Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo

ANDREW LUMSDEN, NICOLA SPRAWSON and ANTHONY GRAHAM

MRC Brain Development Programme, Division of Anatomy and Cell Biology, United Medical and Dental Schools, Guy's Hospital, London SE1 9RT, UK

Summary

A vital dye analysis of cranial neural crest migration in the chick embryo has provided a positional fate map of greater resolution than has been possible using labelled graft techniques. Focal injections of the fluorescent membrane probe Dil were made into the cranial neural folds at stages between 3 and 16 somites. Groups of neuroepithelial cells, including the premigratory neural crest, were labelled by the vital dye. Analysis of whole-mount embryos after 1–2 days further development, using conventional and intensified video fluorescence microscopy, revealed the pathways of crest cells migrating from mesencephalic and rhombencephalic levels of the neuraxis into the subjacent branchial region. The patterns of crest emergence and emigration correlate with the segmented disposition of the rhombencephalon. Branchial arches 1, 2 and 3 are filled by crest cells migrating from rhombomeres 2, 4 and 6 respectively, in register with the cranial nerve entry/exit points in these segments. The three streams of ventrally migrating cells are separated by alternating regions, rhombomeres 3 and 5, which release no crest cells. Rostrally, rhombomere 1 and the caudal mesencephalon also contribute crest to the first arch, primarily to its upper (maxillary) component. Both r3 and r5 are associated with enhanced levels of cell death amongst cells of the dorsal midline, suggesting that crest may form at these levels but is then eliminated. Organisation of the branchial region is thus related by the dynamic process of neural crest immigration to the intrinsic mechanisms that segment the neuraxis.

Key words: neural crest, rhombomere, branchial arch, cell migration, chick, Dil.

Introduction

Strategies employed in development of the vertebrate head differ from those of the trunk in a number of important ways, foremost among which is the deployment of neural crest cells and neurogenic placodes (Gans and Northcutt, 1983). Contributing to the formation of sensory and autonomic ganglia, the cranial neural crest also gives rise to a range of mesenchymal tissues produced elsewhere by the mesoderm (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Noden, 1978, 1983). The cranial crest also has a role in patterning mesodermal derivatives (Noden, 1986, 1988) and appears to be morphogenetically specified before migration (Noden, 1983). The rhombencephalic neural tube, from which much of the cranial crest originates, is transiently subdivided into rhombomeres (Gräper, 1913) – true segments defined by cell lineage restriction (Fraser et al. 1990). There is an organised correlation between specific neuronal populations, their axon projections and the pattern of rhombomeres and interrhombomere boundaries. Neurons that form each of the three principal branchiomotor nuclei, for example, are located within the two rhombomeres that lie adjacent to the corresponding branchial arch (Lumsden and Keynes, 1989). Consistent with early specification (Noden, 1983, 1986) is the possibility that the cranial crest is segmented along with its parent neural tube, thereby establishing a dynamic association between neuromery and branchiomery. The target muscle of a particular branchial arch would acquire its pattern and identity from neural crest-derived connective tissue originating from the same rhombomeric level as its branchiomotor innervation (Lumsden and Keynes, 1989).

The segmented pattern of neural crest cell migration in the trunk appears to be determined solely by the adjacent mesoderm. Crest cells emerge from the neural tube as a continuous column along the dorsal midline but this soon becomes interrupted as crest cells enter distinct pathways lateral to the tube. By far the majority migrate through the rostral half of the dorsal sclerotome (Rickmann et al. 1985). The caudal half sclerotome excludes crest cells and contains molecules that
render it an unfavourable environment for axon outgrowth (Stern et al. 1986; Davies et al. 1990). Enlarged ventral roots and dorsal root ganglia can result from the experimental creation of multiple rostral half somites (Keynes and Stern, 1984).

