well as the range of developmental stages of the embryos within a litter. On E8, a pregnant female was anesthetized with avertin, prepared by mixing 0.5 g of 2,2,2-tribromoethanol (Chemical Dynamics Corporation), 0.31 ml of 2-methyl-2-butanol (Aldrich) and 39.5 ml of distilled water. The dosage in milliliters was determined by the formula: dosage = (0.1 + weight of the mother in grams/50). After deep anesthesia was achieved, the abdomen was swabbed with 70% ethanol and the uterus was surgically exposed. An incision was made in the wall of the uterus on the side opposite the uterine arteries. After the uterus was opened, the mothers were placed in a 37°C hood, where all subsequent manipulations took place.

Those embryos to be used for experiments in culture were removed and placed in dissecting medium consisting of 20% fetal bovine serum (Hyclone), 79% Dulbecco's modified Eagle's medium (DMEM, Whittaker's Bioproducts) and 1% penicillin-streptomycin L-glutamine (GPS, Whittaker's Bioproducts) at 37°C. These embryos were dissected to detach partially their extraembryonic membranes, but both the embryo and the extraembryonic membranes were left attached to the placenta for the entire culture period.

Embryos that were allowed to develop in the mother (*ex vivo* embryos) were left attached to the uterus with their extraembryonic membranes intact. After experimental manipulation of the

embryo, the mother's abdominal cavity was flushed with lactated Ringer's solution (Travenol) to prevent the *ex vivo* embryos from drying out or from adhering to other tissues (Muneoka *et al.*, 1986). The mother then was surgically closed with 5-0 silk (Ethicon) and allowed to recover. After 1 to 5 days of additional development, the mother was again anesthetized, killed, and the embryos removed for analysis.

Preparation of DiI for microinjection

All injections were made with an 0.05% solution (w/v) of 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes), made from an 0.5% stock solution in 100% ethanol, diluted immediately before injection 1:10 in 0.3 M sucrose solution. Micropipets were backfilled with DiI solution and attached to a picospritzer (General Valve). Both the dye and the micropipets were maintained at 37°C during the injections to avoid cold shock to the embryo.

Whole ectoderm labelling

The ectoderm of *ex vivo* embryos was labelled by inserting a micropipet through the decidua capsularis, yolk sac and the amnion into the amniotic cavity and expelling a small amount of dye (Fig. 1). To determine if either the injection or the *ex vivo* technique had any adverse effects on embryonic development, the experimental embryos were compared with embryos that were allowed to develop *in utero*. On the basis of both gross morphology and somite number, embryos that developed *ex vivo* were indistinguishable from embryos that developed *in utero*.

Because the extraembryonic membranes were left intact throughout the injection procedure, it was not possible to visualize the embryos and assess their developmental stage at the time of the injection. The stage at which an embryo was injected was deduced by extrapolating back from the elapsed time and the stage at which the embryo was fixed. Because somite development in the mouse takes approximately 1.5-2 hours per somite pair (Theiler, 1972), 12 to 18 additional somite pairs would be expected to form after an additional 24 hours of development. This estimate of the stage of labelling appeared accurate to within a range of six somites. Because this injection technique labels the neuroectoderm only in the regions of the neural tube that have not undergone neural tube fusion, it also was possible to deduce the stage of injection by observing which regions of the neural tube contained dye. Embryos labelled prior to the formation of the 7th somite (before the fusion of the neural tube begins) contained DiI label all along the neural tube. In embryos that were labelled after the formation of the 7th somite, DiI label was not present in the neural tube at the level of the 4th and 5th somites, where neural tube fusion begins. Embryos labelled after the formation of the 14th somite contained little or no DiI in the neural tube at the cranial levels.

To determine the length of time after injection that the dye remained in the amniotic fluid, several embryos were labelled by injection of the dye into the amniotic cavity *in vitro*. In all cases, the dye was visible at the time of the injection. However, when the amniotic sac was torn open 4 to 5 minutes after the injection, the dye was no longer visible in the amniotic fluid. This suggests that labelling is rapid, occurring transiently only at the time of injection, rather than continuously throughout the experiment. Even if dye were to remain in the amniotic cavity, no artificial labelling would result, as only the cells of the ectoderm and neuroectoderm are in contact with the amniotic fluid during the stages examined here. Although the mechanism responsible for the disappearance of the dye is unknown, two possible explanations are: (1) that the dye becomes sequestered into the cell membranes of the embryo and the extraembryonic structures and/or (2) that dye precipitates due to the salt content of the amniotic fluid.

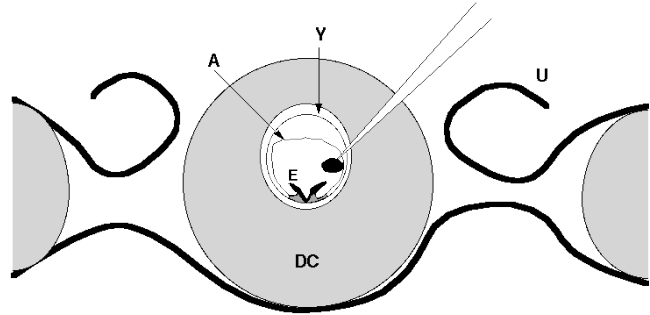


Fig. 1. Schematic diagram of the amniotic labelling technique. An incision is made in the wall of the uterus (U), which is deflected to allow access to the embryos. The micropipet is inserted through the decidua capsularis (DC), yolk sac (Y) and the amnion (A) into the amniotic cavity and a small amount of dye is expelled. The embryo (E) is labelled for orientation.

Regional labelling of premigratory neural crest cells

To label neural crest cells at specific cranial levels, a micropipet was inserted into the developing cranial neural folds of embryos in culture (using a micromanipulator; Marzhauser). When the pipet was positioned at the juncture of the neuroectoderm and the ectoderm, a small amount of the DiI solution was expelled. The fidelity of these injections was determined by examining the embryos both at the time of the injection and at the time of analysis. At the time of the injection, the labelled site was visible through the dissecting scope as a small red spot of dye in the tissue. At the time of fixation, the rostrocaudal extent of the dye within the neural tube was observed using an epifluorescence microscope. Only those embryos in which the DiI was contained within a single region (e.g., the midbrain) both at the time of injection and at the time of fixation were analyzed. Injections in the hindbrain were divided into two groups: (1) large injections, in which the dye spanned two or more rhombomeres; and (2) small injections, in which the dye was contained within a single rhombomere.

Neural tube labelling

In embryos that had undergone partial or complete neural fold fusion in the cranial region, the neural tube was labelled by inserting the micropipet into the lumen of the neural tube at the level of the midbrain using a micromanipulator. In most cases, enough dye was expelled to fill the neural tube.

Embryo culture

Embryos, with their extraembryonic membranes and placenta attached, were cultured for a total of 24 hours in tissue culture-treated cell wells (35 mm diameter; Corning) containing 4 ml of culture medium consisting of 25% rat serum, 25% fetal bovine serum, 49% DMEM and 1% GPS. The embryos were kept in an atmosphere of 5% CO₂ in air at 37°C. After 12 hours, an additional 4 ml of culture medium were added to each well.

Cultured embryos were compared with embryos allowed to develop to similar stages *in utero* to ascertain if the culture period itself affected embryonic maturation. On the basis of gross morphology and somite number, embryos cultured for 24 hours appeared similar to embryos developing *in utero*. Our results agree with previous studies that have shown that mouse embryos develop normally in culture for 24 hours after removal from the mother (New, 1973, 1977; Sadler, 1979; Sadler and New, 1981).

