

Analysis of cranial neural crest cell migration and early fates in postimplantation rat chimaeras

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SUMMARY

Rat embryos were grown *in vitro* during the period of cranial neural crest cell migration. In order to study the pathways and positional fates of cells from different regions of the neural crest, labelled premigratory crest cells from donor embryos were microinjected orthotopically into host embryos of the same developmental stage except for area 1 (forebrain) grafts which were, for technical reasons, injected into area 2. After various periods of time in whole embryo culture, the embryos were examined by immunohistochemical staining in order to determine the new positions of the labelled cells, and a map of their migration pathways was constructed. The observed patterns of migration were consistent with predictions from morphological studies in mammals and with extrapolations from transplantation studies in birds. However, crest cell migratory behaviour in rat and chick embryos was not identical; possible reasons for this are discussed.

INTRODUCTION

Neural crest cells have their origin in the lateral border of the neural folds and migrate to precise locations of the embryo where, in non-mammalian vertebrates, they have been observed to differentiate into a great variety of cell types. These include neurones and glial cells of autonomic and sensory ganglia, skeletal and connective tissues of the craniofacial skeleton, endocrine cells of thyroid, carotid body and adrenal medulla, and pigment cells (for reviews, see Hörstadius, 1950; Weston, 1970; Morriss & Thorogood, 1978; Le Douarin, 1982).

Information concerning the neural crest in mammals is relatively incomplete in comparison with amphibians and birds, and is restricted to a handful of morphological studies which are only able to provide a series of static pictures of a dynamic process (Adelmann, 1925; Bartelmez & Evans, 1926; Holmdahl, 1928; Halley, 1955; Verwoerd & van Oostrom, 1979; Nichols, 1981; Erickson & Weston, 1983; Tan & Morriss-Kay, 1985). Direct evidence of neural crest cell migration in mammalian embryos has been slow to emerge, principally because of the problems involved in manipulating embryos that are effectively isolated within fetal membranes and maternal tissues during the postimplantation phase of gestation.

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An indirect solution has been offered by injecting neural crest cells into the decidual swellings *in utero* on the assumption that there would be a reasonable chance of successfully targeting a number of injections into the conceptus (Jaenisch, 1985). Apart from the drawback of a poor success rate (approximately 15%), this technique also suffers from the more serious disadvantage of invisibility of the injection site, and is thus unsuited to investigating normal migration and differentiation. In addition, the technique is only applicable to the study of a single neural crest derivative (melanocytes).

Any serious attempt to study mammalian neural crest cell behaviour by direct means has to meet two conditions. First, the embryo during organogenesis must be accessible to grafting procedures *ex utero*. Subsequent healing, growth and development of the embryo should be able to proceed normally. This requirement is well satisfied with modern methods of embryo culture, which are now established techniques for growing both rat and mouse embryos *in vitro* (for a review, see New, 1978). In particular, rat embryos may be cultured for up to 4 days from pregastrula and head-fold stages (0–5 somite stage) onwards until the appearance of the forelimb buds (New, Coppola & Cockcroft, 1976a; Buckley, Steele & New, 1978); the greater success of rat compared with mouse embryo cultures makes it the species of choice for *in vitro* manipulations.

The neural crest of the head region in rat embryos does not begin to migrate until the 4-somite stage (Adelmann, 1925; Tan & Morriss-Kay, 1985). Therefore the sustainable culture period is perfectly suited for studying early neural crest development in experimental chimaeras from the time of onset of migration until their colonization in embryonic structures. In practical terms, labelled cells may be injected into unlabelled embryos which are then allowed to develop as *in vitro* chimaeras which may then be analysed at any time during the culture period (Beddington, 1981; Papaioannou & Dieterlen-Lievre, 1984).

The second requirement concerns the availability of an *in situ* cell marker to distinguish donor crest cells from tissues of the host embryo. The ideal cell marker should be cell autonomous, cell localized, neutral, stable and easily detected in tissue sections (McLaren, 1976). There are several genetic markers in mice that approach some of these criteria (Gardner, 1984; West, 1984) but these are unfortunately not applicable to rats except as rat–mouse chimaeras. Although these chimaeras may be produced with some success (Gardner & Johnson, 1973), there is doubt as to whether they reflect the actual events of normal development (Gardner, 1984).

The alternative would be to use exogenous markers (e.g. vital dyes, particulate markers or radioisotopes). These have been well exploited in the past to provide spatial information in a variety of embryonic tissue transplantation experiments (e.g. Detwiler, 1917; Weston, 1963; Campbell, 1973). More recently, there have been reports of a new generation of exogenous cell markers including horseradish peroxidase (Weisblat, Sawyer & Stent, 1978), fluorescein/rhodamine isothiocyanate (Butcher, Scollay & Weissman, 1980), fluorescein–lysine–dextran (Gimlich & Cooke, 1983), and carboxyfluorescein diacetate succinimyl ester (Bronner-Fraser,

1985). These marker molecules have in common their uptake and subsequent localization in cytoplasmic organelles; they are usually large molecules and are therefore unlikely to pass through intercellular gap junctions. Included in this category is a further group of tracer proteins that has enjoyed great popularity in neuronal connectivity and cell plasma membrane investigations (for a review, see Trojanowski, 1983), but remains unexploited for *in situ* cell marking in embryological transplants. This group is the lectins, which include wheat germ agglutinin (WGA, relative molecular mass 35 000) derived from *Triticum vulgare* (see reviews by Sharon & Lis, 1972; Goldstein & Hayes, 1978). Cells exposed to WGA are able to internalize the ligand into their cytoplasm by absorptive endocytosis (Gonatas, Stieber, Olsnes & Gonatas, 1980). The short-term fate of these cells may then be followed in histological preparations by tagging the WGA with a second marker in the form of an enzyme (e.g. horseradish peroxidase), metal (e.g. gold, ferritin), fluorochrome or radioisotope (see Trojanowski, 1983, for references). Alternatively, the ligand may be used on its own and subsequently visualized by immunohistochemical methods using antiserum directed against WGA (Sofroniew, 1983a).

This paper reports the successful use of WGA for short-term experiments using *in vitro* chimaeras. Donor neural crest cells were exposed to WGA and grafted into unlabelled hosts; the migratory positions of the cells, revealed by their incorporated lectin, were scored 24 and 48 h later. Using this approach, we have been able to map the migratory pathways and colonization pattern of rat neural crest cells that arise from the different anteroposterior levels of the head.

MATERIALS AND METHODS

General outline of the experiments

The experimental protocol was adapted from that of Beddington (1981). Head-fold-stage embryos were injected with labelled neural crest cells obtained from other embryos of equivalent stages. Injected embryos were sustained in culture for up to 48 h. The positions of the injected cells in the recipients were then visualized by immunohistochemistry. Others (labelled controls) were exposed to WGA and cultured for 48 h to assess the dilution of the label over the culture period and also the capacity of labelled embryos to undergo normal embryonic development. The development of the injected and labelled embryos was also compared with *in vivo* controls and other unlabelled embryos cultured for a similar period of time. Details of these, together with an outline of the experimental method, are shown in Fig. 1.

Embryo recovery and culture

Random-bred Wistar rats from the animal colony (Human Anatomy, Oxford) were used. Embryos were recovered from day 10 pregnant rats housed in a reversed lighting cabinet (estimated mating at 12.00 h, day 0) and dissected in phosphate-buffered saline (PBS). Reichert's membrane was removed but the visceral yolk sac and ectoplacental cone left intact. Embryos were cultured in groups of five in 50 ml glass bottles containing 5 ml of medium made up of equal volumes of Tyrode's saline and immediately centrifuged, heat-inactivated (56 °C for 30 min) serum. The culture medium contained added penicillin and streptomycin at a concentration of 50 µg ml⁻¹. Culture bottles were rolled at 30 rev. min⁻¹ on a roller system kept at 38 °C and gassed with a mixture of 5 % CO₂/5 % O₂/90 % N₂. The gaseous mixture was changed

to 5% CO₂ in air after 24 h and 5% CO₂/40% O₂/55% N₂ after 36 h. Donor embryos, recipients and injected embryos were all grown in separate bottles.

Labelling of embryos

Donor embryos were labelled with WGA by a two-step procedure. First, a small volume of WGA (0.2 µl of a 0.02% solution, Miles Scientific) in PBS (pH 7.4) was injected into the amniotic cavity and cultured for 30 min prior to microdissection, followed by a further 15 min exposure of the dissected crest cells to WGA (100 µg ml⁻¹) in a drop of PBS at room temperature. Uptake controls were immediately fixed after labelling to provide an initial labelling standard. Labelled controls were injected with lectin into their amniotic cavity and, after 30 min, flushed and replaced with fresh culture medium. Unlabelled controls were cultured in normal medium for 48 h. *In vivo* controls were recovered from pregnant females on the morning of day 12.

Dissection and preparation of donor cells

Neural crest cells were obtained from the lateral margins of the neural folds during early migration. As a precaution against possible contamination by underlying mesoderm, the ectodermal layer was separated from the rest of the donor embryo by proteolytic digestion for 30 min with 0.04% collagenase (Worthington CLS type II, Cooper Medical) dissolved in Hank's Basic Salt solution (Gibco) at room temperature. The reaction was stopped by washing the embryos with 50:50 Tyrode's saline and rat serum. Digested embryos were then dissected with forceps and glass needles in Hank's solution.

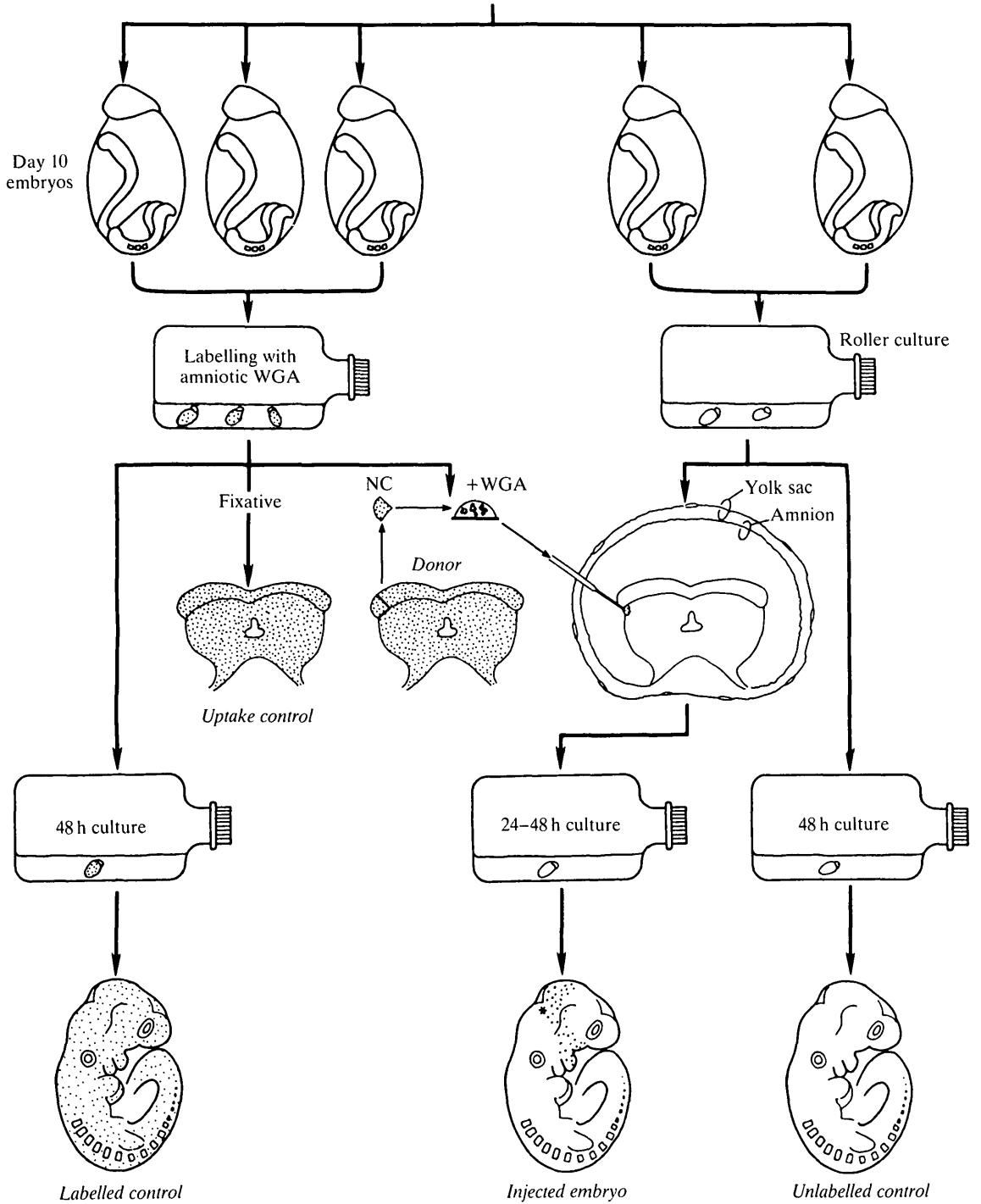
The neural folds together with neighbouring surface ectoderm were peeled off with fine forceps from the rest of the conceptus, leaving the neural crest, but not the subjacent cranial mesoderm, undisturbed at the lateral margins of the fold. Before the appearance of neural crest cells from the neural plate, removal of the mesoderm resulted in a bare ectodermal layer with fine disruption of the basement membrane at the prospective region of neural crest emigration (Fig. 2A). At later stages (4/5 somites) the removal of mesoderm was similarly achieved, but the junction of the neural and surface ectoderm was now occupied by a strip of newly emigrated neural crest cells (Fig. 2B,C,D). Donor crest tissue was obtained by excision along the edge of the fold on either side using glass needles, with great care to exclude cells from the attached but distinct surface epithelium. An imaginary line equal to approximately 10 cell diameters from the lateral margin was used to delineate arbitrarily neural crest tissue from the rest of the neural ectoderm (outlined in Fig. 2D). This conservative estimate was essential to minimize contamination of the graft with neuroepithelial cells not destined to become migratory crest cells.

