Distribution and migration pathways of HNK-1-immunoreactive neural crest cells in teleost fish embryos

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Summary

Whole mounts and cross-sections of embryos from three species of teleost fish were immunostained with the HNK-1 monoclonal antibody, which recognizes an epitope on migrating neural crest cells. A similar distribution and migration was found in all three species. The crest cells in the head express the HNK-1 epitope after they have segregated from the neural keel. The truncal neural crest cells begin to express the epitope while they still reside in the dorsal region of the neural keel; this has not been observed in other vertebrates. The cephalic and anterior truncal neural crest cells migrate under the ectoderm; the cephalic cells then enter into the gill arches and the anterior truncal cells into the mesentery of the digestive tract where they cease migration. These cephalic and anterior trunk pathways are similar to those described in Xenopus and chick. The neural crest cells of the trunk, after segregation, accumulate in the dorsal wedges between the somites, however, unlike in chick and rat, they do not migrate in the anterior halves of the somites but predominantly between the neural tube and the somites, the major pathway observed in carp and amphibians; some cells migrate over the somites. The HNK-1 staining of whole-mount embryos revealed a structure resembling the Rohon-Beard and extramedullary cells, the primary sensory system in amphibians. Such a system has not been described in fish.

Key words: HNK-1 antibody, neural crest cells, Xiphophorus, Oryzias latipes, fish.

Introduction

The neural crest of the vertebrate embryo represents a transient structure that gives rise to cells that migrate along characteristic pathways, localize in particular sites and differentiate into particular cellular phenotypes such as pigment cells in the skin and the iris of the eye, connective tissue in the head and face, neurons and glial cells, neurosecretory cells, etc. (see Le Douarin, 1982). Because of these properties, neural crest cells have been widely used in the investigation of the mechanisms governing cell migration and differentiation. However, these studies have been limited to a few organisms in which grafting experiments have been possible and the transferred cells could be followed by radioactive label (Weston, 1970), dye label (Krotoski et al. 1988) or by differential staining of their chromatin (Le Douarin, 1982; Sadaghiani and Thiebaud, 1987).

The availability of HNK-1 (Abo and Balch, 1981) and NC-1 (Vincent et al. 1983) monoclonal antibodies has provided a more universal and specific tool in the studies of the developmental fate of neural crest cells. These antibodies were initially raised against human natural killer cells and quail ciliary ganglion cells, respectively, but both have been found to recognize the same epitope on the neural crest cells of newt and chicken (Tucker et al. 1984). Using these antibodies, the distribution and the migratory pathways of neural crest cells have been well documented, particularly in chicken (Bronner-Fraser, 1986; Loring and Erickson, 1987), and have been compared with those in rat (Erickson et al. 1989).

There are only a few reports on the formation of the neural crest and the fate of neural crest cells in fish (Newth, 1951, 1956; Lamers et al. 1981; Langille and Hall, 1987, 1988) despite the fact that fish have played and are playing a critical role in the development of several disciplines such as neurobiology, embryology, etc. (see Powers, 1989). Recently, we (Sadaghiani and Vielkind, 1989a,b) have studied the formation of the neural crest and the segregation of neural crest cells in platyfish (Xiphophorus maculatus), swordtails (X. helleri) and Japanese medaka (Oryzias latipes) using scanning electron microscopy. In these studies, we also showed that the HNK-1 antibody recognizes neural crest cells in Xiphophorus. In the present report, we investigated the distribution and migratory pathways of neural crest cells in embryos of these fish using the
HNK-1 antibody and compared our results with those obtained for birds and mammals.

Materials and methods

Fish species and isolation of embryos
Three species of teleost fish, *Xiphophorus helleri* (swordtail), *X. maculatus* (platyfish) and *Oryzias latipes* (Japanese medaka) were used. The swordtail and platyfish species have been kept in our laboratory for many generations, the medaka was obtained from Carolina Biological Supply Company (Burlington, NC).

*Xiphophorus* fishes are viviparous having an ovarian cycle of four weeks and giving birth to 10–40 young. A new set of oocytes matures and is fertilized after 7 days by sperm stored in the ovarian tract (Scrimshaw, 1945; Tavolga, 1949). 11–13 days after the last brood, pregnant females were killed, ovaries obtained and embryos isolated. Stages 8–17 (Tavolga, 1949) were studied directly or after culture for 1–2 days; culture does not affect normal embryogenesis (Vielkind and Vielkind, 1983).

