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Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells

You Mie Lee[1,2,4], Noriko Osumi-Yamashita[1,*], Youichirou Ninomiya[1], Chang Kiu Moon[2], Ulf Eriksson[3] and Kazuhiro Eto[1]

[1]Department of Developmental Biology, Division of Life Science of Maxillo-Facial Systems, Graduate School of Dentistry, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan
[2]Department of Hygienic Chemistry, College of Pharmacy, Seoul National University, San 56-1, Shinlim-dong, Kwanak-Gu, Seoul, Korea
[3]Ludwig Institute for Cancer Research, Box 240, s-17177, Stockholm, Sweden
[4]Department of Toxicology, National Institute of Safety Research, Seoul, 122-020, Korea

*Author for correspondence

SUMMARY

This study investigates the migration patterns of cranial neural crest cells in retinoic acid (RA)-treated rat embryos using DiI labeling. Wistar-Imamichi rat embryos were treated at the early (9.0 days post coitum, d.p.c.) and late (9.5 d.p.c.) neural plate stages with all-*trans* RA (2×10^{-7} M) for 6 hours and further cultured in an RA-free medium. RA exposure stage dependently induced two typical craniofacial abnormalities; that is, at 9.0 d.p.c. it reduced the size and shape of the first branchial arch to those of the second arch, whereas, in contrast, at 9.5 d.p.c. it induced fusion of the first and second branchial arches. Early-stage treatment induced an ectopic migration of the anterior hindbrain (rhombomeres (r) 1 and 2) crest cells; they ectopically distributed in the second branchial arch and acousticofacial ganglion, as well as in their original destination, i.e., the first arch and trigeminal ganglion. In contrast, late-stage treatment did not disturb the segmental migration pattern of hindbrain crest cells even though it induced the fused branchial arch (FBA); labeled crest cells from the anterior hindbrain populated the anterior half of the FBA and those from the preotic hindbrain (r3 and r4) occupied its posterior half. In control embryos, cellular retinoic acid binding protein I (CRABP I) was strongly expressed in the second branchial arch, r4 and r6, while weakly in the first arch and r1-3. CRABP I was up-regulated by the early-stage treatment in the first branchial arch and related rhombomeres, while its expression was not correspondingly changed by the late-stage treatment. Moreover, whole-mount neurofilament staining showed that, in early-RA-treated embryos, the typical structure of the trigeminal ganglion vanished, whereas the late-stage-treated embryos showed the feature of the trigeminal ganglion to be conserved, although it fused with the acousticofacial ganglion. Thus, from the standpoints of morphology, cell lineages and molecular markers, it seems likely that RA alters the regional identity of the hindbrain crest cells, which may correspond to the transformation of the hindbrain identity in RA-treated mouse embryos (Marshall et al., *Nature* 360, 737-741, 1992).

Key words: retinoic acid, craniofacial abnormalities, hindbrain neural crest cells, migration pattern, branchial arch defects, transformation

INTRODUCTION

Vitamin A and its derivatives, collectively referred to as retinoids, play important roles in vertebrate development and in the differentiation of a wide variety of cell types (reviewed in Roberts and Sporn, 1984 and Morriss-Kay, 1992). In particular, retinoic acid (RA), a biologically active retinoid, exerts a broad spectrum of effects.

It is well known that an excess or deficiency of retinoids induces abnormal morphology in mammalian embryos (reviewed in Morriss-Kay, 1992). When rodent embryos are exposed to excess RA during or shortly before neurulation, the preotic hindbrain region is shortened in relation to other head structures (Morriss, 1972; Morriss and Thorogood, 1987). Marshall et al. (1992) showed that RA administration to pregnant mice at 7.5 days post coitum (d.p.c.) induces posteriorization in the body axis, i.e., it alters the anterior structure in the hindbrain region into the posterior one. In contrast, treatment with RA at later stages causes different types of malformations, e.g., reduction and/or fusion of the first and second branchial arches that eventually form the maxilla, mandible, and external and middle ears (Goulding and Pratt, 1986; Webster et al., 1986).

Most of the craniofacial structures in vertebrates, including the branchial arches and cranial ganglia, contain cells derived from the cranial neural crest (reviewed in Le Douarin, 1982;

Morriss-Kay and Tan, 1987; Noden, 1988). The hindbrain neural crest cells, which are thought to be prepatterned or imprinted with positional information (Noden, 1988), migrate to the adjacent branchial arches (I-IV) in a segmental manner (Lumsden et al., 1991; Serbedzija et al., 1992; Matsuo et al., 1993; Osumi-Yamashita et al., 1994). As a parallel event to such migration, a segmented rhombomere structure appears along the anterior-posterior axis, with these rhombomeric boundaries denoting cellular compartments that restrict cell lineage (Fraser et al., 1990; Birgbauer and Fraser, 1994).

In the mouse, *Hox* genes have spatially restricted patterns of expression that appear before the formation of rhombomeric morphology and later map to specific rhombomeric boundaries (Graham et al., 1989). These genes are also expressed in the branchial arches in segment-specific patterns (Hunt et al., 1991a,b; Wilkinson et al., 1989); hence suggesting that the prepatterned information contained in hindbrain crest cells is associated with the expression pattern of *Hox* genes (reviewed in Hunt and Krumlauf 1991; Krumlauf, 1993).

Of particular interest, based on observations of *Hox* marker gene expression and nerve morphology, Marshall et al. (1992) suggested that RA treatment of mouse embryos at 7.5 d.p.c. alters the identity of rhombomeres 2/3 (r2/3) to that of r4/5, as well as altering the identity of the trigeminal nerve to that of the facial nerve. However, since the cranial ganglia contain neurons derived from the hindbrain crest and their nerves innervate into the corresponding branchial arches that are populated by the hindbrain crest cells, a question remains as to whether or not the migration pathway of neural crest cells emerging from the altered hindbrain origins can be changed from a standpoint of cell lineage.

Another interesting phenomenon reported by Brown et al. (1992) is that in rat embryos treated with RA from the headfold stage (a period occurring after the treatment mentioned above), the *Hox* B2 gene, whose expression is normally detected in the second branchial arch and posterior ones, is expressed segmentally in the posterior part of the FBA. It is not known, however, if neural crest cells within the FBA mix or remain distinct.

These two questions led to the present study which elucidates the stage-dependent RA effects on craniofacial pattern formation by investigating the alteration in the migration pathway of cranial neural crest cells from a standpoint of cell lineage and identity. Towards this end, we treated rat embryos in vitro with RA at specific developmental stages and used the vital dye 1,1-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) to monitor the migration of neural crest cells from the anterior hindbrain (the presumptive r1 and r2) and the preotic hindbrain (the presumptive r3 and r4). In addition, to confirm the identity of the hindbrain brachial arches and cranial ganglia, we performed immunostaining of CRABP I and neurofilament on sections and whole-mounts, respectively.

Our results clearly show that RA influences craniofacial development in a stage-dependent manner and that it causes alteration in the identity of branchial arches, cranial ganglia and rhombomeres at morphological and molecular levels. Regarding the migration pathways of hindbrain crest cells, RA treatment at the early stage induced ectopic migration of anterior hindbrain crest cells; thereby suggesting from a cell lineage perspective that RA transforms the regional identities of these crest cells.

MATERIALS AND METHODS

Rat whole embryo culture

Wistar-Imamichi rat embryos were surgically explanted from anesthetized mothers at 9.0 and 9.5 d.p.c. (early and late neural plate stage, respectively; plug day; 0). The time course of the whole embryo culture is shown in Fig. 1. Whole embryos were cultured according to a previously described method (Matsuo et al., 1993; Osumi-Yamashita et al., 1994). Briefly, embryos were placed in 15 ml culture bottles containing 3 ml of culture media consisting of 100% immediately centrifuged rat serum with 2 mg/ml glucose. The culture bottles were attached to a rotator drum and rotated at 20 revs/minute and 37˚C while being continuously supplied with a gas mixture, i.e., 5% O_2/5% CO_2/90% N_2 for the first 36 or 24 hours for early and late stage embryos, respectively, and subsequently with 20% O_2/5% CO_2/75% N_2 for the remaining culture period. The flow rate of gas was increased as necessary.

