RESEARCH ARTICLE

Migratory Patterns and Developmental Potential of Trunk Neural Crest Cells in the Axolotl Embryo

Hans-Henning Epperlein,[1]* Mark A.J. Selleck,[2] Daniel Meulemans,[3] Levan Mchedlishvili,[4] Robert Cerny,[5] Lidia Sobkow,[4] and Marianne Bronner-Fraser[3]

Using cell markers and grafting, we examined the timing of migration and developmental potential of trunk neural crest cells in axolotl. No obvious differences in pathway choice were noted for DiI-labeling at different lateral or medial positions of the trunk neural folds in neurulae, which contributed not only to neural crest but also to Rohon-Beard neurons. Labeling wild-type dorsal trunks at pre- and early-migratory stages revealed that individual neural crest cells migrate away from the neural tube along two main routes: first, dorsolaterally between the epidermis and somites and, later, ventromedially between the somites and neural tube/notochord. Dorsolaterally migrating crest primarily forms pigment cells, with those from anterior (but not mid or posterior) trunk neural folds also contributing glia and neurons to the lateral line. White mutants have impaired dorsolateral but normal ventromedial migration. At late migratory stages, most labeled cells move along the ventromedial pathway or into the dorsal fin. Contrasting with other anamniotes, axolotl has a minor neural crest contribution to the dorsal fin, most of which arises from the dermomyotome. Taken together, the results reveal stereotypic migration and differentiation of neural crest cells in axolotl that differ from other vertebrates in timing of entry onto the dorsolateral pathway and extent of contribution to some derivatives. *Developmental Dynamics 236:389–403, 2007.*
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INTRODUCTION

The neural crest is a transient migratory population of cells found in all vertebrate embryos (Knecht and Bronner-Fraser, 2002). These cells migrate extensively along defined pathways and give rise to diverse derivatives, including neurons and glial cells of the peripheral nervous system, pigment and adrenomedullary cells, the craniofacial skeleton and, in lower vertebrates, mesenchyme of the dorsal fin (Hall and Hörstadius, 1988; Le Douarin and Kalcheim, 1999). Neural crest cells arise in a prospective neural fold area of the ectoderm at the border between prospective neural plate and epidermis (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995). Because the neural folds are contiguous with neural plate and epidermis (Moury and Jacobson (1990), it is not clear where they "end" and neural tissue or epidermis begin.

[1]Department of Anatomy, TU Dresden, Dresden, Germany,
[2]Department of Cell and Neurobiology, University of Southern California Keck School of Medicine, Los Angeles, California
[3]Division of Biology and Beckman Institute, California Institute of Technology, Pasadena, California
[4]Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[5]Department of Zoology, Charles University Prague, Prague, Czech Republic
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***Correspondence to: Hans H. Epperlein, Department of Anatomy, TU Dresden, Fetscherstr. 74, 01307 Dresden, Germany. E-mail: hhepp@rcs.urz.tu-dresden.de**

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Furthermore, single cell lineage analysis in several species suggests that a single neural fold precursor can give rise not only to neural crest but also to neural tube and epidermal derivatives (Selleck and Bronner-Fraser, 1995). Accordingly, Chibon (1966) concluded that Rohon-Beard neurons, missing after neural fold ablation, were neural crest derived.

A major unresolved question in neural crest development concerns the relationship between neural crest and neural tube with respect to the allocation of different lineages and pathways of migration. This question has been extensively studied in the chick embryo, which is amenable to culture and microsurgical manipulation in combination with numerous cell-marking techniques for following neural crest migration (Le Douarin, 1969; Le Douarin and Kalcheim, 1999; Serbedzija et al., 1989; Elena de Bellard and Bronner-Fraser, 2005). The migratory behavior of avian neural crest appears to be highly organized, such that the first cells to exit the neural tube migrate along a ventromedial pathway through the somites, while later migrating cells are restricted to a dorsolateral pathway between the epidermis and somites where they form pigment cells (Le Douarin and Teillet, 1974; Guillory and Bronner-Fraser, 1986; Erickson et al., 1992; Serbedzija et al., 1989). In the chick, it has been proposed that melanocytes are specified before entering the dorsolateral migratory route (Reedy et al., 1998) and that distinct neurogenic and melanogenic sublineages may diverge before or soon after neural crest cell emigration from the neural tube (Luo et al., 2003).

Despite the wealth of information on avian neural crest migration, it remains unknown whether ordered migratory behavior is common to all vertebrate embryos, or whether it is avian specific. Some differences in migratory behavior from those observed in chick have been noted in amphibians like *Xenopus* as well as in mammals like the mouse. In *Xenopus*, neural crest cells including presumptive pigment cells migrate preferentially on the ventromedial route, between the somite and neural tube/notochord and not laterally or between the somites (Macmillan, 1976; Krotoski et al., 1988; Collazo et al., 1993). In the mouse, no timing differences exist between entry onto the dorsolateral and ventromedial pathways (Serbedzija et al., 1990). Thus, species-specific differences exist in some aspects of neural crest migration. There is reason to think that migratory behavior may differ between axolotl (*Ambystoma mexicanum*) and chick as well. In the axolotl, some premigratory neural crest cells, such as presumptive pigment cells, appear to be committed to their fate prior to the onset of migration. Therefore, it is possible that subsequent migration may follow different rules. It is unknown whether the premigratory crest is entirely "mosaic" and contains only unipotent cells or a mixture of cells with different ranges of developmental potential.

Here, we address this issue by studying the migratory patterns and developmental potential of early differentiating trunk neural crest cells in the axolotl such as pigment cells, Rohon-Beard cells, or components of the lateral line. As a first step, we have performed a series of cell-marking and grafting studies using dark wild-type and white mutant embryos, the latter having defective pigment cell migration (Dalton, 1953; Keller et al., 1982; Löfberg et al., 1985). The results show that neural crest cells follow both a dorsolateral and a ventromedial migratory route similar to other vertebrates. However, the time course is different than in other anamniotes or amniotes, since the dorsolateral route is taken first. We confirm that neural crest and Rohon Beard cells share a common lineage within the neural folds and find that only few axolotl neural crest cells contribute to the dorsal fin, which also has a contribution from the dermomyotome (Sobkow et al., 2006). Furthermore, we show that anterior but not mid- or posterior trunk neural folds contribute to the lateral line system. These results reveal interesting similarities and differences in the migratory pathways and derivatives between axolotl neural crest and that of other vertebrates.

RESULTS

DiI labeling was performed at premigratory stages (15–19; 25), early (33), and late migratory (35) stages to analyze the migratory patterns and derivatives of trunk neural crest cells emerging from the neural folds/tube (Fig. 1A–E). DiI injections were performed at different positions along the mediolateral axis (Fig. 1A,F) prior to the onset of neural crest migration. Along the rostrocaudal axis, anterior, middle, or posterior levels of the dorsomedial neural folds or neural tube were injected (Fig. 1B–E;F). In the trunk of early tailbud stage embryos, the neural crest can be morphologically identified as a wedge of cells that forms on the dorsal aspect of the neural tube and expresses snail transcripts (Fig. 1G). Later, an elevated neural crest string is present on top of the neural tube (Fig. 1H). Once neural crest cells migrate, they move along two primary pathways: a dorsolateral pathway underneath the epidermis that is primarily thought to give rise to pigmented derivatives (melanophores and xanthophores) and a ventromedial pathway followed by precursors to sensory, sympathetic, adrenomedullary, and glial cells. It is sometimes difficult to discriminate between laterally migrating neural crest–derived pigment precursors, epidermis, or neural tube on the one hand and between pigment precursors and components of the lateral line on the other. To best illustrate this point, we examined dark instead of white mutant embryos since they have many more laterally migrating cells (Figs. 1I–L). DiI-labeled cells can be distinguished in transverse sections after one anterior (Fig. 1I) or three midtrunk injections (Fig. 1J) into the trunk neural fold from epidermal or neural tube cells by means of their position (Fig. 1K). Pigment precursors and components of the lateral line such as neurons (ganglionic cells), glial cells, and lateral line nerves can be distinguished through the use of specific markers. We found that glial cells are present only in the vicinity of the lateral line primordium or nerve (Fig. 1L). They follow these structures caudally from the posterior cranial neural crest situated at a level caudal to the otic placode (Northcutt et al., 1994; Schlosser, 2002). They do not migrate laterally from mid- or caudal trunk neural crest regions.

