Head morphogenesis in embryonic avian chimeras: evidence for a segmental pattern in the ectoderm corresponding to the neuromeres

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Summary

Areas of the superficial cephalic ectoderm, including or excluding the neural fold at the same level, were surgically removed from 3-somite chick embryos and replaced by their counterparts excised from a quail embryo at the same developmental stage. Strips of ectoderm corresponding to the presumptive branchial arches were delineated, thus defining anteroposterior 'segments' (designated here as 'ectomeres') that coincided with the spatial distribution of neural crest cells arising from the adjacent levels of the neural fold. This discrete ectodermal metamerisation parallels the segmentation of the hindbrain into rhombomeres. It seems, therefore, that not only is the neural crest patterned according to its rhombomeric origin but that the superficial ectoderm covering the branchial arches may be part of a larger developmental unit that includes the entire neurectoderm, i.e., the neural tube and the neural crest.

Key words: quail-chick chimeras, head morphogenesis, ectomeres, neuromeres.

Introduction

The development of the cephalic region of higher vertebrates involves extremely complicated morphogenetic processes. This part of the body has been the site of enormous evolutionary changes, most of which have involved the addition of structures related to the increasing complexity of the brain and associated sensory organs.

The mechanisms involved in assembling the various pieces of the puzzle during ontogeny of the head are still imperfectly understood. This is why we thought that it would be informative to draw up a precise map of the cephalic region at the early stages of neurogenesis and to combine the cartographical study with a topological and dynamic analysis of development. The quail-chick chimera system is ideally suited to such an investigation. The work done in our laboratory and elsewhere concerning the fate of the cephalic neural crest (Le Lièvre, 1974, 1978; Le Lièvre and Le Douarin, 1974, 1975; Johnston, 1966; Johnston and Hazelton, 1972; Noden, 1975, 1984) has already shown that the facial skeleton and dermis do not originate in situ but develop after a phase of dorsoventral migration of cells from the cephalic neural crest. In our previous studies (Couly and Le Douarin, 1985, 1987), we found that the anterior neural fold (located cranially with respect to the prospective neural crest) yields the superficial epidermis covering the forehead and nasofrontal regions. The morphogenetic movements affecting the more rostral parts of the neural fold could be followed from the presomitic stage until the cells derived from this zone were definitively positioned in the head structures.

The present work is essentially concerned with the development of the remaining cephalic ectoderm, from the neurula to the late embryonic stage, and with its relationships to cerebral and neural crest structures.

We report here a striking regionalization of the presumptive facial and hypobranchial ectoderm in areas, arranged in a metameric-like fashion, that we call 'ectomeres' and which parallel the segmentation of the neural anlage into individual neuromeres. This metameration is reflected in neural crest cell migration to specific arch structures, since it is possible to correlate the origin of mesectodermal cells, superficial ectoderm and that of the cranial nerves specifying transverse levels of the head. These ectomeres are oriented cranially at a 45° angle with respect to the embryonic axis at these early stages of neurogenesis.

Materials and methods

Quail and chick eggs from commercial sources were used in these experiments. Microsurgery was performed on embryos at the 3-somite stage (after about 30 to 32 h of incubation in a humidified atmosphere at 38°C for chick and 28 to 30 h for quail eggs). The microsurgical technique involved the selective removal, by means of a microscalpel, of the ectodermal germ
layer from defined areas of the presumptive cephalic territory, including, in some series, the neural fold at the same level (for details, see Fig. 1 and Table 1). Microscalpels are made by sharpening thin needles on an Arkansas stone. An ocular micrometer in the dissecting microscope enables the size of the fragments of neural fold or of ectoderm involved in the experiment to be precisely measured.

Trypsin was not used to separate ectoderm from the underlying mesenchymal cells. However, at the stage considered, only a few of the latter were present in the areas included in the operations since the neural crest cells had not yet migrated.

In all cases, quail embryos were used as donors and chick embryos, from which the corresponding ectodermal area had been removed, as recipients. The operated embryos were killed at embryonic day 3.5 to 8 (E3.5–E8), according to the experimental series, and fixed in Zenker’s fluid.