Retinoic acid treatment

All-*trans* RA (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution (2×10^{-4} M). A 3 μl aliquot was added to 3 ml of culture medium so that the final RA concentration was 2×10^{-7} M. Embryos were exposed to RA for 6 hours and further cultured in fresh medium until they developed to the desired stage (Fig. 1). Vehicle-control embryos were exposed to the same amount of DMSO for 6 hours. After being treated with RA or vehicle, embryos were washed several times with Tyrode's solution and transferred to fresh

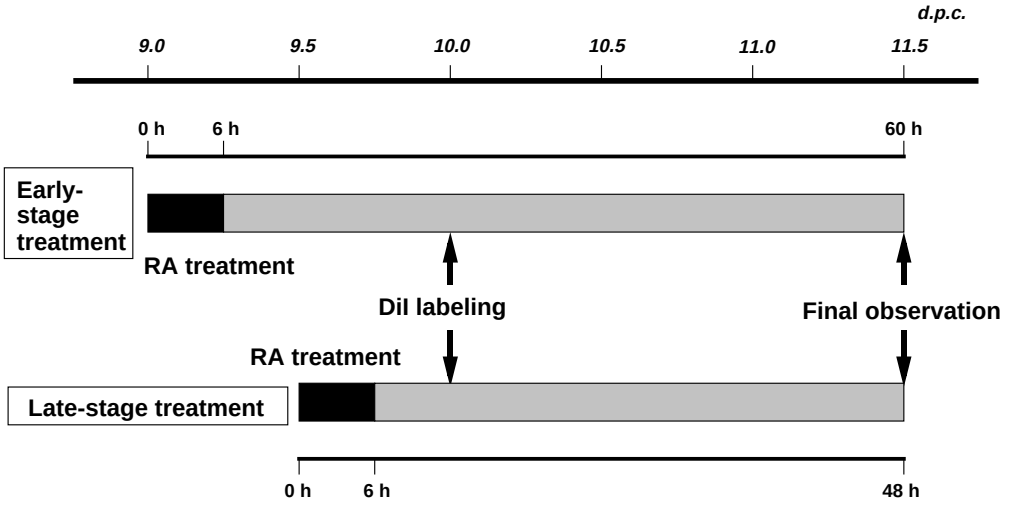


Fig. 1. Experimental time diagram showing the RA treatment and DiI-labeling schedule used for the early and late neural plate stages.

medium for further culture. Because DMSO did not affect normal development of the embryos, inject-control embryos were not treated with the vehicle. The RA concentration, treatment duration and stage at treatment were determined from preliminary experiments, being optimally adjusted so that RA induced to a maximum extent the teratogenic effect in branchial arches.

DiI labeling of cranial neural crest cells

Microinjection of DiI was performed according to the methods previously described (Matsuo et al., 1993; Osumi-Yamashita et al., 1994). Briefly, micropipettes (internal diameter 10 μm) were filled with DiI (Molecular Probes, Inc.) at 0.25% (w/v) in 100% dimethylformamide. Embryos that developed to the 3- to 7-somite stage were selected for DiI labeling (Fig. 1). Focal injections of DiI were performed at the anterior hindbrain (the presumptive r1 and r2) and the preotic hindbrain (the presumptive r3 and r4) in order to label premigratory neural crest cells. All successfully injected embryos were immediately transferred into culture bottles and incubated as described above.

Examination of embryos

After culturing, embryos were assessed for heart beat, yolk-sac and whole body blood circulation, yolk-sac diameter, crown-rump length, somite number and general morphology. To ascertain if the culture period itself affected embryonic development, preliminary experiments were carried out in which cultured embryos were compared with those allowed to develop in utero for the same length of time. Based on the resultant somite number and general morphology, embryos that were cultured for 60 hours from the early stage and 48 hours from the late stage appeared similar to ones developed in utero (data not shown).

Distribution of DiI was photographed (Kodak PKL200) using whole-mount embryos placed under a fluorescent microscope with a rhodamine filter set (Zeiss). Some embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2-4 hours, frozen in OCT compound (Miles Inc.), and serially sectioned (14 μm) at −20˚C. These sections were also subjected to fluorescent microscopy. Data from the microscope were readable on Nikon Coolscan set and images were processed using Adobe Photoshop software (Adobe system, Inc.) on a Macintosh Quadra 840 AV computer.

Scanning electron microscopy

For scanning electron microscopic (SEM) observations, embryos were fixed in 0.05 M cacodylate buffer containing 1% PFA and 1.5% glutaraldehyde (pH 7.35-7.45) for 2 hours at room temperature and rinsed with 0.1 M sucrose in 0.05 M cacodylate buffer. The samples were postfixed with 1% osmium tetroxide in 0.05 M cacodylate buffer, dehydrated in graded ethanol, critical point dried (Critical Point Dryer, Hitachi), sputter coated with Au-Pd (HCP-2, Hitachi) and then observed (JSM-T200, JEOL).

General histological observation

For histological observations, embryos were fixed in Bouin's solution, dehydrated with ethanol series and embedded in paraffin. Serial frontal sections (5 μm) were mounted on glass slides, stained with hematoxylin-eosin, observed under a light microscope (Olympus) and photographed (Fujichrom, RDP).

Immunohistochemistry on sections

The employed antibody was raised against synthetic peptides corresponding to residue 68-81 of bovine CRABP I. The specificity of the antibody to CRABP I is well characterized in rat tissue (Eriksson et al., 1987), and that it has no detectable cross-reactivity to CRABP II (Dencker et al., 1990, 1991; Maden et al., 1991, 1992).

For detection of CRABP I protein, frozen sections of cultured embryos were prepared as described above, quenched with hydrogen peroxide, blocked with normal horse serum and processed overnight for application of the antibody against CRABP I at a dilution of 2 μg/ml and 4˚C. Immunoreactivity was detected using an ABC kit (Vector Laboratorie). Diaminobenzidine (DAB) was used to visualize the reaction product and the sections were counterstained with methyl green.

Immunohistochemistry on whole-mounts

As the primary antibody we used 2H3, an anti-neurofilament monoclonal antibody, which labels 166×10^3 M_r neurofilament raised from adult rat brain membranes. As the secondary antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (NGF 825, Amersham) was used.

For the whole-mount immunostaining, the embryos were further cultured for 9 hours until they developed to the 28-somite stage, and prepared as previously described (Sundin and Eichele, 1990; Kuratani and Eichele, 1993) using the following modification. Cultured embryos were fixed overnight in 4% PFA in PBS at 4˚C and stored in methanol at −20˚C. The samples were subsequently treated with DMSO/methanol and 2% Triton X-100, and then washed with TST (Tris-HCl buffered saline: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100). Next they were treated with 1% periodic acid solution to block endogenous peroxidase activity, sequentially blocked with 5% dry non-fat milk in TST (TSTM), and afterwards incubated overnight at room temperature with 2H3 antibody in spin-clarified TSTM (1:50-100) containing 0.1% sodium azide. Following washing with TST, the samples were treated with HRP-conjugated secondary antibody in TSTM (1:200) overnight at room temperature. Finally, they were preincubated with Tris-HCl-buffered saline (TS) containing DAB (250 μg/ml) for 1 hour, and then reacted with the same concentration of DAB/TS containing hydrogen peroxide (0.05%) at 0˚C for 20 minutes. The reaction was stopped by rinsing the samples with 30% glycerol in distilled water. To obtain transparency, the stained embryos were transferred through a graded series of up to 80% glycerol in distilled water containing a trace amount of thymol.