Fig. 1. Summary of labelling and injection procedures. Embryos at head-fold stages were recovered on the morning of the 10th day of pregnancy and grown in roller culture bottles.

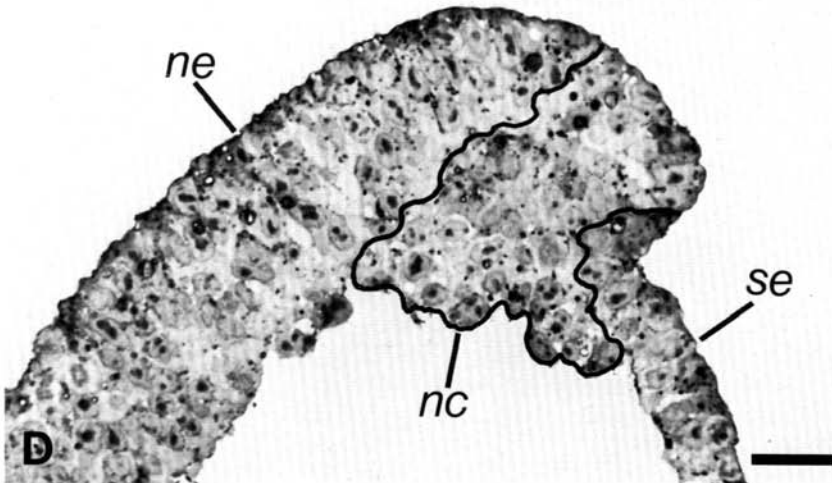
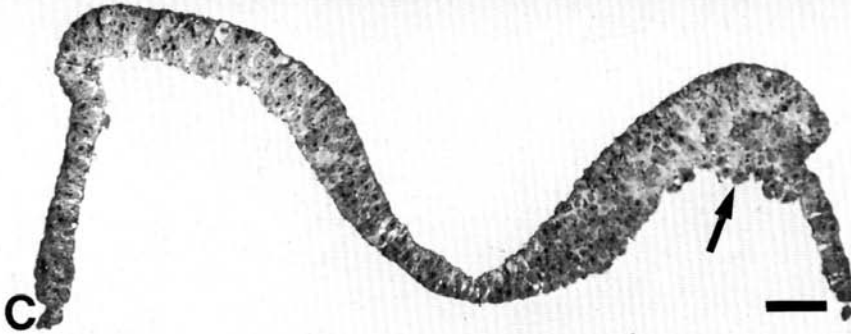
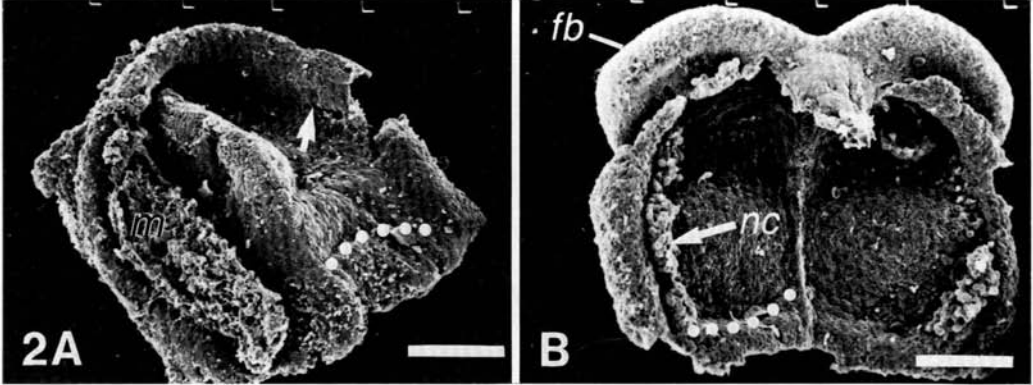
Donor embryos were labelled by the injection of WGA into the amniotic cavity and, 30 min later, flushed with fresh culture medium. Further labelling of donor cells with WGA was carried out on a culture dish just before the injection procedure. Some embryos were fixed immediately after the labelling procedure, providing *uptake controls*. Others were rinsed three times in Tyrode's saline and cultured with fresh medium, to act as *labelled controls*.

Recipient embryos were grown in separate bottles, and at the appropriate moments recovered for microinjection. The *injected embryos* were cultured in fresh medium, and harvested after 24 or 48 h.

Unlabelled controls were provided by litter mates cultured for 48 h under similar conditions in normal culture medium. *In vivo* controls (not shown in diagram) were recovered from pregnant dams whose offspring had continued development *in utero* for a further 48 h.



Donor crest tissue was transferred in a drop of Tyrode's saline to the lid of a plastic culture dish with a mouth-operated hand-drawn Pasteur pipette (overall diameter 100 μm), then placed in a drop of Medium 199+10% fetal calf serum (Gibco) and further subdivided into smaller clumps of 20–30 cells. These clumps were exposed for a further labelling period (15 min) to lectin (100 $\mu\text{g ml}^{-1}$) in a drop of Medium 199 at room temperature. The cells were then washed three times (1 min each) in PBS containing 0.1 M-N-acetylglucosamine (Sigma) to remove any unbound lectin before injection into host embryos.



Microinjection

Micropipettes were made from capillary tubing (internal diameter, 0.85 mm, Drummond, USA) with an electrode puller and the tips broken off to the required diameter and heat polished with a microforge (DeFonbrune, Paris). The micropipettes were connected to micrometer syringes with transparent oil-filled (Boots paraffin oil) tubing. Forward and backward movement of the oil provided the suction and expulsion forces necessary for injecting the cells and for holding the recipient embryos (Gardner, 1978). Injections were carried out on a micromanipulator (Leitz) attached to the micropipette (internal diameter at the tip 20 μm) and activated by a DeFonbrune micropump.

Recipient embryos were held by partway suction of the yolk sac into the tip of a holding pipette (internal diameter 700 μm) controlled by a syringe (Agla). The embryos were refocused to allow a clear perception of the neural/surface ectodermal junction through the fetal membranes. The injection pipette, containing the donor cells, was pushed by a sharp rapid movement through the tensed yolk sac and amnion into the junction of the neural plate and surface epithelium (see Fig. 3, zero time control). The pipette was then slowly withdrawn while simultaneously expelling the donor tissue into the created space.

To assist visualization of the micropipette tip while in the embryo and to ensure accurate positioning of the injected cells, a weak solution (0.01 %) of neutral red in Ringers solution was used to stain supravivally the donor cells immediately before the injection procedure. The whole microinjection process was carried out in a drop of embryo culture medium on a plastic dish. Brightfield illumination was provided by a dissecting microscope (Wild) fitted with 'zoom' facilities. Injected embryos were collected in culture bottles containing fresh medium and gassed. Microinjection was in every case performed on the right side of the embryo while the left side acted as intraembryonic control.

Definition of donor and target sites

The cranial region of the 5- to 7-somite-stage embryo was subdivided into five areas, according to the classical data on the subdivisions of the neural crest in relation to the future forebrain, midbrain and hindbrain (Bartelmez, 1923; Adelman, 1925), and also from the results of a recent study (Tan & Morriss-Kay, 1985) on the timing of neural crest emigration from different regions of the neural plate. With one exception (between areas 2 and 3; see Fig. 4), embryonic landmarks were used to delineate the individual areas. These landmarks (i.e. preotic sulcus, cranial flexure, otic placodes) acted as convenient reference points for visualizing donor

Fig. 2. The appearance of enzyme-digested and mechanically separated embryonic tissues. The scanning electron microscope (SEM) views have been prepared as described previously in Tan & Morriss-Kay (1985).

(A) SEM view of 3-somite-stage embryo showing the undersurface of the neural plate and surface ectoderm. Mesoderm has been removed from one side. The arrow indicates the junction of the surface and neural ectoderm, where the basement membrane has begun to separate, but no neural crest cells were seen to emerge at this stage. The mesoderm (*m*) has been left *in situ* on the other side for comparison. The dotted line indicates the preotic sulcus. Bar, 100 μm .

(B) SEM view of a 4/5-somite-stage embryo where premigratory neural crest cells (*nc*) are seen to occupy a narrow strip between the surface and neural ectoderm. The rudiment of the forebrain (*fb*) has begun to form as a double epithelial layer of neural and surface ectoderm. Dotted line indicates the preotic sulcus. Bar, 100 μm .

(C) Oblique/transverse section of a Spurr-embedded specimen similar to B. Premigratory neural crest cells (arrow) remained attached to the neural plate in the region of the rostral hindbrain, but in the area of the preotic sulcus which is devoid of crest cells (left side) the basal surface of the epithelium is clean. Bar, 50 μm .

(D) Higher magnification of C showing premigratory neural crest cells attached to the neural and surface ectoderm. The inked outline indicates the region from which donor cells were obtained. This specimen has been labelled with WGA, which may be seen as PAP-positive vesicles in all the cells. *nc*, neural crest; *ne*, neural ectoderm; *se*, surface ectoderm. Bar, 50 μm .

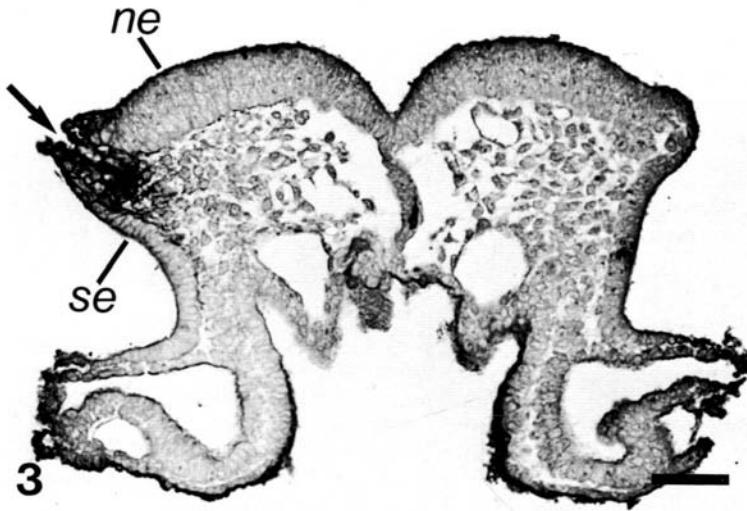


Fig. 3. Zero-time injected embryo. Transverse section of the cranial region of an embryo immediately after injection of WGA-labelled cells (arrow) revealed by immunoperoxidase staining. The point of insertion of the micropipette is visible as a small lesion at the neural/surface ectoderm junction. *ne*, neural epithelium; *se*, surface ectoderm. Bar, 50 μ m.

and recipient sites, while at the same time ensuring that the placement of transplants (indicated by asterisks in Fig. 4) was both precise and repeatable. Thus, the injection sites were as follows: area 2 cells halfway along the midbrain; area 3 cells immediately anterior to the preotic sulcus; area 4 cells immediately anterior to the otic placode; area 5 cells immediately posterior to the otic placode.

Orthotopic cell transfers were carried out in areas 2 to 5 to provide information on the normal migration pathways of midbrain and hindbrain neural crest. Forebrain neural crest (area 1) was studied by heterotopic injection into area 2; this was necessary due to technical difficulties associated with orthotopic injection into the forebrain region, which at this stage of development (5-somite stage), is apposed to surface ectoderm without intervening mesenchyme. Injections into areas 2 and 3 were carried out at the 4/5-somite stages, and into areas 4 and 5 at the 7/8-somite stage. Finally, in order to identify the effects of advancing embryonic age on neural crest migratory potential, area 3 grafts were orthotopically inserted into four synchronous embryos at the 11-somite stage of development (late injections).

Evaluation and fixing of cultured embryos

Upon harvesting, the developmental status of injected and control embryos was recorded. Specifically, embryos were classified as abnormal if they were either dead at the time of harvesting or showed external deformities, e.g. incomplete turning, surface blistering, unclosed neural tube, fusion between embryonic and extraembryonic structures. In addition, the number of somites in normal embryos after 24 and 48 h was noted. Finally, several 48 h embryos from *in vivo* controls and each category of cultured embryos (injected, unlabelled, labelled) were homogenized for protein estimation with the Biorad reagent using the method of Bradford (1976). All normal embryos that were alive at the time of harvesting (with beating heart and yolk sac circulation) were fixed for histological study (except those used for protein estimation). Embryos were fixed with Bouin's fixative for 2 h.

Immunohistochemistry

Immunohistochemical detection of WGA was performed with the indirect technique using polyclonal antibody raised against the lectin (Sofroniew, 1983a) followed by secondary antibody

and peroxidase anti-peroxidase (PAP) (Sternberger, Hardy, Cuculis & Meyer, 1970). After fixation, embryos were placed overnight in 0.1 M-Tris/PBS (pH 7.8) at 4°C to wash out the picric acid. Whole embryos were then incubated in the primary antibody (RB 58, anti-WGA antiserum raised in rabbit, 1 in 400 dilution) for 3 nights. Embryos were then washed six times at half-hourly intervals with Tris buffer before incubation overnight in the secondary antibody (goat anti-rabbit IgG, 1 in 16 dilution; Sigma). After further washing for 3 h, rabbit PAP (1 in 100 dilution; Sigma) was added to the embryos overnight. The peroxidase activity was visualized the next day using 3'3'-diaminobenzidine (DAB, 0.1%; Sigma) substrate and 0.03% hydrogen peroxide (Fisons) catalyst.

All antibodies were diluted in 0.1 M-Tris/PBS (pH 7.8) containing 0.3% lambda carrageenan (Sigma) plus 0.5% Triton X-100 (Sigma) and used at 4°C. Embryos were completely submerged in the antibody in 5 ml glass bottles. After the DAB step, embryos were dehydrated, embedded in paraffin wax and serially sectioned at 7 µm in either sagittal or transverse planes. Transverse sections were cut perpendicular to the long axis of the embryo. Cresyl violet (0.1%) was used as counterstain.

