Neural crest contributions to the lamprey head

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SUMMARY

The neural crest is a vertebrate-specific cell population that contributes to the facial skeleton and other derivatives. We have performed focal DiI injection into the cranial neural tube of the developing lamprey in order to follow the migratory pathways of discrete groups of cells from origin to destination and to compare neural crest migratory pathways in a basal vertebrate to those of gnathostomes. The results show that the general pathways of cranial neural crest migration are conserved throughout the vertebrates, with cells migrating in streams analogous to the mandibular and hyoid streams. Caudal branchial neural crest cells migrate ventrally as a sheet of cells from the hindbrain and super-pharyngeal region of the neural tube and form a cylinder surrounding a core of mesoderm in each pharyngeal arch, similar to that seen in zebrafish and axolotl. In addition to these similarities, we also uncovered important differences. Migration into the

presumptive caudal branchial arches of the lamprey involves both rostral and caudal movements of neural crest cells that have not been described in gnathostomes, suggesting that barriers that constrain rostrocaudal movement of cranial neural crest cells may have arisen after the agnathan/gnathostome split. Accordingly, neural crest cells from a single axial level contributed to multiple arches and there was extensive mixing between populations. There was no apparent filling of neural crest derivatives in a ventral-to-dorsal order, as has been observed in higher vertebrates, nor did we find evidence of a neural crest contribution to cranial sensory ganglia. These results suggest that migratory constraints and additional neural crest derivatives arose later in gnathostome evolution.

Key words: Cranial neural crest, Lamprey, DiI

INTRODUCTION

As the most basal extant vertebrate, the lamprey is in a critical position to provide important insights into our understanding of the evolution of vertebrate traits. Two cell populations that distinguish vertebrates from cephalochordates, closely related non-vertebrate chordates, are neural crest cells and ectodermal placodes. These are both vertebrate-specific populations of cells that arise at the border of the neural plate and epidermis. Placodes invaginate from the surface ectoderm to contribute to the cranial sensory ganglia and sense organs of vertebrates. Neural crest cells arise during neurulation and emigrate from the dorsal region of the neural tube. They migrate extensively along stereotypic pathways and contribute to numerous derivatives, including pigment cells, autonomic and sensory ganglia, and most of the facial skeleton (LeDouarin and Kalcheim, 1999). The migratory patterns of neural crest cells have been well characterized in a few model vertebrates but far less is known about their evolutionary history (Gans and Northcutt, 1983; Baker and Bronner-Fraser, 1997; Hall, 1999; Hall, 2000; Wada, 2001).

In an early attempt to study neural crest derivatives in the basal-most vertebrate, ablation experiments were performed in the lamprey in which the presumptive neural crest was extirpated resulting in lack of cartilage in the branchial arches (Langille and Hall, 1988). However, this approach cannot

distinguish whether neural crest cells themselves contribute to the cartilage of the branchial arches or if interactions between these cells and other tissues are required for cartilage formation. Scanning electron microscopy (SEM) has also provided important insight into the pathways of cranial neural crest migration in the Japanese lamprey, *Lampetra japonica* (Horigome et al., 1999; Kuratani et al., 1999; Kuratani et al., 2001) but does not allow definitive distinction of neural crest cells from other mesenchymal populations. Furthermore, it is not possible to determine the sites of origin or migratory pathways at particular axial levels.

In order to unambiguously follow neural crest migratory pathways in lamprey, it is necessary to perform vital dye labeling of small groups of cells in living embryos, thus allowing one to identify and follow cells from discrete sites of origin to their final destinations. We have undertaken a detailed study of migratory pathways of the cranial neural crest during development in the lamprey, *Petromyzon marinus*, using the lipophilic dye DiI to label small populations in the dorsal neural tube. We have focused on the cranial neural crest because migratory pathways at this axial level are well documented in a number of species, allowing a comparative analysis of these cells and their behavior between agnathans and gnathostomes. We have examined both the superficial migration of neural crest cells and their deeper, internal migration within the branchial arches. The results show that

the general patterns of cranial neural crest migration from dorsal neural tube to ventral branchial arches are conserved throughout vertebrates, with cells migrating in identifiable streams. In particular, migration at the levels of the midbrain and most rostral hindbrain into the first branchial arch and midregion of the hindbrain into the second branchial arch appear very similar between multiple species. However, interesting differences are also observed between agnathans and gnathostomes, particularly at the level of the mid to caudal hindbrain. Neural crest cells arising from the mid-hindbrain displayed extensive caudal migration, and those originating from the more caudal hindbrain migrated in both rostral and caudal directions. Furthermore, neural crest cells from different axial levels populated the same arch. Importantly, no neural crest contribution to cranial sensory ganglia was noted, suggesting they may be entirely placode derived in the lamprey. The results suggest that there are fewer constraints to rostrocaudal migration of cranial neural crest cells in lamprey and that such constraints as well as additional neural crest derivatives may have arisen later in gnathostomes.