RESULTS

General morphology of cultured embryos

The hindbrain region of embryos clearly showed two types of segmental structures after culturing, i.e., a branchial arch structure and a rhombomeric component. In the control embryos cultured from 9.0 or 9.5 d.p.c., the first branchial arch, including the maxillary and mandibular prominences, was larger than the second and posterior arches (Fig. 2A). In contrast, RA induced two typical craniofacial abnormalities depending on stages. RA treatment at the early stage reduced the size of the first branchial arch, including a decrease in the maxillary and mandibular prominences (Fig. 2B; Table 1). This arch did not show the typical bifurcated shape, but instead looked like the second branchial arch. In contrast, the corresponding late-stage-treated embryos (Fig. 2C) had their first and second branchial arches fused together. Although a fused branchial arch (FBA) was usually observed to be bilaterally situated, it was unilaterally situated in a few embryos (Table 2).

The hindbrain morphology also showed interesting differences. The frontal sections of the control embryos had well-developed segmental rhombomeres (Fig. 2D). Although the early- and late-stage RA-treated embryos had a normal total number of rhombomeres, the region with rhombomeres (r) 1-3 was often compact and asymmetric in the early-stage RA-

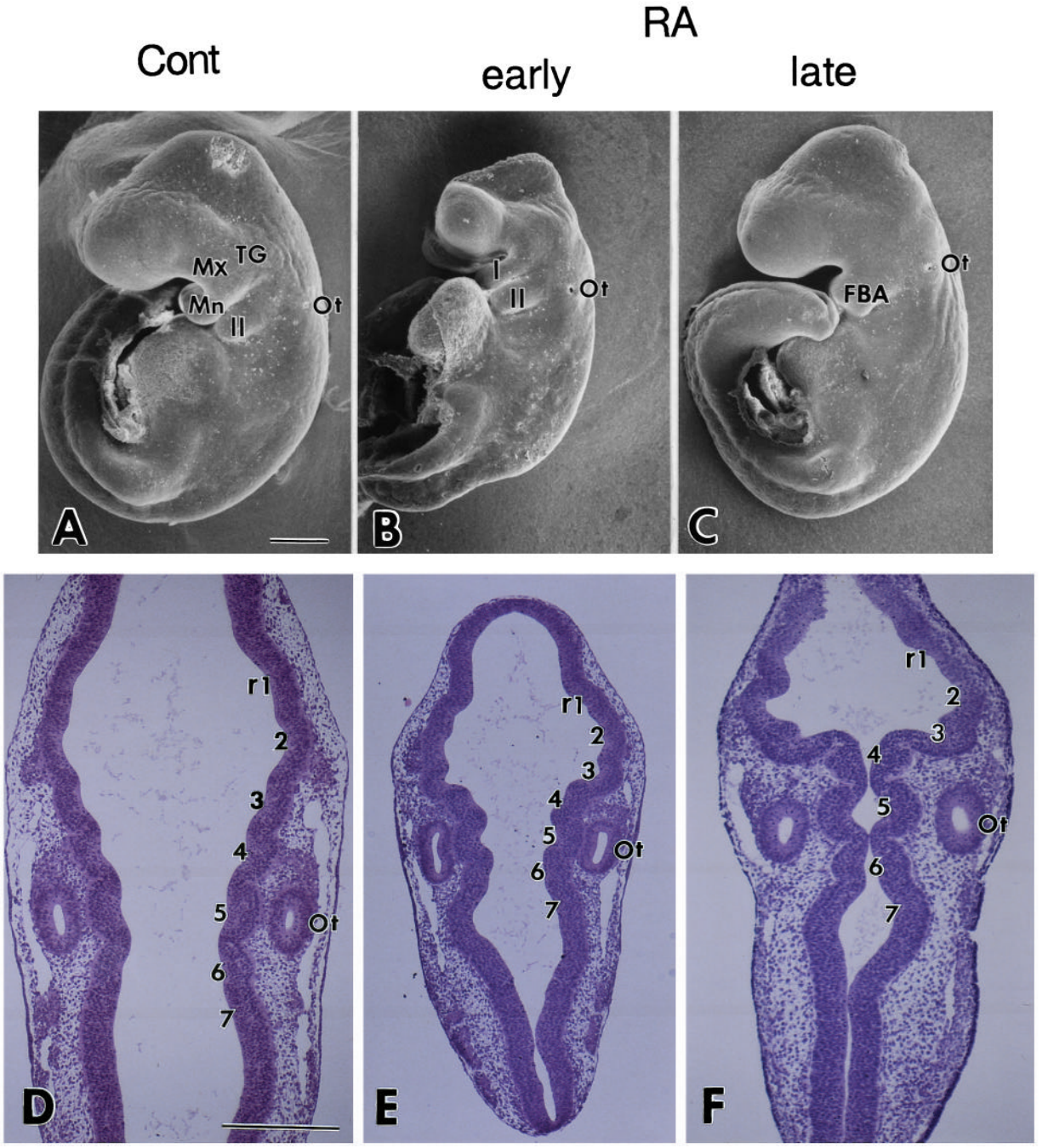


Fig. 2. Typical craniofacial malformations induced in rat embryos by early- and late-stage RA treatments. (A-C) SEMs of the lateral view of embryos. (D-F) Histology showing the rhombomeres of frontal sections of embryos stained with hematoxylin-eosin. (A,D) Morphology of control embryos cultured from 9 d.p.c. for 60 hours. The control embryo shows the normal shape of the large mandibular (Mn) and maxillary prominence (Mx) of the first branchial arch, a small second branchial arch (II), and otocyst (Ot) adjacent to the second branchial arch. In the hindbrain region, normally segmented rhombomeres 1-7 (r1-7) appear. (B,E) The early-stage RA-treated embryos show small mandibullar (Mn) and maxillary prominences (Mx) of the first branchial arch (I), and rhombomeric segmentation (r1-7) is maintained even though the rhombomeres are smaller in comparison with those of the control embryo. (C,F) In contrast, the late-stage-treated embryos have a fused branchial arch (FBA) and show rostrally shifted otocysts due to the compaction of r3 and r4. TG, trigeminal ganglion. Bar, 400 μm.

Fig. 3. Immunohistochemical localization of CRABP I in rhombomeres (A-C) and branchial arches (D-F) of control embryos (A,D), and early- (B,E) and late-stage (C,F) RA-treated embryos. (A) In the control embryo, immunostaining of CRABP I is strong in r4-6, weak in r2, and barely visible in r1/r3. (B) CRABP I distribution is increased in r1-3 due to the early-stage treatment. (C) Immunostaining of CRABP I in the late-stage embryo shows a similar pattern to that shown in the control embryo (A). (D) In normal development, immunoreactivity of CRABP I is high in the mesenchyme of the second (II) and posterior branchial arches, whereas in the first branchial arch (I) it is relatively low. (E) High immnoreactivity in the first and second branchial arches at the early-stage-treated embryo. (F) Weak staining is observed in the anterior half of the FBA, while being strong in its posterior half. Arrow indicates the fused site. Bar, 200 μm.

treated embryos (Fig. 2E), while only the r3-4 region was compact in the late-stage ones (Fig. 2F).

Moreover, the late-stage-treated embryos had their otocyst position shifted rostrally, being due to a shortened preotic