Medaka fishes are oviparous. 10–20 fertilized eggs are attached in a cluster to the female and can be obtained daily 1–2 h after the onset of light. The cluster of eggs was kept in Ringer solution (0.75 % NaCl, 0.02 % KCl, 0.02 % CaCl₂, pH 7.3, (Yamamoto, 1961) 0.0001 % methylene blue to prevent fungal growth (Rugh, 1962)) in which the eggs follow normal embryogenesis. Embryos of stages 19–27 (Matsui, 1949) were used.

Dissection and fixation of embryos
*Xiphophorus* embryos were fixed in 4 % paraformaldehyde in PBS (pH 7.2) for 2–6 h at room temperature or overnight at 4°C and washed in three changes of PBS during which the yolk was removed. Medaka embryos were fixed for 1 h, freed from the thick chorion and further fixed for another hour; the yolk was removed as described during washing for the *Xiphophorus* embryos.

Immunofluorescent staining

Whole-mount preparations

The fixed embryos were incubated for 2–4 days at 4°C with HNK-1 monoclonal antibody (Becton-Dickinson) diluted 1/25 with PBS–0.5 % bovine serum albumin (BSA). They were then washed for 1 h in PBS–BSA, incubated for 1–2 days in rabbit anti-mouse IgM antibody (RAM), stained for 1–2 days with FITC-conjugated goat anti-rabbit antibody (FITC–GAR), rinsed with PBS and mounted in polyvinyl alcohol-based medium (Lennette, 1978) containing 2 % diazobicyclo-octane (DABCO, Aldrich) as an anti-quenching agent. A total of 25 *Xiphophorus* and 10 medaka were investigated.

Sections

The fixed embryos were dehydrated, embedded in paraplast (Lancer) and serially sectioned (6 μm). Sections were deparaffinized, hydrated, washed with PBS–BSA and incubated overnight in 1/50 dilution of HNK-1 antibody at 4°C. After washing with PBS, sections were incubated for 1 h in RAM, followed by 1 h incubation in FITC–GAR, rinsed with PBS and mounted. Sections were done from 53 *Xiphophorus* and 5 medaka.

In control experiments the HNK-1 antibody was replaced with mouse whole molecule IgM. Preparations were analysed and photographed with a Zeiss epifluorescence photomicroscope.

Results

Using the HNK-1 monoclonal antibody that recognizes neural crest cells in many species including these fish (Sadaghiani and Vielkind, 1989a), we have followed the appearance of neural crest cells in whole mounts as well as cross-sections of *Xiphophorus* embryos at stages 8–17 and medaka embryos stages 19–27. These stages were chosen because our previous studies had shown that the first cells segregate from the neural keel at stage 8 and stage 20 in *Xiphophorus* and medaka, respectively. With progression of embryogenesis, the keel becomes hollow in an anterior–posterior fashion and is then referred to as the neural tube. During the following stages, a heavy segregation of neural crest cells was observed in all regions and seemed to terminate at around stage 17 and 27 in *Xiphophorus* and medaka, respectively (for further details see Sadaghiani and Vielkind 1989a,b).

Localization of HNK-1 positive neural crest derivatives in whole-mount embryos

*Xiphophorus*

In the following, we document the results only for the swordtail since the same results were obtained for the platyfish embryos. Immunofluorescent staining can be seen clearly at first in embryos of stage 9 (6 somites) laterally on both sides of the neural keel (Fig. 1A). Faintly HNK-1 stained neural crest cells are recognizable between the optic vesicles and prosencephalon and in the region extending posteriorly towards the trunk region. In the anterior trunk region, posterior to the presumptive otic vesicles, an accumulation of well-stained neural crest cells can be seen gradually decreasing in number in the trunk towards the level of the 2nd somite. A higher magnification of this trunk area at a

