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Migration and Colonization Patterns of HNK-1-Immunoreactive Neural Crest Cells in Lamprey and Swordtail Embryos

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ABSTRACT—Migration and colonization patterns of neural crest cells were analyzed histochemically in embryos of the brook lamprey, *Lampetra reissneri*, and the swordtail, *Xiphophorus helleri*, using HNK-1 monoclonal antibody, which recognizes migratory neural crest cells and crest derivatives in many groups of vertebrates including teleosts. We demonstrated that HNK-1 recognizes a subpopulation of naural crest cells and crest derivatives in the lamprey as well as in the swordtail. In the trunk of lamprey and swordtail embryos, HNK-1-positive cells were observed in the major migratory pathways of neural crest cells, that is ventral and dorso-lateral pathways. In the swordtail embryos, neural crest cells in the ventral pathway were ubiquitously observed along the rostro-caudal axis at the neural tube level, but at the notochord level their migration was restricted to the middle region of the somite. In the lamprey, by contrast, no HNK-1-immunoreactive neural crest cells migrated ventrally beyond the notochord level in the ventral pathway at axial levels behind the gill pouch region. This migration pattern of neural crest cells in the lamprey trunk might be closely related to the absence of anatomically distinct, sympathetic chains in the lamprey, which is one of characteristic features of the lamprey's body organization. Furthermore, we found dorsal pathways, which extended to the embryonic dorsal fin, of neural crest cells in both swordtail and lamprey embryos. In the lamprey, dorsal cells (Rohon-Beard cells) and cells that resemble chromaffin cells immunoreacted with HNK-1.

INTRODUCTION

The neural crest is a unique and transient structure within the vertebrate embryo. Neural crest cells emerge from the dorsal region of the neural tube. Then, they migrate and give rise to a variety of cells, such as pigment cells, the cells of the peripheral nervous system, endocrine cells and ecto-mesenchymal cells (Le Douarin, 1982; Hall and Hörstadius, 1988). Migratory pathways of neural crest cells differ among vertebrates, in particular between anamniotes (Lamers *et al.*, 1981; Krotoski *et al.*, 1988; Sadaghiani and Vielkind, 1989) and amniotes (Bronner-Fraser, 1986; Erickson *et al.*, 1989; Serbedzija *et al.*, 1990; Hou and Takeuchi, 1994; Kubota *et al.*, 1996). The differences in migratory pathways may be related to the variations in spatial arrangements of crest derivatives and, as a consequence, for the difference of pattern formation among various vertebrates (Duncker, 1985; Parichy, 1996). Thus, it is possible that a change in the migratory pathway of neural crest cells might have played a role in the phylogenetic difference of the body organization of vertebrates. The migratory routes of neural crest cells in avian and mammalian embryos have been extensively examined, but relatively little is known about pathways of such migration in anamniote embryos, especially in cyclostome embryos. The developmental capacities of neural crest cells have been analyzed in some studies of lamprey embryos (Newth, 1956; Langille and Hall, 1988), but the migration of these cells remains to be characterized. In the present study, we attempted to analyze the migration and colonization patterns of lamprey neural crest cells and to compare these patterns with those of neural crest cells in the swordtail; the teleost fish in which the migration pattern of neural crest cells has been described in detail (Sadaghiani and Vielkind, 1989, 1990; Sadaghiani *et al.*, 1994). We used an immunohistochemical method with HNK-1 monoclonal antibody (Vincent *et al.*, 1983; Vincent and Thiery, 1984). HNK-1 identifies the carbohydrate moiety on the surface of various cell types including neural crest cells and their derivatives, and on extracellular matrix molecules (Kruse *et al.*, 1984; Hoffman and Edelman, 1987). This antibody has been extensively used for the detection of migratory neural crest cells and the cells derived from the neural crest in embryos not only in amniotes (Vincent *et al.*, 1983; Vincent and Thiery, 1984; Bronner-Fraser, 1986; Erickson *et al.*, 1989; Hou and Takeuchi, 1994) but also in teleosts (Sadaghiani and Vielkind, 1989, 1990; Laudel and Lim, 1993; Sadaghiani *et al.*, 1994).