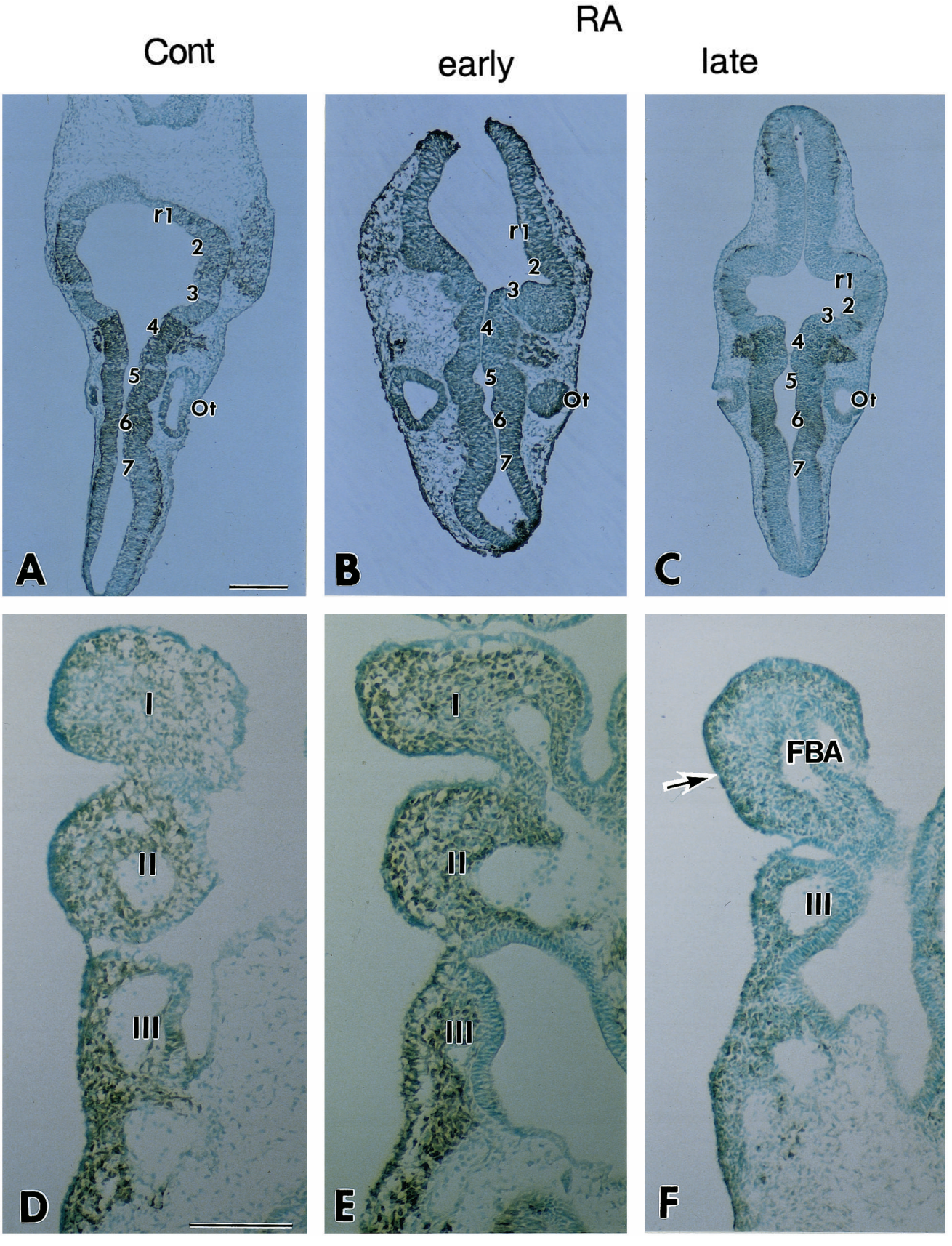


Table 1. Developmental abnormalities observed in cultured rat embryos treated with RA at the early neural plate stage*

	Microcephaly	Small first branchial arch†	Abnormal eye primordium‡	Open neural tube§	Other malformations¶
Control (*n*=38)	0(0)	0(0)	0(0)	0(0)	0(0)
Vehicle control (*n*=8)	0(0)	0(0)	0(0)	0(0)	1(17)
RA (*n*=50)	43(86)	50(100)	38(76)	28(56)	15(30)

*Data are presented as the number of the embryos expressing the abnormalities. Percentages in parentheses.
†Includes small mandibular and maxillary prominences.
‡Includes no optic protrude and abnormal protrusion of the eye primordium.
§Includes open anterior, middle and posterior neural tube.
¶Includes for example incomplete antero-posterior axis rotation and/or a fused branchial arch.

Table 2. Developmental abnormalities observed in cultured rat embryos treated with RA at the late neural plate stage*

	Branchial arch fusion bilateral	Branchial arch fusion right/left	Open neural tube	Other malformations†
Control (*n*=34)	0(0)	0(0)/0(0)	0(0)	0(0)
Vehicle control (*n*=13)	0(0)	0(0)/0(0)	0(0)	0(0)
RA (*n*=38)	34(87)	3/4(11)	17(41)	6(15)

*Data are presented as the number of the embryos expressing the abnormalities. Percentages in parentheses.
†Includes any combination of: a small limb bud, microcephaly, incomplete anterior-posterior axis rotation, or short tail bud.

hindbrain region (Fig. 2C,F). This was not observed in the early-stage-treated embryos, which instead showed a small midbrain and shortened anterior hindbrain (Fig. 2B). In both the RA-treated groups, some embryos had an open neural tube and/or microcephaly (Tables 1, 2).

The effects of RA on general embryonic development were not as severe, yet statistically significant differences were found in comparison to the control (injected or not injected) and vehicle control groups regarding yolk-sac diameter, crown-rump length and number of somites (Tables 3, 4). The toxicity of DiI labeling was evaluated by comparing the injected and uninjected embryos within the control groups and RA-treated groups. Since the parameters for embryonic development did not significantly differ within these groups, this indicates the labeling by microinjection had no effect on general development of embryos (Tables 3, 4).

It should be noted that the early-stage treatment (6 hours) embryos showed very similar craniofacial morphology results to those previously described in which pregnant mice (7.5 d.p.c.) were treated (Marshall et al., 1992). In fact, in additional experiments that exposed the early-stage embryos with RA for only 3 hours, similar abnormalities were obtained, although compaction and asymmetry of the rhombomeres did not occur (data not shown).

Expression of CRABP I in the craniofacial region

In the control groups, immunoreactivity of CRABP I was not detected in r1 or r3 and was weak in both r2 (Fig. 3A) and the mesenchyme of the first branchial arch (Fig. 3D). In contrast, strong immunoreactivity occurred in r4-7 (Fig. 3A) and in the second and posterior arches (Fig. 3D), as well as in the frontonasal mass; similar to previous observations in mouse embryos (Maden et al., 1992; Gustafson et al., 1993).

The expression of CRABP I in the rhombomeres was not different in the late-stage-treated embryos, i.e., the immunoreaction was only strong in r4-6 (Fig. 3C). In the FBA, the posterior region showed intense CRABP I reactivity (Fig. 3F), while being weak in its anterior part; thereby suggesting that the neural crest cells do not freely mix despite the fusion of the arches. The early-stage-treated embryos, however, induced up-regulation of CRABP I immunoreactivity in r1-3, as well as in the mesenchyme of the first branchial arch (Fig. 3B,E), i.e., neural crest cells in their first branchial arch and their associated rhombomeres (r1-3) were strongly stained like those in the second and posterior arches and r4-7.

Phenotypes of cranial ganglia

Whole-mount immunostaining with anti-neurofilament antibody 2H3 clearly visualized the morphology of the trigeminal, acousticofacial, and more posterior ganglia and their

Table 3. Development of cultured rat embryos treated with RA at the early neural plate stage*

Group	Number of embryos	Number of somites	Crown-rump length (mm)	Yolk-sac diameter (mm)
Control intact	19	24.2±0.9	3.25±0.26	4.13±0.17
Cont/DiI Injected	19	23.8±0.9	3.27±0.16	3.98±0.22†
Vehicle control	8	23.5±0.9	3.10±0.23	4.06±0.25
RA intact	20	22.0±1.3†‡§	2.82±0.23†‡§	3.73±0.29†‡§
RA/DiI Injected	30	21.8±1.6†‡§	2.78±0.19†‡§	3.72±0.30†‡§

*Data are expressed as mean ± standard deviation.
†Values are significantly different from the intact control (P<0.01).
‡Values are significantly different from the DiI-injected control (P<0.01).
§Values are significantly different from the vehicle control (P<0.01).

Table 4. Development of cultured rat embryos treated with RA at the late neural plate stage*

Group	Number of embryos	Number of somites	Crown-rump length (mm)	Yolk-sac diameter (mm)
Control intact	14	24.0±0.5	3.23±0.13	4.05±0.21
Cont/DiI Injected	20	23.7±0.9	3.32±0.16	4.08±0.18
Vehicle control	13	24.7±0.9†	3.44±0.24†	4.25±0.24
RA intact	14	22.9±1.0†‡§	3.17±0.12‡§	3.96±0.24§
RA/DiI Injected	24	22.8±1.1†‡§	3.06±0.11†‡§	4.05±0.11§

*Data are expressed as mean ± standard deviation.
†Values are significantly different from the intact control (P<0.01).
‡Values are significantly different from the DiI-injected control (P<0.01).
§Values are significantly different from the vehicle control (P<0.01).

nerve branches in the control embryo at the 28-somite stage (Fig. 4). The ophthalmic branch arose from the trigeminal ganglion extending rostrally toward the eye primordium, while the maxillomandibular branches extended to the first branchial arch. Also the acousticofacial nerve innervated to the second branchial arch and the otocyst (Fig. 4A).