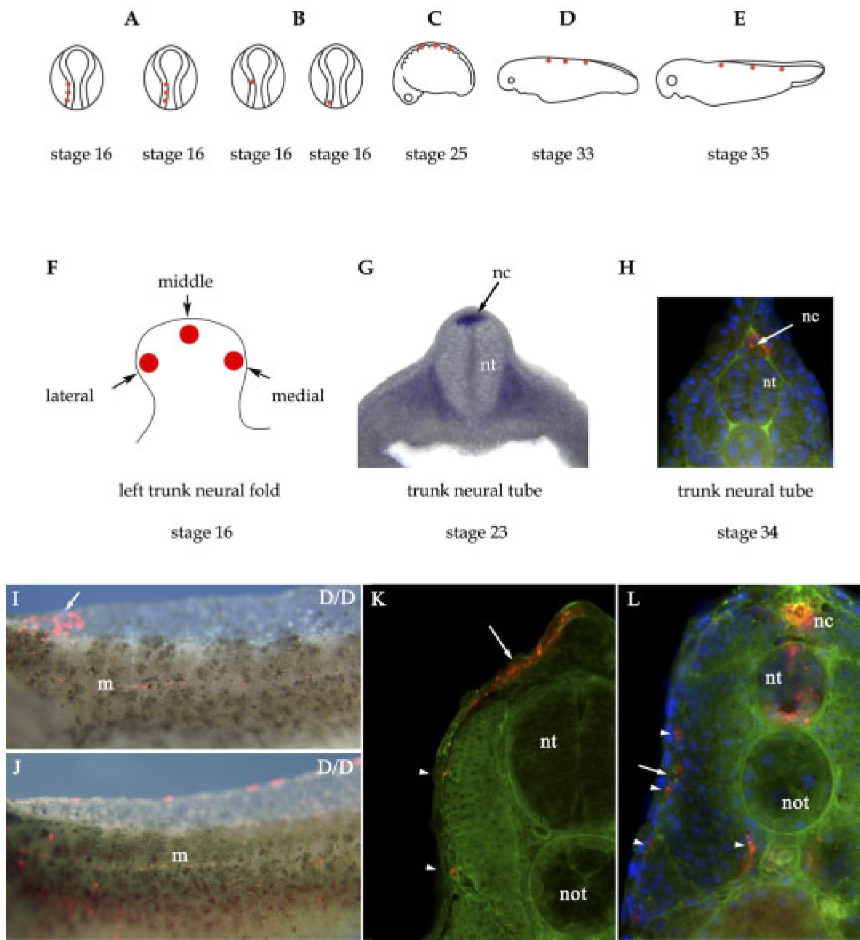


Fig. 1.

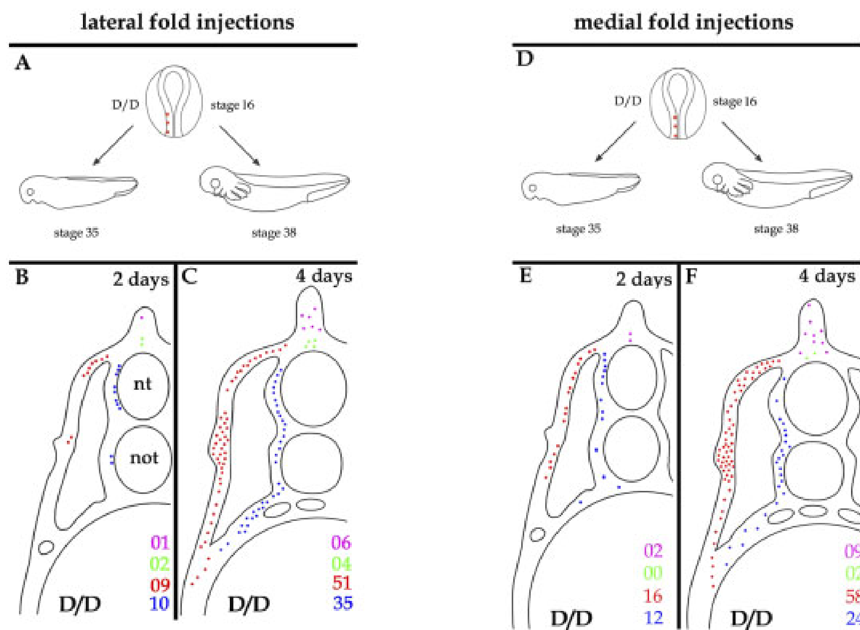


Fig. 2. A–F: Lateral or medial fold injections (average: stage 16). Prior to the onset of neural crest migration in dark (D/D) neurulae (stages 15–19), lateral (A) or medial (D) positions of trunk neural folds were labeled with three focal injections of DiI. Embryos were fixed 2 days (B,E) and 4 days (C,F) after the injection. In either case, after 4 days many more labeled cells were observed on the lateral than on the medial route whereas the distance traversed by neural crest cells was similar on both routes (see also Table 1). The pink numbers indicate DiI-labeled cells in the dorsal fin (df), the green numbers indicate sessile neural crest (nc) cells/derivatives on the neural tube, and the red and blue numbers indicate neural cress cells/derivatives on the lateral (lat) and medial (med) route, respectively.

DiI Labeling at Different Lateral or Medial Positions of the Trunk Neural Folds

At neurula stages, the exact position of the prospective neural crest within the neural fold is not known. To address whether different aspects of the neural folds of neurulae differ in developmental potential, neural folds were labeled laterally or medially along their anterior/posterior axis (Fig. 1F) prior to the onset of neural crest migration (stages 15–19; Figs. 1A, 2A,D; Table 1). Embryos were fixed 2 or 4 days post-injection and the

Fig. 1. DiI labeling and identification of neural crest cells in the axolotl trunk. DiI labeling was carried out at stages 15–19, 25, 33, and 35 to analyze the migratory potential of trunk neural crest. **A:** At stage 16 (mean stage used in this schematic to represent stages 15–19), three focal DiI injections were made into the lateral or medial aspects of the trunk neural folds (F). **B:** At stage 16, the left trunk neural fold was injected anteriorly or posteriorly in a middle position (F). At stages 25 (**C**), 33 (**D**), and 35 (**E**), three positions along the dorsal midtrunk were labeled. **F:** Red dots indicate positions of DiI labeling in the left trunk neural fold at stage 16. **G:** In transverse sections through the trunk of early tailbuds (stage 23), the neural crest (arrow) can be morphologically identified as wedge on top of the neural tube where it stains with the snail riboprobe. **H:** In transverse sections through the trunk of later tailbuds (stage 34), an elevated neural crest string is present on top of the neural tube (DiI labeling of the neural crest, dapi staining, anti-fibronectin staining). **I–L:** DiI labeling of neural fold in dark (D/D) neurulae and identification of neural crest cells in larvae in relation to epidermis and neural tube. I, J show left trunks in larvae (stage 37); in I, one left anterior trunk neural fold, and in J, three left midtrunk neural fold positions were injected with DiI. In I, labeled cells are scattered in the upper anterior trunk and the middle lateral line primordium is stained. The arrow marks the injection site. In J, labeled cells are found throughout the lateral trunk, on top of the dorsal fin, and sparsely in the middle lateral line primordium. Injection sites are not more visible. K: Transverse section through the midtrunk of the larva in I counterstained with anti-fibronectin; in contrast to DiI-labeled epidermal cells (arrow), labeled neural crest cells (arrowheads) are found more internal to the epidermal basement membrane. L: Transverse section through the midtrunk of the larva in J counterstained with anti-fibronectin and dapi. Arrowheads point to DiI-labeled neural crest cells on the lateral and ventromedial pathway. The arrow indicates the middle lateral line primordium; the two labeled cells in its vicinity are very likely glial cells becoming associated with the middle lateral line nerve. m, middle lateral line primordium; nc, neural crest; nt, neural tube; not, notochord.

TABLE 1. Trunk Neural Crest Cell Migration In Axolotl Based on DiI Infections[a]

Groups	Figure	Genotype	Stage at injection	N	Site/number of injections	Time/stage after injection	Neural crest cells				Migrated distance (μm)	
							df	nc	lat	med	lat	med
1	2B	D/D	18/19	5	Lateral fold/3	2d/35	01	02	09	10	450	450
2	2C	D/D	15	5	Lateral fold/3	4d/38	06	04	51	35	1100	1050
3	2E	D/D	17/18	5	Medial fold/3	2d/34–35	02	00	16	12	600	650
4	2F	D/D	15	5	Medial fold/3	4d/38	09	02	58	24	1150	1050
14	3B	d/d	16	5	Anterior fold/1	4d/38	02	04	91	22	800	1200
15	3C	d/d	16	5	Posterior fold/1	4d/38	15	15	23	26	800	950
5	6B	D/D	25	5	Dorsal mid trunk/3	1d/32–33	00	14	05	00	300	0
6	6C	D/D	25	5	Dorsal mid trunk/3	2d/36	00	14	55	07	700	400
7	6E	d/d	25	5	Dorsal mid trunk/3	1d/33	00	00	01	09	350	200
8	6F	d/d	25	5	Dorsal mid trunk/3	2-3d/36–37	05	03	14	28	450	650
9	7B	D/D	33/34	5	Dorsal mid trunk/3	1d/36	02	07	24	07	750	600
10	7C	D/D	33/34	5	Dorsal mid trunk/3	2d/37	03	17	40	44	750	650
11	7E	d/d	33/34	5	Dorsal mid trunk/3	1d/36	00	13	03	02	200	200
12	7F	d/d	33/34	5	Dorsal mid trunk/3	2d/37	02	22	07	31	450	650
13	8B	D/D	35/36	5	Dorsal mid trunk/3	4d/39–40	24	17	22	79	700	1100
16	8C	D/D	35/36	5	Dorsal mid trunk/3	4d/39–40	16	09	32	62	800	1050
	4A	D/D	16	5	Anterior fold/1	4d/38						
	4A	D/D	16	10	Anterior fold/1	5d/39						
	5A	d/d	16	10	Anterior fold/1	2d/32						
	5A	d/d	16	5	Anterior fold/1	4d/38						
	5A	d/d	16	5	Anterior fold/1	5d/39						
	4B	d/d	16	12	Posterior fold/1	2d/32						
	4B	d/d	16	15	Posterior fold/1	4d/38						
	4B	d/d	16	15	Posterior fold/1	5d/39						
	–	D/D	16	5	Posterior fold/1	4d/38						
	–	D/D	16	10	Posterior fold/1	5d/39						

[a]Figures 2,3,6–8 show a quantitative evaluation of DiI-labeled cells. Cell counting in each of 16 groups is based on approximately 20 transverse sections (100-μm thick) through the trunk of embryos until stage 34 and 30 sections until stage 39/40. Five specimens were sectioned per group. Labeled cells in sections through all specimens of a group were counted and mirror-imaged to the left side of a summary section that represented the group (Figs. 2,3,6–8; Table 1). Figures 4A,B and 5A contain data on anterior or posterior trunk neural fold injections with Dil, which were qualitatively evaluated. D/D, wild-type (dark); d/d, white mutant; df, dorsal fin; nc, neural crest; lat, lateral route; med, med at route; n, number of specimens investigated.

distribution of DiI-labeled cells determined (stages 35 or 38; Fig. 2B,C,E,F; Table 1). Because DiI labeling of the neural folds marks epidermis and neural tube in addition to neural crest, neural crest cells were carefully distinguished from epidermal and neural tube using morphological criteria (Fig. 1K).