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MATERIALS AND METHODS

Experimental animals

The embryos of the brook lamprey, *Lampetra reissneri*, and the swordtail, *Xiphophorus helleri*, were used. Sexually mature lampreys were kept in tanks of water at 20°C and embryos were obtained after natural spawning or artificial fertilization. The embryos were staged according to the criteria of Tahara (1988) and they were used at stages 20-30. Swordtails were kept in an aquarium at 25°C and pregnant females were sacrificed at appropriate intervals. Their embryos were used at stages 8-23 (Tavolga, 1949).

Immunohistochemical staining

Embryos were fixed overnight in a 4% solution of paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4°C and then they were washed several times with PBS. The fixed embryos were dehydrated in an ethanol-toluene series, embedded in Paraplast (Oxford Labware, St. Louis, MO) and serially sectioned at 10 μm. Sections were attached to albumin-coated glass slides. They were treated with 1% bovine serum albumin in PBS (BSA/PBS) for 1 hr to block nonspecific binding of antibodies and incubated overnight at 4°C in a solution of HNK-1 monoclonal antibodies (Becton Dickinson, San Jose, CA, Lot 40557) that had been diluted 1/40-fold with BSA/PBS. After washing with PBS, the sections were incubated in FITC-conjugated goat anti-mouse immunoglobulins (IgG, IgA, IgM) (FITC-GAM; Cappel, Durham, NC) for 1 hr at room temperature, rinsed with PBS and mounted in 50% glycerol with 0.1% p-phenylenediamine. In several occasions, the sections were counterstained with Evans blue. When whole-mount immunostaining was performed, fixed embryos were treated with BSA/PBS for 1 day and subsequently incubated with HNK-1 antibodies for 3 days. After extensive washing with PBS, the embryos were incubated in FITC-GAM for 1 day. These samples were observed with a laser confocal microscopic system (Bio-Rad, MRC-500). To observe the distribution of melanophores in lamprey embryos, they were fixed in 4% paraformaldehyde, rinsed in a sucrose solution, and then immersed in increasingly concentrated solutions of glycerol.

RESULTS

Distribution of HNK-1-immunoreactive cells in swordtail embryos

HNK-1-positive cells were first detected in the presumptive cranial ganglia of embryos at stage 12 (Fig. 1A, Table 1). At early migratory phases of cephalic neural crest cells, we did

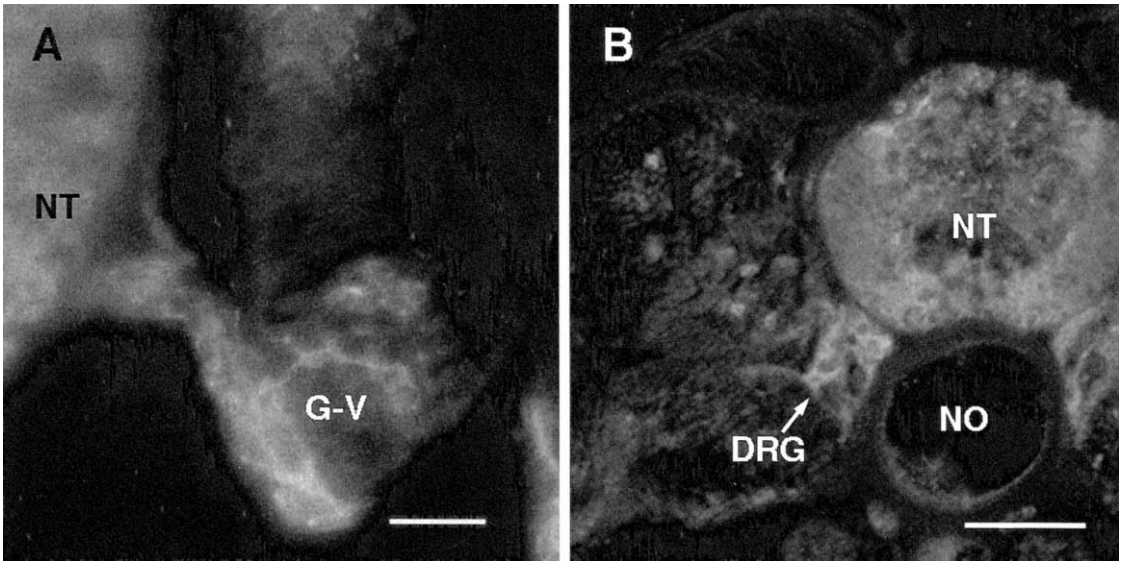

Fig. 1. Distribution of HNK-1-immunoreactive cells in transverse sections of swordtail embryos at stage 16. (**A**) HNK-1-positive cranial (glossopharyngeal-vagus) ganglion (G-V). (**B**) HNK-1-positive dorsal root ganglion (DRG) at the 9th somitic level. Immunostaining with HNK-1 of swordtail neural tubes became detectable at stage 14. NO, Notochord; NT, neural tube. Scale bars: 50 μm.