Chimerism was analysed at the head and neck level on 5 serial sections stained according to the Feulgen-Rossenbeck method, which allows quail cells to be distinguished from chick cells (Le Douarin, 1969, 1973).

Results

Description of the territories involved in microsurgery

The neural fold was divided into anteroposterior segments 150 μm long. Zones A, B and C (Fig. 1a) were investigated previously in 0- to 3-somite stage embryos (Couly and Le Douarin, 1985, 1987). The experiments described in the present article were performed exclusively on 3-somite embryos and involved areas of the head as defined by zones D, E and F in Fig. 1.

Preliminary experiments

In a preliminary experimental series (experiments Pr), rectangular territories of lateral ectoderm perpendicular to the embryonic axis were delineated as indicated in Fig. 2a for levels D and E of the neural fold.

Numerous types of grafts, involving topographically distinct ectodermal areas lateral to areas D, E and F,
were carried out. They will not be described in detail except for the two designated in Fig. 2a as P_rI and P_rII.

In experiment P_rI, involving the D level of the neural fold and ectoderm, the territories occupied by quail cells in the ectodermal area were located in a latero-facial region including the otic and posterior maxillo-mandibular areas (see Fig. 2b). Quail cells invaded the maxillary bud and the mandibular arch with a modest contribution to the second branchial arch derivatives, as seen in Fig. 2c.

In experiment P_rII, transplantation of the ectodermal territory (originating opposite zone E of the neural fold) resulted in the labelling of a small laterocervical region caudal to that labelled in experiment P_rI (Fig. 2b). Neural crest cells migrated only to the 2nd branchial arch (Fig. 2d). Consequently, there is an overlap of neural crest cell migration from zones D and E of the neural fold mainly in the rostral region of the second branchial arch. Similar results were obtained in experiments performed at later developmental stages by Le Lievre and Le Douarin (1975). In these preliminary experiments, the dispersion of the grafted crest cells did not correlate with the distribution of quail cells from the epidermal area.

In the following series of experiments, we aimed at mapping the epidermis covering the maxillary buds and the branchial arches. This led us to identify strips of ectoderm superimposable on neural crest-derived mesenchymal cells carrying the quail marker.

**Detailed experiments**

The appropriate angle of the lateral ectodermal strips was found, on the basis of the results provided by several transplants of different orientations, to be about 45° with respect to the embryo axis (Fig. 1b).

In the following study, two series of experiments, including or excluding the neural fold, were performed (Table 1). The embryos that were studied for chimerism in serial sections showed a normal morphology. Embryos with evident malformations were discarded. The number of embryos analyzed in each experiment is indicated in Table 1. The results described below were strikingly similar for all samples within each series, showing the reproducibility of the experimental design.

**Experiment I: Graft of the ectoderm and neural fold of the prosencephalic and mesencephalic levels (see Table 1)**

In this experiment, the neural fold corresponding to the prosencephalic (zones A,B,C) in the experiments described by Couly and Le Douarin, 1985, 1987) and mesencephalic regions (D) were involved in the oper-

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### Table 1. Experimental designs and number of embryos studied for the mapping of the presumptive facial ectoderm

<table>
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<th>Experiments</th>
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*Refer also to Couly and Le Douarin (1987).*
Fig. 3. Results of experiment I (see Table 1). (a,b) The ectoderm of external structures on the hemifacial region indicated is made up entirely of quail cells. This includes the eyelids and the cornea. (c) Internally, the primary and secondary palate and the floor of the mouth are also of quail origin.

Four embryos were killed at E6 (n=2) and E8 (n=2). The grafted quail cells were distributed within the superficial ectoderm of the right half of the head (Fig. 3a–c). In detail, regions affected by the labelling were the epidermis covering the calvarium, the naso-frontal area, the cornea, the lens, the upper beak as well as the ectoderm of the right nasal cavity along with the right olfactory placode and the right palate. Moreover, grafted ectoderm was also found covering the complex formed by the inferior beak and all but the proximal part of the tongue and the larynx.

Mesenchymal tissues derived from prosencephalic and mesencephalic neural crest were found evenly distributed underneath the ectoderm.