Histology

Embryos were fixed by immersion in 4% paraformaldehyde/0.25%

glutaraldehyde in 0.1 M phosphate buffer (PB) for 3 to 6 hours at 4°C. They were viewed in whole mount through a filter set designed for rhodamine using a laser scanning confocal microscope (Biorad MRC 600 on a Zeiss Axiovert microscope). This allowed the visualization of single focal planes without the interference of scattered light from outside the plane of focus (Fine *et al.*, 1988). Embryos were prepared for cryostat sectioning by washing in 0.1 M PB for 1 hour, followed by soaking in a 15% sucrose solution for 8 to 12 hours at 4°C. They were embedded in 15% sucrose and 7.5% gelatin (Oxiod), rapidly frozen in liquid nitrogen and serially sectioned on a cryostat at 30 microns (HM 500 M, Microm). Sections were coverslipped in gel/mount (Bio-media Corp.) and viewed on an epifluorescence microscope equipped with a light-intensifying camera (RCA-SIT) and an image processing system (Imaging Technologies Series 151), using the VidIm software package (S. E. Fraser, G. Belford and J. Stollberg, unpublished data).

Results

Amniotic injection of DiI

Premigratory neural crest cells were labelled by injecting a solution of DiI into the amniotic cavity of *exo utero* embryos on E8. Because DiI partitions into the membranes of the cells it contacts, injections of DiI into the amniotic cavity at this stage labelled both the ectodermal and the neuroectodermal cells. During neurulation, the neural tube and the neural crest were internalized, leaving the ectoderm (including the placodes) on the surface of the embryo, until approximately E9, when the first placodes invaginate. As a result of this segregation, the only labelled cells present in the mesenchyme were of neural crest or placodal origin. Embryos were maintained in the mother for 1 to 5 days. At the end of this period, the *exo utero* embryos were removed from the mother for analysis. Table 1 summarizes the numbers of embryos analyzed and the distribution of the DiI-labelled cells at the level of the first branchial arch; Fig. 9A schematizes the distribution of labelled cells in embryos for each of the stages examined. The findings are presented below according to the stages at which the embryos were labelled with DiI.

Embryos injected during the 0- to 6-somite stage

At the 6-somite stage, the neural folds have not fused, per-

mitting unhampered access of the DiI to the entire neuroectoderm. Embryos labelled prior to the end of the 6-somite stage and allowed to develop for 4 hours contained DiI-labelled cells in the ectoderm and neuroectoderm exclusively (Fig. 2). This confirmed that the technique labels only those cells in contact with the amniotic fluid. In embryos that developed for 24 hours after the injection, DiI-labelled cells were observed in the cranial mesenchyme and neuroectoderm in regionally distinct patterns (Fig. 3A). At the level of the forebrain, the DiI-labelled cells formed a sheet that extended ventrally from the dorsal portion of the neural tube over the region of the prosencephalon, including the optic vesicle. Transverse sections through the level of the optic vesicle revealed that these DiI-labelled cells were located in the mesoderm between the developing eye and the diencephalon (Fig. 3C). In the midbrain region, the DiI-labelled cells were dispersed individually throughout the mesoderm adjacent to both the ectoderm and the mesencephalon (similar to the pattern shown in Fig. 8B). At the level of the hindbrain, DiI-labelled cells were observed in three sub-ectodermal streams that extended from the fusion point of the neural tube to the distal end of the developing first, second and third branchial arches (Fig. 3B).

Embryos allowed to develop for 48 hours after injection contained DiI-labelled cells throughout the cranial mesenchyme at the levels of the forebrain, midbrain and hindbrain (Fig. 4A), in a pattern similar to that observed 24 hours after injection of DiI. In the forebrain region, DiI-labelled cells were located between the nasal pit and the eye. At the level of the midbrain, DiI-labelled cells were distributed individually throughout the mesenchyme adjacent to the mesencephalon. At the rostral hindbrain level, DiI-labelled cells were located in the region of the developing trigeminal ganglia and in the first branchial arch (Fig. 4B). At the level of the caudal hindbrain, DiI-labelled cells were observed in the second and third branchial arches. In transverse sections taken at the level of the otic vesicle, DiI-labelled cells were present in the developing nodose and petrosal ganglia.

Embryos injected during the 7- to 13-somite stage

After the formation of somite 7, neural tube fusion begins at the level of somites 4 and 5 from where it progresses both rostrally and caudally. Embryos analyzed 24 hours after labelling contained DiI-labelled cells at fore- and midbrain levels in many of the same locations described for embryos injected at earlier stages (Fig. 5A). The level of the caudal hindbrain contained few, if any, DiI-labelled cells, probably because the neural tube had fused there prior to the injection. At the level of the rostral hindbrain, the streams of DiI-labelled cells extended to, but not into, the first branchial arch. Only a few DiI-labelled cells were observed in the branchial arches in any of the embryos injected at this stage. In cross-section, DiI-labelled cells formed a subectodermal stream extending from the dorsal portion of the neural tube but stopping short at the level of the forming trigeminal ganglia (Fig. 5B).

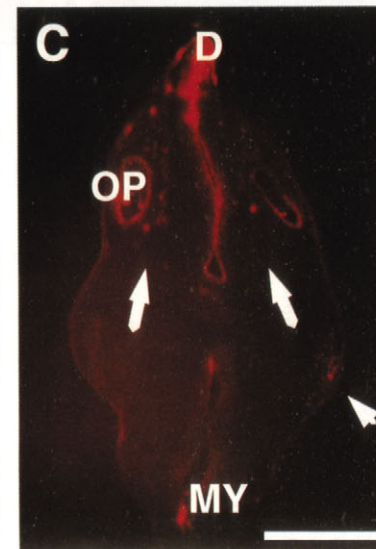
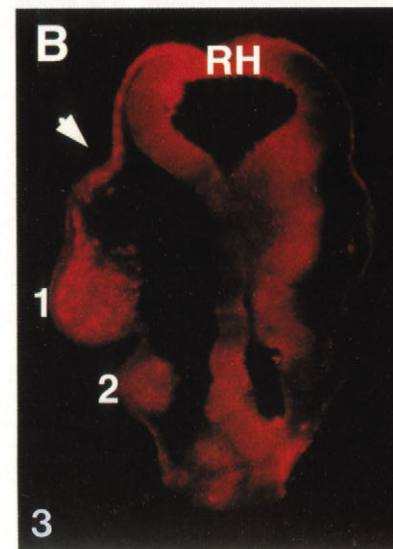
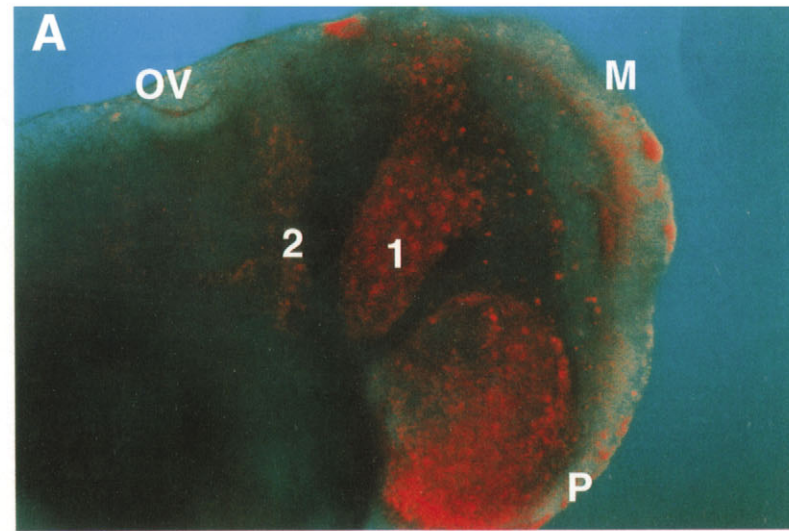
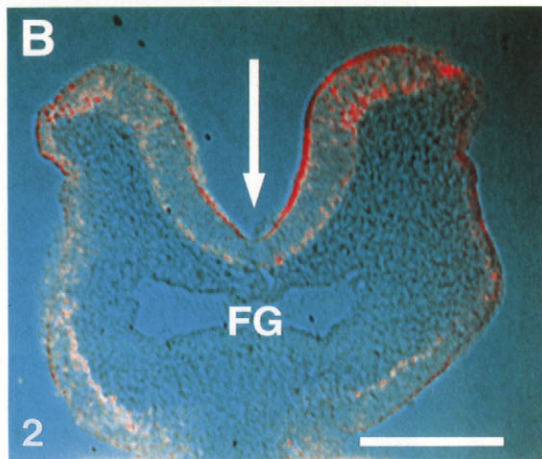
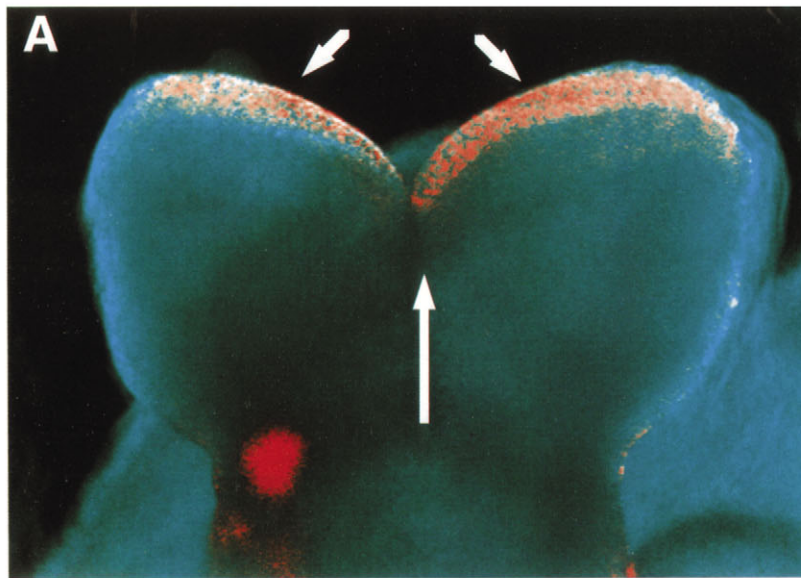
Forty-eight hours after the injection of DiI, the distribution of DiI-labelled cells was similar to that observed in embryos analyzed after 24 hours. Individual DiI-labelled cells were located in the forebrain and the midbrain regions,