Table 1. Appearance of HNK-1-immunoreactive components and melanophores during development of swordtail embryos

	Embryonic stage[¶¶]								
Derivative	11	12	13	14	15	16	17	18	19
Melanophore	–	–	–	–	+	+	+	+	+
Cranial ganglion[¶]	–	+	+	+	+	+	+	+	+
Dorsal recurrent nerve	–	–	–	–	+	+	+	+	+
Dorsal root ganglion	–	–	+	+	+	+	+	+	+
Sympathetic chain	–	–	–	–	+	+	+	+	+
Subepidermal nerve network	–	–	–	–	–	–	–	–	–

+, Positive; –, negative.
[¶] Cranial ganglion represents ganglia of the cranial nerves V + VII, VIII and IX + X.
[¶¶] According to Tavolga (1949).

not find HNK-1 positive cells. In the anterior trunk of embryos at stages 12-14, HNK-1-immunoreactive cells were observed at the medial (Fig. 2A-D) and dorso-lateral (Fig. 2D) sides of somites. The pattern of distribution of these cells was unusual at the medial side. Whereas these cells were ubiquitously observed along the rostro-caudal axis at the neural tube level (Fig. 2A), the distribution was restricted to the middle region of somites at the notochord level (Fig. 2B). HNK-1-imunoreactive cells also colonized the presumptive adrenal region (Fig. 2E) and the region surrounding the dorsal aorta where the sympathetic chains are formed. In the posterior trunk of embryos at stages 14-17, HNK-1-immunoreactive cells accumulated at the dorsal side of the neural tube (Fig. 3A, B). Some of these cells appeared in the dorsal finfold. HNK-1-immunoreactive cells in the finfold disappeared at stage 19. Typical HNK-1-positive dorsal root ganglia (Fig. 1B) and sympathetic chains appeared at stages 13 and 15, respectively (Table 1). The dorsal root ganglia were observed beside the middle region of the somite at the boundary between the neural tube and the notochord. Melanophores appeared at stage 15 (Table 1) and most of them were found in the space between the neural tube and the notochord, below the epidermis, and beneath the coelomic epithelium. The dorsal reccurrent nerve, which is the posterior lateral line nerve, and the lateral line organs were HNK-1-immunoreactive, while no immunoreactive fiber net was detected in the skin (Table 1).

Distribution of HNK-1-immunoreactive cells in lamprey embryos

In lamprey embryos, HNK-1-positive cells became detectable at stage 23 (Table 2). At stage 22, Tahara (1988) observed mesectodermal cells in the head region. In this study, no HNK-1-immunoreactive cells were found in the cranial region at this stage. In the trunk of embryos at stages 24-25, cells immunostained with HNK-1 were seen in the region between the neural tube or the epidermis and the somite, and just under the epidermis of the embryonic dorsal fin (Fig. 4A, B). In the trunk posterior to the gill pouch region, no HNK-1-immunoreactive cells were observed in the region beside the notochord (Fig. 4B). After stage 23, HNK-1-immunoreactive cells were localized in some embryonic organs such as presumptive cranial ganglia (Fig. 5A), the heart primordium, and the pronephros or near blood vessels at axial levels from the caudal gill pouch to the heart primordium (Table 2). HNK-1-positive cells around the heart region were visible as single cell or as aggregates of several cells (Fig. 5B). At stage 23,