In the head region, distinct segmentation of the mesoderm is absent. Although quasi-segmentation of the cranial paraxial mesoderm has been reported (Meier, 1981), the existence of cranial 'somitomeres' has been strongly disputed (Jacob et al. 1986). Furthermore, none of the molecules associated with the guidance of trunk neural crest cells, such as fibronectin (Erickson, 1986), tenascin (Mackie et al. 1988) and peanut agglutinin (PNA)-binding glycoproteins (Stem et al. 1986), is expressed in any spatiotemporal pattern that would suggest an involvement with crest migration in the head (cf. Layer and Alber, 1990). Scanning electron microscope studies have described tongues of crest cells apparently migrating dorsal to the paraxial mesoderm in newt (Jacobson and Meier, 1984), turtle (Meier and Packard, 1984) and chick embryos (Anderson and Meier, 1981), but the described patterns of migration do not allow any consistent relationship to be postulated between the levels at which crest cells exit the neural folds and the rhombomeres.

The dispersal of crest cells in the cranial region of the chick has been studied by orthotopic grafting of labelled regions of the neural primordium (Johnston, 1966; Le Lievre and Le Douarin, 1975; Le Lievre, 1974, 1978; Noden, 1973, 1975; Couly and Le Douarin, 1990). These studies reported that anterior mesencephalic crest cells were the first to migrate, just before closure of the neural folds. The majority migrated rostrally as a sheet of cells beneath the ectoderm and were found throughout the maxillary and mandibular processes. Anterior rhombencephalic crest contributed to the trigeminal ganglion. Posterior region of the mesencephalon migrated ventrally as a sheet of cells beneath the ectoderm and were found throughout the maxillary and mandibular processes. Anterior rhombencephalic crest contributed mainly to the second arch, with a few cells entering the first arch and the trigeminal ganglion. Posterior rhombencephalic crest cells filled the third and fourth arches. However, grafting sizeable brain regions provided fine resolution of crest migration patterns and a relationship to individual rhombomeres could have been missed.

Accordingly, we have used the fluorescent vital dye Dil (Honig and Hume, 1986) to label small regions of the premigratory crest in situ; by analysing the later distribution of fluorescent cells outside the neural tube, we have built up a picture of the migration pathways of cells from discrete mesencephalic and rhombencephalic levels of the neuraxis. In particular, we addressed the question of whether cranial crest cells have a segmented arrangement down the rostrocaudal axis such that those from specific levels migrate to particular branchial arches, or whether the cranial crest is not segmented and therefore funnelled into the correct arch by pathway cues.

Materials and methods

Injections of embryos
Rhode Island Red hen's eggs were incubated at 37°C and 50% R.H., to stages between 8– and 12 (Hamburger and Hamilton, 1951). Eggs were windowed and a small area of vitelline membrane was reflected from over the mid or hindbrain region to allow access for the injection pipette. Solutions of Dil C18 (Molecular Probes, D-282) were made at 3 mg ml⁻¹ in ethanol (Honig and Hume, 1986) or dimethylformamide, or at 0.5 mg ml⁻¹ in 0.3 μsucrose (S. Smith, pers. comm.). Micropipettes were pulled to 1–2 μm tip diameter from thin walled 1.5 mm diameter filament glass. These were tip-filled with the dye solution and introduced through the ectoderm into the dorsal neural tube using a micromanipulator. In neural plate stage embryos, and in those in which the neural tube had not yet closed, the micropipette tip was inserted directly into the neural fold. Dye was ejected with a single 5–10 ms pulse at a pressure of 7 kg cm⁻² (690 kPa) applied to the back of the pipette, delivering a 50–100 picolitre depot within the neuroepithelium (flow rate of 40 μl h⁻¹, calculated from drop diameter). Holding pressure of 70–175 g cm⁻² (6–15 kPa), sufficient to prevent capillary backflow but not to allow dye to weep from the tip, was applied before and after the injection pulse. The position of the injection was noted and the egg sealed with tape and returned to the incubator. Embryos were incubated to stages 14–17, fixed in a solution of 4% formaldehyde/2.5% DABCO anti-fade agent (BDH) at 4°C. Incubation beyond stage 17 resulted in a decrease in the level of fluorescence, and gave no increased resolution of the crest migration pathways. After fixation, embryos were bisected in the median plane and both left and right halves mounted under formaldehyde in cavity slides with their lateral surfaces facing the coverslip.