MATERIALS AND METHODS

Embryo preparation and collection

The sea lamprey, *Petromyzon marinus*, is anadromous and spawns in many tributary streams throughout the Great Lakes. Spawning season is usually in early to mid-June and lasts for 2-3 weeks. Adult lamprey were collected by hand from streams in northern Michigan as they migrated up the streams to breed. They were maintained in the laboratory at Hammond Bay Biological Station, Millersburg, MI in a fresh water flow-through system supplied with lake water from Lake Huron and under ambient light. Eggs stripped from mature female adults were fertilized with sperm from adult males in the laboratory. Eggs and subsequent embryos and larvae were maintained in aerated lake water in a constant temperature bath held at 18°C.

Embryos were staged according to the staging criteria established by Tahara (Tahara, 1988) for *Lampetra reissneri*. Developmental stages between *Lampetra* and *Petromyzon* appear similar in morphology. *P. marinus* zygotes (0-6 hours, stage 2) are 1 mm in size and go through gastrulation by 64-108 hours (stages 12-16). The embryos hatch between days 10 and 13, and are 3-5 mm in length (stages 24-25).

DiI labeling

Fertilization membranes were removed from stage 21 embryos with sharpened forceps. Embryos were then held in place, dorsal side upwards, in 1 mm depressions in an agarose-coated dish. DiI (CM-DiI, Molecular Probes, Eugene, OR) was dissolved in ethanol and diluted in 0.3 M sucrose to 0.5 µg/µl. Dilute DiI was backfilled into pulled glass electrodes and a single bolus was pressure-injected beneath the ectoderm at the dorsal midline of embryos (Fig. 1). Labeled embryos were allowed to develop to stage ~22-23 or to stage ~25-26 (Tahara, 1988) and fixed in 4% paraformaldehyde (PFA). Selected embryos were embedded in 5% agarose and vibratome sectioned (50 µm) to determine accurately cranial neural crest cell migratory pathways internally. Sections (10 µm) were cut from embryos embedded in Epon/Araldite. To determine the timing of late neural crest cell migration, demembranated stage 21 embryos were first allowed to develop 24 hours, to stage 22, then labeled and fixed at stage 25 as described above. Epifluorescence of embryos and sections was imaged using a Zeiss Axioskop 2 containing a rhodamine filter set. Images were collected using a Zeiss Axiocam HRc imaging system.

in situ hybridization

Pax2

Embryos used for in situ hybridization were fixed in MEMFA and held at –20°C in methanol for later use. Lamprey *Pax2* in situ hybridization was performed as described previously (McCauley and Bronner-Fraser, 2002).

AP2

AP2 in situ hybridization was performed as described by Meulemans and Bronner-Fraser (Meulemans and Bronner-Fraser, 2002).

Immunohistochemistry

Embryos fixed in 4% PFA and held in methanol were rehydrated into PBS, and blocked for 1 hour at room temperature in PBS containing 1% heat-inactivated goat serum and 1% BSA (PBS-SB), then treated with pre-adsorbed rabbit α-mouse Sox-10 (1/200; Chemicon) antibody (4°C, overnight). After four 15 minutes washes in PBS, embryos were incubated in alkaline phosphatase-conjugated goat α-rabbit secondary antibody (1/1000; 1 hour, room temperature) and washed in PBS for 2 hours with agitation. Following washes, embryos were developed in NBT/BCIP (Life Technologies) according to manufacturer's instructions. A pan-Dlx antibody (kindly provided by Dr J. Kohtz, from a construct by Dr G. Panganiban) was diluted 1/70 in PBS-SB as described above. DiI-labeled tissue was preincubated 1 hour (room temperature) in PBS-SB, then incubated at 4°C overnight, and rinsed for 3 hours in PBS. Secondary antibody incubation (goat α-rabbit-alkaline phophatase) followed by NBT/BCIP was performed as described above.