Table 1. Summary of embryos analyzed after whole ectoderm labelling

Stage of injection	Hours of additional development	Numbers of embryos analyzed	Distribution of labelled cells at the level of the first branchial arch		
			DBA	PBA	TG
0 to 6 somite	24	17	+	+	+
	48	9	+	+	+
7 to 13 somite	24	15	-	+	+
	48	10	-	+	+
14+ somite	24	8	-	-	-
	48	6	-	-	+/-

DBA, distal portion of the branchial arch; PBA, proximal portion of the branchial arch; TG, trigeminal ganglia.

Fig. 2. Embryos that were analyzed 4 hours after injection of DiI into the amniotic cavity. DiI-labelled cells were observed in the neuroectoderm (short arrows) and the ectoderm, but not in the mesoderm. For orientation purposes, the neural groove (long arrow) and the foregut (FG) are indicated. (A) Whole embryo viewed with fluorescence and dark-field confocal microscopy. The large red patch is intense ectodermal labelling most likely caused by the injection. In sections, such patches do not extend into the mesenchyme. (B) Transverse section through the level of the mesencephalon



viewed with fluorescence and phase-contrast microscopy. Bars: A, 175 μ m; B, 100 μ m.

Fig. 3. Embryos labelled by amniotic injection of DiI at the 0- to 6-somite stage and maintained for 24 hours. (A) A fluorescent and phase-contrast confocal image of an intact embryo allowed to develop for 24 hours after being labelled with DiI. DiI-labelled cells were seen in the cranial mesenchyme and neuroectoderm in the region of the forebrain, midbrain and hindbrain. At the level of the forebrain, a sheet of DiI-labelled cells was observed that extended from the dorsal portion of the neural tube over the prosencephalon (P). At the level of the midbrain, individual DiI-labelled cells were dispersed throughout the mesenchyme overlying the mesencephalon (M). At the level of the rostral hindbrain, DiI-labelled cells were observed in a stream that extended from the fusion point of the neural tube to the distal end of the developing first branchial arch (1). In the caudal region of the hindbrain, two streams of DiI-labelled cells extended into the second (2) and third branchial arch, respectively. The otic vesicle (OV) is indicated for orientation. (B) In a transverse section through the dorsal portion of the first (1) and second (2) branchial arches, DiI-labelled cells filled both of the arches. A subectodermal stream of DiI-labelled cell (short arrow) extended from the dorsal portion of the rostral hindbrain (RH) to the proximal portion of the first branchial arch. (C) In a transverse section through the level of the optic vesicle (OP), diencephalon (D) and myelencephalon (MY), DiI-labelled cells were located in the mesoderm between the developing eye and the diencephalon (long arrows). A few DiI-labelled cells also were present in the dorsal portion of the first branchial arch, adjacent to the ectoderm (arrowhead). Bars: A, 200 μ m; B, C, 125 μ m.

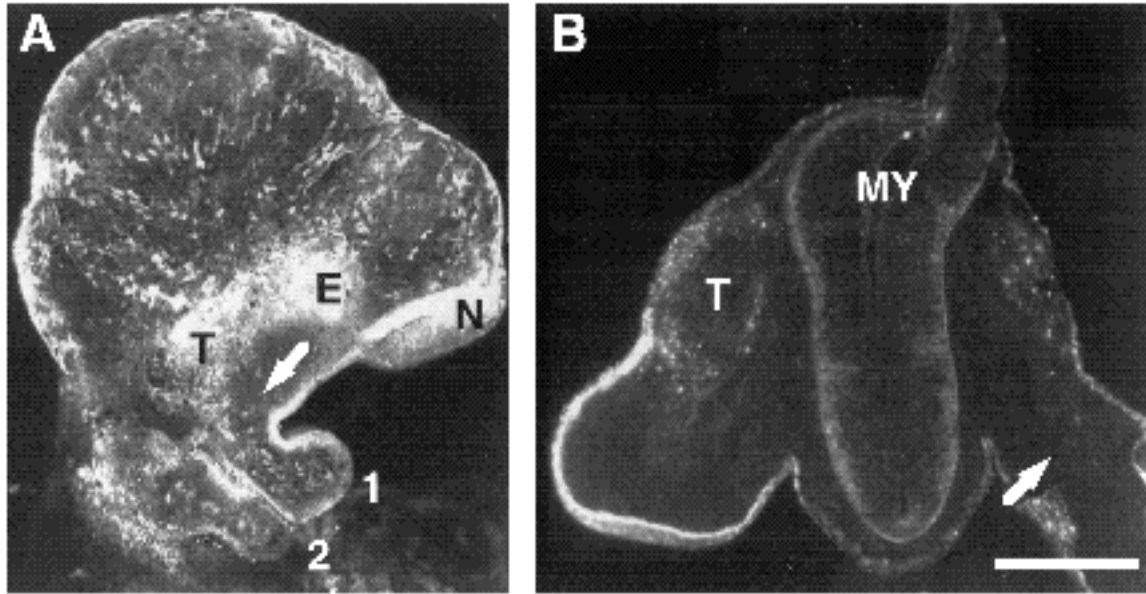


Fig. 4. Embryos labelled by amniotic injection of DiI at the 0- to 6-somite stage and maintained *ex utero* for 48 hours. (A) In this fluorescent confocal image, DiI-labelled cells were located between the nasal pit (N) and the eye (E). At the level of the midbrain, individual DiI-labelled cells were observed throughout the mesenchyme adjacent to the mesencephalon. At the level of the rostral hindbrain, DiI-labelled cells (arrow) were observed in the region of the developing trigeminal ganglia (T) and the first branchial arch (1), as well as in the first and second branchial arches (1 and 2). (B) In transverse sections through the level of myelencephalon (MY), DiI-labelled cells were observed within the developing trigeminal ganglia and in the first branchial arch (arrow). Bars: A, 375 μm ; B, 50 μm .

including the mesenchyme adjacent to the nasal pit, around the eye and overlying the mesencephalon. At the level of the rostral hindbrain, DiI-labelled cells were located at the level of the trigeminal ganglia but not in the branchial arches (Fig. 5C).