Fluorescence microscopy
Whole mounts were viewed under epifluorescence, and combined fluorescence/bright field, using a rhodamine filter set and photographed on 1600 ASA Ektachrome film. For distribution maps, an intensified CCD video camera (Photonic Sciences) was used to produce images of the fluorescent cells and structural outlines in the embryo that were traced directly from the monitor screen. Video images were averaged to reduce camera noise, stored on optical disc and printed via a Mitsubishi C200B(H) video printer.

Visualisation of cell death
Eggs incubated to stages 9–12 were windowed and the embryos were then flooded by a sub-vitelline injection of Nile Blue Sulphate (Saunders et al. 1962. NBS, Sigma N-5632 1:1000 in water). Embryos were immediately dissected out of the egg, viewed as whole mounts under bright-field or dark-field illumination and photographed on Ektachrome 160 film.

Results
The distribution of Dil-labelled crest cells was analysed in 140 embryos (Table 1). Injections of Dil into the neuroepithelium resulted in labelling of all the cells at the injection site including the premigratory neural crest cells. These subsequently detached from the epithelium and migrated laterally and ventrally away from the injection site. The injection of very small volumes of dye ensured that other tissues were not
labelled, with the usual exception of a small area of the epithelium that overlay the injection site. The use of dimethylformamide rather than ethanol (Honig and Hume, 1986) as a vehicle resulted in less dye spread at the injection site, brighter labelling of migrating cells and no detectable effect on cell viability. In many cases our injections were confined to the neural fold of a single rhombomere; in other cases they spanned that of two rhombomeres. In whole mounts, migrating crest cells were evenly dispersed and separately identifiable in the region they had entered at termination (Fig. 1A,B). Both the positions of migrating crest cells and the longitudinal extent of the original injection site, where the neuroepithelium remained brightly fluorescent, could be visualised at once in combined fluorescence/bright-field microscopy. In embryos injected before the appearance of rhombomere boundaries (i.e. before stage 9+) the exact location of the injection site was confirmed retrospectively. Bisection of the embryo prior to observation ensured detection of the crest migration pathways on each side separately. In some embryos, labelled crest cells were located bilaterally, while in others they were unilaterally.

Migration of mesencephalic crest starts at stage 9+ (6 somites) and is complete by stage 10+, rostral rhombencephalic crest migrates between stages 9 and 11+, while crest at the otic level (caudal rhombencephalon) migrates between stages 9+ and 11 (Tosney, 1982). In this study, dye injections were made at appropriate times to mark the earliest stages of migration at all levels of the axis as well as later migrating cells. The earliest embryos injected were at stage 8– (3 somites), before any neural crest cell migration has begun. The latest embryos injected were at stage 12, several hours after the reported completion of neural crest emigration. With spatially overlapping injections at successive stages, it is reasonable to assume that most if not all crest cells within the region of interest have been mapped. Labelling at successive axial levels gave rise to fluorescence in distinct areas of the head. The observed patterns will be described under regional subheadings.

**Midbrain**

Crest populations labelled rostral to the midbrain–hindbrain boundary in the youngest embryos (stage 8– and 8) were observed to have migrated to locations dorsal to the developing eye, presumptive maxillary process and the rostral half of the mandibular process (Fig. 2A,B). In one case, there was also faint speckling throughout the mesenchyme surrounding the diencephalon and telencephalon (Fig. 2B) and, in one case, labelled cells were located in the trigeminal ganglion. In embryos injected later, the whole of the first arch and trigeminal ganglion was filled (Fig. 2C). In embryos injected after stage 10+, only a few labelled cells were located away from the injection site and these remained in the midbrain or scattered around the eye (Fig. 2D).

### Table 1. Number of embryos injected with Dil at each stage (Hamburger and Hamilton, 1951) and neuraxial position

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<th>Neuraxial position</th>
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Embryos were incubated to stage 14–18.
Rhombomere 1

The only injection exclusive to r1 was carried out in an embryo of stage 10. The labelled crest cells were subsequently located in the mmV (Fig. 2G). This observation, coupled with those of injections into the caudal midbrain/r1 and into r1/2, suggests that r1 contributes crest to the mandibular arch.