We already know from our previous experiments that the anterior neural fold of zones A, B and C contributes

Fig. 4. Results of experiment IIA. (a) Epidermal quail cells are mainly located in the maxillary and mandibular buds in the chick host at E4. (b) The neural crest-derived cells from level D have an identical distribution. (c) Transverse section of the head at the level of the mesencephalon. Bar=1 mm. (d) The trigeminal ganglion is entirely of graft (i.e., neural crest and placode) origin. Bar=10 μm. (e) The maxillary and mandibular bud mesenchyme are also composed entirely of quail cells. Bar=10 μm.
Fig. 5. Results of experiment IIB. (a) Scanning electron micrograph of a 3-somite chick embryo showing the transplanted region. (b) Photograph of a chimera at E8 indicating the levels of the sections shown in Figs 5c, 7a, 8a and 9a. In c, the nares and the inferior conchus are of host origin, while the secondary palate ectoderm (sp) is made up entirely of quail cells. Bar=1 mm. (d) Nares at higher magnification. Bar=10 μm. (e) The epithelium of the inferior concha (ic) is made up of chick cells since it arises from the ectoderm of zone B of the neural fold (see Couly and Le Douarin, 1985). Bar=10 μm.
extensively to the epidermis of the nasofrontal ectoderm, which includes the nasal mucosa and the olfactory epithelium, the ectoderm of the upper beak and the ectoderm covering the premaxillary bones and the nasal septum. Therefore, it is not surprising that the lateral cephalic ectoderm of the 3-somite embryo forehead gives rise only to the ectoderm covering the secondary palate, the lateral wall and roof of the mouth and the ectoderm covering the mobile portion of the tongue.

**Experiment II**

(A) *Graft of the mesencephalic neural fold plus a ventrolateral strip of ectoderm (see Table I and Fig. 4).*

This experiment investigated the precise origin of the ectoderm contributing to the floor and roof of the oral cavity and of the tongue and the position of the trigeminal placode (Fig. 4a–e).

The neural fold (Zone D, about 150 μm long) of the mesencephalic area, along with a strip of ectoderm of the same width, oriented at about 45° with respect to the dorsoventral axis, was replaced in the 3-somite chick embryo by its quail counterpart (Fig. 1b). The mesenchyme arising from the mesencephalic neural crest was found in the first arch, in the maxillary and mandibular processes including the primordium of the tongue and, to a lesser extent, in the second branchial arch (see also Le Lièvre and Le Douarin, 1974, 1975 for experiments performed at later stages) (Fig. 4b).

The trigeminal ganglion was entirely of quail origin, (Fig. 4) indicating that the graft included both its neural crest and placodal components (see Narayanan and Narayanam, 1980; Ayer-Le Lièvre and Le Douarin, 1982; D'Amico-Martel and Noden, 1983).

The ectodermal area of graft origin was limited anteriorly as indicated in Fig. 4a, covering maxillary and mandibular buds.

In experiment IIA, the superposition of mesectodermal and ectodermal cells of graft origin is very marked indicating that mesencephalic crest cells migrate laterally along a posteroanterior vector.

(B) *Graft of ventrolateral strip of ectoderm.* This is the same graft as in experiment IIA, without the neural fold (see Table I).

This operation yielded embryos in which quail cells were totally absent from mesenchymal structures and from the trigeminal ganglion. Otherwise, as far as the superficial ectoderm is concerned, no differences were visible between these embryos and those of experimental series IIA (Figs 5–9). Some quail cells form mandibular muscles. Experiments IIB demonstrate that the superficial epithelium covering the tongue is of ectodermal and not of endodermal origin. The tongue epithelium originates from the first branchial arch ectoderm, which, in addition, contributes to most of the oral epithelium. These results also indicate that the presumptive territory of the trigeminal placode is included in the neural fold itself.

**Experiment III**

(A) *Graft of the metencephalic presumptive neural fold plus an anterolateral strip of ectoderm (see Table I).*

The experimental design was similar to that described in experiment II except that it involved the neural fold at the presumptive metencephalic level (Zone E, about 150 μm long) (see Fig. 1b) together with the adjacent superficial ectoderm.