Neural crest cell ablation

The fertilization membranes were first removed from stage 21 embryos with sharpened forceps. Sharpened tungsten needles were then used to make a small longitudinal incision along the dorsal midline and neural crest cells were scraped from the region dorsal to the hindbrain. After the operation, embryos in lake water were held in agar-coated dishes at a constant 18°C and allowed to develop to stage 26, and then were fixed in 4% PFA. Embryos were discarded if the neural tube appeared to have been damaged in the operation. Out of 30 embryos operated, 12 survived that appeared to have a complete neural tube. These were chosen for subsequent observation.

RESULTS

Neurulation in the lamprey

In the lamprey embryo, the neural plate closes to form a neural rod by a folding process that begins around embryonic day 5 (stage 17) and proceeds through day 6 of development (stage 21). Neurulation is first evident at stage 17 when a flattened neural plate forms at the dorsal surface of the embryo. By stage 18, the midline of the neural plate forms a groove. At stage 20, the neural folds rise at the dorsal midline forming dual ridges that subsequently contact each other and fuse to form a solid neural rod (Tahara, 1988). Neural crest cell migation apparently begins at stage 20, and the presumptive branchial arch region is populated with neural crest cells by stage 22 (Horigome et al., 1999), embryonic day 7 in *P. marinus*, suggesting that the migratory process takes several days to complete.

DiI labeling of cranial neural tube reveals axial differences in cranial neural crest migratory patterns

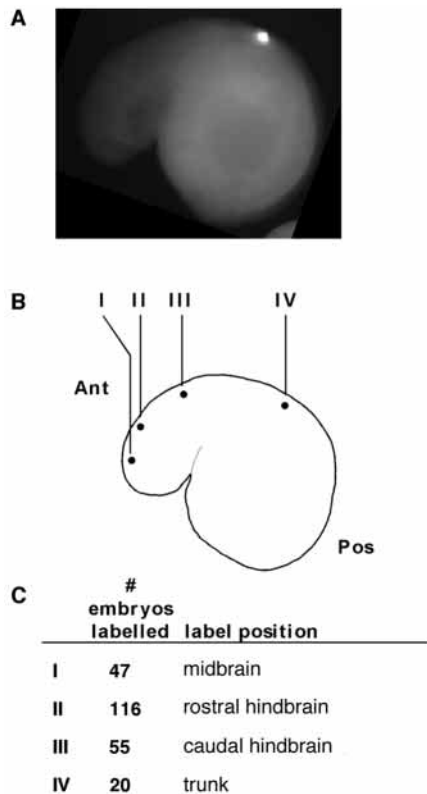In most species, neural crest cells begin their emigration from

Fig. 1. DiI-labeling in the lamprey embryo. Embryos were injected focally with a single bolus of DiI. (A) Embryo fixed immediately after injection to show initial position of labeled cells. (B) Individual embryos were labeled at one of the four positions indicated along the anteroposterior (AP) axis (I, midbrain; II, rostral hindbrain; III, caudal hindbrain; IV, rostral neural tube). (C) Number of embryos labeled at each position and initial positions of labeled cells determined retrospectively after fixation.

the neural tube around the time of neural tube closure. Therefore, we labeled discrete regions of the newly closed dorsal cranial neural tube of lamprey with focal injections of the lipophilic dye, DiI, in order to mark newly formed cranial neural crest cells. A single bolus of DiI was pressure injected beneath the ectoderm at the dorsal midline (Fig. 1A) at selected levels along the rostrocaudal axis (Fig. 1B). Embryos with label in more than one site were discarded. When fixed and examined with epi-illumination immediately after labeling, the focal DiI injections were confirmed to be discrete and to have labeled only a small region (Fig. 1A). For initial experiments, embryos were labeled at stage 21, shortly after neural tube formation, in order to mark early migrating neural crest cells. The migratory pattern of neural crest cells was followed as a function of time, from 2-6 days post-injection, and at different axial levels. The number of embryos examined after injection of DiI at each position along the neural axis is indicated in Fig. 1C.