Rhombomere 1–rhombomere 2

The earliest labelled neural crest cells (stage 8–) were located in the trigeminal ganglion and the caudal half of the mandibular process (Fig. 2H). In early stage embryos there was no labelling in the maxillary process or in the opV. In embryos injected at later stages the trigeminal ganglion and the whole of the mandibular arch were labelled (Fig. 2I,J). Crest cells labelled at stage 9+ onwards filled only the caudal half of the mandibular process and the mmV but not the opV. In one animal (Fig. 2K), a trail of crest cells was observed to extend rostrally into the midbrain, possibly representing the anlage for the trigeminal mesencephalic nucleus. In one late injection (stage 11–), labelled cells within the mandibular process did not extend as far as the most ventral part of the arch (Fig. 2L). No crest became labelled at stages 11–12.

Rhombomere 2

Embryos injected exclusively in r2 at stages 9+, 10– and 10 produced labelled crest cells that filled only the mmV and the caudal half of the mandibular process (Fig. 3A–C). Taken together with the results from r1/r2 injections, this suggests that crest cells from r1 more frequently colonise the rostral part of the mandibular process, while those from r2 more frequently colonise the caudal part. Injections at stages later than 11 failed to label emergent cells.

Rhombomere 2–rhombomere 3

The earliest-labelled cells (stage 8–) filled the mmV and the caudal half of the mandibular process (Fig. 3D). Injecting a stage later, the whole ganglion and both mandibular and maxillary processes became filled (Figs 1C, 3E). By stage 8+, a reduction in the extent of filling the ganglion and presumptive maxillary process was noted (Fig. 3F). A trail of cells extending towards the midbrain from the trigeminal ganglion, possibly cells contributing to the mesencephalic nucleus of the trigeminal, was observed in an embryo that had been injected at this stage (not shown). Labelling at stage 9– and 9+ once again filled only the mmV and the caudal half of the mandibular process (Figs 1D–F; 3G,H). Finally, at stage 11–, only the mmV and a short trail of cells in the mandibular process became labelled (Fig. 3I). Thus early injections labelled the maxillary process whereas later ones (after stage 8) labelled only the ganglion and the mandibular process. Injections to r2/3 and r2 at equivalent stages produced identical patterns (compare Figs 3A and 3H).

Rhombomere 3

Rhombomere 3 cells were labelled exclusively at several stages between 8+ and 12; in all cases, the only labelled cells were those that remained within the hindbrain neuroepithelium (Fig. 3J–L). No emergent cells were labelled, an absence that suggests the labelled cells observed in the first arch after injections to both r2 and r3 (above) derived from r2 alone.

Rhombomere 3–rhombomere 4

Injections at embryonic stages ranging from 8+–11 all resulted in labelled crest cells in the geniculate and vestibuulo-acoustic ganglia and in the hyoid (second) arch. Labelled cells were seen to be distributed throughout the arch, with only slight evidence of the rostrocaudal partitioning seen in the first arch (Fig. 4A,B). Since labelled cells did not emerge from r3 (above), it is likely that all the crest cells in these locations derived from r4 alone.

Rhombomere 4

All crest cells labelled in r4, irrespective of stage, migrated into the hyoid arch and formed the geniculate-vestibuulo-acoustic ganglia (Figs 1G–J). Crest cells labelled at stage 12 still migrated as far as the geniculate ganglion (not shown).

Rhombomere 4–rhombomere 5

One injection, at stage 9+, spanned both rhombomeres. Labelled cells had migrated anterior to the otocyst to fill the second branchial arch (Fig. 1H,I).