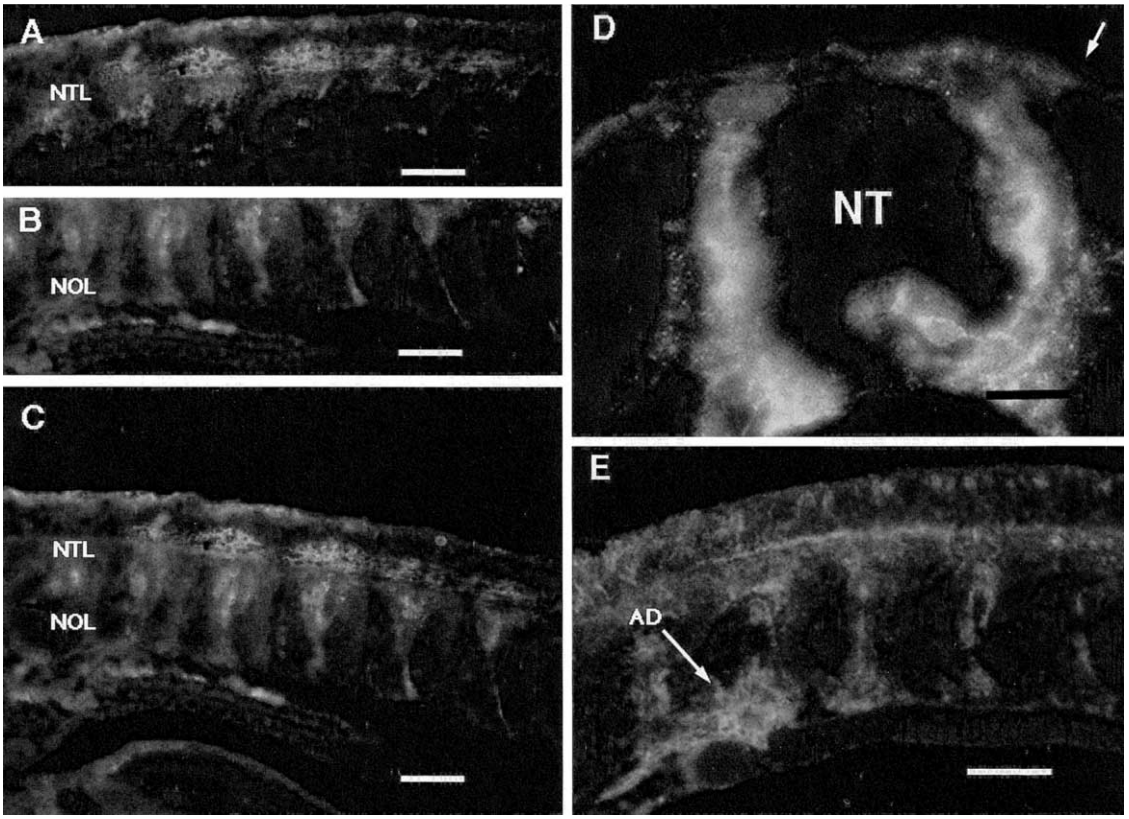

Fig. 2. HNK-1-immunoreactive cells in sections of swordtail embryos. (**A**) Sagittal section of an embryo at stage 12 at the neural tube level (NTL). (**B**) HNK-1-positive cells at the notochord level (NOL) in the section adjacent to (**A**). (**C**) Composite picture of (**A**) and (**B**). (**D**) Transverse section of an embryo at stage 14 at the 11th somitic level. HNK-1-immunoreactive cells are distributed in the dorso-lateral (arrow) and medial portion of the somite. (**E**) Sagittal section of an embryo at stage 12. HNK-1-positive cells have colonized the adrenal region (AD). NT, Neural tube. Scale bars: (**A**, **B**, **C**, **E**) 50 μm; (**D**) 20 μm.