Migration of neural crest cells arising from the midbrain, rostral hindbrain

Segmentation of the embryonic lamprey hindbrain is apparent only transiently (Kuratani et al., 1998a), making it difficult to find morphological markers indicating rostrocaudal position.
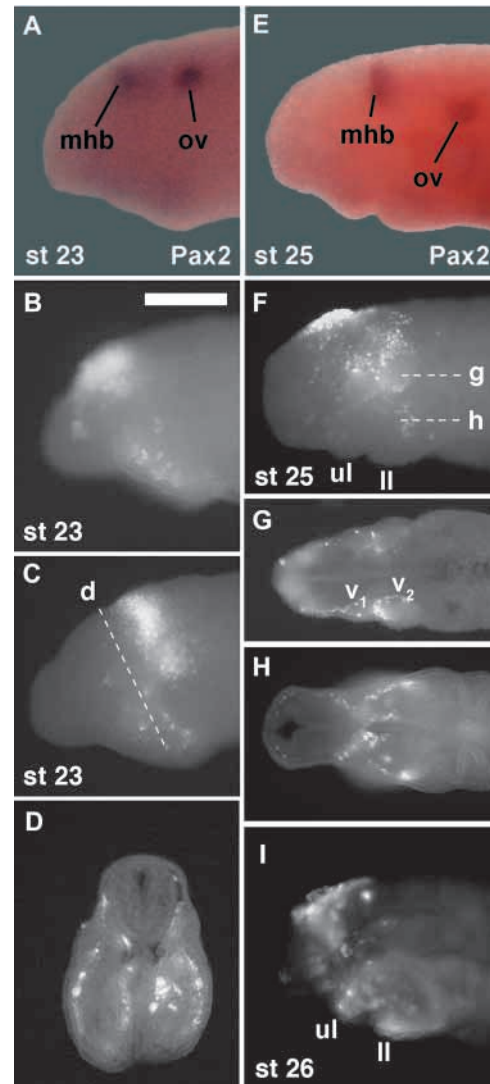

Fig. 2. *Pax2* expression and DiI-labeling of cranial neural crest cells at the midbrain and rostral-most hindbrain. (A) Lamprey *Pax2* expression at stage 23. *Pax2* is expressed at the mid-hindbrain boundary (mhb) and within the otic vesicle (ov). (B) DiI-labeled cells originating from the midbrain region have migrated into the first (mandibular) arch of a stage 23 embryo. (C) Cells originating from the rostral hindbrain have migrated into the mandibular arch. d indicates plane of section shown in D. (D) transverse section through mandibular arch of embryo shown in C in which labeled cells are positioned both medial and lateral to the mesodermal core. (E) *Pax2* expression continued in the mid-hindbrain boundary and otic vesicle at stage 25. (F) Stage 25 embryo labeled in the midbrain; DiI-labeled cells are found in the upper (ul) and lower (ll) lips; g,h indicate planes of section shown in G and H, respectively. (G) Horizontal section indicating labeled cells contributed to the ophthalmic (V_1) and maxillomandibular (V_2) lobes of the trigeminal ganglion. (H) Plane of section through developing lips indicates labeled cells present both medial and lateral to mesodermal core. (I) Stage 26 embryo in which cells originating from midbrain persist in the upper and lower lips. Scale bar: 200 μm.

Therefore, the site of initial DiI injections was determined by comparing labeled embryos with stage-matched companion embryos stained for lamprey *Pax2* expression (Fig. 2). Pax2

marks the mid-hindbrain boundary and the otic vesicle (McCauley and Bronner-Fraser, 2002), thus providing two positional markers for pinpointing the site of the injection.

DiI-labeling performed at the level of the midbrain (Fig. 2B) or immediately caudal to the mid-hindbrain boundary (Fig. 2C) produced marked cells that invaded the first (mandibular) arch. Examination of labeled embryos in wholemount showed migrating cells underneath the ectoderm en route to the mandibular arch. However, transverse sections through embryos fixed 2 days post-injection (stage 23) revealed that neural crest cells migrating to the mandibular arch migrated both subjacent to the ectoderm, as well as along a medial pathway to surround the mesoderm (Fig. 2D), as originally proposed by Damas (Damas, 1944). By stage 25, DiI-labeled cells originating from the midbrain were localized to both the upper and lower lips (Fig. 2F,H) and were localized both medial and lateral to the mesoderm at this time (Fig. 2H). Labeled neural crest cells persisted in the upper and lower lips at stage 26 (Fig. 2I).