Two days following lateral injections into neural folds of wild type embryos, the leading edge of neural crest cells had migrated ~450 μm away from the injection site and ~1,100 μm after 4 days and was found equally far along the dorsomedial and ventrolateral pathways (Fig. 2B,C; Table 1). Following medial injections, neural crest cells had migrated 600–650 μm after 2 days and 1,150/1,050 μm after 4 days (Fig. 2E,F; Table 1). Thus, no major differences were noted for medial versus lateral neural fold injections with respect to the distance traversed by neural crest cells away from the injection site. However, more migrating cells were observed along the dorsolateral pathway after 4 days (Fig. 2C,F).

DiI Labeling of Different Rostrocaudal Axial Levels

Using scanning electron microscopy and dopa-histochemistry, we have previously shown that delamination of neural crest cells starts around stage 28 in the anterior trunk of wild-type embryos, while dorsolateral migration of prospective pigment cells commences by stage 31. Initiation of migration proceeds gradually along the rostrocaudal axis such that by stage 35, migrating pigment cells have spread throughout the flank. It was previously assumed that only pigment cells migrate along the dorsolateral pathway (Löfberg et al. 1980; Epperlein and Löfberg, 1990).

Here, we investigated whether other neural crest populations migrated along the dorsolateral pathway using DiI labeling to mark the neural folds of white mutant embryos, which have a defect in pigment cell migration and thus have only a few melanophores that could obscure recognition of migrating non-pigmented neural crest derivatives. A focal DiI injection was made into one dorsal middle area of either the anterior or posterior trunk neural fold on the left side of a neurula (Fig. 3AB). Previously, the

postotic posterior lateral line placode and associated neural crest have been proposed to contribute to the lateral line system in the trunk (Northcutt et al. 1994; Schlosser, 2002; Collazo et al., 1994).

Following anterior injections into white neurulae (Fig. 3A,B; stage 16, axial level of prospective 2nd somite), the majority of labeled cells migrated along the dorsolateral rather than the ventromedial pathway (e.g., 91 vs. 22 cells for summing 5 representative anterior injections; Table 1). After posterior injections (Fig. 3A,C, stage 16, at the most posterior trunk neural fold level), dorsolateral migration was similar to ventromedial migration (23 vs. 26 labeled cells summing 5 representative posterior injections; Table 1), but lateral migration was fourfold less than after anterior injections.

Interestingly, the front of dorsolaterally migrating neural crest cells appears to stagnate around the middle lateral line nerve regardless of the site of injection. Following anterior injections, there appears to be an additional dorsolateral group of labeled cells at stage 38 (Fig. 3B) corresponding to the position of the dorsal lateral line, which by this stage is present in the anterior but not yet in the posterior trunk (see fig. 16 in Northcutt et al., 1994). Because the number of dorsolateral melanophores in white larvae is ~30 (see "Quantification of DiI-Labeling and Control" in the Experimental Procedures section), the much higher number of labeled cells observed in white larvae for those injected anteriorly at stage 38 can only be ascribed to the presence of many more non-melanophores. This raises the intriguing question if mid and posterior trunk neural fold cells might contribute glia or neurons to the lateral line.

Anterior But Not Mid or Posterior Trunk Neural Folds Contribute to Neurons and Glia of the Lateral Line

In fish and aquatic amphibians, the lateral line system forms part of the VIIIth cranial nerve complex. In the trunk of axolotl larvae, three lines of neuromasts develop, one dorsally, one medially, and one ventrolaterally (Smith et al., 1990). All three are derived from the lateral line placode primordium situated posterior to the otic placode (Northcutt et al., 1994), receive afferent and efferent innervation, and grow along invariant pathways (Schlosser, 2002).

The primordium contains prospective lateral line receptors (neuromast and ampullary primordia) and sensory neurons of lateral line ganglia and glia (Schlosser, 2002; Gilmour et al., 2002). By stage 37, the middle lateral line nerve extends to the tail region, and by stage 41, the dorsal and middle lateral lines fuse above the cloaca (Northcutt et al., 1994).

To address the source of neural crest cells that appear to cease migration in the vicinity of the dorsal or middle lateral line nerve, focal injections of DiI were applied into anterior (Fig. 4A) or posterior (Fig. 4B) neural folds of dark and white neurulae (stage 16; for exact level of injections, see Fig. 3A). This results in random distribution of labeled cells underneath the epidermis of developing larvae. For anterior injections, labeled cells were occasionally found along the middle lateral line (dark larva at stage 37; Fig. 4A) probably depositing the neuromast primordia (Northcutt, personal communication). In contrast, DiI-labeled cells were not found along the dorsal or middle lateral line nerves after posterior injections (white larva at stage 41; Fig. 4B). These results suggest that only anterior trunk neural fold/neural crest cells contribute to the lateral line. To gain better cellular resolution of neural crest contributions to the lateral line, we used a second labeling approach of grafting trunk neural folds from GFP-positive transgenic donor embryos into white hosts. Because white mutants have few melanophores compared with wild-type, pigment cells tend not to obscure the GFP signal. For grafts of the posterior trunk neural fold region, we observed only GFP+ pigment cells, leaving the graft and no fluorescent cells migrating toward or associating with lateral line nerves (data not shown), suggesting that the posterior trunk fails to contribute to the lateral line.

Next, small mid trunk fragments or entire trunk neural folds from GFP+ transgenic neurulae (stage 17) were grafted in place of small left midtrunk fragments or the entire left trunk neural fold, from post-otic region to the tail, of white mutant embryos. By stage 35/36, the primordium of the middle lateral line was labeled intermittently (Fig. 4C). By stage 41, GFP-positive glia cells and neuromasts were observed intermittently along the middle nerve (not visible) on both the ispilateral side bearing the graft (Fig. 4D) as well as the contralateral side (Fig. 4E). In addition, a few GFP-positive pigment and epidermal cells were randomly distributed in the dorsolateral trunk (not shown). In some cases, only the right, unilateral neural fold graft was used to replace the entire host since this often aided in visualizing the GFP-labeled cells on the left side better (no left epidermis labeled). Using this approach, the left dorsal, middle, and ventral lateral line nerves become clearly labeled in larvae at stage 40/41 (Fig. 4F). GFP-positive neural crest cells were observed associated with all three lateral line nerves (Fig. 4G). However, only surrounding the ventral nerve was there a swarm of GFP-positive cells (Fig. 4G) whose origin from the mid-trunk neural crest was questionable. In transverse sections, they proved to be localized more deeply, in the lateral plate mesoderm (Fig. 4H), and, thus, represent neural crest–derived enteric neurons/glial cells migrating on the ventromedial route and turning to the surface from underneath the myotomes. These findings confirm our DiI labeling and suggest that GFP-positive glial cells associated with lateral line nerves at truncal levels arise from anterior neural folds from behind the otic placode.

Immunohistochemistry was used to examine the phenotype of labeled neural crest cells traveling along the nerves. Five days after an anterior injection when neuromasts are present along the dorsal and middle lateral line nerves, the nerves themselves are only lightly labeled with DiI, appearing as a fine continuous line (white larva at stage 39; Fig. 5A,B). In contrast, Schwann cells scattered along the nerves were brightly stained with DiI. Anti-tubulin staining of transverse sections through this larva revealed the nerves as two spots on either side of the dorsal trunk (Fig. 5C). Anti-tubulin staining was observed

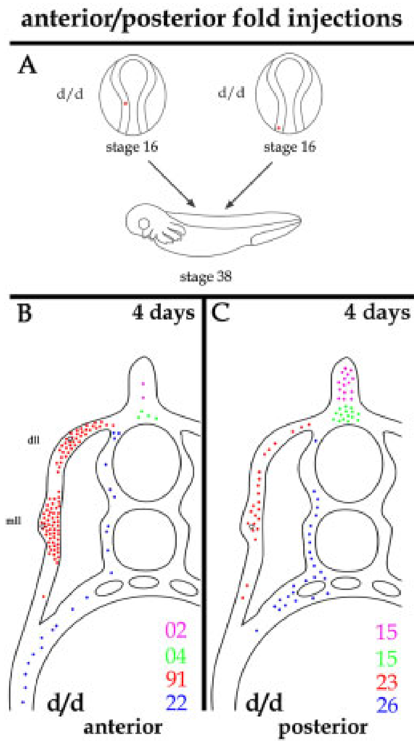


Fig. 3. A–C: Anterior or posterior trunk neural fold injections (stage 16). One focal DiI injection was made into the left trunk neural fold in a middle position either at an anterior or posterior position of a white (d/d) neurula (stage 16) in order to investigate the contribution of neural crest cells to the lateral line system. Following anterior injections, labeled cells greatly favored the lateral pathway over the medial pathway (B). In white embryos injected posteriorly, many fewer labeled cells migrated laterally (C). In either type of injection, the front of lateral migration stagnated in the flank at the level of the middle lateral line nerve (mll); following anterior injections, labeled cells seemed to be grouped also around the dorsal lateral line nerve (dll).

intermixed with DiI staining of putative glial cells on the left side (Fig. 5C). The glia character of these DiI-positive cells (Fig. 5A–C) was shown by anti-GFAP staining of microwave-treated paraffin sections through the midtrunk of stage 41 larvae (Fig. 5D, blue circle) adjacent to anti-tubulin staining of the lateral line nerve (Fig. 5E, condensed brown structure).