Immunoelectron microscopy

Four WGA uptake controls were used for immunoelectron microscopy in order to analyse the localization of the lectin molecule in donor cells. Donor embryos were exposed to the lectin by

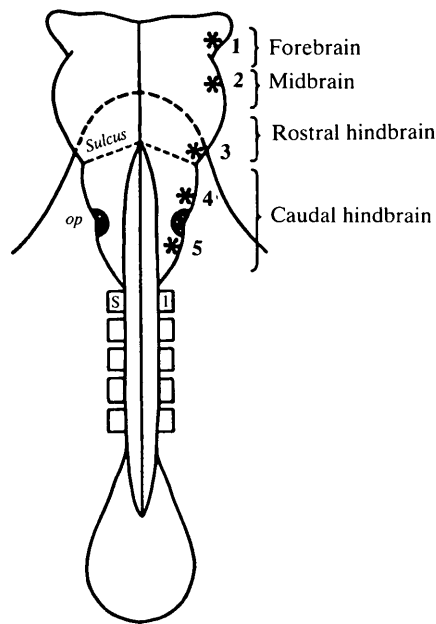


Fig. 4. Diagrammatic representation of a 5-somite-stage embryo showing the division of the premigratory neural crest as defined in this study. The curved line represents the anterior intestinal portal. *Sulcus*, preotic sulcus; *op*, otic placode; *SI*, first somite.

Area 1. Forebrain only; the cranial flexure marks the site of the future forebrain/midbrain junction.

Area 2. Midbrain, injection site is located rostral to a line drawn at equidistance from preotic sulcus to cranial flexure.

Area 3. Rostral hindbrain, injection site is *rostral* to preotic sulcus.

Area 4. Part of caudal hindbrain, injection site is *caudal* to preotic sulcus but anterior to the otic placode.

Area 5. Part of caudal hindbrain, injection site is caudal to the otic placode but rostral to the first somite.

injection into the amnion of $0.2\ \mu\text{l}$ WGA (0.02%) in PBS and cultured. After 30 min the embryos were digested in collagenase to isolate the ectoderm (neural crest attached to neural plate, plus adjacent surface epithelium) from the rest and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M-cacodylate buffer for 1 h (Sofroniew, 1983b). The immunohistochemical procedure for visualizing WGA was carried out as above except for the extra step of post-DAB osmication in 0.2% OsO_4 . The tissues were embedded in Spurr resin (TAAB) and semithin sections cut at $1\ \mu\text{m}$ and stained with methylene blue. Ultrathin sections for electron microscopy were cut with a diamond knife and stained on nickel grids with uranyl acetate and lead citrate (Reynolds, 1963). The specimens were viewed with a JEOL 100S transmission electron microscope.

Criteria for chimaerism in injected embryos

Embryos were judged to be chimaeric if they individually possessed at least 20 labelled cells. An estimate of the total number of labelled cells was achieved by counting every labelled cell in alternate sections ($7\ \mu\text{m}$). As there was no control for the number of donor cells lost to the extraembryonic environment during injection, no attempt was made to quantify the extent of cellular proliferation by injected cells in the host embryo. Moreover, to be able to do this meaningfully would require additional knowledge of cell cycle parameters and mitotic indices; neither of these was investigated in this study. To qualify as a labelled cell, a strong labelling signal over background had to be present. In addition, an individual cell was only considered to be labelled if it was positively scored in two consecutive sections.

Quantitative evaluation of crest cell migration

In an attempt to estimate the maximum distance covered by migrating cells from a single injection site, the spread (**b**) between first and last labelled cells of the grafted population was measured in the longitudinal plane. This was done by adding up the number of transverse sections between first and last labelled cells and multiplying by average section thickness ($7\ \mu\text{m}$). For comparison, the dorsoventral spread between these labelled cells was also estimated with the aid of an eyepiece graticule. These procedures were carried out for seven embryos, each of which had received a graft into area 3 and been cultured for 48 h. Area 3 grafts were chosen for estimation because it was possible to standardize the recipient site (immediately anterior to preotic sulcus) in these embryos.

For an estimate of the initial graft size (**a**), four embryos were injected and immediately fixed at zero time before they were subjected to the same calculation procedure in the longitudinal plane. The ratio (**b/a**) between the two mean measurements therefore provided an estimate of the average increase in size of the grafted cell populations due to actual cell migration plus the effect of longitudinal embryonic growth (calculation procedure set out in Table 1).

Embryonic growth was estimated by measuring the increase in length between two fixed points in four pairs of 48 h and zero-time control embryos. This was done by image analysis (MOP2, Kontron) from photographs of paired embryos fixed at the 5-somite stage and others 48 h later. The distance from the midbrain/forebrain junction to the otic placode or vesicle was measured from the dorsal outline of the developing neural tube (Fig. 5). On the assumption that growth between these fixed points is uniform, the mean ratio (**f**) of the two measurements may be taken to be an estimate of embryonic growth over the 48 h period. The formula (**c-f**) would therefore represent the net increase in size of the original graft due to longitudinal cell migration after allowing for longitudinal embryonic lengthening.

RESULTS

Controls

Embryos that developed in culture were able to undergo extensive growth and morphogenesis at comparable rates to *in vivo* controls of equivalent age (Table 2), confirming similar results obtained by other investigators (New, Coppola &

Table 1. Maximum distance covered by area 3 donor cells after 48 h migration in host embryos

Chimaera no.	Craniocaudal distance (μm)	Dorsoventral distance (μm)
32B	504	330
32C	861	770
32D	987	780
32E	903	730
32F	686	570
34F	658	490
34G	959	710
Mean \pm s.e.	794 \pm 68	625 \pm 63

Craniocaudal migration is significantly greater (*t*-test; $P < 0.001$).

Estimation of craniocaudal migration by area 3 neural crest cells

Calculation of total increase in graft size after 48 h

Mean (\pm s.e.) initial size of graft (at zero time) = 52.5 μm (\pm 4.5) (a).

Mean (\pm s.e.) final size of graft (after 48 h) = 794 μm (\pm 68) (b).

Relative increase in graft size = $\frac{b}{a} = 15.1$ times (c).

Calculation of embryonic lengthening after 48 h

Mean (\pm s.e.) distance from otic placode to MB/FB junction at 5 somites = 11.6 mm (\pm 0.5) (d).

Mean (\pm s.e.) distance from otic vesicle to MB/FB junction after 48 h = 54.4 mm (\pm 0.7) (e).

Relative increase in embryonic length = $\frac{e}{d} = 4.7$ times (f).

Therefore relative increase of graft due to migration = $c - f = 10.4$ times.

Actual extent of craniocaudal migration over 48 h = relative increase in graft due to migration \times mean initial graft size = $(c - f)a = 10.4 \times 52.5 \mu\text{m} = 546 \mu\text{m}$.

Cockcroft, 1976b; Buckley, Steele & New, 1978). Specifically, there were no statistically significant differences in the somite number and protein content between sample embryos from each category of cultured embryos and *in vivo* controls (Student's *t*-test, $P > 0.05$), with the exception of WGA-labelled controls which showed reduced protein synthesis ($P < 0.02$). The histological pictures of labelled controls and injected embryos were similar to that of *in vivo* controls and there was no evidence of structural deformities from marker teratogenicity. The incidence of abnormal embryos was no higher in injected and labelled embryos than in unlabelled controls.

Analyses of labelled controls revealed that signals from the WGA marker remained strong after 48 h and identification of labelled cells was unequivocal (results not shown). These cells were concentrated chiefly in the forebrain and midbrain neural epithelium but were also found in surface epithelium and sub-jacent cranial mesenchyme. (Because of difficulty in distinguishing between neural crest-derived and mesodermal mesenchyme, the use of the term cranial mesenchyme in this report is without connotation of specific origin or fate.)

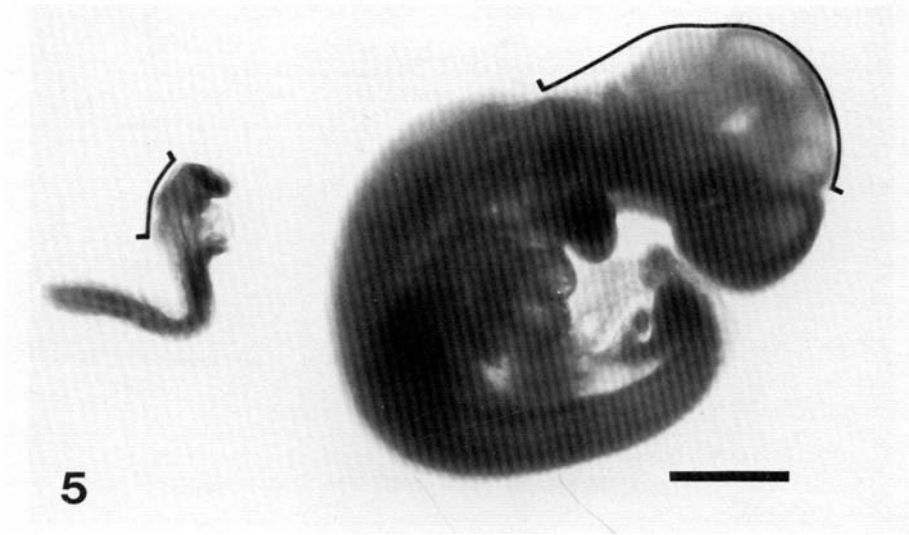


Fig. 5. Live embryos, dissected free of their membranes, showing relative sizes at the time of grafting (left) and after 48 h culture. The line indicates the distance from the midbrain/forebrain junction to the otic placode or vesicle. Bar, 600 μm .

Labelling was patchy in certain parts of the forebrain; however, this may have been due to differential rates of growth during the development of this structure. Labelled cells were seldom present in the ventral part of the hindbrain or trunk neural tube. Their absence here was correlated with the finding that WGA was not being taken up by all the cells of the uptake embryo. Uptake of WGA from the amniotic fluid was selective and restricted to subsurface mesenchyme and ectodermal surfaces that directly faced the amniotic cavity during the labelling process. Hence, parts of the neural ectoderm that were apposed together in the myelencephalon plus the entire trunk neural tube which was shielded by surface epithelium did not show WGA uptake.

Subcellular localization of WGA

After 30 min exposure, WGA was taken up by the surface and neural ectoderm, including cells of the neural crest (see Fig. 2D). In electron micrographs, WGA presented as immunoperoxidase-labelled endosomes and vesicles in a punctate distribution inside the cytoplasm (Fig. 6). Peroxidase staining was never observed inside the nucleus although juxtannuclear labelling was very often present. The stained vacuoles were membrane-bound and rimmed with peroxidase reaction product; the larger ones measured 1–2 μm in diameter and had empty centres which correspond to endosomes identified by other investigators (Gonatas *et al.* 1984; Broadwell & Balin, 1985). Smaller-sized endocytic vesicles and tubules (0.2–0.5 μm wide) were also frequently seen in the immediate vicinity of granular endoplasmic reticulum and Golgi apparatus, although the Golgi cisternae *per se*

Table 2. Comparison of embryonic growth between *in vivo* and cultured embryos

	Total no. of embryos	No. normal	No. abnormal	Somite no. after 48 h (mean \pm s.e.)	Embryo protein (μ g) after 48 h (mean \pm s.e.)
<i>In vivo</i> controls	18	17 (94 %)	1 (5 %)	26.7 \pm 0.5 (n = 17)	229.2 \pm 18.7 (n = 9)
Cultured embryos					
Injected embryos*	167	142 (85 %)	25 (15 %)	27.2 \pm 0.2 (n = 20)	271.0 \pm 6.8 (n = 8)
Unlabelled controls	21	19 (90 %)	2 (10 %)	27.2 \pm 0.3 (n = 19)	226.0 \pm 17.4 (n = 12)
Labelled controls	12	10 (84 %)	2 (16 %)	27.1 \pm 0.7 (n = 6)	161.66 \pm 11.5 (n = 6)

* These have been pooled together from many different experiments; somite no. and protein content were derived from embryos that were at similar stages to those of other categories.

No significant differences in somite no. and protein content between *in vivo* embryos and other categories of cultured embryos (*t*-test; $P > 0.05$). However, significant difference was observed in protein content between *in vivo* and WGA-labelled embryos (*t*-test; $P < 0.02$).

were not labelled. These electron-dense vesicles were never seen in neural crest cells of control embryos that had not been subjected to WGA labelling (Tan, 1986).