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Fig. 1. Distribution of neural crest cells in whole-mount swordtail (*Xiphophorus helleri*) embryos at stages 9 and 10 illustrated by immunostaining with the monoclonal antibody HNK-1. (A,C,E) Dorsal overviews of HNK-1 staining in embryos of stage 9 (6 somites), late stage 9 (11 somites), and stage 10 (14 somites), respectively. (A) The initial faint staining of neural crest cells that are located laterally on the neural keel appears during embryonic development from stage 9 to 10 as bright staining laterally in the head (C,E); the brightly stained crest cells laterally on the neural keel in the trunk (A) extend posteriorly to the 7th (C) and 9–10th somite (E). (B,D,F) Higher magnifications of insets in the overviews. Neural crest cells are located in the trunk on the neural keel (B) and in the head (D) in the area of the optic vesicle, in the primordia of the trigeminal and facial ganglia (arrowheads) and (F) in addition in the primordium of the vagus–posterior lateral line ganglion (arrowhead). Arrows indicate the site of attachment of the trigeminal and facial ganglia to the rhombencephalon. P, T, V-PLL, primordia of facial, trigeminal, and vagus-posterior lateral line ganglia; M, mesencephalon; OpV, optic vesicle; OtV, otic vesicle; P, prosencephalon; Rh, rhombencephalon; arrows 1–4 in 1A and 1–6 in 1E, areas of sections shown in Fig. 4A(E)–D(H) and Fig. 5A–F, respectively. The anterior region of the embryos is on the left side of photographs. Bar, 50 μm.
different focus level reveals that cells that are presumably part of the dorsal neural keel are also HNK-1 positive (Fig. 1B); cells with the same staining behaviour were also seen in the 3rd somite region (data not shown). We assume that these cells are pre-segregating crest cells; support for this interpretation is derived from cross-sections in this area (see below).

Later in stage 9 (11 somites), prominently immunostained neural crest cells can now be seen also in the head region and, in the trunk, they extend to the level of the 7th somite (Fig. 1C). This strong staining reflects the higher number of cells that have been segregating from the neural tube but also a more intense staining of the individual cells, as can be seen at higher magnification of the head region (Fig. 1D). Many neural crest cells can be found in the space between the optic cup and the prosencephalon. Two densely packed cell masses are particularly obvious. The large cell mass stains brightly and is located laterally to the mesencephalon and anterior rhombencephalon while the small cell mass shows a faint fluorescence and is located in front of the otic vesicle (Fig. 1D). These large and small cell masses represent the primordia of the cranial ganglia, i.e. the trigeminal and the facial ganglia,
respectively. They are presumed to be composed of cells of neural crest and placodal origin (Le Douarin, 1982, 1986).

The pattern of neural crest cell distribution in embryos of stage 10 (14 somites) (Fig. 1E) is very similar to that observed in the stage 9 embryos. However, neural crest cells appear in the trunk to the 9–10th somite and appear to accumulate in the dorsal wedges of the somites. The trigeminal ganglion is extended towards the optic vesicle and appears to be attached to the anterior rhombencephalon; the facial ganglia appears to be attached to the rhombencephalon and the otic vesicle (see arrows in Fig. 1F). Numerous faintly stained neural crest cells appear anterior to and behind the optic vesicle. In the anterior trunk region, posterior to the otic vesicle, a considerable number of positive cells can be seen that are connected to the positive cells of the lateral edges of the neural tube (Fig. 1F). This accumulation of cells represents the primordium of the vagus-posterior lateral line ganglion.

With developmental progression of the embryo from stage 10 to stage 11 (19 somites) (Fig. 2A–D), positive staining in the head area is concentrated in a small group of cells caudally to the eye and in the cell masses already described. Posterior to the otic vesicles (presomitic area) chord-like structures originating from the vagus-posterior lateral line ganglia can be seen that extend to the 6th somite and represent the primordia of the posterior lateral line organs. In the somitic area of the trunk HNK-1, positive neural crest cells appear in a segmented pattern to the level of 15th somite (Fig. 2A).