Rhombomere 5

Rhombomere 5 received injections that were exclusive to this area in embryos between stage 9– and 12 (Fig. 4F,G). The only labelled cells were those that
Fig. 5. Nile blue sulphate staining of necrotic cells, dark field illumination. (A) At stage 10 a cluster of stained cells is prominent at the level of r3. Necrotic cells are also distributed sparsely along the dorsal midline of the neuraxis. By stage 10+ (shown), the r3 cluster extends rostrally into r2. Dorsal aspect. (B) By stage 11+, r5 contains a discrete cluster of necrotic cells whereas r4 and r6 contain few. Dying cells can also be seen at the forming r1/2 boundary (arrowed), anterior to the otocyst (asterisk) and at the dorsal midline of the mesencephalon (m). Dorso-lateral aspect. (C) Dorsal aspect of a stage 12 embryo showing these features in relation to the midline.
Fig. 2. Diagrammatic representation of the results at stage 14/15 of local Dil labelling of the neural folds at successive positions down the rostrocaudal axis and at successive developmental stages. Each drawing depicts an individual animal representative of those at each position and stage of injection. (A) Caudal midbrain at st8−. (B) Rostral and caudal midbrain at 8+. (C) Caudal midbrain at st9−. (D) Caudal midbrain at st10−. (E) Midbrain–hindbrain boundary at st8. (F) Midbrain–hindbrain boundary at st9−. (G) R1 at st10. (H) R1/2 at st8−. (I) R1/2 at st8. (J) R1/2 at st8+. (K) R1/2 at st9+. (L) R1/2 at st11−.
Fig. 3. As Fig. 2. (A) R2 at st9+. (B) R2 at st10-. (C) R2 at st10. (D) R2/3 at st8-. (E) R2/3 at st8. (F) R2/3 at st8+. (G) R2/3 at st9-. (H) R2/3 at st9+. (I) R2/3 at st11-. (J) R3 at st9+. (K) R3 at st10-. (L) R3 at st10+.
Fig. 4. As Fig. 2. (A) R3/4 at st9−. (B) R3/4 at st10. (C) R4 at st8. (D) R4 at st10−. (E) R4 at st11−. (F) R5 at st9−. (G) R5 at st10. (H) R6 at st8+. (I) R6 at st9. (J) R6 at st10+. (K) R6/7 at st10−. (L) R4/5/6 at st9+. 
remained in the neuroepithelium. It is unlikely that crest cells from this area had already migrated by 9-- because pre-migratory crest cells can still be labelled in r1 at stage 11+. Furthermore, injections at stages 9-- and 9+ that covered rs 4, 5 and 6 produced crest cells which entered both the hyoid and the third branchial arch, having migrated rostral and caudal of the otocyst (Fig. 4L). Injections into r5 at stage 12 also failed to label emergent cells. Some labelling was observed in the area of the otocyst, where bottle-shaped epithelial cells were sometimes fluorescent (Fig. 4G). These were probably derived from presumptive otic placode ectoderm, which overlaps the neural tube (D’Amico and Noden, 1983), but the possibility cannot be dismissed that these cells derived from the neural crest.

**Rhombomere 6**

Crest cells that were labelled in r6 became located in the superior and petrosal ganglia of the IXth nerve and the third branchial arch (Fig. 4H–J).

**Rhombomere 6--rhombomere 7**

Injections carried out at stages 10-- and 10+ labelled cells which were located in both the third and the fourth branchial arches, and in the petrosal ganglion. Within the branchial region, cells were predominantly located in the third rather than the fourth arch (Fig. 4K). Labelling at stage 12 revealed crest migrating into the proximal (root) ganglion of the IXth nerve.

**Patterns of cell death**

The patterns of cell death in the cephalic region were assessed using Nile Blue Sulphate staining (Saunders et al. 1962; Sulik et al. 1988) during the stages of neural crest production i.e. between stages 9 and 12.

Intense NBS staining in the rhombencephalic region was first evident at stage 10 to 10+, in a single cluster of cells at the dorsal midline region of r3, in the position where neural crest cells would be located (Fig. 5A). By stage 11, a second cluster of NBS staining cells became evident in r5, also at the dorsal midline (Fig. 5B,C). Rhombomere 4 did not display NBS staining of midline cells during the stages examined but, from stage 10+, NBS staining in the r3 region extended more rostrally into r2 territory (Fig. 5A,B). NBS also picked out cells in the dorsal midline of the mesencephalon, forming a long narrow strip from the midbrain–hindbrain boundary up into the rostral half of the mesencephalon (Fig. 5C).

NBS staining in the hindbrain region was not restricted to dorsal midline cells. From stage 11 onwards strong NBS staining was seen within the neuroepithelium at the level of the (presumptive) boundary between r1 and r2 (Fig. 5B,C). Another site of strong NBS uptake, also from stage 11 onwards, was at the rostral end of the developing otocyst (Fig. 5B,C), in the region of the developing VII/VIIIth nerve ganglia.