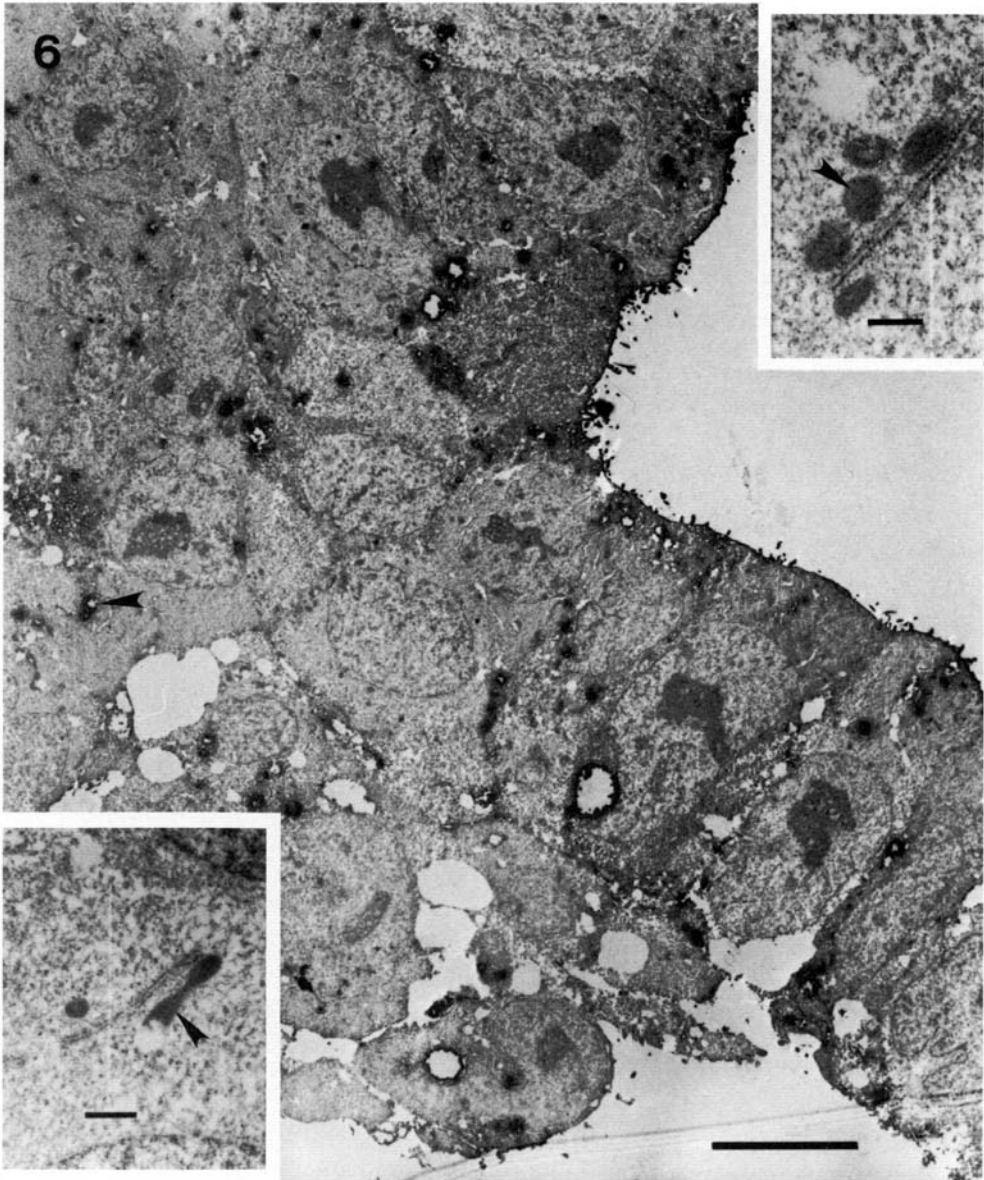


Fig. 6. Transmission electron micrograph of rostral hindbrain neural crest cells and adjacent surface and neural ectoderm (same specimen as Fig. 2D) of an embryo fixed immediately after a 30-min exposure to WGA in the amniotic cavity followed by immunoperoxidase staining. PAP-positive reaction within endosomes (arrow) and smaller vesicles is clear at low magnification. Higher magnification (insets) revealed smaller vesicles of various shapes. Bars: main figure, 10 μm ; top inset, 0.2 μm ; bottom inset, 0.5 μm .

Analysis of WGA-labelled cells in chimaeras

The oldest chimaeras to be analysed were survivors from 72 h embryo culture (unpublished results from pilot study). In these and in 48 h embryos, the development of the facial structures was still in early stages but as in other species of mammalian embryos (e.g. see Hamilton & Mossman, 1972) was sufficiently advanced to permit identification of the presumptive maxillary and mandibular processes of the first arch. The nasal placodes, though still not fully invaginated, also served as landmarks for the division of the mesenchyme that will make up the future medial and lateral nasal processes. However, as the labelled cells had not developed up to the stages of tissue differentiation, they will only be described as mesenchymal cells in relation to the presumptive structures in which they were found.

The pattern of structure colonization by injected cells did not vary significantly between chimaeras cultured for the two time periods (24 and 48 h), so the two sets of observations are considered together. A proportion of injected embryos failed to give rise to any sort of cell labelling and these were embryos assumed to have lost the grafts either by sequestration into the extraembryonic environment or *via* the circulation into the yolk sac.

General behaviour of orthotopic transplants

By serial reconstructions and analyses in both transverse and sagittal planes, cell migration from a given site may be traced in the lateral, dorsoventral and rostrocaudal planes. Cells in the ventral pathway did not constitute a narrowly defined stream but instead occupied a variety of positions inside the lateral space bounded by neural tube and surface epithelium. This notwithstanding, migrating cells were frequently observed close to the basilateral surface of the neural tube and underneath the surface epithelium, giving the impression that these surfaces may have acted as substrata for cell locomotion.

Most of the injected cells tended to stay together during the early stages of migration but upon reaching the main part of the cranial mesenchyme were quickly dispersed among the host mesenchyme. Thus, the pattern after 48 h consisted mainly of individual marked cells either incorporated into host structures (e.g. ganglion, blood vessel walls) or assimilated into pharyngeal arch mesenchyme. However, these structures were not all colonized in every successful chimaera examined: a few chimaeras showed heavy colonization of a select number of sites (e.g. pharyngeal arch mesenchyme, blood vessel walls) while others showed cell labelling in surface epithelium and ganglionic anlagen only.

The second dimension of crest cell migration was seen in the rostral pathway. These cells travelled in the longitudinal axis, to be distributed in more anterior locations than their places of origin. They were closely associated with the dorsal and lateral aspects of the neural tube and did not achieve very much ventral migration into deeper parts of the embryo. In addition, cells associated with the

neural tube were often viewed in caudal migration away from the graft (see areas 4 and 5).

Migration in areas 2 and 3

Neural crest cells in these areas originate from positions anterior to the preotic sulcus and were chiefly concerned with contribution to first pharyngeal arch structures and neighbouring periocular mesenchyme. There was some overlap in the distribution of cells from the two areas in the caudal regions of the optic vesicles and, to a lesser extent, the maxillary process.

The migration of area 2 cells was confined mainly to the mesenchymal tissues surrounding the forebrain expansions (Fig. 7). After 24 h, donor cells had moved ventrally on the sides of the midbrain tube and also around the optic stalk and vesicle on their dorsal, ventral, caudal and rostral surfaces (Fig. 7A). There was some condensation of labelled cells where they meet the anterior cardinal vein, at the site of the presumptive trigeminal ganglion (Figs 7C,D). By 48 h, the nasal placode had formed at the rostral extremity of the forebrain expansion and, although not fully invaginated, it served to divide the surrounding labelled cells into prospective medial and lateral nasal mesenchyme. Other donor cells had travelled *via* the rostral pathway around the rostral extremity of the forebrain and were localized in the narrow strip of mesenchyme between surface and forebrain epithelium (Fig. 7B). Generally, cells from area 2 were not found in the mandibular process and their distribution in other structures is summarized in Table 3.

The distribution of area 3 cells in the ventral pathway was closely, though not entirely, allied to the structures associated with the first pharyngeal arch (Fig. 8). A small percentage had migrated into periocular areas normally served by area 2 neural crest cells (Fig. 8A). A few cells had penetrated the dorsal surface epithelium overlying the closed neural tube. These cells did not migrate very far away from the dorsal midline and similar invasion of embryonic epithelium in other areas by donor cells was not observed.

Cells that made up the ventrally directed population did not all migrate into the pharyngeal arch mesenchyme: a small percentage of cells frequently ceased migration upon reaching the anterior cardinal vein (Fig. 8B). Here, on the lateral

Fig. 7. Migration from area 2.

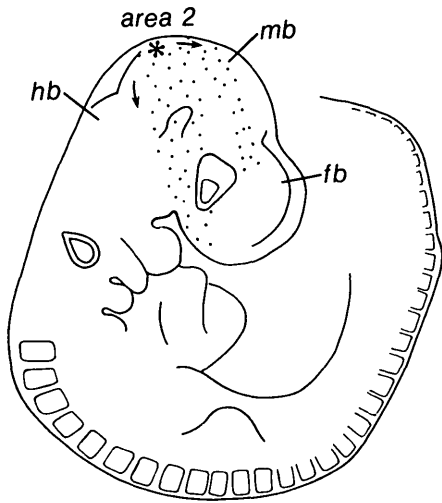
(A) Diagram summarizing final locations of labelled cells after rostral and ventral migration (arrows) from the site of injection (asterisk). These diagrams are compiled from the scoring of labelled cells in different embryos, therefore the number of dots does not correspond to the number of cells, only to their final locations. *fb*, forebrain; *mb*, midbrain; *hb*, hindbrain.

(B) Parasagittal section showing labelled cells (arrows) rostral and ventrolateral to the injection site 24 h after culture. *op*, optic vesicle. Bar, 100 μ m.

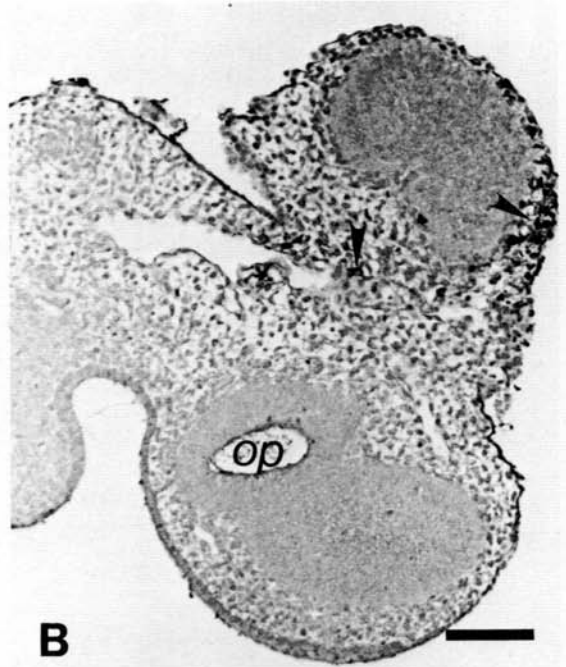
(C) Diagrammatic transverse section summarizing locations of labelled cells in this plane. *fb*, forebrain; *mb*, midbrain; *acv*, anterior cardinal vein; *lens pl.*, lens placode; *nasal pl.*, nasal placode. Dotted line indicates outline of trigeminal ganglion.

(D) Example of one of the sections from which C is compiled. Labelled cells, two of which are indicated by arrows, may be seen dorsal and ventral to optic vesicle (*op*) 24 h after injection. Bar, 200 μ m.

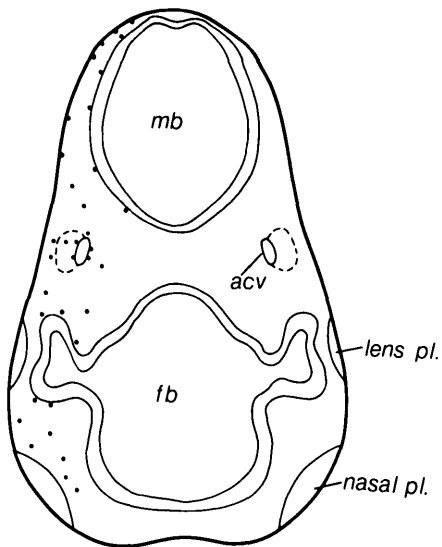
aspect of the blood vessel, cells participated in the aggregation of a closely knit structure in the space between the vein and surface epithelium, forming the primordium of the trigeminal ganglion. This condensation frequently distended into the lumen of the anterior cardinal vein whose walls were also infiltrated by



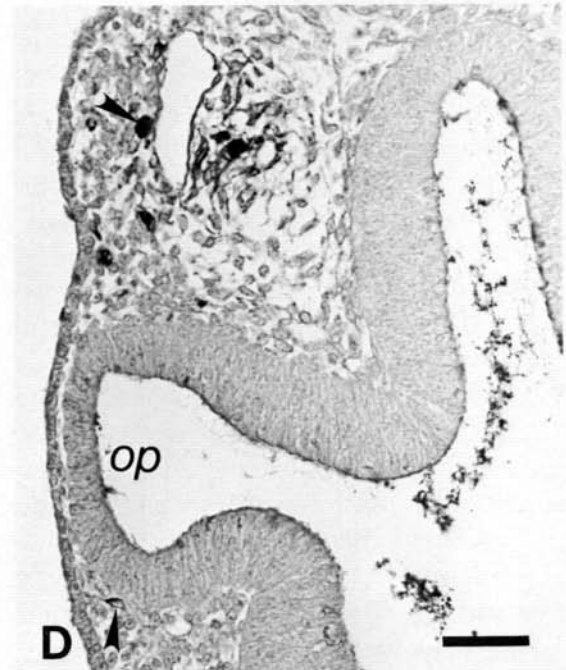
7A



B



C



D

Table 3. Donor cell distribution in host structures after orthotopic injection into areas 2, 3, 4 and 5

	Area 2	Area 3	Area 4	Area 5
No. injected*	4	30	9	4
No. successful	4	19	8	4
Structures colonized†				
Dorsal surface epithelium	—	12 (63 %)	5 (62 %)	3 (75 %)
Ant. cardinal vein	4 (100 %)	18 (94 %)	6 (75 %)	4 (100 %)
Trigeminal ganglion	4 (100 %)	17 (89 %)	—	—
Periocular mesenchyme	4 (100 %)	—	—	—
Maxillary process	3 (75 %)	19 (100 %)	—	—
Dorsal aorta	—	12 (63 %)	6 (75 %)	3 (75 %)
Prox. mandibular process	—	18 (94 %)	8 (100 %)	—
Dist. mandibular process	—	13 (68 %)	—	—
Acousticofacial ganglion	—	—	7 (87 %)	—
Prox. hyoid arch	—	—	8 (100 %)	3 (75 %)
Dist. hyoid arch	—	—	6 (75 %)	—
Vagal ganglion	—	—	—	2 (50 %)
3rd pharyngeal arch	—	—	6 (75 %)	3 (75 %)
Contact with gut wall	—	—	—	2 (50 %)

* Pooled together from 24 and 48 h embryos.