DiI-Labeling After Neural Tube Closure Reveals Differences in Timing of Neural Crest Entry Onto Lateral Versus Ventromedial Routes

We next investigated the choice of pathway and timing of migration of

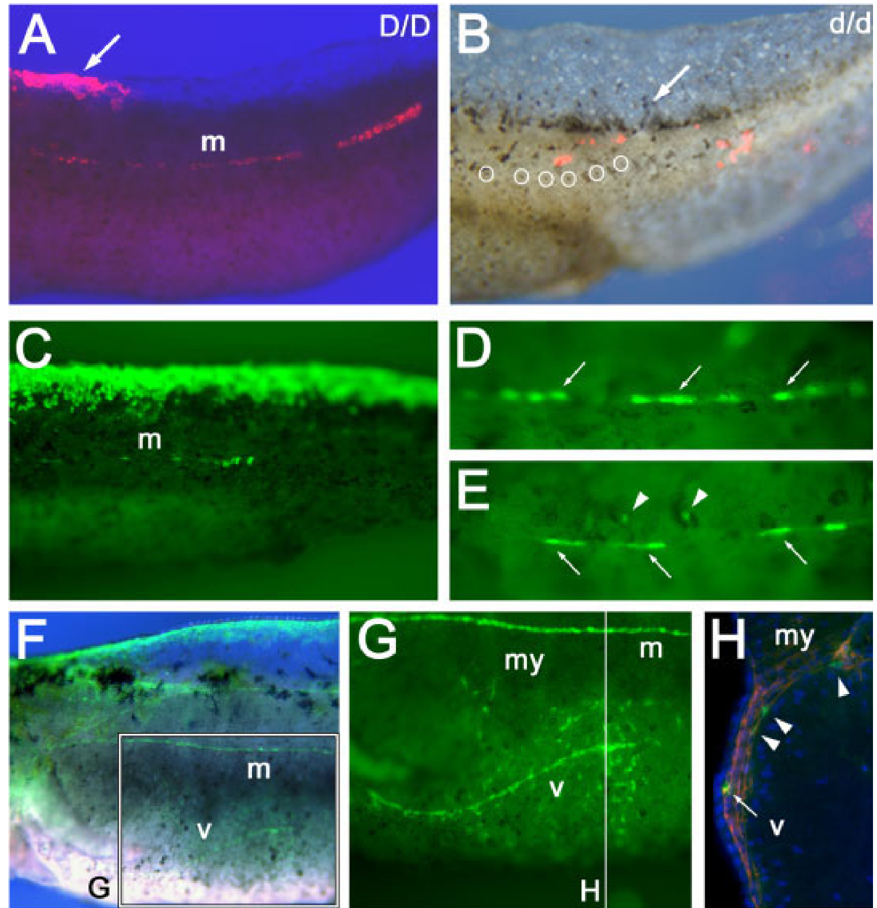


Fig. 4.

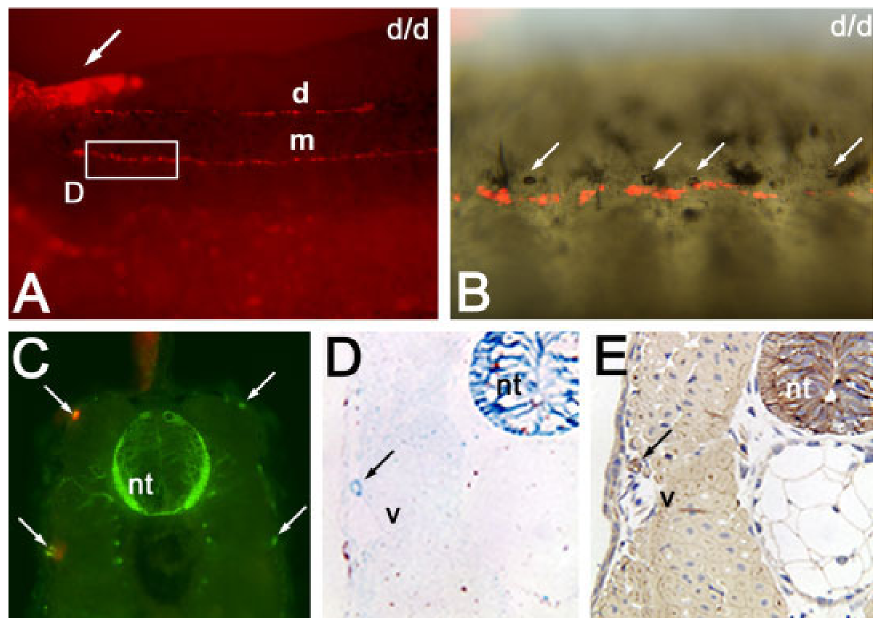


Fig. 5.

neural crest cells by labeling the dorsal neural tube after its closure but prior to the onset of neural crest emigration. Results were compared between wild-type and white mutant embryos. DiI was injected at three sites along the middle of the trunk neural tube of stage 25 wild-type and white mutant embryos (Fig. 6A,D) and the results examined 1 or 2–3 days later in fixed embryos (stages 32–33 or 36–37). As in DiI labeling of lateral and medial trunk neural folds, we also carefully distinguished between neural crest and epidermal cells when counting neural crest cells on the dorsolateral and ventromedial pathways.

In dark embryos, the front of neural crest cells on the lateral route had moved about 300 μm after 1 day (5 cells) and 700 μm after 2–3 days (55 cells; Figs. 6B,C; Table 1). On the ventromedial pathway, labeled cells were absent after 1 day but had moved maximally 400 μm away from the injection site after 2 days (7 cells), suggesting that this pathway opens secondarily (Fig. 2A,B; Table 1). In the white embryo, dorsolateral migration was reduced (1 cell after one day, 14 cells after 2–3 days; Fig. 6E,F) compared to the wild type. Labeled cells in the mutant appeared stagnant in their migration ~450 μm distant from the injection site. Again, their site of arrest corresponded to the position of the middle lateral line nerve.

Taken together, these results suggest that neural crest cells in wild type embryos labeled at stage 25 migrate first on the lateral route where they are more numerous than those migrating ventromedially. In white mutants, in contrast, lateral migration is reduced whereas ventromedial migration (stage 36–37, Fig. 6F) resembles that in the wild type (stage 36, Fig. 6C; stage 37, Fig, 7C).

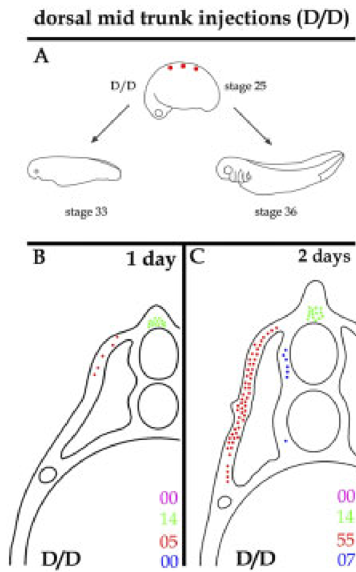
DiI-Labeling at Early and Late Migration Stages

To determine how long neural crest migration persists on different migratory pathways, cell marking was carried out at progressively later stages ranging from early (stage 33; Fig. 7A–F) to late migration (stage 35; Fig. 8A–C). Each embryo received focal injections of DiI at three rostrocaudal locations on the dorsum of the neural tube and was examined one day (stage 36), two days (stage 37), or four days (stage 40) post-injection.