**Discussion**

The principal finding described here is that neural crest cells are segregated into groups that populate discrete segmental levels down the rhombencephalic neuraxis. Between the mesencephalic–rhombencephalic boundary and the first somite, three groups of crest cells could be delineated. The first, originating from r1/2 populated the trigeminal ganglion and the mandibular arch, with fewer cells contributing to the maxillary process. The second, originating from r4 and migrating rostral to the otocyst, populated the geniculate/vestibular ganglia and the hyoid arch. The third, originating from r6 and migrating caudal to the otocyst, populated the superior and petrosal ganglia and the third branchial arch. These three regions alternate with two that appear to release no crest, r3 and r5.

These results refine those of previous studies that used orthotopic grafts of the neural primordium. Using orthotopic grafts from tritiated thymidine-labelled chick embryos into unlabelled hosts, Noden (1973, 1975) mapped the migration of cranial crest from five regions. The more caudal of these were the mesencephalon (corresponding to rs 1, 2 and 3) and the rostral myelencephalon (corresponding to rs 4 and 5). He found that crest cells from the metencephalon populated the trigeminal ganglion and the mandibular arch, while those of the rostral myelencephalon populated the geniculate ganglion and hyoid arch. These regions were not exclusively populated by crest originating in the hindbrain, however, since some cells from the caudal mesencephalon contributed to the mandibular arch. Our results also contrast markedly with those of other grafting studies. Using the quail/chick technique, Le Lievre and Le Douarin (1975) made chimaeras with grafts of the prosencephalic, mesencephalic, and the anterior and posterior rhombencephalic neural crest regions. In agreement with Noden, crest from mesencephalic levels was found to colonise the mandibular process, in addition to the trigeminal ganglion, maxillary process and the area surrounding the developing eye. Cells from the rhombencephalon entered the second, third and fourth branchial arches. However, only a few cells from these levels were detected in the first arch, or in the trigeminal ganglion. Finally, Couly and Le Douarin (1990) have more recently described the crest from r4 entering the first arch and that of r5 entering the second arch.

Discrepancies between these results may reflect individual variation in the methodologies; grafting allows the possibility of introducing abnormalities by spatial and/or temporal mismatch between graft and host. Wounding the neural epithelium and enforcing its repair may disrupt the environment and migration of the crest cells (Serbedzija et al. 1989). The time required for repair and consolidation of grafts also means that the early postoperative period cannot be examined. Finally, and most pertinent, grafting sizeable regions of the neural primordium does not allow fine resolution; in previous labelled graft experiments, the pathways of crest migration could not be mapped with sufficient
precision to reveal that crest deployment is rhombo-
meric.

**First arch: maxillary process**

Of cells labelled at stage 8—, only those in the midbrain migrated to fill the maxillary process of the first arch. By stage 8, injections into the midbrain, midbrain–hindbrain boundary and rs 1 and 2 also produced labelling here. By stage 9—, the midbrain again became the only region to generate crest cells for the maxillary process. After this stage, injections in these regions did not label cells in the maxillary process at all. Assuming a rostral to caudal maturation in the onset of migration, all the crest cells that fill the maxillary process have already left the neural tube by stage 9+.

**First arch: mandibular process and trigeminal ganglion**

Labelled cells were present in the mandibular process after injections into the midbrain, the midbrain–hindbrain boundary and rs 1 and 2, each region contributing to a different extent with time. When labelled between stages 8— and 8+, the midbrain and midbrain–hindbrain boundary regions produce cells which migrated into just the rostral half of the process. Later, at 9— and 9+, their cells fill the entire process, while at stage 10 midbrain crest no longer contributes to the mandibular process. Injections into rs 1–3 at stage 8— labelled cells which filled just the caudal half of the process. Then, at stages 8 and 8+, cells from these regions filled the whole of the mandibular process and the trigeminal ganglion. Later, cells labelled in rs 1–3 again filled only the caudal half of the process. While early injections resulted in labelling of the entire trigeminal ganglion, later injections tended to result in labelling only of the maxillo-mandibular lobe. There was also a marked ventral to dorsal filling of the arch as crest cells labelled in later staged embryos occupied progressively more dorsal regions of the arch. Thus, crest cells emerging at later times had an increased probability of assuming a gangliogenic rather than an ectomesenchymal fate, as judged by their position.