Migration of neural crest cells arising from the mid-region of the hindbrain

We next examined neural crest migration from the hindbrain near the otic vesicle. This region probably corresponds to the fourth to fifth hindbrain rhombomere, though the unavailability of hindbrain molecular markers in the lamprey prevents precise identification of the rhombomere of origin. Labeled hindbrain neural crest cells were found within the second (hyoid) arch at stages 23 (Fig. 3A) and 25 (Fig. 3F). Transverse sections through a stage 23 embryo showed that migration in the hyoid arch occurred in the lateral region subjacent to the ectoderm (Fig. 3E). In horizontal sections through stage 23 embryos, neural crest cells appeared to envelop the mesoderm of the hyoid arch (Fig. 3C) both medially and laterally. Unexpectedly, we found that in numerous cases, cells had migrated caudally along the entire length of the presumptive pharyngeal region (Fig. 3B,C). At the time of fixation (stage 23), the mandibular and hyoid regions had formed discrete arches as assessed by distinct endodermal outpocketings that separate these arches and partition labeled cells. However, the more caudal presumptive pharyngeal arch-forming region contained a column of labeled cells that extended caudally beyond the posterior limit of the developing pharynx (Fig. 3C). Outpocketing of the caudal endoderm into pharyngeal pouches had not begun in this region 48 hours after migratory cells were labeled (stage 23). A change in the distribution of labeled neural crest cells within the arches was observed by stage 25. In contrast to cells enveloping the mesodermal core both medially and laterally, at this later stage, most labeled cells were now restricted only to the region lateral to the mesoderm and adjacent to the ectoderm (Fig. 3G).
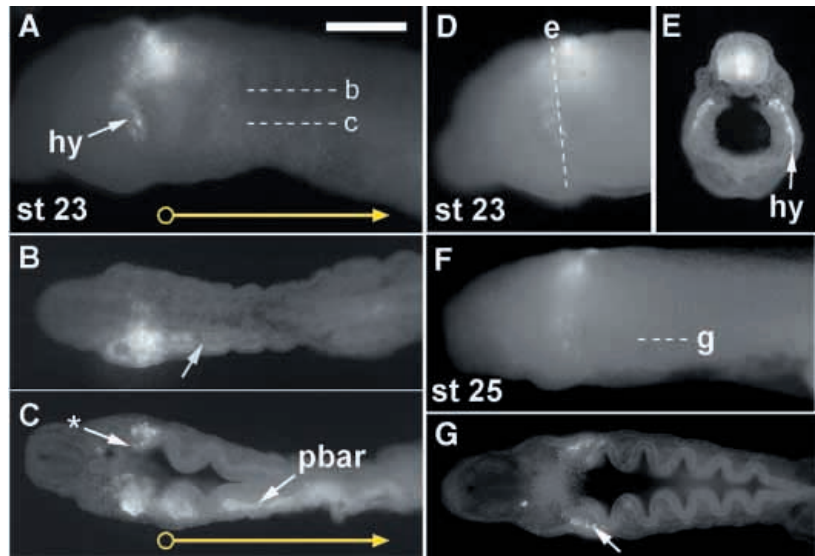

Fig. 3. Migration of cranial neural crest cells from hindbrain. (A) Lateral view of stage 23 embryo showing initial hindbrain position of DiI-labeled cells and migration into the hyoid arch (hy). b and c indicate horizontal planes of section shown in B and C, respectively. (B) Migration of cells caudal to the initial site of labeling in the dorsal pharyngeal region. Arrow indicates position of labeled cells caudal to the original position of the DiI label. (C) Ventral section through developing branchial arches. Two most rostral arches containing labeled cells, the hyoid and first branchial arch, have been segregated by the outpocketing of the pharyngeal endoderm in these regions. Labeled cells in the caudal presumptive branchial arch region (pbar) are in a contiguous stream. DiI-labeled cells surround the mesodermal core of the hyoid arch (arrow pointing from white asterisk). (D) Lateral view of stage 23 embryo shown in cross-section in E. (E) Cross-section through hyoid arch of D at position marked e. Labeled neural crest cells have migrated ventrally beneath the ectoderm. (F) Lateral view of stage 25 embryo labeled in the hindbrain region. Neural crest cells have migrated ventrally into the hyoid arch. g indicates plane of section shown in G. (G) Horizontal section of stage 25 embryo shows neural crest cells in the hyoid arch are located in a lateral position beneath the ectoderm (arrow). A few cells can still be seen adjacent to the endoderm. Yellow circle indicates the initial position of the DiI injection. Yellow arrow indicates the direction and distance of migration along the rostrocaudal axis. Scale bar: 200 μm.

Migration of neural crest cells arising in the caudal hindbrain and rostral trunk

Neural crest cells originating from caudal hindbrain regions migrated ventrally to populate the caudal branchial arches (Figs 4, 5) but demonstrated a variety of migratory patterns en route to the caudal pharyngeal region. In some embryos, caudal hindbrain neural crest cells were later found rostral to the site of injection, suggesting a rostral movement (compare Fig. 4A with 4D; see also Fig. 5D,E). In other embryos, labeled cells moved ventrally but not rostrally or caudally (Fig. 4E-G). Still other embryos demonstrated a caudally directed migration (Fig. 5) similar to that observed in neural crest cells emigrating from more rostral hindbrain regions (Fig. 3C). We also observed embryos with both rostrally and caudally directed neural crest migration (Fig. 5D,E).