Embryos labelled at 14- or more somites

Neural tube fusion in the cranial region is completed at approximately the 16-somite stage. Embryos labelled after the formation of somite 14 and allowed to develop for 24 or 48 hours contained only a few DiI-labelled cells in the cranial mesenchyme. These few cells were adjacent to the dorsal portion of the neural tube. One embryo, labelled after the fusion of the neural tube was complete, contained a few DiI-labelled cells in the trigeminal ganglia. These DiI-labelled cells probably were of placodal origin since the neural crest cells are not labelled by amniotic injection after the fusion of the neural tube. The labelled processes of these trigeminal ganglia cells could be seen extending from the ganglia into the first branchial arch (Fig. 5D).

Long-term survival of embryos injected at E8

In order to identify the final destinations of the cranial neural crest cells, embryos were labelled by amniotic injection of DiI on E8 after which they were allowed to develop for 3 to 5 days before analysis. All of the somites are formed by embryonic day 11; therefore, it was not possible to deduce the exact stage at which these embryos were labelled on the basis of the exact number of somites present at the time of fixation. In addition, due to the size of the embryos and the increased opacity of their tissues, it was not possible to analyze the embryos in whole mount after

embryonic day 11. Therefore, all observations were made on sectioned material.

In all of the 22 embryos that were allowed to develop for 3 or more days after labelling, large numbers of DiI-labelled cells were observed in the trigeminal, the superior, the jugular, the nodose and the petrosal ganglia (Fig. 6). DiI-labelled cells also were observed in several neural tube-derived structures, including the olfactory bulb and the optic stalk. Unlike the neural derivatives, the mesenchymal derivatives of the neural crest contained only a few detectable DiI-labelled cells in embryos examined at E11 and no detectable DiI-labelled cells at later stages. This probably was due to dilution of the dye by the extensive cell division in the mesenchymal derivatives.

Regional labelling

In order to confirm the above results and to examine axial differences in the migration of cranial neural crest cells, subsets of the neural crest were labelled by focally injecting small amounts of DiI into the juncture of the neuroectoderm and the ectoderm. To permit adequate visualization and control of the injection site, these injections were performed in culture. In addition, this allowed the exact stage of the embryo at the time of injection to be determined. Two types of injections were made: (1) larger injections, which labelled most or all of a particular region (e.g. the entire midbrain or forebrain) and (2) smaller injections, which labelled relatively few cells and were contained within a specific region (e.g. in the hindbrain injection sites were contained within a single rhombomere). By virtue of their position, these injections labelled both the neural crest cell precursors within the neural fold and the newly emerg-

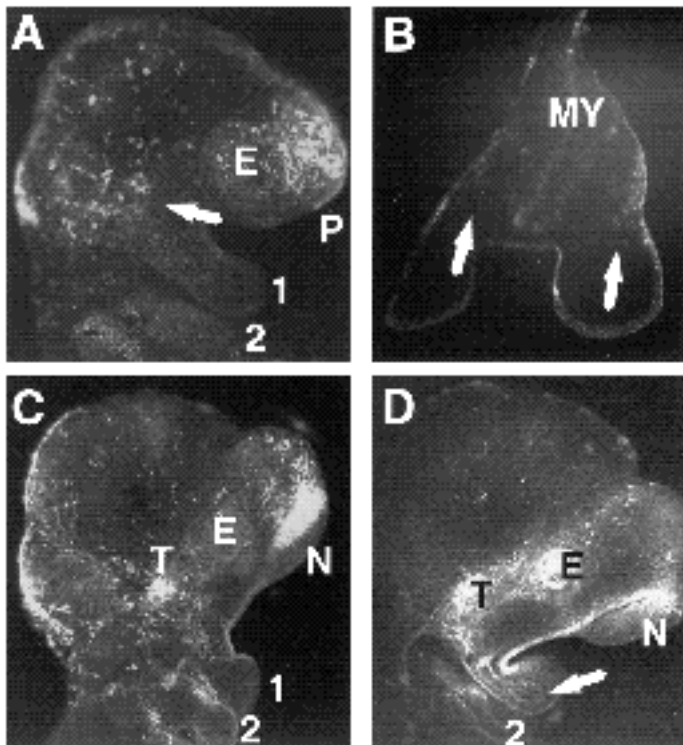


Fig. 5. Embryos labelled by amniotic injection of DiI at the 7- to 13-somite stage and maintained *ex vivo* for 24 and 48 hours. (A) After 24 hours, embryos contained a sheet of DiI-labelled cells extending temporally from the dorsal portion of the neural tube in the forebrain, over the prosencephalon (P) toward the developing eye (E). In the midbrain region, DiI-labelled cells were dispersed throughout the mesenchyme overlying the mesencephalon. In the hindbrain, DiI-labelled cells were present in the dorsal portion of the branchial arches (1 and 2), in the region of the presumptive trigeminal ganglion (arrow), and in the region overlying the mesencephalon. (B) A transverse section through the level of the first branchial arch and the myelencephalon (MY) contains DiI-labelled cells in the developing trigeminal ganglia (arrows). (C) Embryos that developed for 48 hours after the injection contained DiI-labelled cells in the mesenchyme adjacent to the nasal pit (N), around the eye (E) and overlying the mesencephalon. At the level of the rostral hindbrain, DiI-labelled cells were located in the trigeminal ganglia (T) and the dorsal portion of the branchial arches (1 and 2). (D) An embryo labelled by amniotic injection of DiI at the 14+ somite stage after fusion of the neural tube was completed up to the level of the midbrain, contained DiI-labelled cells in the trigeminal ganglia and DiI-labelled processes (arrow) extending from the trigeminal ganglion (T) into the first branchial arch. The eye (E), the trigeminal ganglion (T), the nasal pit (N), the prosencephalon (P), the myelencephalon (MY) and the first and second branchial arches (1 and 2) are indicated for orientation. Bars: A, 100 μm ; B, 50 μm ; C, D, 200 μm .

ing neural crest cells. Following injection, embryos were cultured for 24 hours before analysis. Table 2 summarizes the number of embryos analyzed and the presence of labelled cranial neural crest cells outside the neural tube; from these data, the stages when cells emigrate from the different axial levels of the neural crest were deduced (Fig. 9B). Table 3 summarizes the number of embryos that received injections into individual rhombomeres.

Table 2. Summary of embryos analyzed 24 hours after regional or neural tube labelling

	Stage at time of injection	Number of embryos analyzed	Migrating cells observed
Forebrain labelling	0 to 5 somite	5	+
	6 to 10 somite	10	+
	11 to 15 somite	11	+
	16+ somite	8	+/-
Midbrain labelling	0 to 5 somite	10	+
	6 to 10 somite	12	+
	11 to 15 somite	9	+/-
	16+ somite	3	-
Rostral hindbrain labelling	0 to 5 somite	7	+
	6 to 10 somite	13	+
	11 to 15 somite	10	-
	16+ somite	4	-
Caudal hindbrain labelling	0 to 5 somite	5	+
	6 to 10 somite	11	+
	11 to 15 somite	10	+/-
	16+ somite	6	-

Embryos injected at the 0- to 5-somite stage

Before the 5-somite stage, focal injections of DiI at any cranial level resulted in labelled cells in the cranial mesenchyme. With injections at the level of the forebrain, DiI-labelled cells were found a short distance from the neural tube toward the developing eye. Injections into the midbrain gave rise to dispersed labelled cells in the mesenchyme overlying the mesencephalon (Fig. 7A). Injections into the rostral hindbrain produced a stream of cells extending from the neural tube toward the developing first branchial arch (Fig. 7B). At the level of the caudal hindbrain, injections gave rise to a stream of labelled cells that extended into the second or the third branchial arch. For example, injections slightly caudal to the level of the otic vesicle gave rise to a stream of labelled cells extending into the third branchial arch (Fig. 7C). The distribution of labelled cells in each region was similar to that observed in embryos labelled by amniotic injection at comparable stages.