Partitioned filling of the mandibular process illustrated the different positional fates of cells at different axial levels. Crest cells labelled in the midbrain and at the midbrain–hindbrain boundary populated the rostral half of the process while those cells labelled in r1 and r2 populated the caudal half of the process. The motor axons of the trigeminal nerve, which exit at r2, also grow through the caudal half of the process where the branchiomiocer muscles lies. These observations suggest that parts of the mandibular arch are attractive to invading neural crest cells from a particular rhombomere. The degree to which the arch is attractive to migrating cells could vary according to rostrocaudal sequence coordinated with that of crest maturation. Alternatively, a more passive mechanism might operate whereby newly emigrating crest cells simply fill the closest available space.

**Second, third and fourth arches**

The hyoid arch was filled with labelled crest cells exclusively from the fourth rhombomere injected at stages 8–11. Within this arch, there was little evidence of rostrocaudal partial filling. The third and fourth branchial arches were filled by cells injected in r6/r7. Injections that were made exclusively into r6 resulted in cells populating only the third arch. It seems likely, therefore, that cells from r6 populate the third arch while those from r7 populate the fourth arch.

Our observations show that there is a defined spatial and temporal sequence of crest migration within the cranial region. The phenomenon of what might be termed rostrocaudal filling could, however, be accounted for in several ways. First, a single region could give rise to crest cells that populate a particular structure and as time progresses, cells from this region are progressively fated to migrate to more caudal regions of that structure. Second, there is a rostrocaudal wave of migration within the neural crest and cells could simply migrate out to populate whatever tissue space lies adjacent at the same axial level. Third, the phenomenon is most marked in the first arch, where crest cells derived from a considerable length of the axis converge on a comparatively narrow entrance. The pathway to the hyoid process, by contrast, is more parallel than convergent and cells would be less funnelled on entry to the process. In addition, emigrating crest cells colonised first ventral and then progressively more dorsal regions, as has been shown for trunk crest (Serbedzija et al. 1989).

**Rhombomeres 3 and 5**

Neural crest cells labelled at r2 and r3 levels were found only in locations normally populated by cells from r2 alone. Crest cells labelled at r3 and r4 levels populated the same region as cells originating from r4 alone. Similarly, labelling r4/5 and r5/6 produced identical patterns to labelling r4 and r6 alone. Combined with the observation that injections into r3 or r5 did not give rise to labelled cells outside the neural tube, this strongly suggests that rhombomeres 3 and 5 are areas that do not produce migratory crest.

SEM studies have previously noted the lack of crest cells emerging from the region of the neural tube adjacent to the otocyst (Anderson and Meier, 1981) but the precise neuraxial location of this region and the reason for its lack of emergent crest have remained undescribed. A possibility is that crest cells from rs 3 and 5 are divided in the same manner as those opposite the caudal part of the sclerotome (Teillet et al. 1987). Those situated rostrally in rhombomere 3 might migrate rostrad to join cells emerging from r2, while those situated caudally in the rhombomere would migrate caudal to coalesce with crest cells from r4. If this were the case, however, cells labelled at the level of r2/3 would have been seen in the hyoid arch and cells labelled from r3/4 would have been detected in the mandibular arch. In the case of r5 crest, the otocyst might act as an obstruction to crest migration.
However, the otocyst is centred on the boundary between r5 and r6, and would thus be expected to inhibit the migration of r6 crest in the same way. Remaining possibilities are that r3 and r5 do not produce neural crest cells at all, or that they do produce crest cells that either fail to migrate or die.

Our observations on Nile blue sulphate staining patterns suggest that cell death is enhanced in r3 and r5. We noted a rostral to caudal appearance of NBS staining cell clusters at the dorsal midline, first in r3 at stage 10 and then in r5 at stage 11, a temporal difference which may reflect a general feature of neural crest development (Tosney, 1982). These staining patterns are consistent with the possibility that r3 and r5 do produce neural crest cells but that these subsequently undergo cell death. While suggesting a mechanism which would allow r3 and r5 to be crest free, it remains to be determined whether the observed extent of cell death could account for the total lack of emergent neural crest cells from these segmental levels. The spread of r3 staining into r2 may involve r3 crest cells which migrate rostrally, although it is more likely that r2 also contains a small population of necrotic neural crest cells.