† These represent number and percentage of embryos that showed colonizing cells in these structures.

labelled cells. In more caudally located regions, the presumptive ganglion was found closer to the basal surface of the neural tube as the intervening vein became ventrally displaced. In this position, labelled cells from the periphery of the developing ganglion were viewed in close contact with the basal lamina of the neural tube.

On the medial and ventral aspects of the anterior cardinal vein, labelled cells were never densely grouped but became widely scattered as a dispersed population (Fig. 8C,D,F,G). Labelled cells frequently lodged in the walls of the anterior cardinal vein, dorsal aorta and pharyngeal arch arteries. The remainder of the donor population was well mixed with host cells of the maxillary and mandibular processes but it was not obvious whether these cells had ceased migratory activity or were still capable of further movement. Since development had not progressed to the stage of histodifferentiation, cells in this region may be identified only as belonging to the maxillary or mandibular process. To distinguish further between proximal and distal parts of the mandibular process, an imaginary line was drawn across the dorsal border of the foregut in transverse sections (Fig. 8D). The final distribution of area 3 cells in the various structures is summarized in Table 3.

The rostral component of area 3 migration extended right up to the midbrain region where labelled cells were distributed in mesenchyme surrounding the brain tube (Fig. 8E). A small number of labelled cells had migrated rostral to the level of

the developing eye but in most specimens labelled cells were found only in the caudal half of the optic vesicle and adjacent maxillary process.

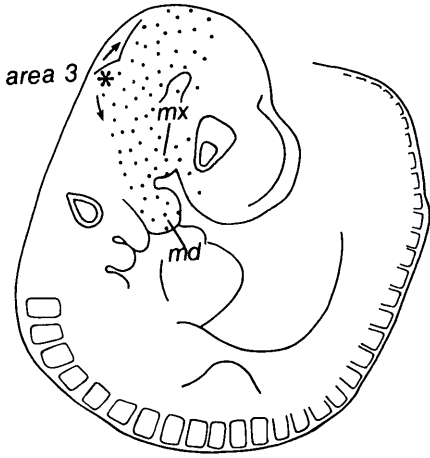
The two separate vectors of migration in area 3 showed statistically significant variation: craniocaudal migration was more extensive than dorsoventral migration (Table 1; *t*-test, $P < 0.001$). This phenomenon may not be entirely due to embryonic growth in the anteroposterior direction. The extent of the rostral migration in terms of dimensional increase over the original graft size was calculated to be 15.1 times. Over the same period, the cranial embryonic length was found to have increased by 4.7 times. Therefore, it may be construed that the area distribution of donor cells due to actual cell movement had increased by an average of 10.4 times the initial graft size ($52.5 \mu\text{m} \pm 4.5 \text{ S.E.}$) or $546 \mu\text{m}$ in real terms. However, the exact events must be more complicated than this, and these calculations are only able to demonstrate that the spreading of area 3 cells cannot always be accountable by passive displacement alone.

Migration in areas 4 and 5

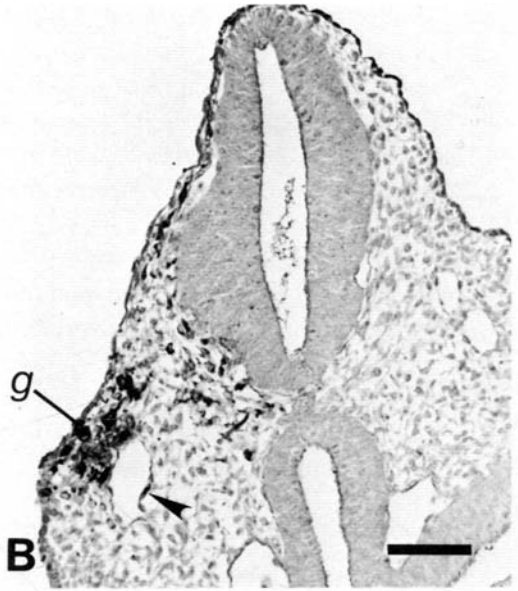
The behaviour of area 4 neural crest cells may also be followed in the transverse and longitudinal planes (Fig. 9). At the level of the second pharyngeal (hyoid) arch, the ventral route was divided into two separate pathways by the intervening otic vesicle (Fig. 9A). Labelled cells in the medial pathway were sandwiched between neural tube and otic vesicle as a single layer in intimate contact with the neural tube (Fig. 9F,G). The lateral pathway between otic vesicle and surface epithelium was occupied by labelled cells which were associated with the anterior cardinal vein and second arch mesenchyme. There was condensation of area 4 cells on the lateral aspects of the anterior cardinal vein to participate in gangliogenesis (Fig. 9B). This condensation was more apparent in the rostral half of the second arch (rostral to the otic vesicle) where the two separate pathways had merged (Fig. 9D,E). The extent of structure colonization by area 4 cells is summarized in Table 3.

Rostral migration of area 4 cells was seen to extend into the proximal mandibular process. This meant that the population of cells that was normally identified with the second arch also contributed to first arch mesenchyme. In addition, area 4 cells were observed in the blood vessel walls associated with the first arch region. There was also migration of area 4 cells into the mesenchyme of the third and fourth pharyngeal arches (Fig. 9C). These cells were also commonly found within and adjacent to the walls of the regional arteries and veins. This pattern of distribution implies that the otic vesicle did not act to obstruct cell migration from the site of injection into the more caudally situated pharyngeal arches.

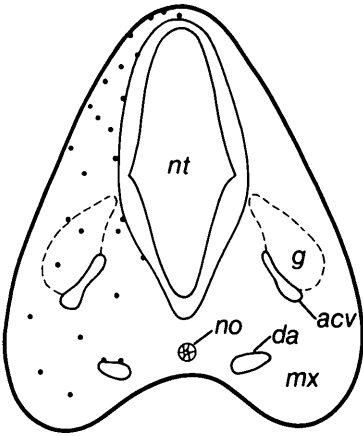
The distribution of area 5 cells followed the same basic pattern of migration into third and fourth pharyngeal arch structures (Fig. 10; Table 3). The rostral pathway carried cells into the second arch mesenchyme which, taken together with data from area 4, indicate an extensive degree of cell mixing by the migratory populations from areas 4 and 5 (Fig. 10A). Once again, rostrally migrating cells were closely associated with the walls of the neural tube and blood vessels. There was



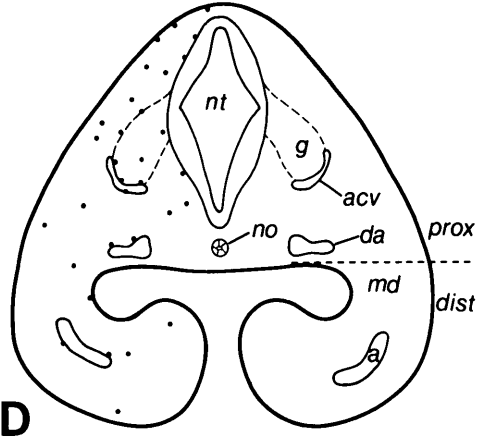
8A



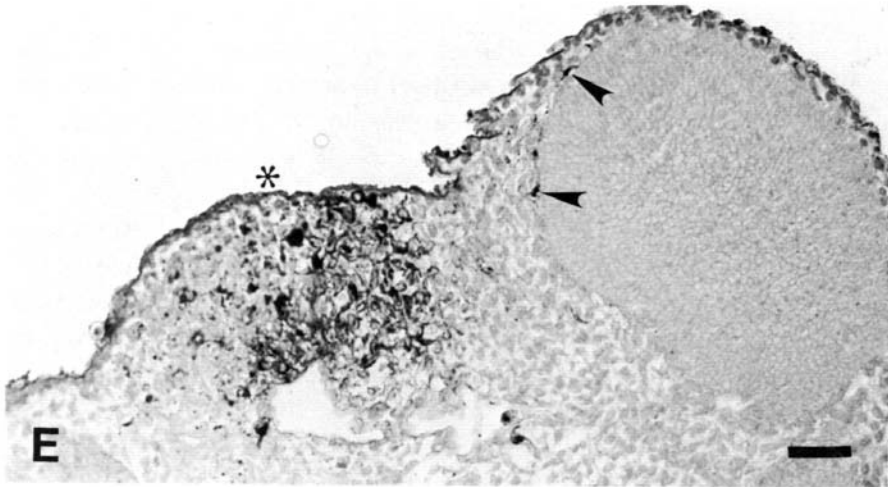
B



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D



E

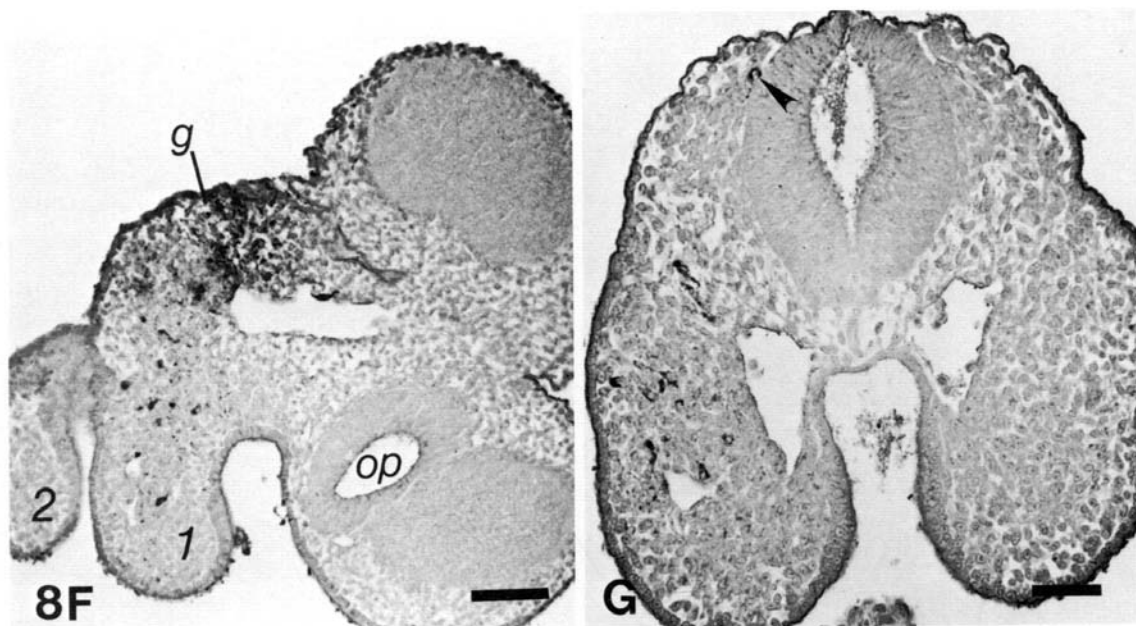


Fig. 8. Migration from area 3.

(A) Diagram summarizing final locations of labelled cells (arrows) after culture. *mx*, maxillary process; *md*, mandibular process.

(B) Transverse section through maxillary process after 24 h culture. Labelled cells are situated around the hindbrain and notochord, and in the region of the presumptive trigeminal ganglion (*g*) between anterior cardinal vein and surface ectoderm. The arrow indicates a labelled cell in the endothelium of the vein. Bar, 70 μm .

(C) Locations of labelled cells compiled from transverse sections passing through the maxillary region. *nt*, neural tube; *g*, trigeminal ganglion primordium; *no*, notochord; *da*, dorsal aorta; *acv*, anterior cardinal vein.

(D) Locations of labelled cells compiled from sections passing through the mandibular arch. Dotted line demarcates proximal (*prox*) from distal (*dist*) parts of the mandibular arch process. *a*, first aortic arch artery. Other labels as above.

(E) Another section from the specimen illustrated in F, showing rostral migration by a small number of cells found lying against the midbrain neuroepithelial basement membrane. The asterisk points to the approximate site of injection. Bar, 50 μm .

(F) Parasagittal view of the head region and optic vesicle with positively labelled crest cells in the mandibular arch, trigeminal ganglion and anterior cardinal vein, 24 h after injection. 1, 2, first and second pharyngeal arches; *g*, ganglion; *op*, optic vesicle. Bar, 100 μm .

(G) Transverse view of mandibular arch, with the majority of labelled cells in the distal region of the mandibular arch. A single labelled cell (arrow) may also be seen in the presumptive trigeminal ganglion. Bar, 50 μm .

similar caudally directed migration into the mesenchyme of the fourth pharyngeal arch up to the level of the first somite (Fig. 10B).