Embryos injected at the 6- to 10-somite stage

All focal injections at this stage produced DiI-labelled cells that migrated away from the site of the injection. Injections at the level of the forebrain gave rise to DiI-labelled cells in the region of the developing eye. Embryos labelled at

Table 3. Summary of embryos analyzed after individual rhombomere labelling

Stage of injection	r1	r2	r3	r4	r5	r6	r7	r8
6 to 10 somite	3	3	2	1	NA	2	3	2
11 to 15 somite	NA	4	1	2	2	1	2	2

r, rhombomere.

Fig. 6. This fluorescent and phase-contrast image of a transverse section through the level of rhombomere 6 of an embryo allowed to develop for 3 days. DiI-labelled cells are observed in the superior (S) and petrosal (P) ganglia. In addition, a few DiI-labelled cells were observed in the mesenchyme, adjacent to the anterior cardinal vein (AC). The neural tube (NT) is indicated for orientation. Bar, 150 μ m.

Fig. 7. Fluorescent and phase-contrast confocal images of embryos labelled in specific regions of the neural folds at the 0- to 5-somite stage. (A) Injection into the midbrain at the 4-somite stage gave rise to DiI-labelled cells dispersed throughout the mesenchyme overlying the mesencephalon (M). (B) Embryos labelled in the rostral hindbrain at the 4-somite stage contained a sheet of DiI-labelled cells extending from site of injection (arrow) into the first branchial arch (1). (C) Injections into the caudal hindbrain at the 5-somite stage gave rise to cells extending from the site of injection (arrow) into the third branchial arch. For orientation purposes, the prosencephalon (P), mesencephalon (M), otic vesicle (OV), heart (H) and first branchial arch (1) are indicated. Bars: A, B, C, 250 μ m.

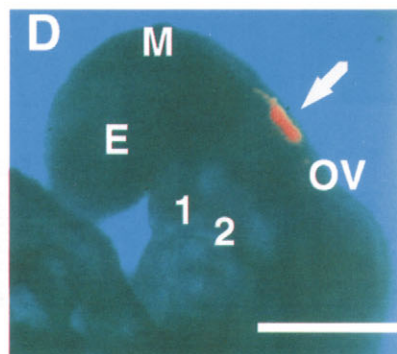
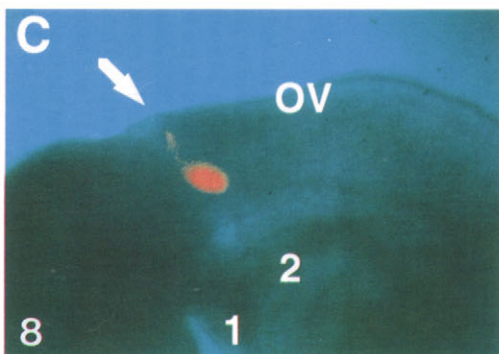
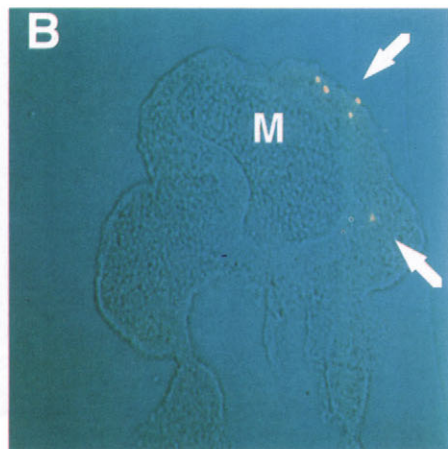
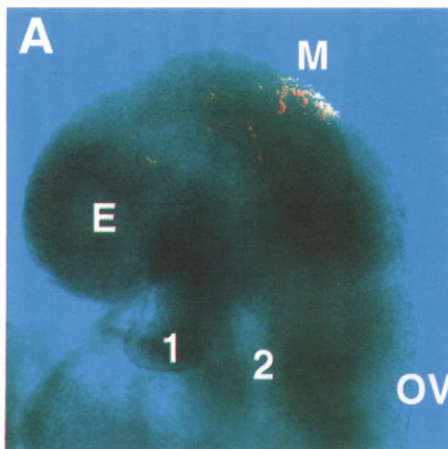
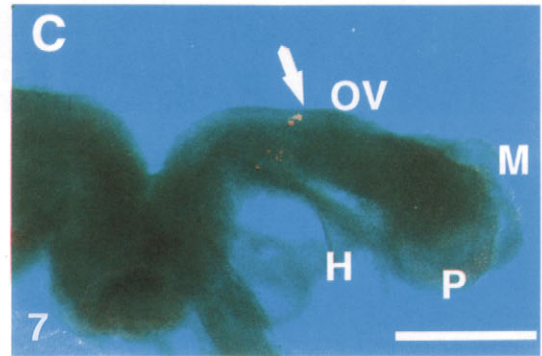
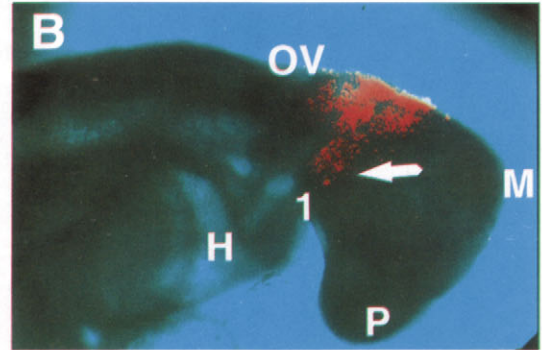
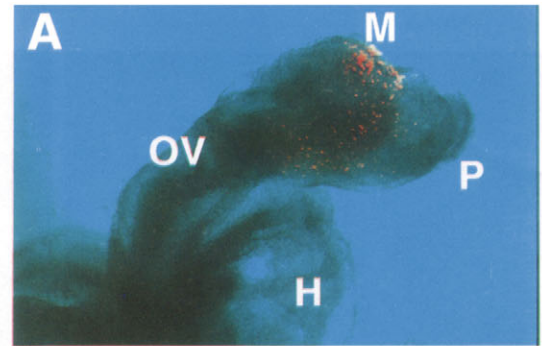
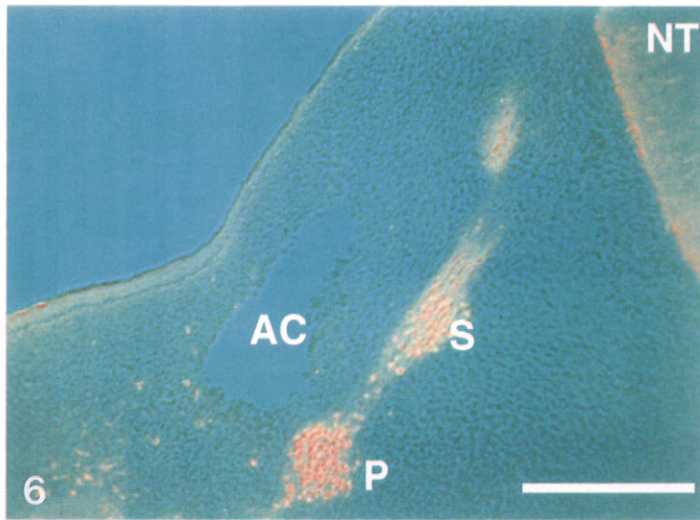