In wild-type embryos labeled during early migratory stages (Fig. 7A), many more labeled cells were observed on the dorsolateral than the ventromedial pathway one day after injection (Fig. 7B; Table 1) but numbers were approximately equal by two days after injection (Fig. 7C; Table 1). The front of dorsolaterally migrating cells was about 750 μm away from the injection site at both 1 and 2 days. Ventromedial migration was about 600 μm away from the injection site after 1 day and 650 μm after 2 days. In white mutant embryos (injection sites Fig. 7D), few labeled cells were present on either route after 1 day (Fig. 7E; Table 1). After 2 days, a few more labeled cells were observed on the dorsolateral pathway but appeared to stagnate in their migration (Fig. 7F; Table 1). Those on the ventromedial pathway, however, had increased considerably in number and traveled 650 μm. Thus, in dark embryos dorsolateral cells migrate first and are more frequent than those on the ventromedial pathway similar to embryos injected at stage 25. In white mutant embryos, dorsolateral migra-

Fig. 4. Contribution of neural crest cells to the lateral line. **A:** Left side of a dark axolotl larva at stage 37, 4 days after injection of DiI into an anterior location of the left trunk neural fold. DiI likely labels the main trunk lateral line placode depositing the neuromast primordia for the middle lateral line (m). **B:** Left tail region of a white axolotl larva at stage 41, 5–6 days after injection of DiI into a posterior location of the left trunk neural fold. Neuromasts of the middle lateral line nerve are encircled. A few labeled cells spread laterally from the injection site but do not associate with the middle or dorsal lateral line nerves. **C:** Left side of a white larva (stage 35/36) bearing a trunk neural fold graft from a GFP+ transgenic neurula on its left side. The dorsal neural tube and covering epidermis are heavily labeled. The primordium of the middle lateral line nerve (m) migrates posteriorly and has reached a level above the cloaca. The intermittent labeling along the nerve is very likely due to glial cells migrating posteriorly. From the labeled neural tube, no labeled neural crest cells were observed to migrate laterally and associate with the middle lateral line nerve. **D:** Lateral aspect of left midtrunk of white larva (stage 41) whose entire left trunk neural fold was replaced with a GFP+ fold. Elongated fluorescent glial cells (arrows) were present intermittently along the middle lateral line nerves of the ipsi- (D) and contralateral side (**E**). Arrowheads in E point to GFP+ centers of neuromasts where possibly supportive cells are stained. From the GFP+ grafted neural fold (dorsal, outside the image), no lateral migration of labeled neural crest cells was observed towards lateral line nerves. **F:** Left side of a white larva (stage 40/41) following removal of both trunk neural folds and replacement of the right fold with a GFP+ fold. The dorsal and middle lateral line nerve and the advancing ventral nerve are labeled. **G:** Enlarged inset from F showing the enlarged middle (m) and ventral (v) nerve. The advancing tip of the ventral nerve is surrounded by a swarm of GFP+ cells. Only those at the nerve or in close vicinity to it seem to be glial cells. GFP+ cells further apart are very likely enteric neurons/glial cells that migrated into the lateral plate on the ventromedial route. **H:** Transverse section through larva in F,G in the plane indicated by a white vertical line in G; GFP+ cells in the epidermis were observed only in the position of the ventral lateral line (v). All other GFP+ cells were lying in the lateral plate mesoderm (arrowheads) and had reached this position after migrating on the ventromedial pathway below the myotomes (my). Immunostaining with primary anti-fibronectin and secondary Cy3-conjugated antibody to reveal basement membranes; dapi staining.

Fig. 5. Identity of lateral line nerves and glia. **A:** Left side of a white larva at stage 39, 4 days after injection of DiI into an anterior location of the left trunk neural fold. Irregular labeling of the dorsal (d) and middle (m) lateral line nerve with DiI. **B:** Enlarged inset from A showing neuromasts (arrows) dorsal to the middle lateral line nerve; irregular DiI-labeling along the nerve stains possibly neuromast primordia and glial cells. **C:** Immunostaining of a transverse vibratome section through the midtrunk of the larva in A with a primary anti-tubulin antibody and a FITC-conjugated secondary antibody reveals two green fluorescent subepidermal spots on either side of the dorsal trunk that label the dorsal and midde lateral line nerve. Green spots on the left side colocalize with DiI-labeled glial cells. **D:** Immunostaining of transverse microwave-treated paraffin sections through the trunk of a larva (stage 41) with a primary anti-GFAP-antibody, a secondary biotinylated antibody, and a tertiary avidin-peroxidase complex (Vectastain ABC-Kit). Glia (arrow; blue color) around the middle lateral line "nerve" and in the neural tube (nt) reacted positive and (**E**) colocalized with the middle lateral line nerve (arrow, brown color) dorsal to a blood vessel (v) in a similar transverse microwave-treated paraffin section (stage 41 larva) that reacted positive with a primary anti-tubulin antibody, a secondary biotinylated antibody, and a tertiary avidin-peroxidase complex (Vectastain ABC-Kit).



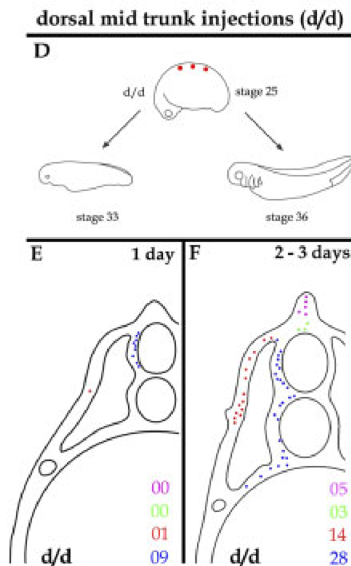
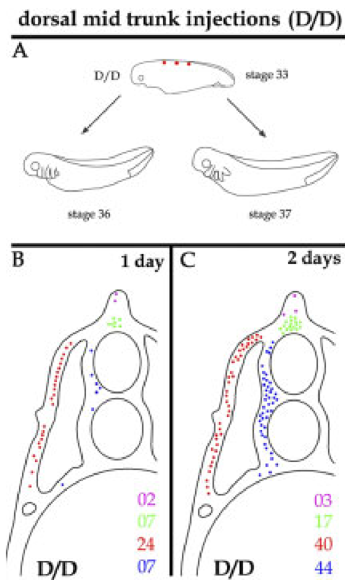


Fig. 6. A–F: Dorsal mid trunk injections (stage 25, premigratory stage). Three separate focal injections of DiI were applied along the mid trunk neural tube/premigratory neural crest of wild type(D/D;6A) and white mutant (d/d) embryos (6D) at stage 25. Neural crest cells in the wild type migrate first on the lateral route where they are more numerous than on the medial pathway. In the white mutant, lateral migration remains strongly reduced. The front of cells coincides with the positon of the middle lateral line nerve. Medial migration dominates and resembles that in the wild type (compare F with C).



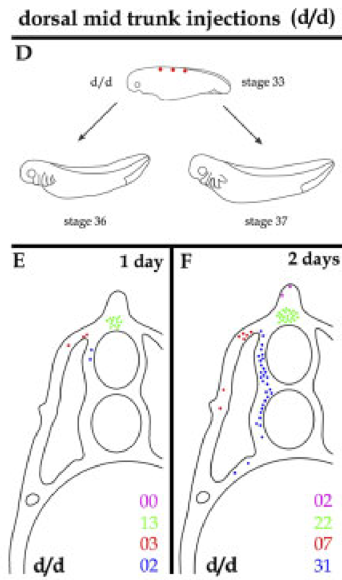


Fig. 7. A–F: Dorsal mid trunk injections (stage 33, early migration stage). Three separate focal injections of DiI were carried out along the mid trunk neural tube/early migratory neural crest of wild type (D/D; A) and white mutant (d/d; D) embryos at stage 33. In wild type embryos one day after the injections, many more labeled cells were observed on the lateral than on the medial pathway (B). By 2 days (C), numbers were approximately equal. In white embryos one day after the injection, few labeled cells were found on both routes (E). By 2 days, many more labeled cells were found on the ventromedial route but their number stagnated laterally (F).

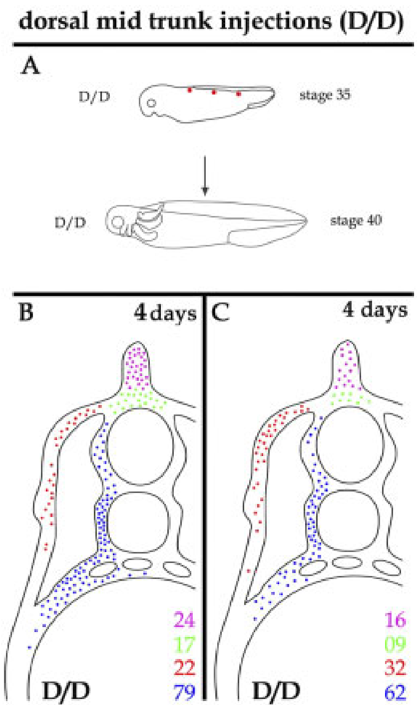


Fig. 8. A–C: Dorsal mid trunk injections (stage 35, late migration stage). Wild-type (D/D) embryos (two groups, B and C, with 5 individuals in each group) were injected with DiI into the dorsal neural tube/neural crest at late migration (stage 35) and fixed 4 days post-injection (stage 40). At stage 40, ventromedial migration and migration into the dorsal fin dominate.

tion appears to be compromised compared with that in wild-type counterparts.

To examine late stages of neural crest migration, wild type embryos were injected as above, but at stage 35 (Fig. 8A) and fixed 4 days post-injection (stage 40). As late as stage 40, DiI labeling resulted in many labeled migratory neural crest cells (Fig. 8B,C). The majority of labeled cells moved along the ventromedial pathway or into the dorsal fin with some migration observed along the lateral pathway.

It is difficult to perform DiI labeling of the trunk neural tube much later than stage 35 due to the impenetrable nature of the epidermis. Based on larvae injected at stage 35 (Fig. 8A) and investigated 4 days later (stage 40), it is likely that neural crest cells are still emigrating from the neural tube after stage 35 (Fig. 8B,C; Table 1).

Pigment cells (melanophores, xanthophores) start to visibly differentiate under the epidermis by stage 35. By stages 38–40 neurons and glial cells are condensing into dorsal root ganglia at the ventrolateral margin of the neural tube as revealed in transverse sections stained with anti-tubulin and anti-GFAP (not shown), respectively. In 14-mm-long larvae, tyrosine hydroxylase (TH)-positive chromaffin cells start to appear, preceding TH+ sympathetic neurons that differentiate in larvae >24 mm (not shown; see also Vogel and Model, 1977).