Within the third arch, a large number of labelled cells were located near the site of the developing glossopharyngeal–vagal ganglia situated between neural tube wall and anterior cardinal vein (Fig. 10C). The remainder of the donor population was divided into two ventrally migrating streams: one hugging close to the neural tube wall and the other dispersed inside the mesenchyme of the somatopleure and

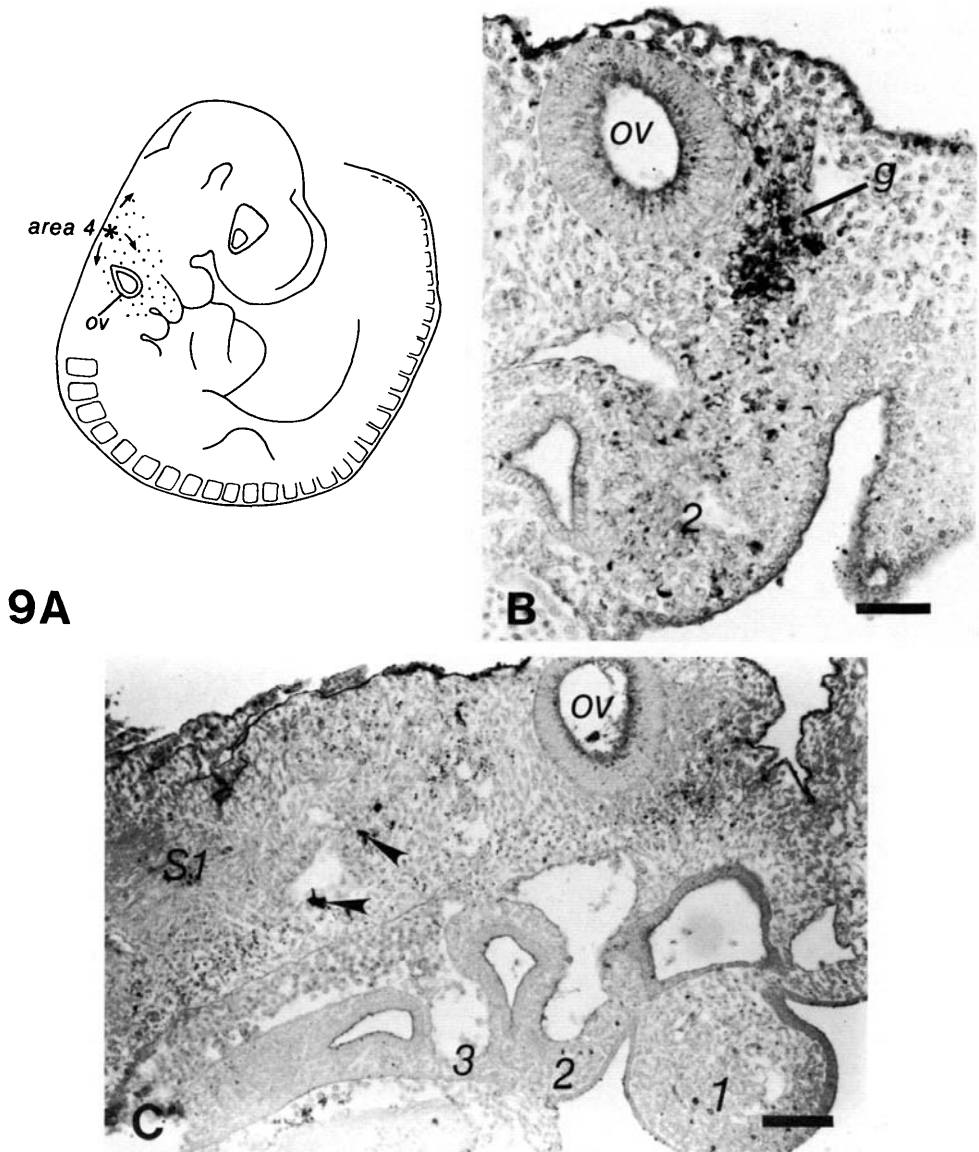


Fig. 9. Migration from area 4.

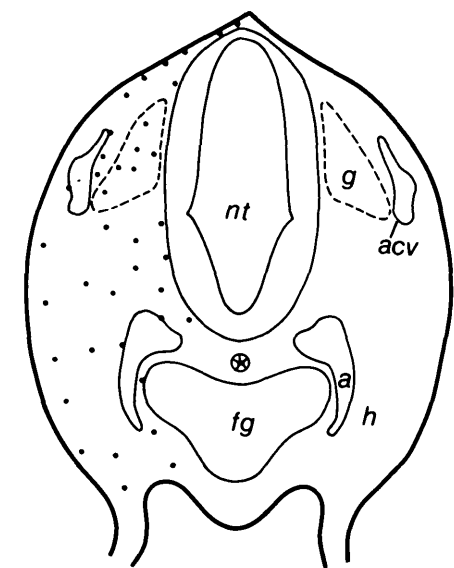
(A) Diagram summarizing final locations of labelled cells (arrows) after culture. Cells pass both rostral and caudal to the otic vesicle (*ov*).

(B) Parasagittal section showing a group of cells that have condensed in the region of the presumptive acousticofacial ganglion while others are dispersed within hyoid arch mesenchyme 24 h after injection. *g*, ganglion; *ov*, otic vesicle; 2, hyoid arch. Bar, 50 μ m.

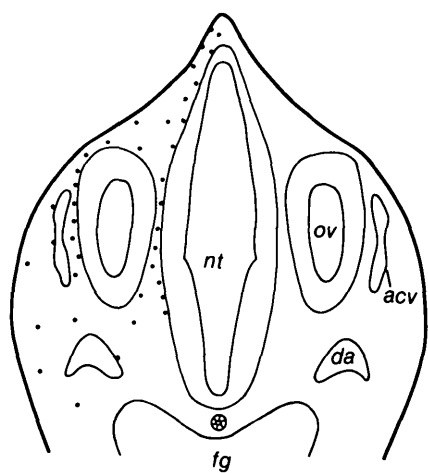
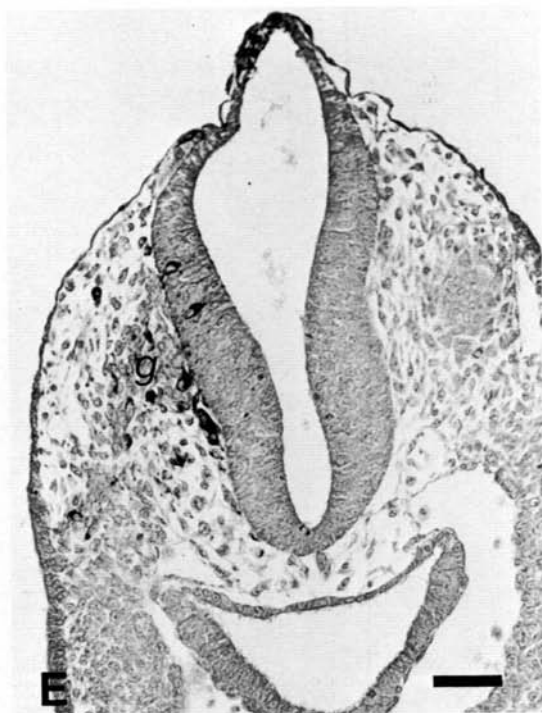
(C) Parasagittal section showing a group of cells close to the area 4 injection site and others which have migrated caudally (arrows) almost to the level of the first somite (*S1*) 48 h after injection. 1, 2, 3, first, second and third pharyngeal arches; *ov*, otic vesicle. Bar, 100 μ m.

(D) Locations of cells compiled from sections in the plane rostral to the otic vesicle. *fg*, foregut; *h*, hyoid arch; other labels as in Fig. 8C.

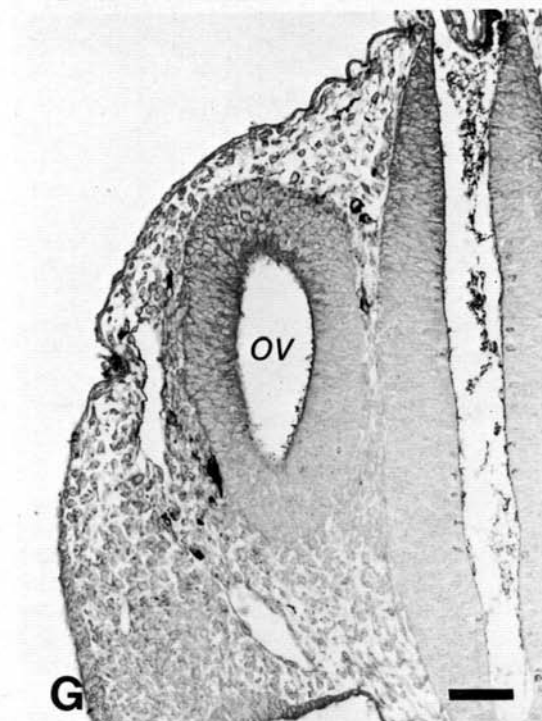
(E) A transverse section rostral to the otic vesicle showing labelled cells in the acousticofacial ganglion condensation (*g*) and two labelled cells which have remained within the neural epithelium 24 h after injection. Bar, 50 μ m.



9D



F



G

Fig. 9. (F) Locations of cells compiled from sections in the plane of the otic vesicle. *ov*, otic vesicle.

(G) A transverse section passing through the otic vesicle (*ov*), 48 h after injection, showing labelled cells both medial and lateral to the vesicle and others within the wall of the cardinal vein. Bar, 50 μ m.

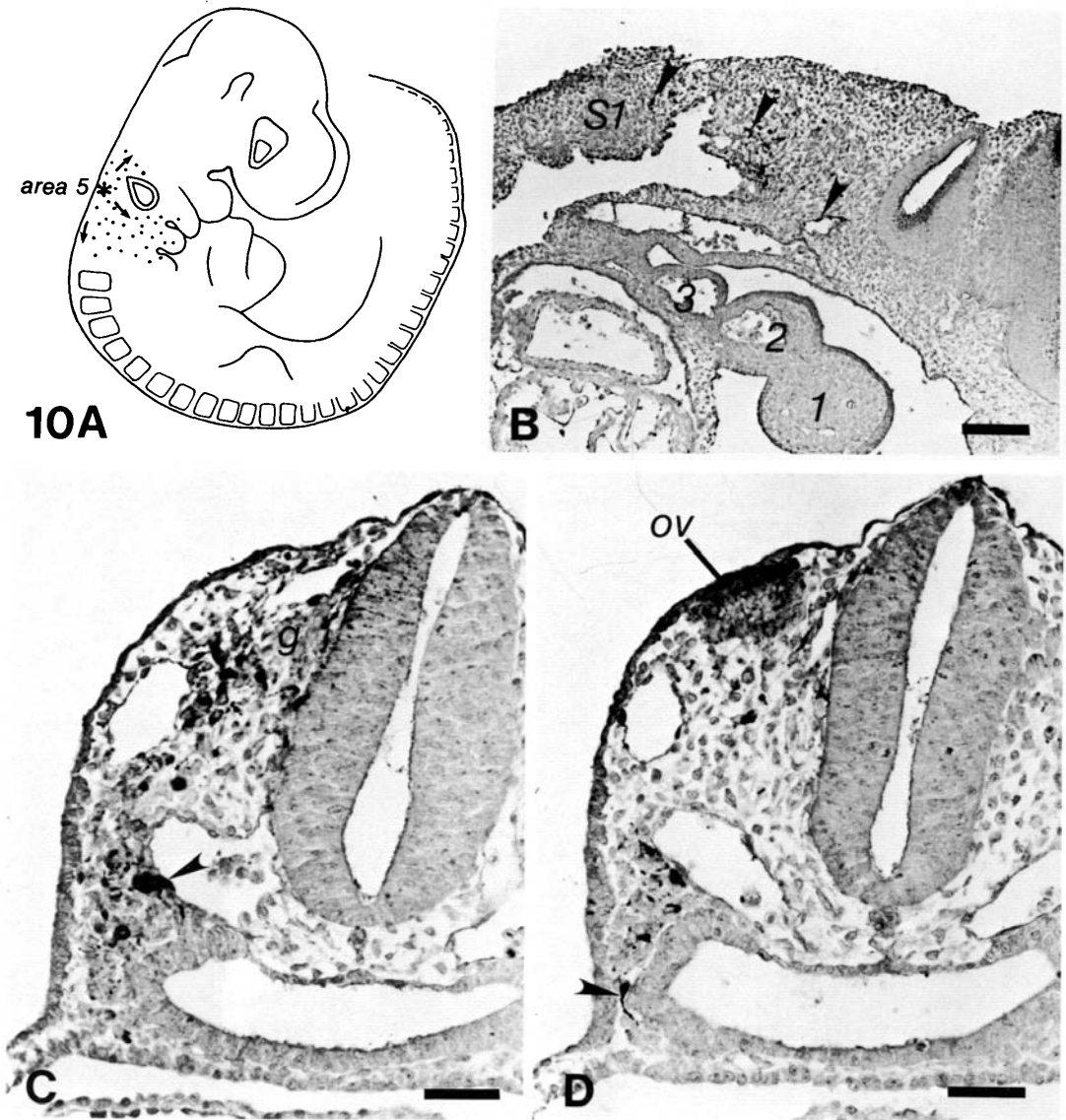


Fig. 10. Migration from area 5.

(A) Diagram summarizing the final locations of labelled cells after culture.

(B) Parasagittal section showing a group of cells (arrows) which have migrated caudally, some to the level of the first somite, 48 h after grafting. 1, 2, 3, first, second and third pharyngeal arches; S1, first somite. Bar, 100 μ m.

(C) Labelled cells found 24 h later within dorsal surface epithelium, anlage of the vagal ganglion (g), anterior cardinal vein and dorsal aorta (arrow) walls, and third pharyngeal arch mesenchyme. Bar, 50 μ m.

(D) Labelled cells associated with the caudal border of the otic vesicle and adjacent mesenchyme, and in contact (arrow) with the foregut epithelium. ov, otic vesicle. Bar, 50 μ m.

splanchnopleure. In this latter site, labelled cells sometimes approached and established contact with the foregut (Fig. 10D) but there was no penetration into the enteric walls. Labelled cells were also seen within the dorsal surface epithelium and the walls of the anterior cardinal vein and aortic arch arteries, including dorsal and ventral aortae.

Area 1 injected into area 2

The behaviour of area 1 cells in over half of the embryos (five out of nine) was striking in their failure to be assimilated into host structures. Instead, the transplanted cells displayed autonomous development into one or two hollow vesicles per embryo. These vesicles were either attached to the host midbrain neural tube or to surface epithelium and were pseudostratified (Fig. 11A–C). The size of the vesicles varied, some approaching the size of the developing optic vesicle. Despite direct contact with host tissues, the marker was not seen to have been released into surrounding cells. In the remaining four cases that did not form ectopic vesicles, injected cells were well mixed together with host cells although the pattern of dispersion was often random and did not resemble the behaviour of any particular area. Specifically, they were predominant in surface and neural epithelium, but were also extensively distributed into cranial mesenchyme of no special demarcation.

Late injections

The results from late transplantation experiments demonstrate that crest cells from the 11-somite embryo have reduced dispersal capacities. When area 3 cells were introduced synchronously into orthotopic sites, they failed to achieve as much ventral migration as earlier grafts (Fig. 12A). The bulk of injected cells remained close together underneath the dorsal surface of the embryo with a small minority of cells localized in the adjacent surface epithelium (Fig. 12B). Occasionally, some labelled cells were seen to have moved away from the main mass as individual cells in host mesenchyme (Fig. 12C). The rostrocaudal vector of migration in these grafts was also reduced and very few cells were seen any distance away.