The mesectoderm arising from zone E of the neural fold was distributed primarily in the 2nd branchial arch with a few quail cells sparsely distributed in the 3rd arch (Figs 10a and 11a,c). The geniculate ganglion was entirely of quail origin, indicating that precursors of both its neuronal (from the placode) and non-neuronal (from neural crest) contingents were included in the graft (Fig. 11e), as was the case for the trigeminal ganglion in experiment II. Moreover, the cranial part of the auditory placode was of graft origin.

The superficial ectoderm arising from the graft was located caudally to the strip labelled in experiment II and covered the areas of the 2nd branchial arch ventrally (Fig. 10).

(B) *Graft of anterolateral strip of ectoderm (no neural fold).* Only the superficial ectodermal component described above was found in embryos resulting from experiment IIIB (Figs 12, 13a).

The geniculate ganglion in this series was formed by host (chick) non-neuronal cells, while the neurons were entirely of quail type. Therefore, in contrast to the trigeminal, the placode is situated laterally and externally with respect to the neural fold at this stage (3-somite).

The auditory placode and ganglion were partly of quail origin so indicating that they are located more
Fig. 7. See legend opposite
Fig. 8. For legend see p. 552
Fig. 9. For legend see p. 552
Fig. 8. Experiment IIB (continued). (a) Section as indicated in Figure 5b. Bar=1 mm. (b) Quail cells in the ectodermal layer overlying the mandible stop at the midline. The following ectodermal structures are composed entirely of quail cells in the chick host: (c) the lateral mandibular facial area; (d) the lateral wall of the mouth (m) and the anterior part of the tongue (t); (e) the secondary palate (sp) up to its junction with the endoderm (End). (f) In the absence of grafted quail neural crest, the chondrocytes that form Meckel's cartilage are of host origin. b-f, bar=10 μm.

Fig. 9. Experiment IIB (continued). (a) Section as indicated in Fig. 5b. Bar=1 mm. The following ectodermal structures in the maxillary region are composed entirely of quail cells in the chick host: (c) the lateral midline. The following ectodermal structures are composed entirely of quail cells in the chick host: (c) the lateral ectoderm of the 1st endodermal pouch covering the endodermal layer of the pouch; (d) the caudal portion of the floor of the mouth (m, mouth mucosa; t, tongue mucosa); (e) the posterolateral wall of the palate; (f) all ectoderm in the mandibular region. (g) The base of the tongue is of host origin. b-g, bar=10 μm.

Laterally than the trigeminal placode at this early stage of neurogenesis.

**Experiment IV**

(A) Graft of the myelencephalic neural fold with the adjacent ectoderm down to the level of the 3rd somite. The myelencephalic region, extending to the level of somite 3, included both the neural fold (Zone F) and the superficial ectoderm (Fig. 1b, Table 1).

The fate of the ectodermal germ layer of this particular area was carefully analyzed for its possible contribution to the glandular derivatives of the 3rd and 4th pharyngeal pouches from which the thymic rudiments and the parathyroid glands originate (Figs 13b, 14).

The epithelial component of the parathyroid and thymus was of chick origin in all 8 embryos observed at E6 and E8, at which time these structures are fully identifiable. The thymic rudiment was composed only of chick cells except for mesenchymal layers surrounding the thymic glandular cord, which were derived from donor neural crest cells (see also Le Douarin and Jotereau, 1975, for experiments done at later developmental stages).

![Fig. 10. Results of Experiment IIIA at E3.5. The mesenchyme (a) and the ectoderm (b) are entirely of quail origin in the 2nd branchial arch region.](image)

The parathyroid gland was entirely formed of chick cells on the operated side, with a few quail mesenchymal cells between the epithelial cords. The epidermis, of quail origin, formed a dorsolateral strip located at the level of the more caudal part of the neck and of the cervicothoracic junction, reaching ventrally the mediadorsal line.

Migration of the myelencephalic neural crest could be followed in these birds. Crest cells from this level contributed to the dermis in a territory corresponding roughly to the quail ectodermal area.

As expected (Le Douarin and Teillet, 1973), neural crest cells from this level also gave rise to enteric ganglia. The superior–jugular ganglionic complex (of neural crest origin) as well as the petrosal and nodose ganglia, the neurons of which originate from epibranchial placodes and the non-neuronal cells from the neural crest were all made up of quail cells in these birds (Fig. 14).