In embryos fixed at stage 23, the caudal migration of cells was not yet impeded by formation of pharyngeal pouches (Fig. 5E). Later (stage 25), cells that had migrated in a more caudal direction, were separated into the caudal branchial arches (Fig. 5B). Neural crest cells in the embryo shown in Fig. 5D,E
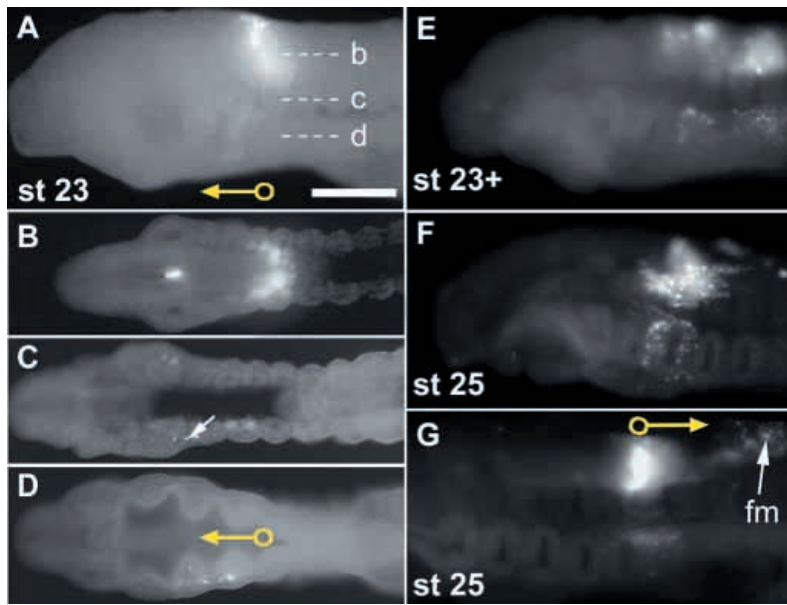
Fig. 4. Neural crest cell migration at hindbrain and rostral trunk levels. (A) Stage 23 embryo in which cells originating from the caudal hindbrain region are located rostral to the site of injection within the rostral branchial arches; b-d indicate planes of section in B-D, respectively. (B-D) Neural crest cells from the mid hindbrain region have migrated ventrally and rostrally to populate the rostral branchial arches. (E) Late stage 23 embryo labeled at the hindbrain and rostral trunk neural tube level; by stage 23, labeled cells have migrated ventrally towards the caudal presumptive branchial arches. (F) At stage 25, labeled cells from the caudal hindbrain region are located throughout two branchial arches, having completely filled both arches along the dorsoventral axis. (G) Neural crest cells originating from the caudal hindbrain to rostral trunk level migrated ventrally toward the most caudal presumptive arch and migrated dorsocaudally into the dorsal fin. fm, fin mesenchyme; white arrows, final position of labeled cells; yellow circle, initial position of DiI; yellow arrow, direction and distance of migration away from initial site. Scale bar: 200 µm.

cartilages form medial to the mesodermal core. By contrast, Meulemans and Bronner-Fraser (Meulemans and Bronner-Fraser, 2002) found that *AP2* is expressed in lamprey cranial neural crest within each of the arches surrounding the mesodermal core.

To resolve this discrepancy, we carefully examined sections through the arches as a function of time and also compared the migration of DiI-labeled cells with Dlx expression in the branchial arches (Figs 4, 5). Consistent with the findings of Damas (Damas, 1944) and Meulemans and Bronner-Fraser (Meulemans and Bronner-Fraser, 2002), we found that DiI-labeled neural crest cells migrating into the presumptive branchial arches were positioned in a ring completely surrounding the mesodermal core in each of the branchial arches (Fig. 4D, Fig. 5B,E). To determine accurately the neural crest identity of cells in DiI-labeled embryos, we compared the location of DiI-labeled cells with Dlx protein distribution within an embryo. Interestingly, in our hands, a pan-Dlx antibody indicated the presence of Dlx protein throughout the arches both lateral and medial to the mesoderm in an overlapping pattern with DiI-labeled cells (compare Fig. 5B with 5C, see also Fig. 5F). This differs from the report of Neidert et al. (Neidert et al., 2001) who showed *Dlx* expression only lateral to the mesoderm in the lamprey. Taken together, these findings suggest that medial migration of neural crest cells within the caudal branchial arches is not a new feature of gnathostomes (Kimmel et al., 2001), but instead, the position of the branchial arch cartilages depends on a mechanism independent of neural crest cell migration.

migrated rostral to the site of injection, as well as in a caudal direction. In the more rostral position, these cells became segregated by the formation of endodermal pouches. However, at more caudal positions, migratory cells were still in a continuous stream extending to the posterior limit of the presumptive pharynx. Interestingly, migration in the rostral direction did not appear to be as extensive as the caudal movement of the cranial neural crest cells either originating from the rostral (Fig. 3) or the caudal (Fig. 5) hindbrain.