Fig. 8. Embryos labelled by injection of DiI into specific regions of the cranial neural crest. (A) A fluorescent and phase-contrast image of an embryo injected at the level of mesencephalon (M) at the 9-somite stage. DiI-labelled cells were dispersed over the mesencephalon. (B) A fluorescent and phase-contrast image of a transverse section through the level of mesencephalon (M) of an embryo similar to the one in A shows that DiI-labelled neural crest cells are present in the mesoderm adjacent to the ectoderm and in the mesoderm adjacent to the neuroectoderm (arrows). (C) In this fluorescent and phase-contrast image of an embryo labelled at the level of rhombomere 3 (arrow) at the 10 somite stage, DiI-labelled cells are present in the proximal portion of the second branchial arch (2). (D) Fluorescent and phase-contrast image of an embryo labelled by injection of DiI into the rostral hindbrain at the 11-somite stage. DiI-labelled cells are present only at the site of the injection (arrow). The eye (E), otic vesicle (OV), mesencephalon (M), and first (1) and second (2) branchial arches are indicated for orientation. Bars: A, B, C, 75 μ m; D, 125 μ m

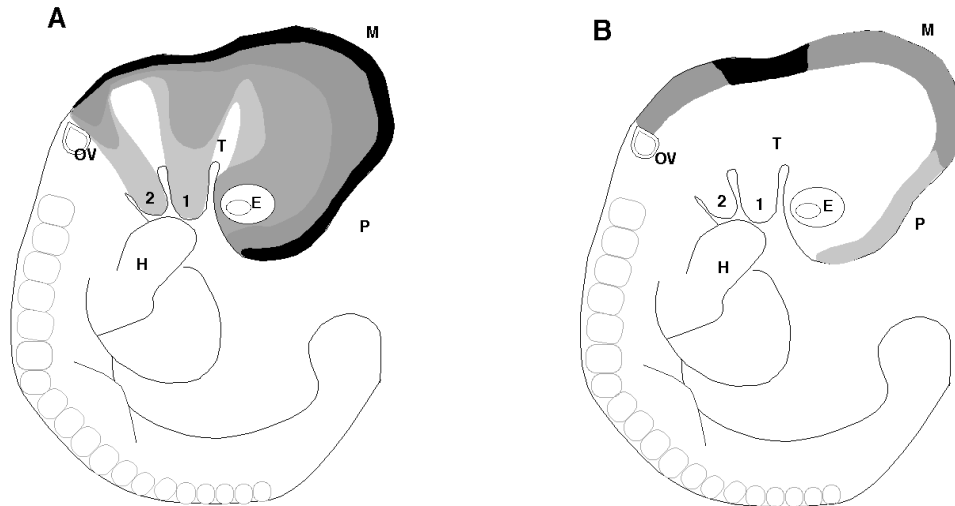


Fig. 9. (A) Schematic representation of the location of DiI-labelled cells after amniotic injection of DiI into progressively older embryos. Light gray represents the distribution of DiI-labelled cells in embryos injected at early stages of migration (0- to 6-somite stage). At the level of the forebrain, a sheet of DiI-labelled cells is observed that extends ventrally from the dorsal portion of the neural tube to encompass the developing eye (E). At the level of the midbrain, individual DiI-labelled cells are dispersed over the mesencephalon (M). At the level of the rostral hindbrain, DiI-labelled cells are observed in a stream, which extended from the fusion point of the neural tube to the distal end of the developing first branchial arch (1). In the caudal region of the hindbrain, a stream of DiI-labelled cells is observed extending into the second arch (2). Embryos injected at slightly later stages (7- to 10-somite stage) contain DiI-labelled cells in similar locations; however, the cells do not extend as far ventrally (medium gray). Embryos labelled at late stages of migration (11- to 16-somite stage) contain DiI-labelled cells only along the dorsal portion of the head (dark gray). Embryos labelled after cranial neural crest cell emigration is complete contain DiI-labelled cells only in the neural tube (black). (B) Schematic representation of the pattern in which neural crest cells stop emigrating from the neural tube. By the 11-somite stage, DiI-labelled cells no longer emigrate from the region of the rostral midbrain (black). DiI-labelled cells in the midbrain and caudal hindbrain stop emigrating from the neuroectoderm by the 14-somite stage (dark gray). In the forebrain, emigration of DiI-labelled cells ends at the 16-somite stage (light gray). The prosencephalon (P), mesencephalon (M), eye (E), otic vesicle (OV), trigeminal ganglion (T), first (1) and second (2) branchial arches and heart (H) are labelled for orientation.

the level of the midbrain contained dye-labelled cells in the mesenchyme between the mesencephalon and the ectoderm (Fig. 8A and B). Focal injections into the region of the neural tube which will develop into the 1st or 2nd rhombomeres (r1 or r2) produced a stream of DiI-labelled cells that extended from the site of the injection to the level of the first branchial arch. Injections into r3, r4 or r5 gave rise to labelled cells extending from the dorsal portion of the neural tube into the second branchial arch (Fig. 8C). Injections into r6, r7 or r8 gave rise to a stream of cells extending from the injection site toward the third, and possibly the fourth branchial arch.

Embryos injected at the 11- to 15-somite stage

Before the 14-somite stage, injections at the level of the forebrain, midbrain or r2-8 gave similar patterns of DiI-labelled cells to those described above. In contrast, embryos injected at the level of rhombomere 1 contained no DiI-labelled cells outside the injection site (Fig. 8D), suggesting that all of the neural crest cells at the level of the first rhombomere had completed emigration by the 10-somite stage. After the 14-somite stage, DiI injections at the level of the midbrain, or r2-8 no longer gave rise to labelled cells outside the injection site, suggesting that emigration from these axial levels was completed by the 14-somite stage. In contrast, at the level of the forebrain, emigration continued through the 14- and 15-somite stage.