(B) Ectoderm down to 3rd somite level. The structures containing the quail nuclear marker in this experimental series (4 embryos) were the same as in experiment IVA except that no mesectodermal cells of quail origin were present. Moreover, only the neurons and not the non-neuronal cells were of donor type in the petrosal and nodose ganglia.

**Conclusions and discussion**

In this work, we define segmentally distributed strips of ectodermal areas in the cephalic region at the late neurula stage of the avian embryo. These strips correspond to the first, second, third and fourth branchial arches. The ectoderm of these regions covers the following structures in a cranio-caudal sequence.

1. The first branchial arch, yielding the maxillary and the mandibular buds plus the lateral wall and floor of the mouth and the mobile part of the tongue (experiment II, corresponding to area D of the neural fold) (Fig. 15). This region includes the presumptive territory of the trigeminal placode, which is located at the external aspect of the neural fold itself and extends on the lateral ectodermal area as indicated in Fig. 4.

   The region occupied by quail crest cells originating from zone D of the neural fold corresponds to the extension of the region innervated by the trigeminal nerve (D'Amico-Martel and Noden, 1983).

2. The second branchial arch, which is covered by epidermis arising from the ectoderm indicated as zone E in Figs 10–13 (experiment III).
Fig. 11. See legend opposite.
Fig. 12. See legend opposite
established between our ectodermal segments and the recently (Lumsden and Keynes, 1989; Wilkinson et al., 1918; Vaage, 1969) and further documented of the epidermis is labelled, as is a small area of the neural folds. Moreover, these antero-posterior spond strikingly well with the spatial distribution of F of the neural fold is transplanted. Considering that the superfi- cial ectoderm overlying the 2nd branchial arch.

(3) The third and fourth arches are included in experiments in which ectoderm corresponding to zone F of the neural fold is transplanted. Considering that this involves an embryonic area equivalent in surface to that of zones E and D, the contribution to superficial ectoderm is smaller. The posterior cervicolateral region of the epidermis is labelled, as is a small area of ventrolateral skin in the thoracic region.

As could be expected (D’Amico-Martel and Noden, 1983; Le Douarin et al. 1986), the territories of the 1st and 2nd epibranchial placodes are lateral and quite distinct from the neural fold at that stage.

The ectodermal strips that we have defined corre- spond strikingly well with the spatial distribution of neural crest cells arising from the corresponding levels of the neural folds. Moreover, these antero-posterior 'segments' of superficial ectoderm parallel the discrete metamerisation of the hindbrain described by pioneering embryological workers as rhombomeres (Orr, 1887; Neal, 1918; Vaage, 1969) and further documented recently (Lumsden and Keynes, 1989; Wilkinson et al. 1989). Fig. 16 indicates the correspondence that can be established between our ectodermal segments and the rhombomeres (see Vaage, 1969). This is of particular interest since the neuromeric metamerisation of the hindbrain seems to be genetically determined, as suggested by the restricted expression of genes of the Hox-2 cluster in well-defined segments of the embryonic mouse brain correlating with the previously de- fined rhombomeres and to the corresponding neural crest derivatives (i.e., cranial nerve ganglia, Holland and Hogan, 1988; Wilkinson et al. 1989).

It therefore appears that not only is the neural crest patterned according to its rhombomeric origin (Lumsden and Keynes, 1989; Wilkinson et al. 1989) but that, in fact, the entire ectodermal layer at this level of the body, including the presumptive epidermis may form a genetically defined developmental unit. It is important to remember that cephalic neural crest cells migrate superficially as a multisheet of cells underneath the ectoderm (Johnson, 1966; Noden, 1975; Thiery et al. 1982). Interestingly, it has already been suggested that superficial ectoderm may provide directional guidance for crest migration (Löfberg et al. 1985).