Migration of neural crest cells medial to the branchial arch mesoderm

Ambiguity exists in the literature regarding patterns of neural crest migration into the branchial arches of the lamprey. Damas (Damas, 1944) reported ectomescenchymal cells between the mesoderm and endoderm within the arches. However, Neidert et al. (Neidert et al., 2001) found lamprey Dlx genes expressed in the neural crest laterally between the ectoderm and a central core of mesoderm within each arch. Kimmel et al. (Kimmel et al., 2001) suggested that in lamprey, neural crest cells involved in the formation of branchial arch cartilages may migrate only lateral to the mesoderm in the caudal branchial arches, in contrast to gnathostomes, where neural crest cells completely surround the mesoderm (Miller et al., 2000), and branchial arch

Later DiI labeling suggests little spatiotemporal changes in migratory pattern

In higher vertebrates, DiI labeling performed at progressively later stages in chick and mouse (Serbedzija et al., 1989; Serbedzija et al., 1990) has revealed that neural crest cells fill their derivatives in a ventral-to-dorsal order, such that the earliest migrating cells populate the most ventral derivatives. To test whether there was an orderly contribution of neural crest derivatives in lamprey, DiI labeling was performed at progressively later times. After labeling at stage 22, neural crest cells originating from hindbrain (Fig. 6B) or rostral spinal cord regions (Fig. 6C; compare to Fig. 6A) continued to undergo ventral migration toward the pharyngeal arches and at stage 25 were distributed similarly to those in embryos labeled at earlier stages (see above). In two cases of labeling hindbrain neural crest cells, we found extensive migration of cells into caudal branchial arches (Fig. 6B). In addition to ventral migration, caudal movement of cells appeared to continue at these stages, with labeled migratory cells apparent in arches caudal to the initial injection site (Fig. 6B). This suggests that rostrocaudal migration of neural crest cells continued even in late stages of migration. These results suggest that cranial neural crest cells in lamprey do not fill their derivatives in a ventral to dorsal order as they do in gnathostomes. Rather, both

Fig. 5. Rostrocaudal movement of cells originating from the hindbrain. (A) Lateral view of stage 25 embryo. Neural crest cells populate arches ventral and caudal to the site of injection. b indicates horizontal plane of section in B in which neural crest (nc) cells are also found medial to the mesoderm (m). (C) Pan-Dlx immunolabel of section shown in B. Black arrow indicates Dlx expression co-localizes with DiI labeled cells surrounding the mesodermal core. (D) Stage 23 embryo in which neural crest cells labeled at the hindbrain level have migrated ventrally in both rostral and caudal directions. e indicates plane of section in E, in which rostal labeled cells are segregated into arches while caudal cells are still in a continuous stream; neural crest cells are found medial to the mesodermal core. (F) Section (10 μm) of boxed region in C; arrowhead indicates Dlx positive cells excluded from mesodermal core. Yellow circle, initial position of DiI dorsally; yellow arrows, direction and distance of migration of labeled cells. Scale bars: in B, 200 μm for A-C; in D, 200 μm for D,E.
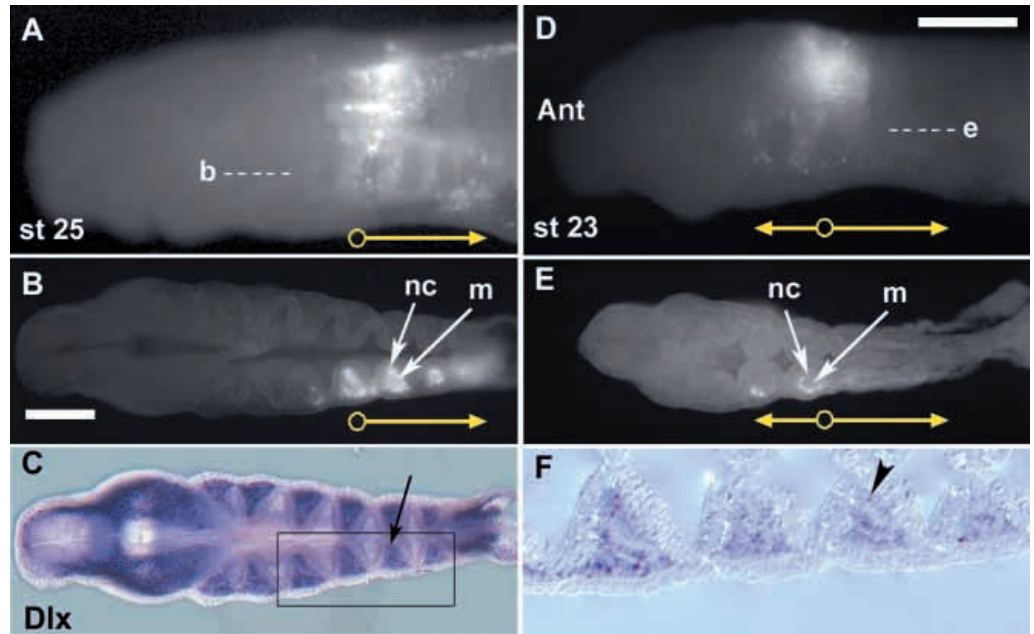
ventral and caudal migration appear to continue in cells that emigrate from the neural tube at late times.