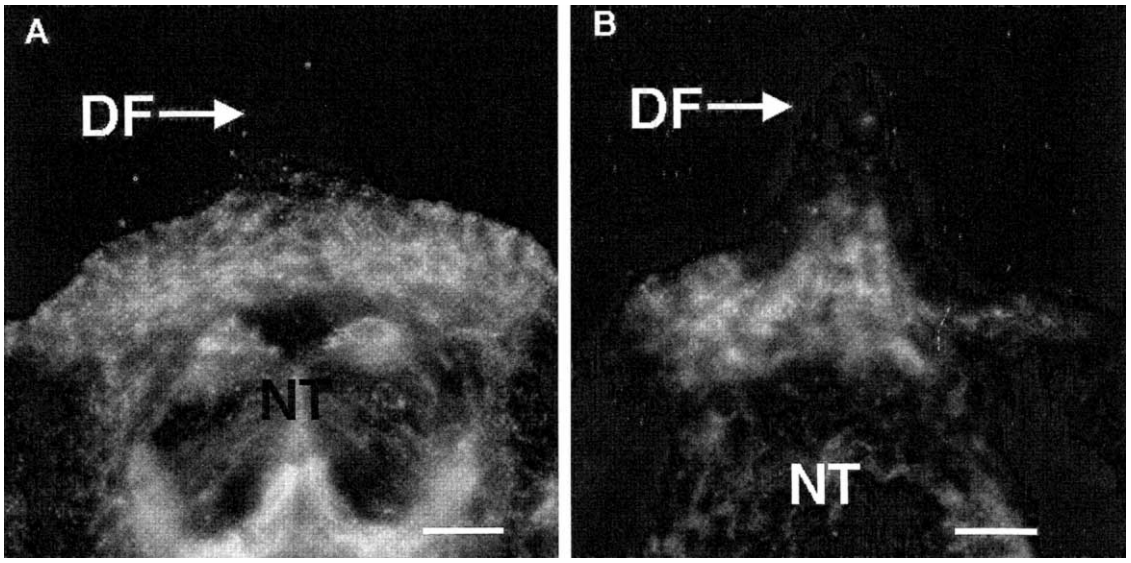

Fig. 3. HNK-1-immunoreactive cells in transverse sections at the 24th somitic level of swordtail embryos at stage 14 (**A**) and stage 15 (**B**). DF, Dorsal finfold; NT, neural tube. Scale bars: 20 μm.

Table 2. Appearance of HNK-1-immunoreactive components and melanophores during development of lamprey embryos

	Embryonic stage¶¶						
Derivative	22	23	24	25	26	27	28
Melanophore	–	–	–	+	+	+	+
Cranial ganglion¶	–	+	+	+	+	+	+
Dorsal recurrent nerve	–	–	+	+	+	+	+
Dorsal root ganglion	–	–	–	+	+	+	+
Dorsal cell (Rohon-Beard cell)	–	+	+	+	+	+	+
Chromaffin cell	–	+	+	+	+	+	+
Subepidermal nerve network	–	–	–	–	+	+	+

+, Positive; –, negative.
¶ Cranial ganglion represents ganglia of the cranial nerves V+VII, VIII and IX+X.
¶¶ According to Tahara (1988).

"dorsal cells" in the neural tube became HNK-1-immunoreactive (Fig. 5C). An immunoreactive fiber was occasionaly traced from a dorsal cell to the dorsal root of the spinal nerve. HNK-1-positive dorsal root ganglia were observed in the intersomitic space after stage 25 (Fig. 5D). The size of dorsal root ganglia of the lamprey was much smaller than that of the swordtail (Fig. 1B). Melanophores appeared at stage 25 (Table 2). Most of them were found beneath the coelomic epithelium, between the neural tube and somites, and just under the epidermis (Fig. 5E). Under the dorsal epidermis, melanophores were localized on the boundaries between somites. We observed melanophores at the ventral side of the notochord in transverse sections of larva after stage 28. At axial levels behind the gill pouch region, however, no melanophores were seen in the space between the notochord and somites (Fig. 5E). In the skin, an HNK-1-positive fiber net appeared at stage 26, as observed in whole-mount preparations (Fig. 5F). Transverse sections revealed that the fibers were located subepidermally (Fig. 4B).

DISCUSSION

HNK-1-immunoreactive migratory cells were observed near to the medial and dorso-lateral portions of somites at the neural tube level in both lamprey and swordtail embryos. These embryonic regions correspond to major migratory pathways of neural crest cells, namely the ventral and dorso-lateral pathways, not only in amniotes and amphibians (Le Douarin, 1982; Hall and Hörstadius, 1988) but also in teleosts including the swordtail (Sadaghiani and Vielkind, 1989, 1990; Sadaghiani *et al.*, 1994). Therefore, it is highly probable that HNK-1 recognizes migratory neural crest cells in the trunk of lampreys. This antibody also recognized their possible crest derivatives such as cranial ganglia and dorsal root ganglia. We did not find early migratory neural crest cells that immunoreacted with HNK-1 in the cephalic region in lamprey