A higher magnification of the somitic area (Fig. 2B) shows that this pattern is due to cells accumulating in the dorsal wedges between the somites. A few individual cells with several short cell processes can be observed on the somites (Fig. 2B). In the posterior trunk region some cells, residing on the neural tube and also at the edge of the neural tube, bear very long cell processes which run under the ectoderm (Fig. 2B,C). A lateral view of the somitic area (somite 1–5) (Fig. 2D) reveals that the most dorsal cells in the wedges are connected to the neural tube. They seem to have segregated anteriorly to the somite and migrated ventrally but along the medial portion of the somite.

In embryos of stage 12 (22 somites), we observed a network of cell processes under the ectoderm which stained positive (Fig. 2E). The posterior lateral line organ is also stained (data not shown). We did not observe immunofluorescent staining on other structures or in other areas, which is likely due to the fact that the antibody cannot penetrate through the tissues, since in the cross-sections positive staining was observed; the use of membrane permeabilizing agents such as dimethyl sulfoxide (DMSO) did not lead to a different result. Embryos older than stage 12 also showed no positive staining.

**Oryzias latipes**

The HNK-1 staining of the whole medaka embryos revealed a similar pattern of immunoreactivity to that in *Xiphophorus*. Fig. 3A shows a stage 22 (10 somites) embryo in which the positive staining can be seen with cells on the lateral sides of the brain in the head and with the cells on the neural keel in the anterior trunk region (compare with Fig. 1A). In an embryo of stage 24 (20 somites) (Fig. 3B), the positive cells are similarly located in the lateral part of the brain, and in the trunk they have accumulated in the dorsal wedges of somites and more positive cells have appeared laterally to the neural tube, caudally to the level of 15th somite (compare with Fig. 1E). The notable difference that we observed in medaka is that positive cells, which seem to have migrated laterally over the somites at stage 22, have settled at the lateroventral part of the somites (Fig. 3C). By stage 24 they increase in number (Fig. 3D) and are still apparent by stage 27 (26 somites) when HNK-1-negative pigment cells appear in the same location (Fig. 3E).

**Distribution of HNK-1-positive neural crest cells in sections of embryos**

*Xiphophorus*

Cross-sections of embryos of early stage 9 (4 somites) and late stage 9 (8 somites) stained with the antibody are shown in Fig. 4A–D and 4E–H, respectively. For cross-reference, the areas that are shown in the cross-sections are indicated in the whole mounts (Fig. 1A).

In early stage 9 embryos, faintly stained neural crest cells are located between the neural tube and the optic vesicle, as well as ventrally to the optic vesicle (Fig. 4A). In the presumptive meso- and rhombencephalic regions, positive cells are located dorsolaterally over the mesoderm (Fig. 4B). In the anterior trunk region (presomitic region), a single layer of neural crest cells appear dorsally on the neural keel and are connected to a group of strongly stained neural crest cells that appear dorsolaterally of the neural keel (Fig. 4C). In the somitic area, a bright fluorescence is associated with the dorsal part of the neural keel (Fig. 4D). In general,

**Fig. 2.** Distribution of neural crest cells in whole-mount swordtail embryos at stages 11 and 12 illustrated by immunostaining with the monoclonal antibody HNK-1.