Embryos injected at the 16+ somite stage

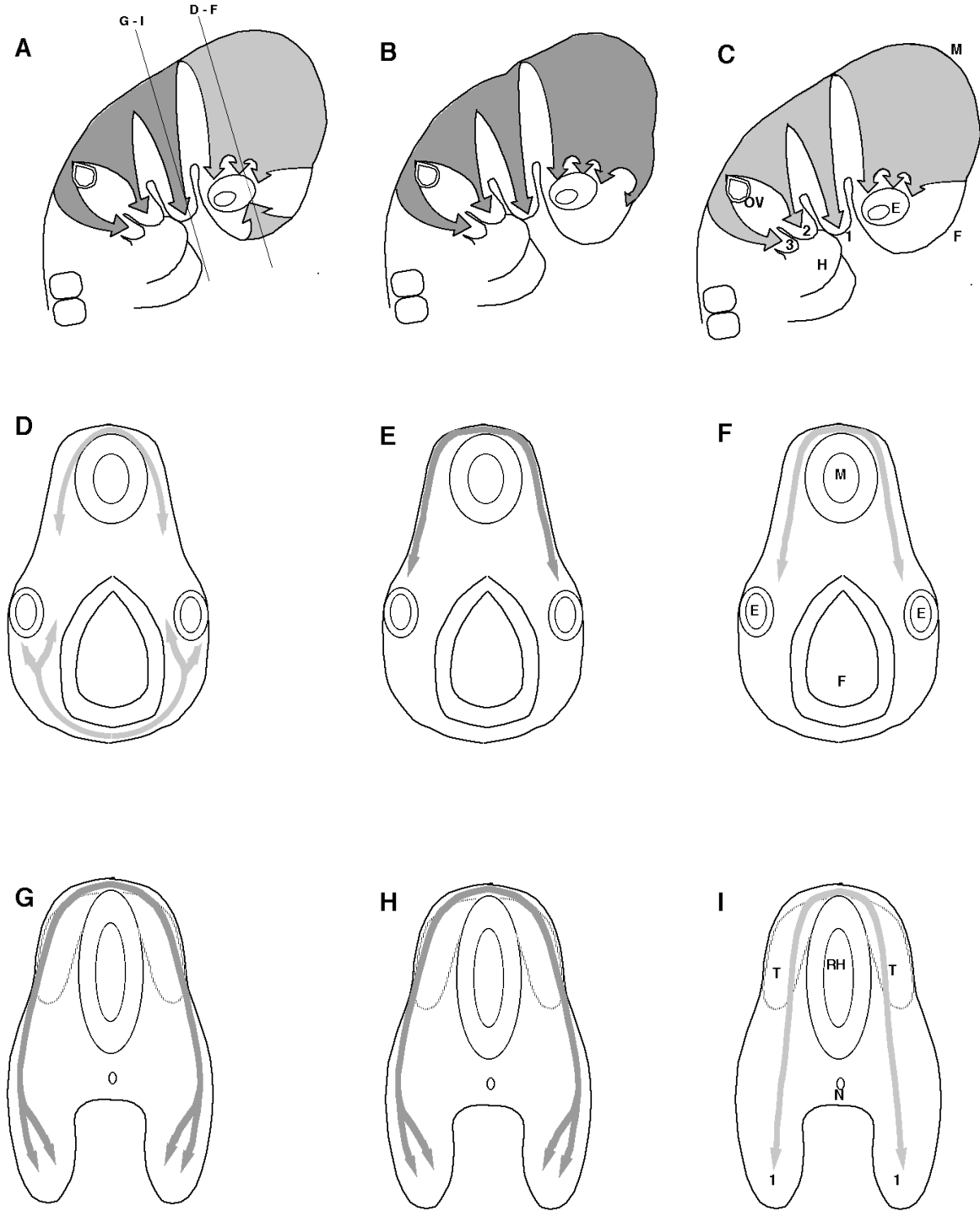
Embryos that were labelled at the levels of the forebrain, midbrain or hindbrain after the formation of somite 16 contained no DiI-labelled cells outside the site of injection.

Neural tube labelling

At approximately the 16-somite stage, neural tube fusion is complete, or nearly complete, in the head of the mouse. Therefore, it was possible to label the cells of the neural tube, including any premigratory neural crest cells, by injecting DiI into the lumen of the neural tube of embryos in culture (Serbedzija *et al.*, 1990). Embryos were labelled by intraluminal injection of DiI and then cultured for 24 hours before analysis. The neural tube at the level of the forebrain, midbrain and hindbrain contained DiI-labelled cells. However, only those embryos that were labelled prior to the formation of somite 16 contained DiI-labelled cells outside the neural tube and these were found only at the level of the forebrain.

Discussion

DiI labelling of premigratory and migratory neural crest cells in *ex vivo* and cultured mouse embryos provided a direct assay of the pattern and timing of cranial neural crest cell migration. Three distinct patterns of cranial neural crest



cell migration were observed. Neural crest cells from the level of the forebrain migrated ventrally through the mesenchyme in a sheet extending from the dorsal portion of the neural tube to the level of the optic vesicle and populated the mesenchyme around the eye. In contrast, neural crest cells at the level of the midbrain appeared to migrate ventrolaterally as dispersed cells through the mesenchyme between the lateral surface of the mesencephalon and the ectoderm. These cells populated the region overlying the mesencephalon. At the level of the hindbrain, neural crest

cells migrated ventrolaterally along three segmentally distributed subectodermal streams, from the dorsal portion of the neural tube to the distal portion of the first, second and third branchial arches. In addition to the branchial arches, these cells populated the trigeminal, superior, jugular, petrosal and nodose ganglia.

The order in which neural crest cells populated their derivatives was determined by labelling embryos at different stages of development. Injection of DiI into the amniotic cavity labelled only cells that are continuous with the

Fig. 10. Schematic representations showing the pathways of neural crest cell migration in the mouse, chick and rat embryos. Because the labelling and the data analysis techniques varied for each of the species represented here, a small amount of artistic license was used for the sake of comparison. Light gray represents neural crest cells that migrate through the mesenchyme. Dark gray represents neural crest cells that migrate along subectodermal pathways. (A-C) Whole embryo view showing the origins and overall pathways of neural crest cell migration. (A) In the mouse embryo there are three regionally distinct pathways of neural crest cell migration. Neural crest cells from the level of the forebrain migrate ventrally in a sheet extending from the dorsal portion of the neural tube to the level of the eye (E). Neural crest cells from the level of the midbrain migrate ventrolaterally toward the maxillary process and the eye (E). At the level of the hindbrain, neural crest cells migrate ventrolaterally in three streams, from the dorsal portion of the neural tube to the distal portion of the first (1), second (2) or third (3) branchial arches. (B) In the chick embryo, only the midbrain and hindbrain give rise to cranial neural crest cells. Neural crest cells from the level of the midbrain migrate ventrolaterally toward the maxillary process and the eye (E) and rostrally toward the nasal process (N). At the level of the hindbrain, neural crest cells migrate ventrolaterally in three streams, from the dorsal portion of the neural tube to the distal portion of the first (1), second (2) or third (3) branchial arches. (C) In the rat, migratory neural crest cells arise at the level of the midbrain and hindbrain. However, it is not clear whether or not migratory neural crest cells arise from the level of the forebrain. At the level of the midbrain, neural crest cells migrate ventrolaterally toward the maxillary process and the eye (E). Neural crest cells from the hindbrain migrate ventrolaterally in three streams extending from the dorsal portion of the neural tube to the distal portion of the first (1), second (2) or third (3) branchial arches. (D-F) A cross-sectional view through the level of the eye. (D) In the mouse, neural crest cells from both the midbrain and the forebrain migrate through the mesoderm. In addition, neural crest cells from the forebrain migrate between the eye (E) and the forebrain (F). (E) In the chick, neural crest cells migrate along a subectodermal pathway from the dorsal portion of the midbrain neural tube (M) to the eye (E). (F) In the rat, neural crest cells migrate through the mesenchyme from the dorsal portion of the midbrain neural tube (M) toward the eye (E). (G-I) A cross-sectional view through the level of the first branchial arch. (G and H) In the mouse and the chick, neural crest cells migrate along a subectodermal pathway that extends from the neural tube into the first branchial arch. (I) In the rat, neural crest cells migrate through the mesenchyme as dispersed cells from the neural tube into the first branchial arch. The forebrain (F), midbrain (M), rostral hindbrain (RH), eye (E), notocord (N), otic vesicle (OV), trigeminal ganglion (T), first (1), second (2), third (3) branchial arches and heart (H) are labelled for orientation.

neuroectoderm and migratory neural crest cells are left unlabelled; making it possible to mark temporally distinct subsets of the neural crest cells. Because of the dramatic changes in the anatomy of the head during these stages of development, the order in which cranial neural crest cells populated their derivatives was best illustrated at the level of the rostral hindbrain. Embryos labelled during the earliest stages of neural crest cell migration contained DiI-labelled cells throughout the first branchial arch, as well as in the region of the trigeminal ganglia (Fig. 9B, light gray). In contrast, embryos labelled at slightly later stages contained DiI-labelled cells at the level of the trigeminal gan-

glia, but not in the first branchial arch (Fig. 9B, medium gray). Embryos labelled at still later stages contained DiI-labelled cells adjacent to the dorsal portion of the neural tube, but not at the level of the trigeminal ganglia or in the first branchial arch (Fig. 9B, dark gray). Therefore, cranial neural crest cells populated their derivatives in a ventral-to-dorsal order, similar to that observed for neural crest cells in the trunk (Serbedzija *et al.*, 1990).