Some Neural Crest Cells Migrate Contralaterally

In birds and frogs, some neural crest cells cross the midline to migrate on the contralateral side (Hall and Hörstadius, 1988; Couly et al., 1996; LeDouarin and Kalcheim, 1999). In axolotl, this possibility was first investigated by Hörstadius and Sellman (1946) using vital dyes. To extend this work with higher resolution labeling methods, we performed unilateral grafts of left neural folds that had been injected with fluorescent dextrans at the two-cell stage, thus labeling all neural fold cells in the graft.

Prior to the onset of trunk neural crest migration (stage 30/31), fluorescent cells derived from a rhodamine dextran–labeled neural fold were confined to the epidermis and neural tube on the grafted side and to a premigratory neural crest on top of the neural tube, which also contained unlabeled cells presumably from the contralateral neural fold (Fig. 9A). The neural crest was surrounded by basement membranes as revealed by anti-fibronectin staining (Fig. 9A). At early migratory stages (stages 32–34), fluorescent neural crest cells from a left, FITC-labeled neural fold entered the ventromedial pathways on both the ipsilateral and contralateral sides (Figs. 9B,C). When the left trunk neural fold contains a FITC- and the right a rhodamine dextran graft (Fig. 9D), contralateral migration of neural crest cells from each fold was observed. We show rhodamine dextran–labeled neural crest cells on the lateral and ventromedial pathway of the contralateral side (Fig. 9D).

In horizontal sections (Fig. 9F) through the dorsal trunk of an embryo at stage 35 bearing a FITC-dextran graft on its left side (Fig. 9E), single labeled cells were clearly identified on the dorsolateral and ventromedial pathway on both sides of the embryo. Interestingly, those cells moving ventromedially were observed within the narrow sclerotome (Fig. 9B), although no labeled neural crest cells were observed migrating through the myotome as proposed by Vogel and Model (1977). These results show that neural crest cells can migrate to the contralateral side of the embryo and then follow analogous migratory pathways.

Both Neural Crest and Rohon-Beard Neurons Originate From Neural Folds

Rohon-Beard cells are transient sensory neurons in the dorsal trunk neural tube of fish and amphibian embryos (Hall and Hörstadius, 1988; Le Douarin and Kalcheim, 1999). They form a primary sensory system that degenerates when dorsal root ganglia develop (Hughes, 1957; Roberts and Patton, 1985) and persist in axolotl at least through larval stages (DuShane, 1938).

Rohon-Beard cells were identified using a combination of anti-tubulin staining and DiI injections in sections through the trunk of embryonic (4 mm) to larval (17 mm) stage axolotls (Fig. 10A). In larvae, Rohon-Beard cells appeared as large neurons at the dorsal-most aspect of the spinal cord (Fig. 10B), in contrast to interneurons that are localized more laterally (Roberts, 2000). Both cell types were about 2–3 times larger than neighboring cells of the spinal cord. In younger larvae (9 mm; stage 39/40), several large cells with a rectangular or rounded shape were observed; these can be distinguished from Rohon-Beard cells because they reach from the ependymal layer to the outer surface of the tube and have nuclei at different levels from the center (Fig. 10C,D; see Sauer, 1935). Thus, they are likely to be mitotic neuronal precursors.

To examine the origin of Rohon-Beard cells in the axolotl, DiI was injected into the left trunk neural fold of neurulae (stage 16), similar to those injections performed to examine neural crest migratory patterns. Embryos were subsequently fixed at embryonic stage 33–34. In horizontal sections through the dorsal neural tube, several giant cell bodies at the left margin of the neural tube were labeled with DiI and appeared several times larger than adjacent neurons. Furthermore, they extended nerve fibers laterally into the intersomitic clefts (Fig. 10E,F), characteristic of Rohon-Beard neurons (for details see Hughes, 1957; Taylor and Roberts, 1986; Roberts, 2000). The same injections also gave rise to neural crest derivatives and epidermis. This result suggests that the Rohon-Beard cells, epidermis, and neural crest cells share a common origin from the dorsal neural folds (Fig. 10E,F).

DISCUSSION

Neural Crest Cells From Different Sites Within the Neural Fold Have Equivalent Developmental and Migratory Potential

In amphibian embryos, it has been proposed that different neural crest derivatives may arise from different lateral or medial positions within the neural folds. Testing this proposal in axolotl, Brun (1985) had shown that melanophores differentiate from the medial cranial neural fold ("L.N.F." in Brun, 1985) but not from lateral epidermis adjacent to lateral cranial neural fold. Furthermore Northcutt et al. (1996) had demonstrated in axolotl that the medial wall of (cranial) neural folds forms neural crest and tube derivatives, whereas the lateral wall differentiates into dorsal and dorsolateral ectoderm including neurogenic placodes. The latter authors emphasized that "the neural folds . . . .must be qualified by division into medial and lateral fields which each give rise to multiple lineages" (Northcutt et al., 1996). It also has been reported that neural plate adjacent to neural fold in axolotl gives rise to melanophores whereas epidermis adjacent to fold contributes to spinal and cranial ganglia (Moury and Jacobson, 1990).

Because previous studies did not definitively address which neural crest populations differentiate from positions within medial or lateral neural fold positions, we re-examined this issue in the axolotl trunk. However, we failed to find any difference in the pathways chosen, distances traversed, or derivatives produced by neural crest cells derived from either medial or lateral trunk neural folds. Thus, there was no evidence for obvious mediolateral differences or of sublineages within the premigratory neural crest in the trunk neural folds.

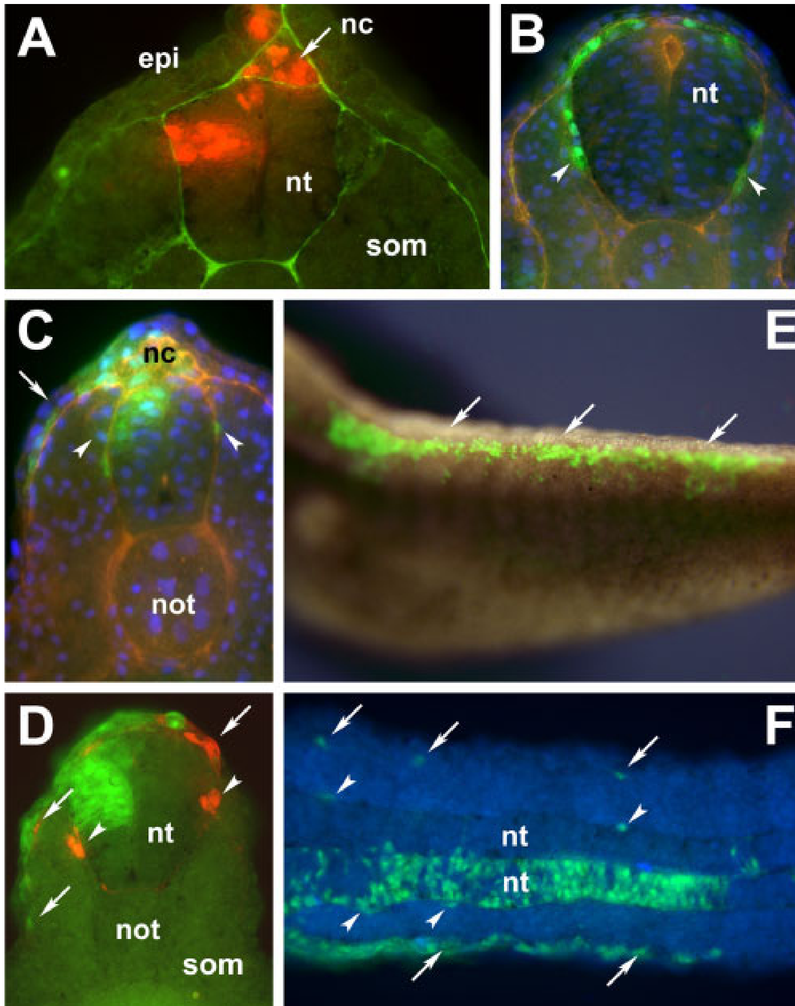


Fig. 9.

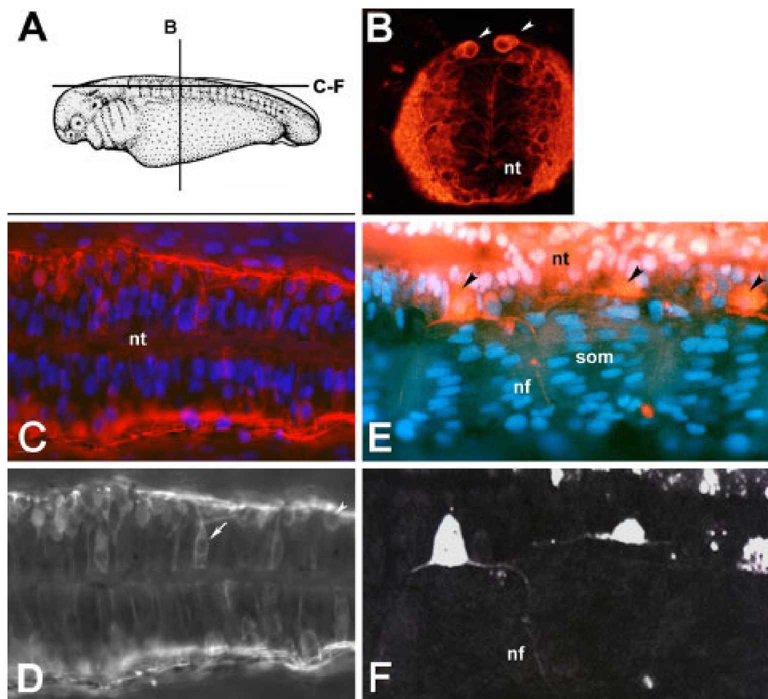


Fig. 10.