(A) A dorsal overview of a stage 11 (19 somites) embryo illustrating a similar distribution of staining to that observed in stage 10. In addition, neural crest cells appear in the primordium of the posterior lateral line organ (arrowheads); the areas of higher magnification shown in Figs. 4–6 are indicated. (B,C) Dorsal views of trunk area in which the positive staining can be seen with cells on the lateral sides of the brain in the head and with the cells on the neural keel in the anterior trunk region (compare with Fig. 1A). In an embryo of stage 24 (20 somites) (Fig. 3B), the positive cells are similarly located in the lateral part of the brain, and in the trunk they have accumulated in the dorsal wedges of somites and more positive cells have appeared laterally to the neural tube, caudally to the level of 15th somite (compare with Fig. 1E). The notable difference that we observed in medaka is that positive cells, which seem to have migrated laterally over the somites at stage 22, have settled at the lateroventral part of the somites (Fig. 3C). By stage 24 they increase in number (Fig. 3D) and are still apparent by stage 27 (26 somites) when HNK-1-negative pigment cells appear in the same location (Fig. 3E).
late stage 9 embryos the positive neural crest cells have increased in number in all areas. In the head region, the cells stain more strongly (Fig. 4E,F) as compared to those in early stage 9 (Fig. 4A,B). In mes- and rhombencephalic regions (Fig. 4F), positive cells are located in a more lateral position between the mesoderm and the ectoderm; in the pre-somatic region (Fig. 4G) they are located over the mesoderm. More posteriorly, in the somitic area (Fig. 4H), staining is associated with a single layer of cells on the neural keel and with the cells between the neural tube and somites. Comparing the somitic region of early and late stage 9 embryos (Fig. 4D,H), it is obvious that positive cells have moved dorsolaterally between the neural tube and the somites (Fig. 4H) from their earlier position on the neural keel (Fig. 4D) where we assume that they were pre-segregating neural crest cells. In the late stage 9 embryo, staining can also be seen laterally on the wall of the prosencephalon and the optic vesicles (Fig. 4E) which will be discussed below.
HNK-1-stained cross-sections of stage 10 embryos are shown in Fig. 5 (for cross-reference of areas see Fig. 1E). A faint staining can be seen on a group of neural crest cells between the brain and the optic vesicles and a strong staining can be seen dorsally on the lens presumably stemming from precipitated vitreous humor (Fig. 5A). In the mesencephalic area, a bright staining can be seen under the ectoderm to be associ-
ated with migrating neural crest cells but presumably also with cells representing the trigeminal placode (Fig. 5B) which showed HNK-1 reactivity in chicken. Medially to this placode, some cells among the mesenchymal cells display a speckled fluorescence (Fig. 5B) and may represent neural-crest-derived mesenchymal cells losing their reactivity. Posterior to the area shown in Fig. 5B, the presumptive trigeminal placode enlarges and connects with the trigeminal nerve protruding from the rhombencephalon (Fig. 5C). A second HNK-1-positive cell mass, representing the facial ganglion, can be observed located caudally to the trigeminal ganglion and anteriorly to the otic vesicle (Fig. 5D). Both cell masses extend lateroventrally into the gill arches which are formed by the ectoderm and the cranial portion of the endoderm (Fig. 5D). In the pre-somitic area, many brightly stained cells can be seen packed in two or three groups that are attached laterally to the neural tube and ventrally make contact with the newly formed intestine (Fig. 5E). These groups of cells take part in the

Fig. 4. For legend see p. 204
formation of the nervous system of the intestine and the posterior lateral line organs. In the somitic area, brightly stained neural crest cells appear dorsally on the neural tube and between the neural tube and the somites (Fig. 5F).

In embryos of stages 11 and 12, a similar pattern of staining can be observed to that described for embryos of stage 10. However, the cell masses representing the primordia of the cranial ganglia decreased in volume and the ventral portion of these cell masses, which could be observed in association with the gill pouches, gradually have lost their reactivity (data not shown). In the trunk region, neural crest cells are located between the neural tube and the somites extending ventrally (Fig. 5G). Stages 13 and 14 are marked by the additional formation of positively labelled cell masses ventrally to the somites representing the sympathetic ganglia (Fig. 5H) and similar cell masses attached to the ventral part of the neural tube representing the primordia of the sympathetic ganglia. These masses of cells have migrated in the trunk region of an embryo at stage 12 (22 somites). Neural crest cells are found: (H) in the anterior trunk region in the primordia of the sympathetic ganglia; (I) in the somitic area in the primordia of the spinal ganglia. Note positive staining in the wall of the intestine (H) and in the wall of the neural tube (H, I). DA, dorsal aorta, En, endoderm, In, intestine; M, mesencephalon; OpV, optic vesicle; N, notochord, NT, neural tube, P, prosencephalon; Rh, rhombencephalon; S, somite. Bar, 50 μm.