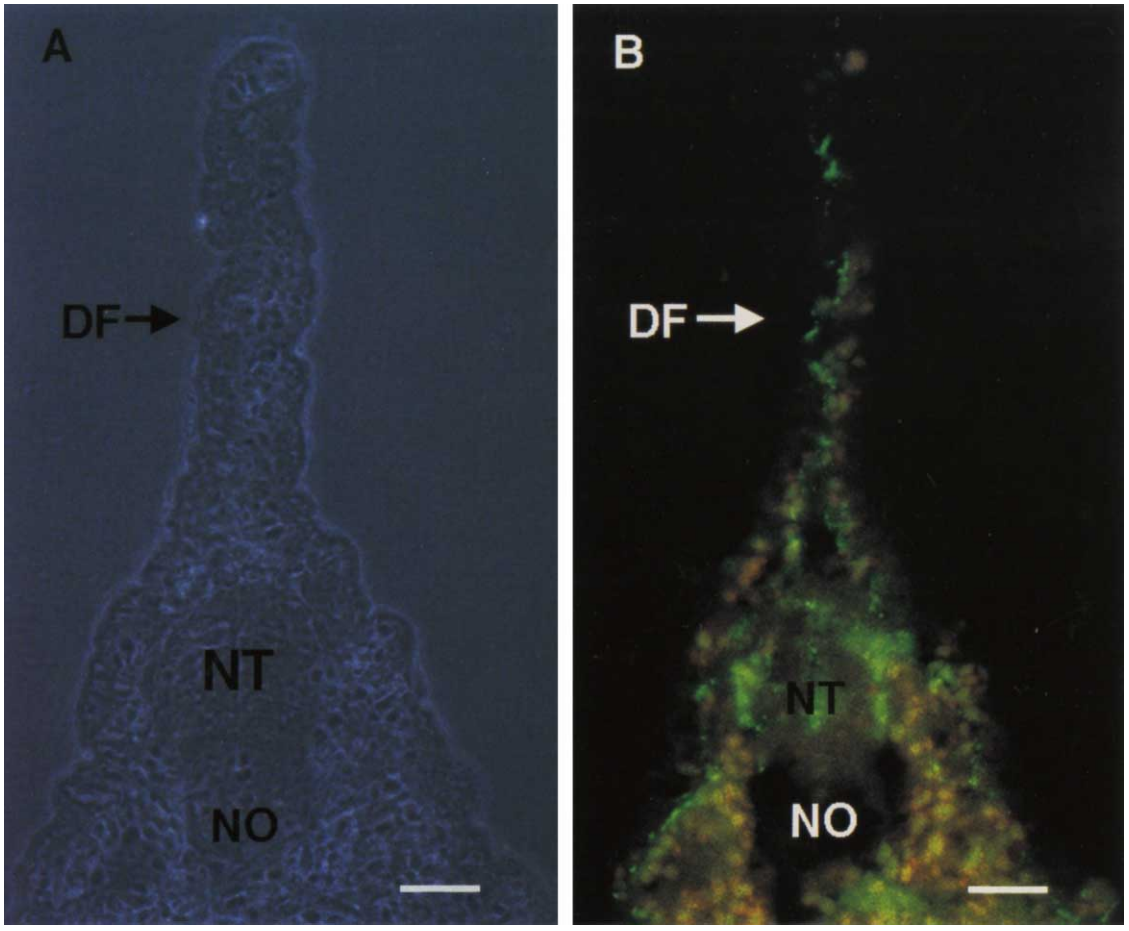

Fig. 4. HNK-1-immunoreactive cells in a transverse section at the trunk level of a lamprey embryo at stage 24. (**A**) Phase-contrast micrograph. (**B**) Fluorescent micrograph. The weak orange fluorescence in (**B**) is due to the counterstaining by Evans blue. DF, Dorsal fin; NO, notochord; NT, neural tube. Scale bars: 20 μm.

and swordtail embryos even though crest-derived cranial ganglia were immunoreactive. In zebrafish embryos, the cell type restriction occurs early during the neural crest development (Raible and Eisen, 1994; Schilling and Kimmel, 1994). It is also well known that the varieties of crest-derived cell types are different between the head and the trunk (Le Douarin, 1982; Hall and Hörstadius, 1988). These observations are not incompatible with the results that the cranial neural crest in lamprey and swordtail embryos does not contain HNK-1-positive cells in contrast to the trunk neural crest in the premigratory and/or early migratory stages.