Fig. 9. Migration of trunk neural crest to the contralateral side. The left trunk neural fold of wild-type (D/-) hosts (stages 15–17) was replaced orthotopically by a neural fold from dark donors (same age) labeled with FITC or rhodamine dextran. **A:** Dorsal part of transverse section through the trunk of a stage 30/31 embryo to demonstrate the distribution of labeled tissues that were derived from the left, grafted rhodamine labeled neural fold before the onset of neural crest migration. Labeled cells from the left neural fold graft were observed in the left epidermis (epi) and left neural tube (nt). The neural crest (nc, arrow) cannot be divided into left or right portions; it is partially labeled and contains also unlabeled cells from the right fold. **B–D:** Transverse sections through different embryonic trunks at early migratory stages of the neural crest (stages 30/31–34). Embryos contain either one FITC-dextran labeled neural fold on their left side (B,C) or one FITC-dextran- on the left and one rhodamine-dextran labeled fold on their right side (D). Sections were immunostained with a primary polyclonal anti-fibronectin antibody visualized by a FITC-conjugated (A) or a Cy3-conjugated secondary antibody (B,C). Fluorescent neural crest cells entered ventromedial (arrowheads) and lateral pathways (arrows) on the ipsi- and contralateral side. In B, fluorescent neural crest cells on the left medial pathway pass through the sclerotome. **E:** Left side of a larva (stage 35) bearing a FITC-dextran-labeled trunk neural fold graft on the left side. **F:** Horizontal section through larva shown in E at a middle neural tube level. Single labeled cells are visible on the medial (arrowheads) and lateral pathway (arrows) of both the ipsi- and contralateral side. B,C,F: counterstaining with dapi.

Fig. 10. Origin and distribution of Rohon-Beard neurons. Reinvestigation of Rohon-Beard cells on sections through the axolotl trunk from embryos (stage 33) to larvae (17 mm length). **A:** Schematic showing planes of sectioning in an axolotl embryo at stage 34. **B:** In transverse sections through the trunk of larvae (12 mm), large neurons (arrowheads) were observed at the dorsal-most aspect of the spinal cord following immunostaining with anti-tubulin, which fulfills the criteria of Rohon-Beard cells. **C,D:** Horizontal section through the dorsal neural tube of a younger larva (stage 39/40). C: Immunostaining with primary anti-tubulin and secondary Cy3-conjugated antibody followed by dapi staining. D: Same section uncolorized. Large cells with a rectangular (arrow) or rounded shape (arrowhead) are mitotic precursors and not Rohon-Beard cells because they are continuous from the ependymal layer to the outer surface of the neural tube and have their nuclei at different distances from the central lumen. **E,F:** Horizontal section (E, DiI, and dapi; F, laser scanning micrograph) through the dorsal neural tube of an embryo (stage 33–34), which had received DiI injections into the left trunk neural fold. Three giant labeled cell bodies at the left margin of the neural tube extend nerve fibres laterally into the intersomitic clefts. They are likely Rohon Beard cells. nt, neural tube; som, somite; nf, nerve fibre.

Anterior Trunk Neural Fold Gives Rise to More Laterally Migrating Cells Than Posterior Fold

In dorsal trunks of white mutant embryos injected with DiI at stage 25, a quantitative investigation showed that labeled cells were distributed on the lateral migratory route at stage 36–37 and stopped in an area of the middle lateral line nerve (Fig. 6F). These DiI-labeled cells (14 per 5 individuals) formed only a fraction (about 10%) of all lateral melanophores counted in white controls (~30/individual); however, it was unclear whether their arrest at the lateral line nerve was coincidental or reflected a contribution to derivatives of the lateral line system. To distinguish between these possibilities, we injected DiI focally into either anterior or posterior positions of the trunk neural fold (stage 16). The number of labeled cells on the lateral route was fourfold higher for anterior than posterior injections. While anterior injections contributed labeled cells to the lateral line primordium (Northcutt et al., 1994), posterior injections failed to do so. Thus, labeled cells migrating from an injected anterior neural fold may contain neuronal (ganglionic) and glial precursors for the lateral line in addition to few pigment cells.

Further evidence for this assumption was corroborated by a qualitative characterization of the labeled cells distributed, either after injecting DiI into anterior or posterior trunk neural fold as before (Figs. 4A, 5A) or by grafting GFP transgenic neural folds into white hosts (Fig. 4C–H). The latter experiments, in particular, left no doubt that midtrunk neural crest does not supply glial and neuronal cells for the lateral line. The results confirmed the view that these cells appear to arise from anterior trunk neural fold levels behind the otic placode (Northcutt et al., 1994), which migrate posteriorly together with or in the vicinity of lateral line primordia/lateral line nerves.

Neural Crest Migrates First Laterally and Then Medially

DiI labeling of premigratory stages (stage 25) and early migratory stages (stage 33) of the trunk neural crest in axolotl revealed that neural crest cells in wild type embryos migrate first dorsolaterally and then ventromedially and are more numerous on the former route. In contrast, dorsolateral migration is restricted in the white mutant whereas ventromedial migration (Fig. 6F) resembles that in the wild type (Figs. 6C, 7C). At late-migratory stages (stage 35), there was still moderate migration of neural crest cells along the dorsolateral pathway, but most labeled cells moved along the ventromedial pathway or into the dorsal fin.

Our results contrast with previous findings by Vogel and Model (1977), who, based on (^{3}H)thymidine-labeling of neural folds, suggested that ventromedial migration of presumptive neurons and glial cells at stage 34 had advanced similar to pigment cell migration along the dorsolateral pathway (staging according to Schreckenberg and Jacobson, 1975; stage 34 is equivalent to stage 32 in the table of Bordzilovskaya et al., 1989; see Epperlein and Junginger, 1982, for comparison). Similarly, we found no evidence for migration of neural crest cells through the somite (stage 30) as Vogel and Model (1977).

The early migration of axolotl neural crest along the dorsolateral pathway contrasts with that in several other amniotes/anamniotes. Dorsolaterally, neural crest migration is about 300–400 μm advanced compared to medial migration. Dorsolateral cells need about 1 day to cover such a distance (Fig. 6B,C). One possible explanation for this earlier lateral migration is that presumptive pigment cells may be committed to their fate prior to initiation of migration (Epperlein and Löfberg, 1984) and that this prespecification is essential for migrating dorsolaterally. Thus, pigment cells in axolotl might follow the "phenotype-directed model" for neural crest migration as proposed for pigment cell precursors in the chick (Erickson and Reedy, 1998). However, results by Wakamatsu et al. (1998) argue against such a general prespecification of the neural crest. Chicken neural crest cells migrating dorsolaterally appear initially to include neuronal cells in addition to melanocytes and these are later eliminated by apoptosis. Similarly, we observed glial cells and neurons migrating dorsolaterally at least from anterior trunk neural folds in the axolotl.

The migration pathways followed by neural crest cells in axolotl are surprisingly similar to those previously described in the chick. For example, axolotl neural crest cells migrate laterally between the epidermis and a narrow dermomyotome (referred to as "dermatome" in Sobkow et al., 2006; Epperlein et al., unpublished data), comparable to the dorsolateral migration between the epidermis and dermomyotome observed in chick (Erickson et al., 1992). Also as in chick (Bronner-Fraser, 1986), medial neural crest cells in axolotl migrate through a sclerotome (Fig. 9B), albeit very narrow.

It is uncertain whether an earlier lateral migration of pigment cells is an evolutionary advantage for axolotl embryos. Pigment cells might provide the larvae with disguise. On the other hand, Rohon-Beard cells constitute a primary sensory system in axolotl, perhaps making formation of derivatives along the ventromedial pathway less crucial at early times in development.

Dual Neural Crest and Somite Origin for the Mesenchyme of the Dorsal Fin

Classically, the mesenchyme of the dorsal fin in lower vertebrates has been assumed to be neural crest derived. The dorsal fin epidermis is induced by the neural crest and removal of the neural folds leads to the absence of the dorsal fin (Raven, 1931; DuShane, 1935; Bodenstein, 1952). Following injections of DiI into dorsal mid trunks of axolotl tailbuds at stage 25 (Fig. 6A–F) and 33 (Fig. 7A–F), we observed only a few labeled cells in the developing dorsal fin after 2 days (stages 36–37). When DiI was injected into larvae at stage 35 (Fig. 8A–C), the dorsal fin mesenchyme was abundantly labeled after 4 days (stage 40). Although DiI injections hit only a fraction of all neural crest cells present at a certain stage, we found the number of labeled mesenchymal cells 2 days after an injection (stages 36–37) surprisingly small.

By grafting single GFP+ labeled somites into unlabeled hosts, we recently found that the dermomyotome participates in early stages of mesenchyme formation of the dorsal fin (Sobkow et al., 2006). Thus, in axolotl neural crest appears to make only a limited contribution to the mesenchyme of the developing dorsal fin at early larval stages; later its contribution appears to increase as shown by DiI labeling of dorsal trunks at stage 35 (Fig. 8B,C).