**HNK-1 reactivity with other structures**

As observed in chicken (Vincent and Thiery, 1984) the HNK-1 monoclonal antibody exhibited reactivity in these fish not only with neural crest cells but also with other cells and structures such as the otic vesicles (Figs 1F; 2A), the lateral wall of the neural keel/tube (Figs 4E,F; 5H,I), the retina (Fig. 6A), and on the notochord (data not shown). In addition, the HNK-1 showed reactivity with other structures that had not been described before; some reactivity was found on structures that may be of importance to the fate of

**Fig. 3.** Distribution of neural crest cells in whole medaka (Oryzias latipes) embryos at stages 22, 24 and 27 illustrated by immunostaining with the monoclonal antibody HNK-1. (A,B) Dorsal overviews of stages 22 and 24. (A) In a stage 22 (10 somites) embryo, neural crest cells are located in the head laterally and in the trunk dorsally on the neural keel. (B) In a stage 24 (20 somites) embryo, a similar staining is observed except that more cells appear in the trunk and have accumulated in the dorsal wedges of the somites. (The staining under the head is an artifact due to yolk sac rests (arrow) in preparation of the whole mounts.) (C–E) Lateral views of the anterior trunk at higher magnification of stages 22 (10 somites), 24 (20 somites) and 27 (26 somites). Neural crest cells appear: (C) on the neural tube and lateroventrally of the somites (arrows); (D) as a segmented pattern in the wedges of the somites (open arrows) and the cells located lateroventrally have increased in number (arrows); (E) staining appears in the posterior lateral line organ (open arrow) and the lateroventral cells (arrows) are aligned with the melanophores (arrowheads). NT, neural tube. The anterior region of the embryos is on the left side of photographs. Bar, 50 μm.

**Fig. 4.** Distribution of neural crest cells in cross-sections of swordtail embryos at early and late stage 9 illustrated by immunostaining with the monoclonal antibody HNK-1. (A–D) Immunostained cross-sections of a swordtail embryo at early stage 9 (4 somites), the planes are indicated 1–4 in Fig. 1A; (E–H) Cross-sections in equivalent planes of an embryo at late stage 9 (8 somites). (A,B and E,F) In the head faintly stained neural crest cells have migrated (A) between the optic vesicles and the prosencephalon (arrows); (B) in the rhombencephalic region dorsolaterally over the mesoderm (arrows). These cells appear more brightly stained in the late stage 9 embryo (E,F) and additional staining appears on the lateral wall of the optic vesicles and the neural keel. (C,G) In the anterior trunk region (C) neural crest cells appear in early stage 9 in a thin layer dorsally in the neural keel and some segregated dorsolaterally from it; (G) in the late stage 9 more cells segregated dorsolaterally from the neural tube. (D,H) In the somitic region neural crest cells appear (D) in the early stage 9 in layers in the dorsal part of the neural keel and (H) as a thin layer dorsally and in masses dorsolaterally on the neural tube in the late stage 9 embryo. Ms, mesoderm; OpV, optic vesicle; NK, neural keel; NT, neural tube, P, prosencephalon; Rh, rhombencephalon; S, somite. Bar, 50 μm.
Fig. 6. HNK-1 immunoreactivity in structures of different or possible neural crest origin in cross-sections of swordtail embryos of various stages. (A) Optic region of an embryo at stage 10 (14 somites): bright staining can be seen on the cornea and lens epithelial cells (arrow) and in the vitreous humor (arrowhead). (B) Posterior trunk region of an embryo at stage 11 (22 somites): staining can be seen associated with fibrils in the extracellular spaces around the notochord and under the ectoderm as well as around the mesonephric duct (small arrow) and the intestine. (C) Cardiac region of an embryo at late stage 9 (8 somites): staining can be seen on the endoderm (arrows) associated with the heart mesoderm. (D) Posterior trunk region of the same embryo as in C: staining can be seen on the endoderm (arrows) as well as around the notochord. (E) The third gill area of an embryo at stage 17–18: heavy staining can be seen on the pharyngeal teeth (arrows) epithelium. (F) Abdominal region of an embryo at stage 17–18: a heavy staining can be seen on the mesentery of the swim bladder. En, endoderm; In, intestine; MD, mesonephric duct; N, notochord; NCC, neural crest cells; NT, neural tube; Ph, pharynx; S, somite. Bar, 50 μm.