The neural crest cells originating from levels D, E and F migrate into well-defined facial and hypobran- chial structures (i.e., the maxillary buds and the branchial arches) and not only form the skeleton and dermis of these regions (Johnson, 1966; Johnston et al. 1974; Johnston and Hazelton, 1972; Johnston and Listgarten, 1972; Le Lièvre and Le Douarin, 1975) but also provide their sensory and motor innervation (V, VII, IX, X). This is why we propose the term of 'ectomere' for the bands of ectoderm described above, whose orientations with respect to the embryonic axis correspond to a roughly 45° caudocranial angle at the late neurula stage (precisely at the 3-somite stage). This does not imply in our view that the superficial ectoderm constituting these ectomeres is committed to a specific spatial fate in the embryos. Such a possibility however should not be ruled out since it has been extensively documented that chordogenesis of the Vertebrate skull is elicited by epitheliomesenchymal interactions (see Hall, 1988; Thorogood, 1988 for reviews). Whether the patterning of the facial and hypobranchial skeletal structures is specified by the neural crest cells, as suggested by Noden (1983), by the ectoderm or by both is now under investigation in our laboratory. Namely, experiments in which heterotopic grafting of the presumptive ecto- meres are being performed to answer this question.

Finally, the work reported in our previous articles (Couly and Le Douarin, 1985, 1987; Le Douarin et al. 1986), together with the results described here, allow us to visualize the prospective fates of different regions of the superficial head ectoderm at the neurula stage (early somitic embryo). The anterior neural fold pro- vides the adenohypophysis medially and the epithelium of the olfactory organ laterally (internal nasal ectoderm including the sensory olfactory placode) plus a large area of epidermis covering the anterior cephalic region (roughly corresponding to the area occupied by the telencephalon) the nose, the upper beak and primary palate.

Posteriorly, the neural fold cells located caudal to the epithysis anlage (region C in Couly and Le Douarin, 1987) are endowed with migratory properties and become the neural crest, which later contributes to the skeleton and dermis of the face and the ventral aspect of the neck.

Our results, based on experiments carried out at an earlier developmental stage than those described pre- viously (Johnston, 1966; Le Lièvre and Le Douarin, 1975), confirm the neural crest cell distribution but give a more detailed picture of the fate of different presump-

Fig. 12. Results of experiment III B. (a) Chimeric embryo at E6. (b) Frontal section of the same embryo showing the geniculate ganglion [magnified in c], and views of the upper limit (d), the middle (e), and lower (f) limit of the cervical ectoderm overlying the 2nd branchial arch.

Fig. 13. Schematic representation of the results of experiments III and IV. (a) At a more advanced stage (E8), the ectoderm corresponding to the 2nd branchial arch labelled in experiment III is located craniolaterally on the neck (hatched region) which is entirely of donor origin. (b) In a similar manner, transplants involving the 3rd branchial arch (experiment IV) yield the ectoderm of the cervicothoracic region.
Fig. 14. Results of experiment IV. (a,b,c) Frontal sections of a chimeric embryo at E6 showing that the lateral ectoderm (arrow: transition with host's ectoderm) and the nodose ganglion are entirely composed of quail cells. (d,e,f) Horizontal sections through the cervical region of a chimeric at E8 embryo showing quail cell labelling in the posterior (e) and anterior (f) ectoderm (arrows transition with host's ectoderm). a, bar=1 mm; b-f, bar=10 μm.
Fig. 16. Evolution of the ectodermal territories in the chick from the 3-somite-stage (A) to the eight-day embryo (B). Pink, nasal mucosa; green, ectoderm of upper beak; orange, ectoderm covering the anterior calvarium; grey, ectoderm of eyelids and cornea; red, ectoderm of the 1st arch; yellow, ectoderm of the 2nd arch; blue, ectoderm of the 3rd and the 4th arches. (C) Rhombomeres and associated cranial nerves (R1...R8) with the branchial arches that they innervate in a 3.5 day chick embryo. R1–3 correspond to the trigeminal nerve which innervates the first branchial arch. R4–5 correspond with the facial nerve, which innervates the 2nd branchial arch, and R6–8 correspond to the glossopharyngeal and vagal nerves, which innervate the 3rd and 4th branchial arches. (Arrows: correspond to direction of neural crest cell migration). V, Trigeminal nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagal nerve; bm, maxillary bud; 1, first arch; 2, second arch; 3, third arch; 4, fourth arch.
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