In late-stage RA-treated embryos at the same stage, compression of the preotic hindbrain region resulted in the fusion of the trigeminal and acousticofacial ganglia near their proximal portions, though the typical features of the ganglia and branching patterns were retained (Fig. 4C). In contrast, these ganglia remained separated in early-stage RA-treated embryos with the same number of somites. Interestingly, however, the morphology of the trigeminal ganglion was clearly altered in this case; the ophthalmic branch innervated the eye region as in the control, whereas the typical axonal projections into maxillary and mandibular regions had vanished (Fig. 4B). The morphology of the facial nerve and its ganglion did not change in early-stage RA-treated embryos.

Migration pathways of hindbrain crest cells

In situ labeling was carried out at the 3- to 7-somite stage based on results by Tan and Morriss-Kay (1985) and Osumi-Yamashita et al. (1994) which clarify when the emigration of

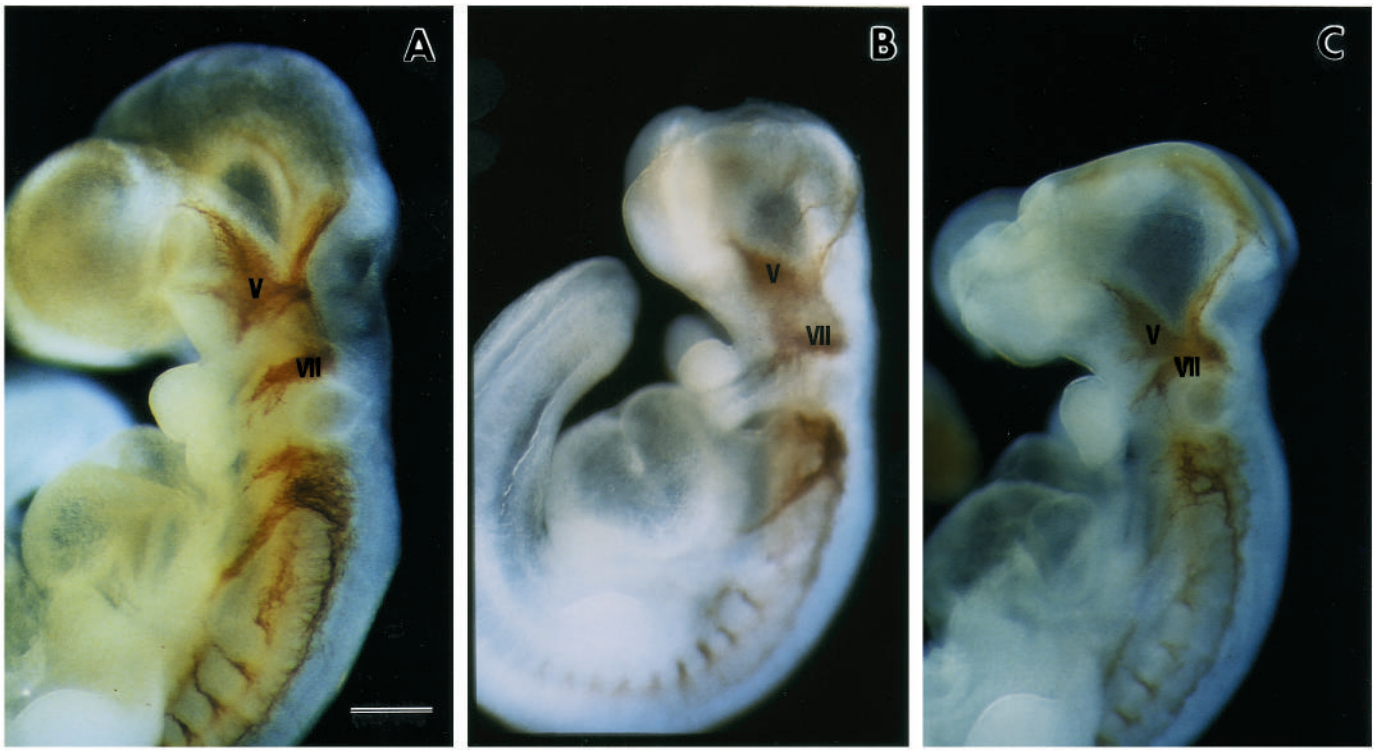


Fig. 4. Lateral views of embryos at the 28-somite stage stained with anti-neurofilament antibody. (A) Control embryos show the typical shape of axons of the trigeminal (V) and acousticofacial (VII) ganglia. The ophthalmic branch arises from the trigeminal ganglion extending rostrally toward the eye primordium, while the maxillomandibular branches extend to the first branchial arch. The acousticofacial nerve innervate to the second branchial arch and the otocyct. (B) In early-stage RA-treated embryos, these ganglia remain separated, but the morphology of the trigeminal ganglion is clearly altered; as in the control, the ophthalmic branch innervated the eye region, whereas, the typical axonal projections into maxillary and mandibular regions had vanished. The morphology of the facial nerve and its ganglion is not changed. (C) In late-stage RA-treated embryos, however, typical features of the trigeminal and acousticofacial ganglia are seen similar to the control embryo, though both ganglia are fused together near their proximal portions. Bar, 100 μm.