neural crest cells. For example, in the posterior portion of the trunk in embryos of stages 9–12 staining can be observed to be associated with abundant fibrils in the extracellular spaces between the somites and neural tube as well as under the ectoderm and also with presumably the basement membrane of the mesonephric duct and the intestine (Fig. 6B); interestingly the staining of these structures was observed prior to the segregation of the crest cells from the neural keel/tube and thus the stainable region decreases in an anterior–posterior fashion in parallel with the anterior–posterior gradient of segregation. At late stage 9, staining can be seen in the endoderm that is associated with the heart mesoderm located ventrally of the pharynx (Fig. 6C) and also on the lateroventral endoderm in the posterior trunk region (Fig. 6D). HNK-1 reactivity can be seen
on various structures in early development of the eye such as on the lateral wall of the optic vesicles where the lens placode will form (Fig. 4E), on the epithelial cells of the lens (Fig. 6A), on the cornea (Fig. 6A), and on the vitreous humor, which usually collapses during fixation (Figs 5A; 6A). In embryos of stage 17–18 during formation of the pharyngeal teeth, a positive staining can be seen on the teeth epithelial cells (Fig. 6E) and also on the mesentery of the swim bladder (Fig. 6F).

**Discussion**

Using the ability of the HNK-1 monoclonal antibody to recognize neural crest cells in a variety of vertebrates including *Xiphophorus* fish (Sadaghiani and Vielkind, 1989a), we have studied the distribution and the migratory pathways of neural crest cells in embryos of *Xiphophorus maculatus* (platyfish), *X. helleri* (swordtail) and *Oryzias latipes* (medaka). Whole-mount preparations were used to obtain a quick overview of the appearance and location of the crest cells while the cross-sections were necessary for greater detail. In general, we did not find differences in the appearance and distribution of neural crest cells in these three fish species, perhaps reflecting the close similarity of embryogenesis. The only exception was the occurrence of positive cells under the ectoderm in the medaka (see below). Minor differences were observed in the strength of the staining between the platyfish/swordtail and the medaka; the *Xiphophorus* fish stained strongly, similar to the staining in chicken. In addition, for as yet unknown reasons the cross-sections of the medaka did not stain. Since we observed similar staining in whole-mount medaka and *Xiphophorus* fish, and since we did not observe differences in neural crest development between these fish in previous scanning/light microscopic studies, we assume that the details of the migratory pathways in medaka are very similar, if not identical, to those in the platyfish/swordtails.

HNK-1 staining of neural crest cells followed an anterior–posterior gradient reflecting the anterior–posterior gradient of the appearance of migrating cells. This is similar to the observations made in chick and rat. However, when the earliest staining occurred in the head in these fish, strong staining was simultaneously observed in the anterior trunk region. This staining stemmed from cells which still resided in the dorsal part of the neural keel; such an observation is in striking contrast to those reported in other vertebrates where the migration of neural crest cells has been studied with the HNK-1 antibody. Thus, the trunk cells express the HNK-1 epitope earlier than those in the head. Therefore, it seems that expression of the epitope is not a trigger for segregation and early migration of neural crest cells but may be necessary in conjunction with other signals (see below). This would explain that although in previous studies (Sadaghiani and Vielkind, 1989a,b) we found that the neural crest cells in the platyfish segregate earlier than in the swordtail, a time difference in the staining of neural crest cells in platyfish and swordtails was not evident. With progression of migration all non-neural derivatives of the neural crest lose their reactivity with the HNK-1 antibody as reported for chicken (Vincent and Thiery, 1984). Therefore, in older embryos staining is retained only in the brain and in peripheral nerves.

Migration is observed first in the optic region where dorsolaterally segregated cells at the border of pro- and mesencephalon (Sadaghiani and Vielkind, 1989a) migrate ventrally. Once they encounter the optic stalk the cells subdivide in two streams. One stream migrates further ventrally along the neural keel, the other moves rostrally over the optic stalk. The two streams of neural crest cells rejoin ventral to the optic vesicle and fill the empty space in this area. The cells in the mes- and rhombencephalic regions move lateroventrally over mesenchymal cells. The few positive cells that we observed between the mesenchymal cells, may represent neural crest cells that participate in the formation of the cephalic skeleton. This lateroventral migratory pathway ends in the pharyngeal gills. These cephalic pathways of neural crest cell migration seem to be similar to those observed in *Xenopus* (Sadaghiani and Thiebaud, 1987) and in chicken (Johnston, 1966; Noden, 1975; Duband and Thiery, 1982; Tosney, 1982).