EXPERIMENTAL PROCEDURES

Embryos

Wild-type (dark, D/-) and white mutant (dd) embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained from the axolotl colony in Bloomington, Indiana, or from our own colonies in Dresden. GFP embryos were spawned from a β-actin promoter-driven GFP germline transgenic animal that had been produced by plasmid injection (Sobkow et al., 2006). Embryos were kept in tap water at room temperature or at 7–8°C and were staged according to the normal table of Bordzilovskaya et al. (1989). Before being used for grafting, embryos were washed thoroughly with tap water and sterile Steinberg solution (Steinberg, 1957) containing antibiotics (Antibiotic-Antimycotic, Gibco) and then decapsulated mechanically.

DiI-Injections

CellTracker CM-DiI (C-7000; Molecular Probes, Eugene, OR) was dissolved in absolute ethanol to a concentration of 1 mg/ml and further diluted in 4 or 9 parts of 10% sucrose in water just before injection. For injection, either glass micropipettes were used that were backfilled with DiI solution and attached to a Parker Hannifin Corporation Picospritzer II assembly or glass capillaries that were connected to a IM 300 injector (Narshige). DiI was injected into embryos as indicated below and monitored with different stereo and epifluorescence microscopes.

Trunk neural fold/neural tube.

DiI injections were made into three different rostrocaudal locations of the left lateral or medial trunk neural fold of dark neurulae (stages 15–19) to determine whether regional differences exist in migratory potential and cell fate at different mediolateral positions within the neural folds (Figs. 1, 2A–F; Table 1). Injected embryos were fixed 2 or 4 days post-injection and vibratomesectioned (transverse sections, see below).

DiI was injected into one middle anterior or posterior location of the left trunk neural fold of dark and white neurulae (stage 16) to examine whether and in which way trunk neural crest in these sites contributes to lateral line nerves (Figs. 1, 3A–C, 4, 5; Table 1). The anterior injection site was localized at the level of the prospective 2nd somite, the posterior one in the most posterior positon of the left fold at the level of still unsegmented mesoderm. White injected embryos were fixed 2, 4, and 5 days post-injection, dark ones after 4 and 5 days and mostly investigated as whole mounts. Some embryos fixed 4 days after the injection were vibratomesectioned (transverse sections, see below). If positions or identity of labeled cells were unclear, sections were counterstained with anti-fibronectin to visualize tissue borders.

DiI injections were made into three different rostrocaudal locations of the dorsal midtrunk neural tube of dark and white embryos at stages 25, 33, and 35 to examine whether there were different migratory potentials of trunk neural crest cells (Figs. 6A–F, 7A–F, 8A–C; Table 1). Embryos were fixed at different times post-injection and vibratomesectioned (transverse sections, see below).

Quantification of DiI-labeling and controls.

Quantification. For a quantitative evaluation of the distribution of DiI-labeled neural crest cells, approximately 20 transverse sections (100 μm thick) were cut through the trunk of DiI-labeled embryos until stage 34 and 30 until stage 39/40. Five specimens were sectioned per group. The labeled cells in sections through all specimens of a group were counted and mirror-imaged to the left side of a summary section that represents the group (Figs. 2,3,6–8, Table 1).

Controls. The distribution of melanophores in whole white larvae was determined between gills and cloaca at stages 36 and 42 in order to obtain a measure that would allow comparison with the migration of Di-labeleld cells in sectioned white embryos. At stage 36, six larvae were used and subjected to Dopa treatment (Epperlein and Löfberg, 1984) in order to enhance the visibility of melanin pigment in melanophores. At stage 42 when melanophores are clearly visible, we used 10 larvae. In both stages, we found a mean value of ~30 melanophores in the dorsolateral trunk.

FITC- and Rhodamine Dextran Injections

Dark axolotl embryos at the two- to four-cell stage were kept at room temperature in a deepening of an agar dish filled with 5% ficoll in 1/10 Steinberg solution containing antibiotics. One blastomere was injected with FITC- or rhodamine dextran (Molecular Probes; 50 mg/ml Steinberg solution; 50 nl/injection) through the egg membrane using an IM 300 injector (Narshige). After one day, the embryos were transferred into normal strength saline. At the neurula stage parts of fluorescent trunk neural folds were used for grafting.

Axolotl Embryos Expressing Green Fluorescent Protein (GFP)

White axolotl embryos transgenic for GFP were produced by mating a male GFP transgenic with a normal white female (Sobkow et al., 2006). β-actin promoter-driven GFP expression first became clearly visible in the neurula stage. Trunk neural fold material was grafted into white donors in order to investigate a possible contribution of neural crest derivatives to the lateral line system (see below).

Neural Fold Grafting Experiments

Head and trunk neural fold material pre- or postlabeled in different ways

(see below) from sterile neurulae (stages 15–17) was grafted isochronically into orthotopic locations of dark or white hosts. Before the operations, host neurulae were placed into a deepening of an agar dish filled with cold Steinberg saline. Grafting was performed under sterile conditions using tungsten needles.

Whole left trunk neural folds were transplanted orthotopically from dark FITC- dextran (n=5) or rhodamine dextran (n=2) labeled neurulae into dark unlabeled hosts or from white GFP+ transgenic neurulae (n=6) into white unlabeled hosts. In addition small left midtrunk (n=5) or posterior trunk neural fold fragments (n=5) were grafted orthotopically from white GFP+ donors into white hosts. In some cases, both trunk neural folds of dark embryos were removed and the left fold replaced with a FITC- and the right one with a rhodamine dextran–labeleld neural fold fragment. Finally, both trunk neural folds of white hosts were removed and only the right fold replaced with a GFP+ fold (n=10). Donor and hosts were at stages 15–17. After 2–4 days, migration of labeled trunk neural crest cells through somites (FITC-label) or contribution to the lateral line (GFP+ transgenic) was analyzed.

Fixation, Embedding, and Sectioning

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight and kept either in fixative for a later use in histology or were transferred directly into 100% methanol and kept there at −20°C before use in in situ hybridization. Some individuals were examined in an epifluorescence microscope for the distribution of DiI- or GFP-labeled cells. For histology, specimens were washed in PBS for 1 h, sectioned with a vibratome (vibratome series 1000 sectioning system, Ted Pella, Inc.) in transverse or horizontal planes at 100 μm thickness, stained as indicated below, and examined with an epifluorescence microscope. Some PFA-fixed embryos were embedded into paraffin for microwave-treatment of transverse sections (5 μm), which were used for immunohistochemical demonstration of lateral line nerves and glia.

Histochemistry/Histology/ Immunostaining

White larvae at stage 35/36 were subjected to the Dopa reaction (Epperlein and Löfberg, 1984) in order to enhance the visibilitiy of melanophores and facilitate their exact counting. The numbers of melanophores were needed for assessing the numbers of DiI labeled cells in white embryos.

Some vibratome sections (100 μm) were cut through PFA-fixed DiI-, FITC- or rhodamine dextran-, or GFP-labeled embryos and counterstained with an anti-fibronectin antibody to visualize tissue borders. Sections were then incubated with a polyclonal anti-fibronectin antibody (Dako, Hamburg, Germany) followed by a FITC- or Cy3-conjugated goat-anti-rabbit secondary antibody (Dianova, Hamburg, Germany). Some sections were stained with dapi (0.1–1 μg/ml in PBS) to mark cell nuclei. Vibratome sections through larvae that had received one focal DiI injection into the left anterior trunk neural fold were also stained for anti-tubulin or anti-GFAP to reveal lateral line nerves or glia, respectively. For anti-tubulin staining, a primary monoclonal antibody (W. Halfter, Pittsburgh) was used followed by a FITC-conjugated goat-anti-mouse secondary antibody (Dianova). Staining of glia was carried out with a primary polyclonal GFAP-antibody (Dako) followed by a FITC-conjugated goat-anti-rabbit secondary antibody (Dako). Because staining with fluorescence techniques was sometimes weak (anti-tubulin) or had high background (anti-GFAP), some unlabeled control larvae of a similar age as DiI-injected specimens were embedded into paraffin. Microwaved transverse sections (5 μm) were stained with the same primary antibodies but with biotinylated secondary antibodies and tertiary avidin-peroxidase complexes (Vectastain ABC-Kit).

In Situ Hybridization

Axolotl Snail riboprobes were prepared as previously described (Epperlein et al., 2000). In situ hybridization was performed on albino and wild-type axolotl embryos as described by Henrique et al. (1995), with the addition of an overnight wash in MAB-T. In situ–hybridized wild-type embryos were washed for 2 hours in several changes of PBS and bleached for 1–2 hr in 1% H_2O_2/5% formamide/.5×SSC under intense illumination to reveal hybridization

Laser Scanning Microscopy

Horizontal vibratome sections (100 μm) through the dorsal trunk of DiI-injected embryos (stage 33–34) were also investigated in the laser-scanning microscope (Zeiss; ×10 objective) to gain a better resolution of Rohon Beard cells and their nerve fibres.

Image Analysis

Whole embryos and sections were analyzed with an epifluorescence microscope and images were captured with a Spot digital camera (Visitron). Using Adobe Photoshop software, the contrast and brightness of captured images were optimized, and bright field images of whole embryos were combined with corresponding fluorescence images. Separate images were captured from sectioned material labeled with DiI, fluorescent-dextrans or GFP (neural crest cells), anti-fibronectin (tissue borders), anti-tubulin (lateral line nerve), anti-GFAP (glial cells), and dapi (cell nuclei), before combinations of two or three of them were superimposed into a single image.

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