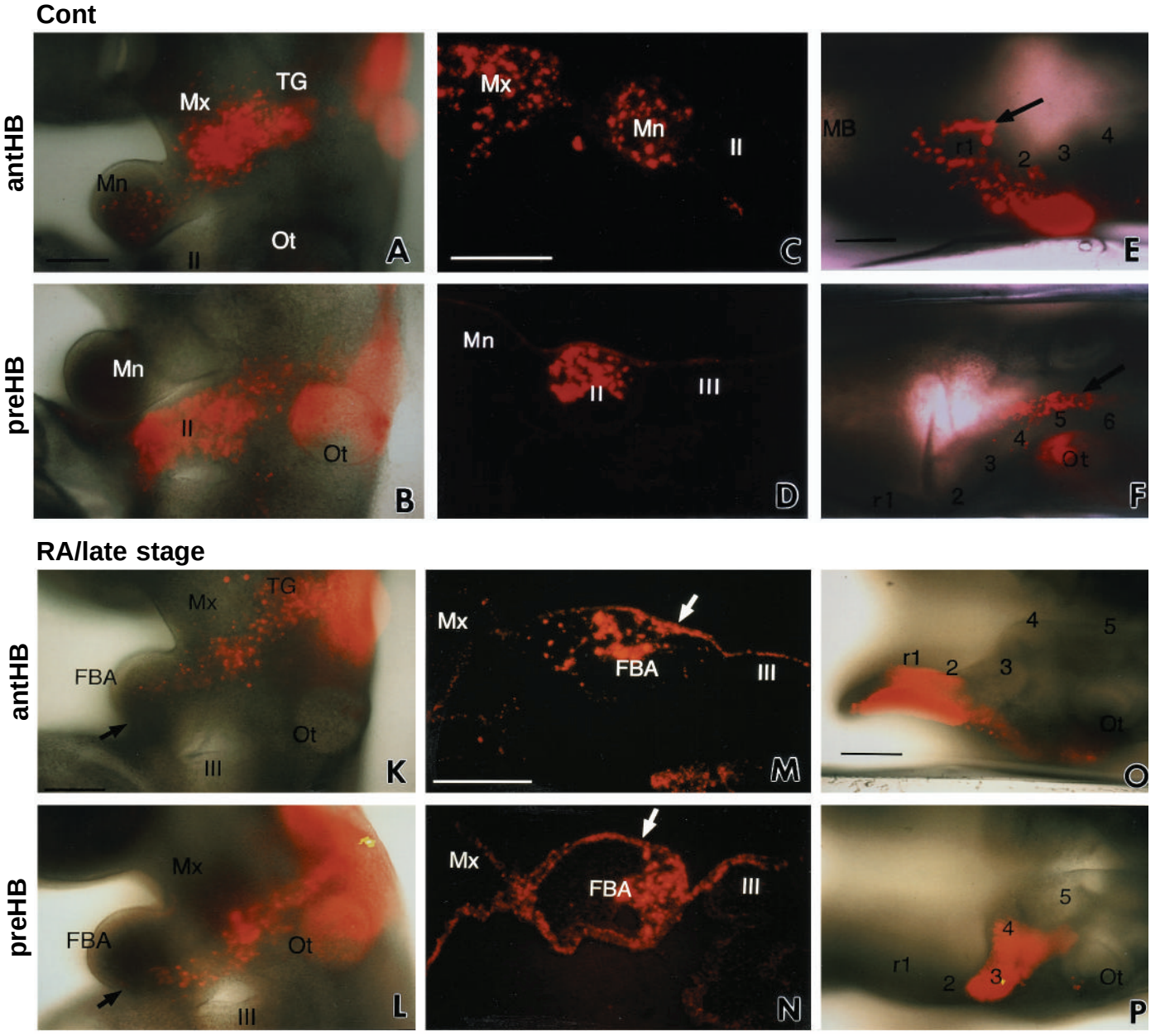
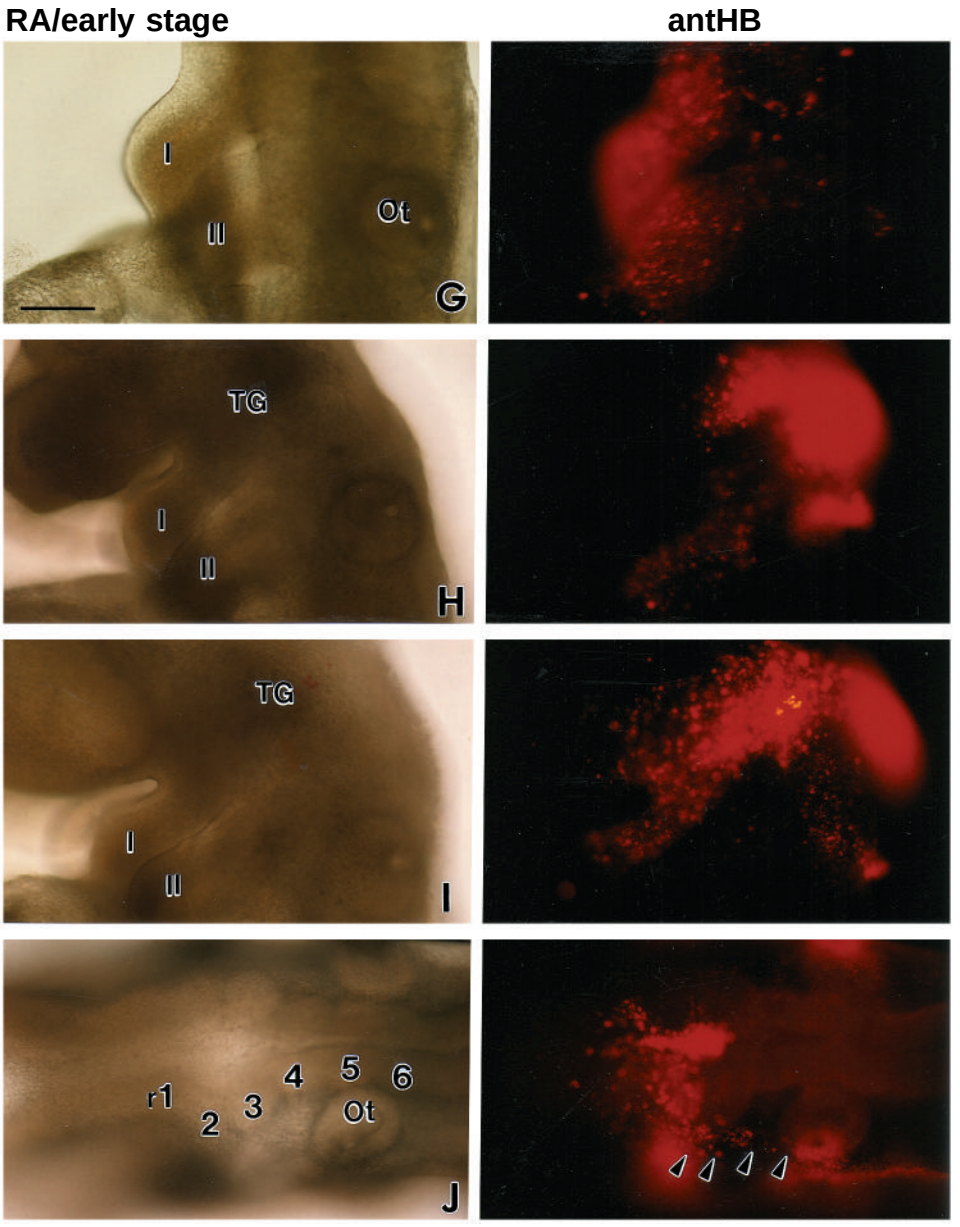


Fig. 5. Migration patterns of DiI-labeled neural crest cells observed in whole-mounts (A,B,G-I,K,L, lateral view, E,F,J,O,P, dorsal view) and sectioned (C,D,M,N) embryos at the end of culture. In G-J, left columns are micrographs of bright-field images and the right ones are corresponding dark-field images. (A-F) Normal migration pattern of hindbrain crest cells in control embryos. (A,C,E) Cells labeled at the anterior hindbrain are distributed in the maxillary (Mx) and mandibullar (Mn) prominences in the first branchial arch (I) and trigeminal ganglion (TG), as well as in rhombomere (r) 1 and 2. (B,D,F) Labeled cells that emigrated from the preotic hindbrain populate the second branchial arch (II) and acousticofacial ganglion adjacent to the otocysts (Ot). Labeled neuroepithelial cells are observed in r4. (G-J) Distribution of labeled cells that emigrated from the anterior hindbrain of early-stage RA-treated embryos. (G) Labeled crest cells are mainly distributed in the first branchial arch and also ectopically in the second arch (II). (H) Labeled cells are localized in the trigeminal ganglion and also ectopically in the second branchial arch and acousticofacial ganglion. (I) Labeled crest cells are in the first branchial arch and trigeminal ganglion, and in addition have ectopically migrated to the acousticofacial ganglion. (J) Labeled neuroepithelial cells are located in r1 and 2, while some are also in the dorsal unsegmented region corresponding to r3. Labeled crest cells from r2 are clearly shown to have migrate into the region rostral to the otic vesicle (arrowheads). (K-P) Labeled cells that emigrated from the anterior (K,M,O) and preotic (L,N,P) hindbrain of late-stage-treated embryos show segmental migration patterns within the FBA, and also have the same labeling pattern as control embryos in the rhombomeres. (K) Labeled anterior hindbrain crest cells are only in the anterior half of the FBA, trigeminal ganglion and maxillary prominence. (L) Labeled cells in the preotic hindbrain are only distributed in the posterior half of the FBA and acousticofacial ganglion. (M,N) Corresponding frontal sections of the embryos in K and L, respectively, where no mixing of crest cells appears in the FBA. Arrows indicate the fused site. Even though an open neural tube exists and compaction has occurred in r3 and 4, labeled cells at the anterior hindbrain populate r 1 and r2 (O), while those labeled at the preotic hindbrain populate r3 and r4 (P). Bar, 200 μm.



hindbrain crest cells begins. The premigratory neural crest cells were labeled at the anterior hindbrain (similar to rhombomere A in Bartelmez and Evans, 1925, corresponding to presumptive r1 and r2) and preotic hindbrain (rhombomere B, the presumptive r3 and r4).