DISCUSSION

Methodological considerations

A method has been described whereby WGA was successfully used as an exogenous cell marker for studying the early stages of neural crest development in rat embryos. Whole embryo culture was used to sustain development of post-operative embryos up to the limb-bud stage, a period that proved adequate for monitoring the earliest phases of crest development including onset of migration, pathway selection and homing. Nevertheless, it cannot be assumed without question that the injected cells behaved in precisely the same manner as adjacent

host crest cells. In contrast to the avian transplantation technique, cells were added without the removal of an equivalent piece of tissue. However, the number of cells injected was small, so the problem of additional material is negligible.

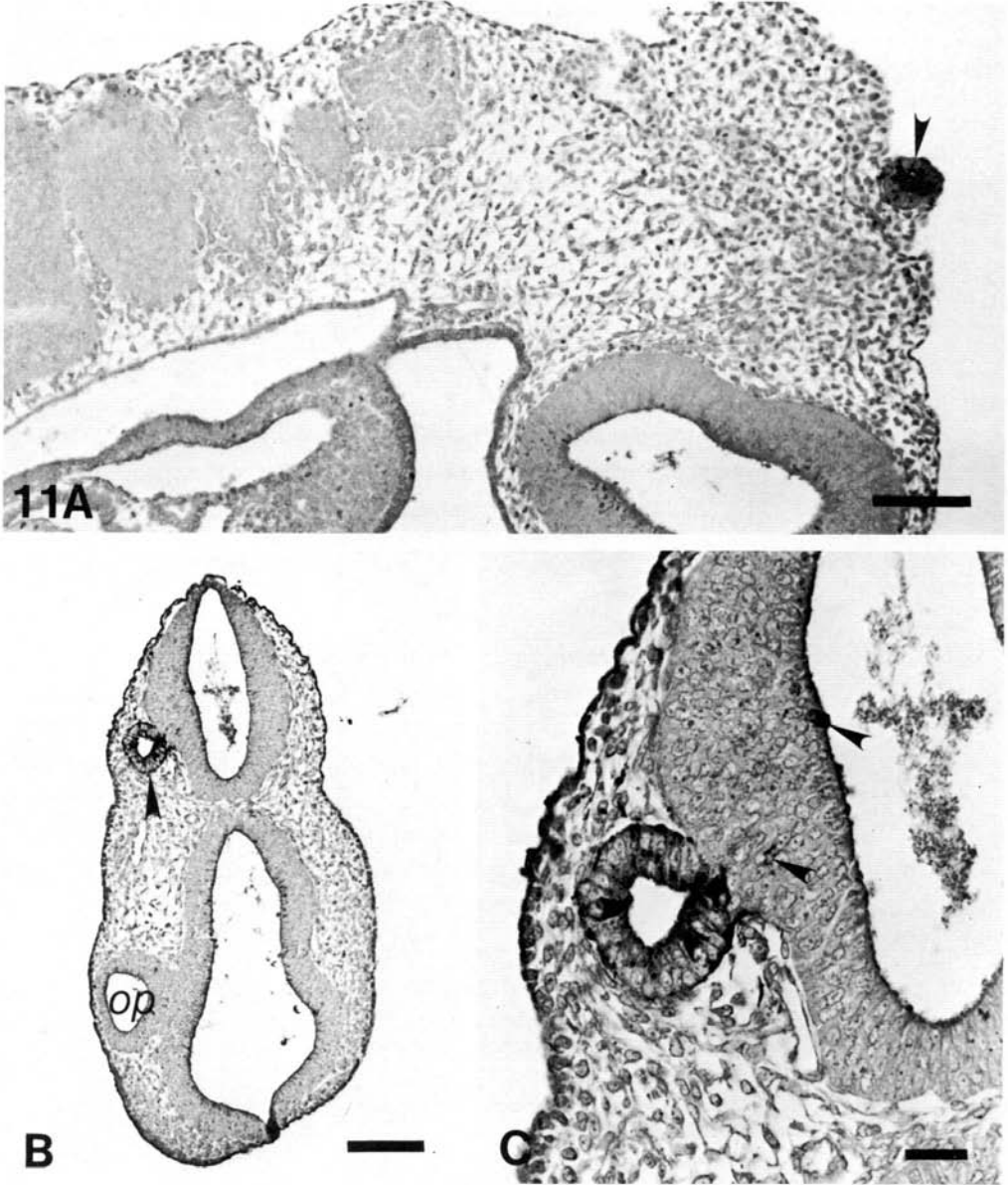


Fig. 11. Heterotopic transplants: area 1 to area 2.

(A) Small vesicle (arrow) containing labelled cells close to the site of injection; parasagittal section. Bar, 100 μ m.

(B) Vesicle of labelled cells, similar in form to the optic vesicle (op), attached to the midbrain neural tube. Bar, 100 μ m.

(C) Higher magnification; two labelled cells (arrows) have been incorporated into the neural epithelium. Bar, 25 μ m.

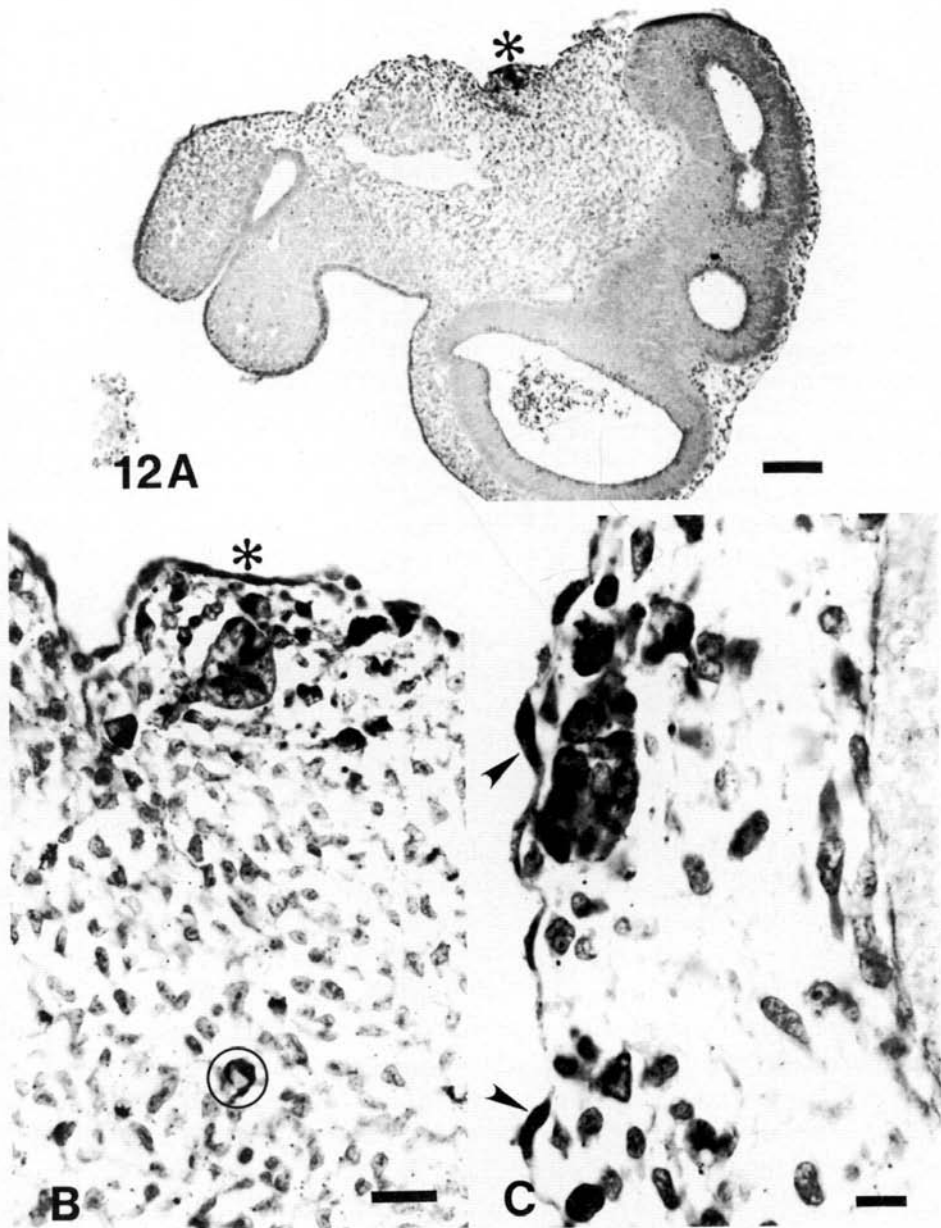


Fig. 12. Late injections into area 3, analysed 24 h later.

(A) Cells have migrated poorly and remained at the injection site (asterisk). Bar, 100 μm .

(B) Occasionally, a single labelled cell (circled) may be seen to have migrated away from the main mass of stationary cells. Bar, 10 μm .

(C) At higher magnifications, labelled cells are found grouped together in the subectodermal location and a few have been incorporated into the surface ectoderm (arrowed). Bar, 25 μm .

Trauma during insertion of the injection micropipette was also minimal; 85 % of injected embryos continued to develop normally, and the remaining 15 % was excluded from analysis. Evidence that the injected cells did participate in normal neural crest cell behaviour comes from their postmigratory distribution. First, they migrated to sites predicted from non-mammalian studies; second, they did not remain as a clump but dispersed in a manner suggestive of integration with a larger population of similar cells.

The use of WGA for cell marking may be justified on the following grounds. The binding affinity of WGA for *N*-acetyl glucosamine residues present on the cell surface is specific (Nagata & Burger, 1974), with the possibility of some nonspecific binding to sialic acid (Nicholson, 1974). The binding complexes are internalized by adsorptive endocytosis, as part of the process of membrane renewal and turnover (Gonatas *et al.* 1980), remaining inside cytoplasmic organelles. There is some evidence that WGA is not recycled to the plasma membrane even though the accompanying receptors may be resurfaced (Gonatas *et al.* 1984) and therefore minimizing the risk of WGA transfer to neighbouring cells. There is unfortunately no available information on the kinetics of WGA release from dying cells and the possibility of WGA uptake by other cells needs to be further determined. With regard to marker toxicity, WGA has been safely used as a marker molecule for studying neuronal axonal transport; in those studies, WGA did not pose any toxicity problems when used at prudent concentrations (Harper, Gonatas, Stieber & Gonatas, 1980; Gonatas & Gonatas, 1983; Trojanowski & Gonatas, 1983). Further evidence is available from cell agglutination studies of chick embryonic neural retina (Kleinschuster & Moscona, 1972). These workers showed that WGA binding to disaggregated cells did not cause irreparable damage as the agglutinated cells were able to recover after 24 h, retaining their normal developmental organization.

When control embryos were exposed to WGA and cultured for 48 h, they were found to be normal, possessing anatomical and histological features that were developmentally appropriate, including the correct number of somites when compared with *in vivo* embryos of equivalent age (Table 2). The single exception was seen when the protein content of these embryos was analysed: WGA-labelled controls had slightly lower protein levels compared to all other experimental categories, and *in vivo* embryos. However, it would be difficult to say whether this is due to retardation in embryonic growth from the effects of WGA *per se* or due to temporary nutritional setback suffered during the labelling procedure when, for 30 min, the amniotic fluid is replaced by WGA-containing PBS. Whichever the case may be, it may be assumed that the growth retardation is from a systemic influence, and therefore would not invalidate the cell injection experiments where only 20–30 WGA-labelled cells were deposited into unlabelled recipients.

There are several lines of evidence to confirm that WGA as an *in situ* marker is stable and cell localized. First, immunoelectron microscopy showed that the ligand had been endocytosed as membrane-bound vesicles, tubules and endosomes into the cytoplasm and juxtannuclear areas. These data corroborate other observations

of WGA and WGA-HRP endocytosis in cultured cells and neurones of the adult nervous system (Harper *et al.* 1980; Gonatas *et al.* 1984; Broadwell & Balin, 1985). Second, data from double-injected chimaeras (where two separate populations of labelled neural crest cells, one labelled with WGA and the other with tritiated thymidine, were injected into a single embryo) demonstrate that there was no transfer of marker from one population into the other even after the two populations were mixed together (Tan, 1986). Third, donor cells that had formed into a coherent structure in the shape of an epithelioid vesicle (area 1 injection) did not show release of WGA to host tissues that were in direct contact.

There have been previous attempts to study neural crest cell migration in gastrula- and neurula-stage mouse embryos by injecting WGA (conjugated to gold) into the amniotic cavity (Smits-Van Prooije, Poelmann & Vermeij-Keers, 1984; Smits-Van Prooije *et al.* 1986). Those studies were based on the assumption that the marker is able to diffuse from the amniotic fluid into the adjoining embryonic ectoderm, from which labelled neural crest cells would carry the marker with them as they migrate into deeper layers of the embryo. However, this approach is seriously limited by the lack of specificity in the type of cells that are labelled, i.e. difficulty in distinguishing labelled neural crest cells from other migratory populations that have also taken up the label, e.g. ectodermal placodes, primitive streak and tail bud cells. As the marker is freely available to cells along the entire length of the embryo, the technique is also poorly suited for studying longitudinal migration pathways as crest cells from different loci in the longitudinal axis become mixed together. Furthermore, as the present study has demonstrated, subepithelial mesoderm can become labelled after intra-amniotic injection of WGA. The possibility therefore exists for false positive results from these mesodermal cells which cannot be distinguished from veritable mesoderm.

Likewise the method of Johnston & Krames (cited in Johnston & Pratt, 1975) is fraught with difficulties of accurately interpreting the results. In that method, a solution of tritiated thymidine was carried on a sable hair tip and delivered into the region of neural crest emigration. Although authentic neural crest cells may be adequately traced using this marker, the difficulty of excluding adjoining mesodermal cells which may have also taken up the diffusible thymidine solution still remains.