In addition to the ventral and dorso-lateral pathways, HNK-1-positive cells were found in the embryonic dorsal fins of both the lamprey and swordtail. Krotoski *et al.* (1988) revealed the migratory pathways of neural crest cells into the dorsal fin, namely, the "dorsal pathways" in *Xenopus* embryos. It is possible that such dorsal pathways are also present in swordtail and lamprey embryos. These HNK-1-positive cells may differentiate into mesenchymal cells in their dorsal fins.

The migration of trunk neural crest cells in the ventral pathways differed between the swordtail and the lamprey. In the swordtail, HNK-1-positive neural crest cells were ubiquitously observed along the rostro-caudal axis at the neural tube level, and were found in the middle region between adjacent somitic boundaries at the notochord level. This migration pattern of trunk crest cells was similar to that observed in zebrafish embryos (Raible *et al.*, 1992). In the lamprey, however, no HNK-1-immunoreactive cells that migrated ventrally beyond the notochord were observed at axial levels posterior to the gill pouch region. Melanophores originate from neural crest cells even in lampreys (Newth, 1956). No melanophores were found in the region between the notochord and the somite at axial levels posterior to the gill, although melanophores colonized the ventral side of the notochord. Sadaghiani and Thiébaud (1987) suggested that some neural crest cells in *Xenopus* embryos deviate from the dorso-lateral pathway and locate in the position where the sympathetic nervous system is formed. It is conceivable that melanophores observed at the ventral side of the notochord of lampreys might be derived from neural crest cells migrated in the dorso-lateral pathway and no crest cells in the ventral pathway might move ventrally beyond the notochord. Alternatively, their melanophores might be derived from neural crest cells which moved in the ventral pathway and lost the