The thin strip of NBS staining at the dorsal midline of the mesencephalon indicates that necrosis can involve only a small subpopulation of the indigenous neural crest cells.

The region of necrotic cells at the boundary between r1 and r2 is interesting because it appears at the time cells are becoming positionally restricted at this boundary (Fraser et al. 1989a). Domain expression in the neural tube is one mechanism almost certainly does not apply to other rhombomere boundaries (which do not display noticeably higher levels of cell death during their formation); more likely it reflects the special nature of r1 as compared to the other rhombomeres (Lumsden, 1990).

A mechanism whereby crest cells fail either to segregate or to emigrate, instead pursuing a different fate, would act to maintain the separate streams of crest cells. Segmentation of the neural crest and the hindbrain serves to produce distinct groups of cells to populate and then innervate the branchial arches (Lumsden and Keynes, 1989). There are no obvious extrinsic influences beside the hindbrain that may produce this segmentation, compared to the sclerotomal influences in the trunk (Teillet et al. 1987). In the pre-otic region of the higher vertebrate head, the mesoderm is unsegmented; an alternation of crest-permissive and crest-inhibitory paraxial mesoderm (i.e. rostral and caudal somite halves) is not available to channel the neural crest outflow into the segmented streams and its 'sclerotomal' component forms a single block of cartilage, the parachordal. Thus, whereas trunk neural crest cells are produced down the rostrocaudal axis without interruption, it would be expected that this production would be discontinuous in the head. The surface ectoderm may provide directional guidance for crest migration (Löfberg et al. 1985) and, at the hindbrain level, specific local cues may be available to consolidate the migration paths. The ectodermal appears to be regionalised into segmental domains registering with the rhombomeres (Couly and Le Douarin, 1990), but it is not yet known whether these 'ectomeres' are true segments defined by lineage restriction. There must be, additionally, an intrinsic process that influences the segmental generation of neural crest cells from the rhombomeres. Our results suggest that the neural crest populations are allocated before their emigration from the neural folds to migrate into specific branchial arches. This would be predicted by Noden's (1983) observations that the cranial crest is specified to pattern the structures of a particular branchial arch before migration begins.

Concluding remarks

The segmental specification of neural crest cells in the hindbrain could be linked to the expression of homeobox-containing genes and other genes involved in patterning. Genes at the 3' end of the Hox 2 cluster have domains of expression in the mouse CNS that terminate rostrally at rhombomere boundaries. 

Hox 2.8, for example, is expressed up to a sharp limit at the r2/3 boundary, and Hox 2.7 expression extends up to the r4/5 boundary (Wilkinson et al. 1989b). These genes are also expressed in the neural crest as it migrates out into the branchial arches (Hunt et al. 1991). However, expression in the neural tube is one segment out of register with that in the arches. Thus, Hox 2.8 is expressed in the second arch, up to a level that coincides with the r3/4 boundary rather than the r2/3 boundary. Hox 2.7 shows the same expression pattern two segments caudally. The absence of crest migration from r3 and r5 makes sense of this pattern, by matching the expression of the genes in the neural tube and in the periphery. Another gene capable of regulating transcription, the zinc-finger-containing gene, Krox 20, is also expressed in a segment-specific manner. Expression is restricted to two precise regions in the CNS, rhombomeres 3 and 5, and to the cranial ganglia opposite the alternate, even-numbered segments (Wilkinson et al. 1989a). Krox 20 is expressed during a time interval consistent with a role in segmentation, but the genes that it regulates remain to be identified.

The subdivision of the neural crest into distinct segmental subpopulations thus provides an unifying feature of head development. Early migrating crest cells populate the branchial arches, while later ones form the sensory ganglia in combination with cells derived from the ectodermal placodes. The existence of two crest-free segments, alternating with crest-producing ones, causes the separation of rhombencephalic crest into three distinct streams. This results in specific filling of the corresponding arches and in the organisation of cranial ganglia and the entry/exit points for the peripheral nerves. It is probable that segmentation of the hindbrain neural tube is a conserved and crucial feature of development in the vertebrate head, project-
ing its pattern into subjacent branchial region by means of the migrating neural crest.

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References


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