Mammalian cranial neural crest cell pathways

This study provides direct evidence for an important contribution of neural crest to craniofacial development in mammals. It therefore confirms what has long been assumed, that the role of the neural crest has maintained a constant relationship to craniofacial development throughout vertebrate evolution (for a review, see Gans & Northcutt, 1983). Nevertheless, there appear to be class-related differences in the rules that govern the spatial distribution of cranial neural crest cells in different vertebrates. The most prominent is that cranial crest cell migration in mammals is not restricted to the characteristic subectodermal pathway described for the

cranial crest in birds (Johnston, 1966; Noden, 1975; Duband & Thiery, 1982). Tangential migration away from the top of the neural tube, which has been reported for nonmammalian crest (Noden, 1975; Lofberg, Ahlfors & Fallstrom, 1980; Tosney, 1982), is also less obvious in the mammalian embryo. This is because the majority of the cranial crest cells in mammals are released from wide-open neural folds (Adelmann, 1925; Holmdahl, 1928). This may also be the reason why migration across the embryonic midline was never exhibited by the injected cells. In contrast, migration across the dorsal midline normally occurs among cranial neural crest cells of the avian embryo (Noden, 1975; Nakamura & Ayer-Le Lievre, 1982), which provides a mechanism, unavailable to mammals, for cell mixing and regulative development.

Other differences between mammalian and avian cranial neural crest behaviour may be seen in their migratory patterns: rat neural crest cells appear to take a multitude of routes, migrating as diffuse streams through the mesenchyme, rather than in compact masses, to their destinations. The absence of a single well-defined pathway during rat neural crest cell dispersion in the transverse plane is probably a reflection of extensive cell mixing with host mesenchyme (of either neural crest or mesodermal origin) and argues against precise, region-specific pathways. In this respect, crest cells from the rat cranial region share with those from the chick trunk area the attribute of penetration into mesodermal mesenchyme (Weston, 1963; Rickman, Fawcett & Keynes, 1985).

There are two possible explanations for the observed differences in migratory patterns between chick and rat embryos. First, in the cranial region of chick embryos, a subectodermal cell-free space rich in hyaluronate normally appears next to the source of emigrating crest cells (Pratt, Larsen & Johnston, 1975). Second, fibronectin, a glycoprotein that is known to promote crest cell migration (Rovasio *et al.* 1983), has been shown to be present at significantly higher levels in subepithelial channels that serve as migratory routes (Newgreen & Thiery, 1980; Duband & Thiery, 1982). These factors, together with concomitant separation of previously fused basement membranes between neural tube and surface ectoderm (Anderson & Meier, 1981; Tosney, 1982), suggest that migrating crest cell populations in the chick may be preferentially localized in the subectodermal route. These observations are in contrast with the lack of cell-free spaces in rat cranial neural crest pathways (Tan & Morriss-Kay, 1985) and the ubiquitous nature of fibronectin distribution in rat cranial mesenchyme (Tuckett & Morriss-Kay, 1986).

It might be argued, however, that the difference could be one of methodology and that the diffuse neural crest cell distribution is the result of an artefact created by the transplantation procedure. The depth of injection is critical and uncontrolled release of donor cells too deep into host mesoderm could give rise to false positive migratory patterns. This is unlikely, in view of the extreme care taken during the injection procedure to ensure that the grafts were correctly positioned at the neural crest/surface epithelium junction. We favour the explanation that a small number of donor cells would be likely to become separated from each other as they confront a larger number of host neural crest cells during the course of

migration and homing. Evidence for intermingling of migrating neural crest cells was obtained from double-injection experiments (Tan, 1986) which showed two separately labelled neural crest cell populations (one labelled with WGA and the other with tritiated thymidine) in a mosaic arrangement. Thus, conventional transplantation techniques with avian embryos, where a considerable length of neural fold or neural tube is usually replaced, are less likely to reveal scattering of labelled cells. In agreement with this point, a recent study that made use of small clusters of avian pigmented neural crest cells for grafting also reported donor cell dispersion within host cranial mesenchyme (Erickson, 1985).

An exception to the general migratory pattern was found among area 4 cells migrating near the otic vesicle. Migration in this region appeared to be channelled into lateral and medial routes by the intervention of the vesicle in the paraxial space, which, in this respect, functions in a similar way to somites in their determination of the early phase of trunk neural crest cell migration (Holmdahl, 1928). This migratory pattern suggests that structural heterogeneities may act to confine the direction of migrating crest cells and perhaps influence their subsequent localization in terminal structures (Ris, 1941; Fox, 1949; Thiery, Duband & Delouvee, 1982). Other structures that also appear to function by this mechanism include the walls of the neural tube and blood vessels; these structures were consistent in their association with migrating crest cells. Apart from achieving a blockade effect, these structures may also have acted through a contact guidance effect (Weiss, 1958; Noden, 1975). At the same time, the role of the neural tube as a source of positional and differentiation cues cannot be discounted in the light of chick experiments that suggest a possible role for the neural tube in skeletogenesis (Benoit & Schowing, 1970); melanogenesis (Glimelius & Weston, 1981), and sensory and adrenergic neurone differentiation (Cohen, 1972; Norr, 1973; Teillet & Le Douarin, 1983; Howard & Bronner-Fraser, 1985).

This study makes no claims on the nature of the topographical relationship between neural crest and mesodermal mesenchyme. Although labelled cells were generally scattered across the head mesenchyme, we were unable to resolve the exact source of this mesenchyme that surrounds donor crest cells. Avian data suggest that there is a strict demarcation, evident 1–2 days after neural crest emigration, between mesodermal and crest-derived mesenchyme, the latter being localized chiefly beside and ventral to the level of the foregut (Le Lièvre & Le Douarin, 1975; Noden, 1982, 1984*a,b*). This study did not permit the neural crest/mesodermal mesenchyme boundaries to be defined in the rat embryo, nor was it possible to follow the labelled cells to the point of differentiation of cartilaginous pharyngeal elements.

Previously, our knowledge of mammalian neural crest cell behaviour was based on descriptive studies of fixed embryos and this static approach has not revealed a true picture of the essentially dynamic events. The weakness of the morphological approach may be illustrated by considering the results of area 1 (fore-brain) transplants. The homologue of the neural crest in this region is normally tightly apposed to the adjacent surface ectoderm, without the intervention of

mesodermal cells; cross-sectional observations do not show delamination of cells from the lateral edges. However, previous descriptive studies have credited forebrain crest with migratory capabilities across a variety of species, including rats, mice, guinea pigs and humans (Da Costa, 1921; Bartelmez & Blount, 1954; Bartelmez, 1962; Nichols, 1981). When area 1 cells were injected into area 2, five out of nine cases failed to disperse but instead showed autonomous development in their new site, re-establishing polarity and forming epithelioid vesicles. Given that the ability to migrate in foreign locations is a general property of neural crest cells (see heterotopic grafts in Noden, 1975; Tan, 1986) it is unlikely that the observed results of area 1 transplants are artefacts of the transplantation procedure or due to foreign environmental influences. Moreover, the injected cells have proliferated and continued on their developmental pathway as epithelial cells within ectopic sites, a phenomenon never shared by other types of orthotopic or heterotopic transplants (Tan, 1986). Thus, it would appear that the reluctance of neuroepithelial cells from the forebrain to undergo mesectoderm conversion is highly conserved, echoing the general observations in chicks and urodeles, where the neural crest homologue from the extreme anterior end of the neural tube is similarly static (Johnston, 1966; Noden, 1975; Hörstadius & Sellman, 1946).

The behaviour of area 1 grafts in the remaining cases is puzzling. Although they were found predominantly as cells incorporated into surface and neural epithelia, a small percentage of cells were also dispersed within mesenchyme. It is likely that as a result of being introduced into a conducive environment, some of these cells have destabilized to become mesenchymal. This idea may be supported by *in vitro* experiments on adult epithelia from a variety of sources: when plated onto collagen gels, they lost their polarity and exhibited mesenchymal characteristics including cell migration (Greenberg & Hay, 1982).

The role of morphogenetic movements

Previous observations (references above) of the cranial crest in a variety of mammalian species (mouse, rat, cat, human) have been unanimous in pointing out that the departure of neural crest cells from the neural folds is precocious with respect to the timing of tube closure in the mesen- and metencephalon. Unlike chick embryos, mammalian crest cells from these regions emigrate very early on while the folds are wide open. This may be a consequence of evolutionary specialization of the mammalian crest, expressed as an earlier process of commitment by neuroepithelial cells to become neural crest while the folds are still flat. Alternatively, it might be because of the evolution in mammals of a larger brain which requires a longer time to close. Whatever the case may be, this unique behaviour of mammalian crest (compared to that of other vertebrates) endows it with distinct advantages from the point of view of the actual migratory distances that these cells have to cover in order to arrive at their terminal end points. As neurulation proceeds, the lateral edges of the neural folds move dorsally and then medially to appose in the midline (Morris-Kay, 1981). Premigratory crest cells are transported dorsomedially as part of this epithelial movement; those that emigrate

relatively late start from a dorsal position, while those that emigrate early avoid the 'transportation effect' and begin their migratory journey from a more ventral position.

It has been shown previously that different phases of neural crest cell emigration may be related to different stages of neural fold morphology along the length of the cranial region (Tan & Morriss-Kay, 1985). The present study has demonstrated that the earliest cells to leave from area 3 at the 5-somite stage (while the neural folds are widely open) are more likely to contribute to the formation of ventrally derived structures, e.g. mesenchymal derivatives of the distal mandibular process. In contrast, later departing cells in 11-somite embryos (from nearly apposed neural folds) did not migrate very far and instead became situated in the dorsal cranial mesenchyme. Clearly, other mechanisms (filling of distal niches, abolition of pathways) (Noden, 1975; Weston & Butler, 1966) may be partly responsible for the observed behaviour of neural crest cells transplanted at later stages. Similar reduction in migratory potential with increasing embryonic age has also been reported using melanocyte injections into the trunk region of chick embryos (Bronner-Fraser & Cohen, 1980*a*). These authors conclude that changes in the environment that lead to reduced migration are a consequence of developmental age and perhaps provide a mechanism for the selective trapping of crest cells in different ganglionic (sensory or autonomic) sites (Bronner-Fraser & Cohen, 1980*b*).

The above illustrates the importance of morphogenetic movements during crest cell dispersion. In the chick embryo, a comparable scenario has been documented in which the neural crest cell population together with surrounding tissues undergoes a ventral shift during the period of neural crest migration (stages 10–14, somites 10–20) (Noden, 1984*a*). Once again, it demonstrates that crest cells are not moving through a stationary environment and, as discussed by Noden (1984*a*), the two processes might fulfil the same function in the two types of embryo. This should not be taken to be an attempt to negate the necessity for cranial crest cells to migrate actively relative to neighbouring tissues; the forebrain transplants in this study indicate that certain types of non-migratory cells do remain static even in the presence of a shifting environment. Measurements performed for area 3 cells suggest that embryonic expansion alone cannot account for the total migratory distances observed. These serve to illustrate that tissue movement is only one component of a complex but integrated set of events that result in the physical displacement of crest tissue from one point to another in the developing embryo.

This study has highlighted the importance of longitudinal cranial crest cell migration as a route for cells from area 2 to furnish the periocular and forebrain regions with mesectoderm. At more caudal levels, the longitudinal pathway may provide the means for crest cells to cross the boundaries between pharyngeal arch regions and regulate cell numbers as the lateroventral head mesenchyme becomes segmented by intervening pouches. There is certainly support from work in other vertebrates that crest cell populations from different pharyngeal arch levels may

after all be continuously linked (Noden, 1983) and this pathway would appear to supply such a mechanism. This crossing of crest cells from one pharyngeal arch level into the next has also been observed for specific regions of avian neural crest cells during migration (Le Lièvre & Le Douarin, 1975; Noden, 1975) and in the formation of skeletal structures by crest cells originating from different arch levels (Le Lièvre, 1978; Noden, 1983; also demonstrated for amphibian trabeculae cranii, Hörstadius, 1950; Chibon, 1966). Perhaps the most dramatic demonstration of cooperation between avian neural crest cells of different axial levels is seen after surgical ablation of the midbrain crest (McKee & Ferguson, 1984). Although the surviving embryos exhibited cervical deformities, facial development in these embryos was remarkably normal and histological examination suggests that the defect was restored by longitudinal migration from adjacent cranial levels. In contrast, there is some evidence that mammalian neural crest cells do not show regulation when they migrate to inappropriate pharyngeal arches. When rat embryos are exposed to excess vitamin A at the primitive streak stage, the cranial neural plate and neural tube which develop subsequently are reduced in size and shifted rostrally in relation to the pharyngeal endoderm; neural crest cells rostral to the otocyst consequently have their migration pathways shifted rostrally. In addition to the rod-like Meckel's cartilage, a rod-like cartilage also forms in the maxillary region, which is normally devoid of cartilage (Morris & Thorogood, 1978). Hence, although we have observed in the present study that neural crest cells may change their pharyngeal level during migration, it is likely that the longitudinal pathways are precisely programmed elements of the overall migration pattern.

In avian embryos, longitudinal migration has been reported to be restricted to the midbrain crest (Noden, 1975). In the present study we observed longitudinal migration in hindbrain crest also, where it occurred in both rostral and caudal directions. These discrepant results may be partly due to differences in technique: a longitudinal pathway may not be revealed by transplantation of a length of neural tube with its attached neural crest, in contrast to the precise tracing of cell displacement which is possible when a small number of cells is injected into a specific site. Or more extensive longitudinal migration may be a characteristic feature of the mammalian neural crest, together with emigration from widely open neural folds, lack of mixing across the midline and diffuse migration through the fibronectin-rich cranial mesenchyme. Some lack of homology of neural crest behaviour between the different classes of vertebrates is only to be expected at these early stages, in view of the differences in cranial structure that are evident later on.

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