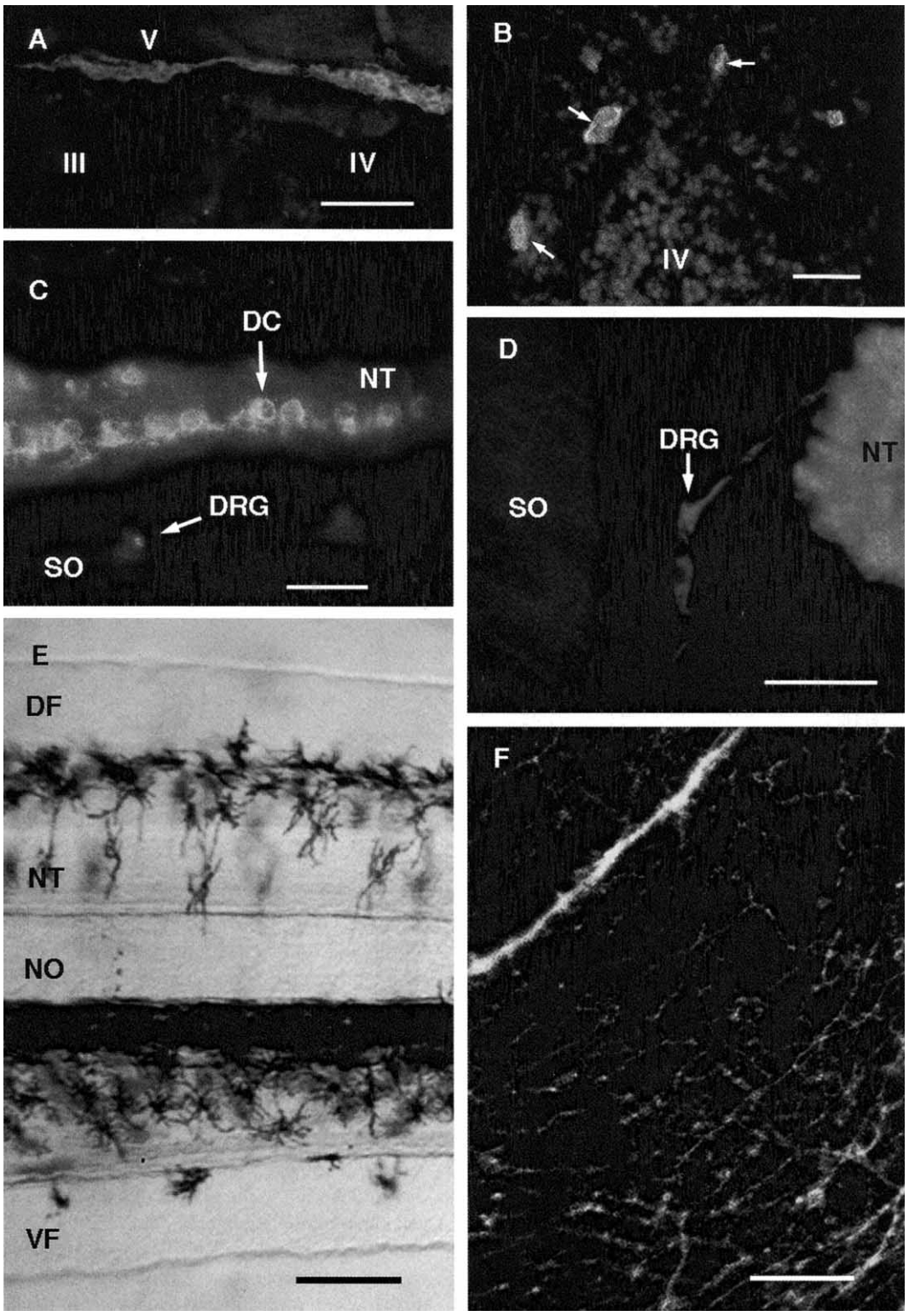

Fig. 5. Distribution of HNK-1-immunoreactive cells and melanophores in lamprey embryos and ammocoetes. (**A**) HNK-1-positive cranial (vagus) ganglion (V) of an embryo at stage 27. III, Third branchial arch region; IV, fourth branchial arch region. (**B**) HNK-1-immunoreactive cells that resemble chromaffin cells (arrows) in the fourth (IV) gill pouch region of an embryo at stage 24. (**C**) HNK-1-immunoreactive dorsal cells (DC) of an embryo at stage 27. (**D**) HNK-1-immunoreactive dorsal root ganglion (DRG) of an ammocoete at stage 30. In the lamprey, the neural tube started to be immunostained with HNK-1 at stage 28. (**E**) Melanophores in the trunk of a larva at stage 30. Rostral is to the left. (**F**) The HNK-1-positive subepidermal nerve network in the caudal fin of an ammocoete at stage 30. DF, Dorsal fin; DRG, dorsal root ganglion; NO, notochord; NT, neural tube; SO, somite; VF, ventral fin. Scale bars: (**A**, **B**, **C**, **D**, **F**) 50 μm; (**E**) 100 μm.

HNK-1 immunoreactivity at the boundary between the neural tube and the notochord. It is possible that lamprey neural crest cells do not develop into typical sympathetic nerve cells for the deficiency of the developmental potentials and/or the environmental cues. This possible feature in neural crest development of the lamprey might be closely related to the absence of typical sympathetic ganglia (Healey, 1972). Evolutionary changes in migration patterns and/or developmental capacities of neural crest cells are likely to be responsible for, or at least related to, the significant difference in pattern formation and body organization between the cyclostome and the teleost, for example, the absence or the formation of typical sympathetic chains.

In lamprey embryos, HNK-1-positive cells were scattered in the heart primordium, the pronephros and around blood vessels at axial levels from the caudal gill pouch to the heart primordium. It has been determined anatomically and histochemically that cells containing catecholamines (chromaffin cells) are scattered in the heart, kidney, intestinal wall and in the vicinity of blood vessels in larval (ammocoetes) and adult lampreys (Healey, 1972). Thus, these HNK-1-immunoreactive cells might have been precursors to chromaffin cells. It has been revealed in chick embryos that neural crest cells migrating to the heart and neighboring organs originate from the level of the vagus nerve (Le Douarin, 1982; Kirby, 1987). It is conceivable that chromaffin cells in lamprey embryos might originate from neural crest cells formed at particular axial levels from the gill pouch region to the heart primordium.

We found HNK-1-positive cells within the neural tube in the trunk of lamprey embryos. These cells can be regarded as "dorsal cells" and/or their precursors, as judged from the morphology of the cells and their location within the neural tube (Healey, 1972). It has been claimed that dorsal cells in lamprey embryos are morphologically and functionally homologous to intramedullary sensory neurons called Rohon-Beard cells in gnathostome embryos (Hardisty, 1982) and, moreover, that amphibian Rohon-Beard cells are derived from the neural crest (Du Shane, 1938; Le Douarin, 1982). Therefore, it is probable that the HNK-1-immunoreactive dorsal cells in lamprey embryos might originate from the neural crest. The relationship of these cells to the HNK-1-positive subepidermal nerve net and to lateral line organs remains to be clarified.

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