Previous studies, using either ultrastructural characteristics (Nichols, 1981, 1986) or topical wheat germ agglutinin labelling (Chan and Tam, 1988) to identify neural crest cells, indicated that neural crest cell migration begins at approximately the 5-somite stage in the rostral hindbrain (Nichols, 1981, 1986; Chan and Tam, 1988). Emigration follows in the midbrain, the caudal hindbrain and finally the forebrain (Nichols, 1981). These previous studies also suggested that neural crest cell emigration at the rostral hindbrain level is complete by the 10-somite stage. By labelling premigratory neural crest cells with DiI at progressively later stages of development, we were able to make a direct determination of the stages at which neural crest cells cease emigrating from the neural folds not only at the level of the rostral hindbrain but also at each of the other axial levels (Fig. 9B). Focal injections of DiI into the juncture of the neuroectoderm and the ectoderm labelled both the neural crest cell precursors within the neural fold and the newly emerging neural crest cells. In the most rostral hindbrain (r1), neural crest cells ceased to emigrate from the neural folds by the 11-somite stage. In the midbrain and caudal hindbrain regions, neural crest cell emigration from the neural folds ended by the 14-somite stage. In the forebrain, however, neural crest cell emigration from the neural folds continued until the 16-somite stage. Because the DiI-labelling technique marks both emigrating and soon-to-emigrate neural crest cells, it is difficult to determine exactly the time of initiation of neural crest emigration by this method. However, the previous ultrastructural studies (Nichols, 1981, 1986) were well-suited to determining the stage of neural crest emigration, and generated results consistent with our own. By combining the data from these various approaches, it was possible to deduce the duration of neural crest cell emigration at each axial level. Neural crest cell emigration from the rostral hindbrain begins at the 5-somite stage and ends by the 10-somite stage, approximately 9 hours later. At other axial levels, the timing of initiation of migration and cessation of emigration paralleled one another, suggesting that the duration of cranial neural crest cell emigration at all axial levels was approximately 9 to 12 hours.

The final destinations of the DiI-labelled cranial neural crest cells were examined by allowing some of the embryos labelled by amniotic injection on E8 to develop for an additional 3 to 5 days. DiI-labelled cells were found in all of the neuronal derivatives of the cranial neural crest, including the trigeminal, the superior, the jugular, the nodose and the petrosal ganglia. Surprisingly, no DiI-labelled cells were detected in any of the mesenchymal derivatives after E11, even though DiI-labelled cells were observed in the mesenchymal derivatives of embryos examined 1 to 2 days after the injection. Because a fixed amount of DiI was injected, the dye could have been diluted by extensive cell division.

Consistent with this possibility, cranial ganglion cells in avians do not undergo extensive division (see review by Le Douarin, 1982), while the cells that contribute to the mesenchymal derivatives of the cranial neural crest proliferate extensively (Noden, 1988).

Cranial neural crest cells in the region of the hindbrain migrate in a segmented pattern that corresponds to the location of the branchial arches. This pattern consists of three separate streams of cells, lateral to r2, r4 and r6, each of which extends from the neural tube into the adjacent branchial arch. The high degree of segmentation in the hindbrain that becomes apparent at later stages might suggest that the formation of the neural crest also may be segmented, directly contributing to this pattern. For example, the streams at r2, r4 and r6 might be created by the failure of other axial levels of the neural tube (e.g. r3 and r5) to give rise to migrating neural crest cells, similar to the pattern recently reported to occur in avians (Lumsden *et al.*, 1991). However, injections of DiI into the mouse neural crest at each level of the hindbrain, from rhombomere 1 through 8, gives rise to labelled cells outside the neural tube. In addition, recent experiments suggest that there may be a small, but significant contribution of neural crest cells from r3 and a large contribution from r5 in the avian embryo as well (J. Sechrist, G. N. Serbedzija, S. E. Fraser, T. Scherson and T. Bonner-Fraser, unpublished data). This casts doubt on the simple hypothesis that segmented migration reflects segmental origin and suggests that the segmented pattern of mouse neural crest cell migration at the level of the hindbrain may be imposed by factors extrinsic to the neural tube, as is the case for the relationship between trunk neural crest cells and the somitic mesenchyme (Bronner-Fraser and Stern, 1991).

Although the DiI-labelling technique permits defined groups of cells to be marked, it does have some limitations. First, because the intra-amniotic injection labels the whole ectoderm, the ectodermal placodes also are labelled. Because the placodes contribute to the cranial ganglia, the possibility exists that some of the labelled cells found in the mesenchyme are of placodal rather than neural crest origin (see Fig. 5D). Second, this technique does not permit the analysis of cranial neural crest cell emigration from regions where the neural tube has fused. To minimize these potential pitfalls and to confirm the results, embryos in culture were labelled by small injections of DiI into specific axial levels of the neural tube. The results from this regional labelling are consistent with the results obtained with the whole ectoderm labelling, suggesting that the whole ectoderm labelling approach gives a realistic view of neural crest cell migration in the mouse embryo.

The pattern described here for mouse cranial neural crest cell migration shares some, but not all, characteristics with the patterns described for both chick and rat neural crest cell migration. At the level of the hindbrain, mouse and chicken cranial neural crest cells migrate ventrolaterally along three subectodermal streams that extend from the neural tube into each branchial arch (Fig. 10A, B, G and H; Chan and Tam, 1988; Lumsden *et al.*, 1991). In the rat, neural crest cells from the level of the hindbrain appear to

migrate through the mesenchyme as dispersed cells (Fig. 10C and I; Tan and Morriss-Kay, 1986). At the midbrain level, neural crest cells of the mouse and rat migrate through the mesenchyme (Fig. 10A, C, D and F), while chick neural crest cells migrate along more superficial subectodermal pathways (Fig. 10B and E; Le Douarin, 1982; Tan and Morriss-Kay, 1986; Noden, 1988). At the level of the forebrain, we find that mouse embryos do have emigrating neural crest cells in support of previous ultrastructural results (Fig. 10A and D; Nichols, 1986); in contrast, neither chick nor rat embryos appear to have a contribution from the forebrain to the cranial neural crest cells (Fig. 10B, C, E and F; Johnston, 1966; Noden, 1975; Tan and Morriss-Kay, 1985). Some of these apparent differences may be due to the different labelling techniques and analytical approaches used to study neural crest cell migration in each of the three species. Further comparative analyses of apparent similarities and differences of neural crest cell behavior in these three species may provide insights into the mechanisms that control the development and migration of cranial neural crest cells.

In summary, by labelling premigratory cranial neural crest cells with DiI, the patterns and timing of cranial neural crest cell migration in the mouse were determined. Our observations confirm and extend the observations of both ultrastructural (Nichols, 1981, 1986) and topical labelling studies (Chan and Tam, 1988). Because our approach permitted the labelling of embryos *in vivo* rather than in culture, it circumvents many of the potential difficulties encountered in previous studies (Nichols, 1981, 1986; Chan and Tam, 1988) and allows the analysis of neural crest cell migration over much more extended periods of development than previously possible. The use of DiI-labelling in intact embryos has permitted, for the first time, direct analyses of the migration pathways, the duration of emigration and the order of contribution to derivatives of the mouse cranial neural crest. By defining the normal pattern of cranial neural crest cell migration, the results presented here provide a solid foundation for further experimental analysis of the factors affecting both neural crest cell migration and differentiation. This approach does not require specific mouse strains; furthermore, it can be applied to a variety of species. Future experiments using DiI-labelling to define the spatial and temporal aspects of neural crest migration in genetically manipulated embryos should permit decisive test experiments and provide new insights about the mechanisms of neural crest cell migration and differentiation in higher vertebrates.

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