At the junction of the head and trunk (anterior trunk region), a similar lateroventral migration, which stops in the intestinal wall can be seen.

As indicated above, the truncal neural crest cells retain their reactivity with the HNK-1 antibody after segregation and thus migration could also be followed. In whole mounts, the segregated neural crest cells seem to accumulate in the dorsal wedges of somites as was also observed in chicken (Loring and Erickson, 1987) and rat (Erickson et al. 1989) before they migrate ventrally between the neural tube and the somites as seen in cross-sections. Such a migratory pathway has been reported for carp (Lamers et al. 1981) and amphibians (Loebberg and Ahlfors, 1978; Loebberg et al. 1980; Sadaghiani and Thiebaud, 1987; Krotoski et al. 1988). In chicken and rat (Erickson et al. 1989), only a few cells are observed in this pathway; some cells appear in the intersomitic space but the majority of cells migrates in the anterior half of each somite on the basal surface of dermomyotome (Rickmann et al. 1985; Erickson et al. 1989). This migratory pathway of neural crest cells has been correlated to the distribution of acetyl- and butyrylcholinesterase (Layer et al. 1988) within the anterior halves of somites. Although the neural crest cells in these fish do not follow this pathway, they segregate and initiate their migration between the neural tube and the anterior half of somites. Whether or not similar molecules are located in the anterior somites of these fish is unknown.

In previous studies, we provided evidence that some neural crest cells, presumed to represent premelanoctyes, migrate under the ectoderm (Sadaghiani and Vielkind, 1989a,b). In agreement with other studies (Vincent and Thiery, 1984; and our observations of cultured neural crest cells (Sadaghiani and Vielkind,
1990) that premelanocytes lack HNK-1 immunoreactivity, we did not find HNK-1-positive cells under the ectoderm except those that presumably represent cells of the posterior lateral line rather than migrating neural crest cells (Sadaghiani and Vielkind, 1989a). Individual, positive cells on the apaxes of somites observed in whole-mount preparations may represent migrating, presumptive premelanocytes. Owing to the fact that premelanocytes and melanocytes are believed to lack the HNK-1 epitope, we do not believe that the positive cells observed only in medaka embryos aligned with the lateral stripe of melanocytes are precursors pigment cells but rather cells of unknown identity.

In whole-mount preparations of *Xiphophorus* positive cells were observed in the dorsal part of the neural tube bearing long, stained processes that run between the ectoderm and the somites. These cells resemble the neural-crust-derived Rohon–Beard and extramural cells, the primary sensory system in amphibians (Hoerstadius, 1950) believed to innervate the skin (Taylor and Roberts, 1983). Such a system has not been reported previously in fishes. The fact that we did not observe such cells in the medaka may be due to the lesser staining in this species as compared to that in *Xiphophorus*.

We also observed other tissues and structures that are not of neural crest cell origin but showed a positive staining with the HNK-1 antibody; similar observations were reported in chicken (Vincent and Thiery, 1984). This is not surprising because this antibody recognizes a carbohydrate epitope of several cell surface glycoproteins which belong to a family of neural cell adhesion molecules including, for example, the neural cell adhesion molecules (N-CAMs) (Cole and Schachner, 1987), the myelin-associated glycoprotein (MAG) (Kruse et al., 1984), the integrins, receptors for fibronectin and laminin (Pesheva et al., 1987), etc. Because all members of this family are involved in cell–cell and cell–substrate interaction, it has been suggested that the epitope recognized by HNK-1 antibody has a functionally important role in early developmental processes (Canning and Stern, 1988). The HNK-1 staining that we observed on the ECM fibrils prior to segregation and its diminishment once the cells have segregated may indicate a change in the state of the ECM that may allow or induce segregation and subsequent migration. A maturation of the ECM with regard to segregation/migration has been suggested by Loefberg et al. (1985). However, it is also conceivable that the expression of the HNK-1 epitope on the neural crest cells may exert an influence on the surrounding ECM or cells among which the neural crest cells migrate or may be the consequence of inductive outside influences. Such an interplay of the appearance of HNK-1 has recently been described as a result of hypoblast–epiblast induction in the early chick embryo (Canning and Stern, 1988).

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