Crest cells labeled in the anterior hindbrain of the control groups were observed in the first branchial arch and the trigeminal ganglion, while those labeled at the preotic hindbrain were distributed in the second branchial arch and acousticofacial ganglion (Table 5; Fig. 5A-D). Such segmental migration patterns are identical to those observed in previous studies (Serbedzija et al., 1992; Matsuo et al., 1993; Osumi-Yamashita et al., 1994). Neuroepithelial cells labeled at the anterior hindbrain distributed in r1 and r2, while those labeled at the preotic hindbrain in r3 and r4 (Fig. 5E,F). Labeled neuroepithelial cells were sometimes observed crossing over the rhombomeric boundaries in the dorsal unsegmented region (arrows in Fig. 5E,F).

In 18/18 cases of the early-stage RA-treated embryos, crest cells labeled in the anterior hindbrain ectopically migrated into the second branchial arch and/or the acousticofacial ganglion in addition to their original destinations, i.e., the first branchial arch and the trigeminal ganglion (Fig. 5G-I; Table 5). Of importance, labeled neuroepithelial cells derived from the anterior hindbrain were observed in r1 and r2, but not in r3 or r4 (Fig. 5G-J), which suggests that the exit point of anterior hindbrain crest cells remains unchanged and that these crest cells directly migrate into the ectopic regions. This is typically indicated in the dorsal view of the embryo since it simultaneously shows labeled neuroepithelial cells in r1 and r2 and labeled crest cells in the acousticofacial ganglion (arrowheads in Fig. 5J). In this embryo, some labeled cells were also observed in the dorsal midline corresponding to r3 but, because this region was completely unsegmented, it should not be considered to be a rhombomere. The migration pathways of the crest cells derived from the preotic hindbrain (presumptive r3 and r4) were not much different from the control group's, although 4 of 12 embryos showed ectopic migration from the preotic hindbrain into the trigeminal ganglion (Table 5). A particular issue of interest in these observations is that the preotic sulcus may possible serve as a lineage restriction in the early-stage RA-treated embryos.

Surprisingly, the migration patterns were segmental in the late-stage RA-treated embryos even though FBA occurred. Labeled cells from the anterior hindbrain populated the anterior half of the FBA and trigeminal ganglion (Table 5, Fig. 5K,M), whereas those from the preotic hindbrain populated the posterior half of the FBA and acousticofacial ganglion (Fig. 5L,N). This behavior is noteworthy because cells from the different prorhombomeres (anterior and preotic hindbrain, i.e., rhombomere A and B) did not mix in the FBA of these embryos, despite the fact they have adjacent positions in the same arch. Like the control groups, no cells originating from different neuroepithelial areas were observed to be mixed in hindbrain axial level. Moreover, the labeling patterns in rhombomeres were the same as those in control embryos even though they showed the compacted rhombomeres; i.e., labeled cells at the anterior hindbrain distributed in r1 and r2, while those at the preotic hindbrain in r3 and r4 (Fig. 5O,P). Consequently, the segmental migration pattern of the late-stage embryos was not affected by the RA treatment, nor were the

Table 5. Distribution of labelled cells in the control (cont) and RA-treated (RA) embryos cultured from the early and late neural plate stages

Stage of treatment	Early neural plate				Late neural plate				Stage of treatment
Injection site*	ant HB		pre HB		ant HB		pre HB		Injection site*
Treatment	cont	RA	cont	RA	cont	RA	cont	RA	Treatment
Number of embryos injected	11	18	8	12	10	11	10	13	Number of embryos injected
1st branchial arch									*Fused branchial arch (FBA)*
Maxillary prominence	10	8			4	3			Maxillary prominence
Mandibular prominence	9	10			5	9			Anterior part of FBA
Trigeminal ganglion	11	13	0	4	8	8			Trigeminal ganglion
2nd branchial arch	0	6	8	12			10	12	Posterior part of FBA
Acousticofacial ganglion	0	12	7	11			10	11	Acousticofacial ganglion

*Embryos were injected with DiI at the anterior hindbrain (ant HB) or preotic hindbrain (pre HB).

regional properties affected of neural crest cells originating from different hindbrain regions; suggesting that the treatment did not change their identities.

DISCUSSION

Stage-dependent induction of craniofacial abnormalities

Although RA treatment induced compaction of rhombomeres in the late-stage embryos and sometimes in the early-stage ones, their number was the same in both groups. These results are in agreement with the effects of RA treatment on frog, fish and mouse embryos (Papalopulu et al., 1991b; Holder and Hill 1991; Marshall et al., 1992), which suggest that such changes are in common with vertebrates. Morriss-Kay et al. (1991) and Wood et al. (1994) observed unsegmented-type rhombomere malformation in maternally treated mouse embryos, which we did not observe in cultured rat embryos: a discrepancy that may be due to using different experimental systems.

In all the RA-treated embryos, we found an altered structure of the branchial arches in addition to the rhombomeric defects. Early-stage treatment specifically affected the first branchial arch, since it appeared to have a similar size and structure to the second branchial arch. Marshall et al. (1992) reported a corresponding finding in mouse embryos that were maternally treated with RA at a similar stage. In the late-stage-treated embryos, however, another type of arch defect typically occurred, i.e., fusion of the first and second branchial arches (FBA). The treatment also concomitantly induced an anterior shift of the otocysts caused by shortening of the anterior hindbrain region. A similar FBA has also been observed in RA-treated mouse embryos (Goulding and Pratt, 1986; Webster et al., 1986).

We also found differential effects of RA on cranial ganglia formation. Whole-mount immunostaining of neurofilament in early-stage-treated embryos clearly demonstrated that the trigeminal ganglion changed its morphological structure, though it developed independently from the acousticofacial ganglion. A similar situation has been reported by Marshall et al. (1992) who found that the trigeminal nerve adopted a facial nerve identity with respect to the location of nerve cell bodies and their axonal projection after RA administration. In contrast, in our system, the late-stage treatment induced a fusion of trigeminal and acousticofacial ganglia. It should be noted that, genetic disruption of *Krox-20* produces a similar fusion of these ganglia in mouse embryos (Schneider-Manoury et al., 1993; Swiatek and Gridley, 1993). However, such fusion seemed to be due to the loss of r3 (Schneider-Manoury et al., 1993; Swiatek and Gridley, 1993), which is likely to be caused

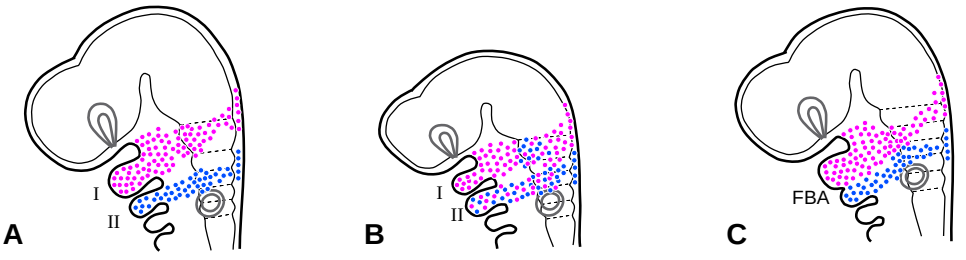


Fig. 6. Diagram summarizing the migration pattern of neural crest cells. Neural crest cells from the anterior hindbrain are marked with pink dots and those from the preotic hindbrain with blue dots. RA stage-dependently alters the identity of cranial neural crest cells from a standpoint of cell lineage. (A) In control embryos, hindbrain crest cells are segmentally distributed; i.e., crest cells from the anterior hindbrain migrate to the first branchial arch (I), including the maxillary and mandibular prominences, and the trigeminal ganglion; and those from the preotic hindbrain migrate to the second branchial arch (II) and acousticofacial ganglion. (B) In the early-stage-treated embryos, the migration patterns of the hindbrain crest cells are changed since some of the anterior hindbrain crest cells alter their migration pathway posteriorly to the second branchial arch and acousticofacial ganglion. (C) In late-stage-treated embryos, even though the FBA and fusion of trigeminal and acousticofacial ganglia are present, the segmental migration pattern of neural crest cells remains the same as in the control embryos, namely, the labeled cells from the anterior hindbrain migrate to the anterior part of the FBA including the trigeminal ganglion, while those from the preotic hindbrain migrate to the posterior part including the acousticofacial ganglion.

by a different developmental mechanism than ours since r3 exists in our late-stage-treated embryos.

Taken together, the effects of RA on embryonic patterning are obviously stage-dependent. The treatment at the early neural plate stage induces malformation specifically in the first branchial arch and trigeminal ganglion, which may correspond to the transformation of the anterior hindbrain in RA-treated mouse embryos as shown by Marshall et al. (1992). In contrast, later-stage treatment caused entirely different phenotypes, i.e., fusion of the first and second branchial arches and that of the cranial ganglia.

Stage-dependent effects on neural crest cell migration

It is well known that the migration of hindbrain crest cells is segmental in normal development, i.e., those derived from the anterior hindbrain migrate to the first branchial arch and never mix with the pathway of the preotic hindbrain crest cells migrating to the second arch (Lumsden et al., 1991; Serbedzija et al., 1992; Matsuo et al., 1993; Osumi-Yamashita et al., 1994).

However, early-stage RA treatment altered such segmental migration patterns of the hindbrain crest cells by inducing ectopic pathways in all embryos investigated. Anterior hindbrain crest cells posteriorly migrated to the second branchial arch and acousticofacial ganglion, as well as to their original destination, the first arch and trigeminal ganglion. Conversely, the preotic hindbrain crest cells had their pathways anteriorly altered to the trigeminal ganglion, which is normally occupied by anterior hindbrain crest cells (Fig. 6B).

In the ectopic migration of anterior hindbrain crest cells, most of neuroepithelial cells, including premigratory crest cells, did not move posteriorly over the preotic sulcus, i.e., labeled anterior hindbrain neuroepithelial cells were restricted in r1 and r2. Thus, RA treatment at the early neural plate stage induces distortion of the neural tube, though it does not alter the location of the exit points of the neural crest cells, instead only altering the identity of the anterior hindbrain into that of the preotic hindbrain before migration begins. In other words, RA treatment causes premigratory and/or migrating anterior hindbrain crest cells to have a common identity with the preotic hindbrain crest cells, and then these altered anterior hindbrain crest cells ectopically migrate directly to the second branchial arch and/or the acousticofacial ganglion. Regarding the crest cells emerging from the preotic hindbrain in early RA-treated embryos, they migrated to the second branchial arch (ventral structure), and to the trigeminal and acousticofacial ganglia (dorsal structure), though they were never observed in the first branchial arch. One possible explanation is that a discrepancy exists in the emigrating time of crest cells derived from the anterior hindbrain and preotic hindbrain, i.e., the former emigrate earlier than the latter (Tan and Morriss-Kay, 1985) and occupy the first arch before the latter enter it. Further support of this possibility is that a ventral-to-dorsal migration pattern is a natural feature of crest cells (Serbedzija et al., 1992; Osumi-Yamashita et al., 1994).

In contrast, the late-stage RA treatment did not change the normal segmental migration from different hindbrain crests in the FBA (Fig. 6C). The anterior half of the FBA was populated by anterior hindbrain crest cells, whereas the posterior half by preotic ones. Cranial ganglia were also segmentally populated, i.e., from the anterior hindbrain to the trigeminal ganglion and from the preotic hindbrain to the acousticofacial ganglion, despite the fact that both ganglia were fused.

These results clearly indicate that the migration pattern of the hindbrain neural crest cells is stage specifically affected by exogenous RA because segmental hindbrain crest cell migration is perturbed by the early treatment but not by the late one. We should point out that this is the first evidence showing that RA treatment alters the hindbrain identity by affecting the lineage of hindbrain crest cells.

Another important conclusion drawn here is that the function of the branchial arches is not solely to separate different populations of hindbrain crest cells. We base this on the fact that these crest cells did not freely mix in the FBA of the late-stage RA-treated embryos, whereas in the early-stage ones, changing the identity of the cells allowed them to mix or take alternate pathways in the branchial arches. Thus, migration of the neural crest cells into specific arches is not a completely passive process, but instead somewhat dependent on the ability of the cells to interpret migratory cues.

Stage-dependent effects at the molecular level

The observed stage-dependent effects of RA on the migration pattern of neural crest cells are consistent with the results of Conlon and Rossant (1992) in that the expression of *Hox-B1* (*Hox* 2.9) and *Hox-B2* (*Hox* 2.8) genes are responsive to RA in the early neural plate stage, though by the early somite stage, expression within the neural tube no longer occurs.

RA treatment of mouse embryos at the preheadfold stage (roughly corresponding to the early neural plate stage in the present experiment) induces the anteriorly shifted expression of *Hox-B1* and *Hox-B2* and *Krox 20* genes in rhombomeres and branchial arches (Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Marshall et al., 1992; Wood et al., 1994). Segment-specific expression patterns of these genes in rhombomeres and branchial arches are suggested to impose a regional identity in prospective craniofacial structures (Hunt et al., 1991a,b). Therefore early stage-RA treatment possibly induces a homeotic transformation in the identity of rhombomeres and branchial arches with respect to gene expression.

In contrast, RA treatment at the late neural plate stage may not change the regional identities of the branchial arches and rhombomeres that have already been established by the time of the treatment. Supportive evidence of this is provided by Brown et al. (1992) who found that *Hox*-B2 expression only takes place in the posterior part of the FBA in cultured rat embryos treated with RA from the late-head-fold stage.

In accordance with the stage-dependent alteration of gene expression described above, we found that CRABP I distribution was changed by the early treatment, but not altered by the late one. In normal development, CRABP I is intensely expressed in r4-6 and in the second and posterior arches, while it is not specifically detected in r1 and r3, and is weak in r2 as well as in the first arch (see our results; Maden et al., 1992; Gustafson et al., 1993; McKay et al., 1994). Thus CRABP I should be regarded as another suitable marker for identifying the hindbrain regions. Early RA treatment was found to upregulate CRABP I in the first branchial arch and r1-3 to a similar degree as that in the second branchial arch and r4. This observation corresponds well with the altered expression of *Hox* and *Krox-20* genes in RA-treated embryos (Morriss-Kay et al.,

1991; Marshall et al., 1992; Wood et al., 1994), and provides molecular evidence pointing towards the stage-dependent effects of RA affecting the rigional character of the hindbrain and their crest cells.

Possible mechanism for transformation of hindbrain and branchial arch identity

Taken together, our results and those of other systems (Marshall et al., 1992; Wood et al., 1994) suggest that RA mediates head development in vertebrates by controlling cell lineage as well as through specific gene expression. We do know that RA can activate *Hox* genes in vitro in a colinear manner (Simeone et al., 1990, 1991; Papalopulu et al., 1991a,b). In fact, *Hox-B1* gene has RARE at its 3′ end, with mutation analysis of RARE showing that it is essential for establishing early *Hox-B1* neural expression (Marshall et al., 1994). In addition, RA is known to induce both *Hox-B1* and *Hox-A1* genes through the RARE elements (Studer et al., 1994). Most recently, it was shown that RA activation of *Hox-A1* gene may have created a part of the RA-induced phenotypes, i.e., when the *Hox-A1* construct is injected into transgenic mice it induces *Hox*-B1 expression in r2, leading into the transformation of the identity of r2 to r4 (Zhang et al., 1994). Thus, RA can induce rapid changes in *Hox* gene expression, which alters the regional identity of both the hindbrain neuroepithelium and crest cells. This in turn would alter the migration patterns of the hindbrain crest cells and result in the abnormal patterning of their derivatives. It may be too premature at present to conclude that the altered morphology of the first branchial arch and trigeminal ganglion is the transformation as seen in the experiments of Marshall et al. (1992). However, the altered expression of CRABP I in the branchial arches and hindbrain and changes in migration patterns of the hindbrain crest cells may possibly imply that RA can also cause transformation in the rostral branchial region. Further experimental studies on the mechanisms involved in the transformation of hindbrain and branchial arches should contribute to a better understanding of craniofacial development in vertebrates.

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