Neural crest cells originating at the level of the rostral trunk neural tube of stage 22 embryos migrated ventrally, but only into the superbranchial region just dorsal to the caudal branchial arches (Fig. 6C). Labeled cells did not appear to populate the most ventral region of these arches, though migration toward the pharyngeal arches indicated that ventral migration still occurred even at this later stage at the level of the trunk. Caudal migration into the dorsal fin was also seen in these embryos (Fig. 6C; see also Fig. 4G), as was suggested by Newth (Newth, 1956). Attempts to label the midbrain neural crest at these late stages were unsuccessful (data not shown) though we cannot rule out the possibility that emigration at these axial levels may still continue at this time. However, SEM studies on stage 22 embryos of *Lampetra japonica* (Horigome et al., 1999) suggest that neural crest cells are no longer present above the dorsal midline by these later stages.

Stage 23 embryos were also injected in the caudal hindbrain region but in no case did we observe any migration away from the labeled site. This suggests that cranial neural crest cell emigration from the dorsal midline is completed by this stage (data not shown).

Ablation of the neural folds supports neural crest cell requirement for melanocytes and dorsal fin

The DiI-labeling results above suggest that neural crest cells originating from the caudal hindbrain and rostral trunk contribute to the dorsal fin. To test this further, we removed a small region of the neural crest at the hindbrain level in 30 stage 21 embryos. This neural crest removal resulted in reduction of the dorsal fin (Fig. 7) and a concomitant loss of pigmentation in the affected area in the 12 surviving embryos

fixed at stage 25 (Fig. 7B) when compared with control embryos (Fig. 7A).

Both DiI-labeling and Sox10 staining suggest that lamprey neural crest do not contribute to cranial sensory ganglia

Although many injections into stage 21 embryos selectively labeled the dorsal neural tube, in other cases both the dorsal neural tube and overlying ectoderm were labeled. Interestingly, we found that cranial sensory ganglia only contained DiI-labeled cells in those embryos in which the ectoderm was labeled (Fig. 8A,B; see also Fig. 2F,G). This correlation between ectodermal and ganglionic labeling suggests that the cranial sensory ganglia in lamprey may solely derive from ectodermal placodes. By contrast, in higher vertebrates, many of these ganglia receive a dual contribution from the neural crest and the placodes.

To explore this relationship further, we used an antibody against Sox10 as a neural crest marker in lamprey. This transcription factor is expressed by migrating neural crest cells in numerous species, including rodent and chick. (Kuhlbrodt et al., 1998; Cheng et al., 2000). We found that a mouse Sox10 antibody recognized migratory neural crest cells in the lamprey (Fig. 8C-F), similar to the patterns observed with this same antibody in bird embryos (Cheng et al., 2000). Interestingly, Sox10 positive cells appeared to be excluded from the cranial ganglia (Fig. 8D,E) though other Sox10-positive cells were localized more ventrally in the branchial arches (Fig. 8F). Taken together with our DiI labeling results, this raises the intriguing possibility that the cranial sensory ganglia do not receive a neural crest contribution in lamprey. In fact, no labeled neural crest cells (with either DiI or Sox10) were observed in any ganglionic structures in lamprey, suggesting that neural crest-derived
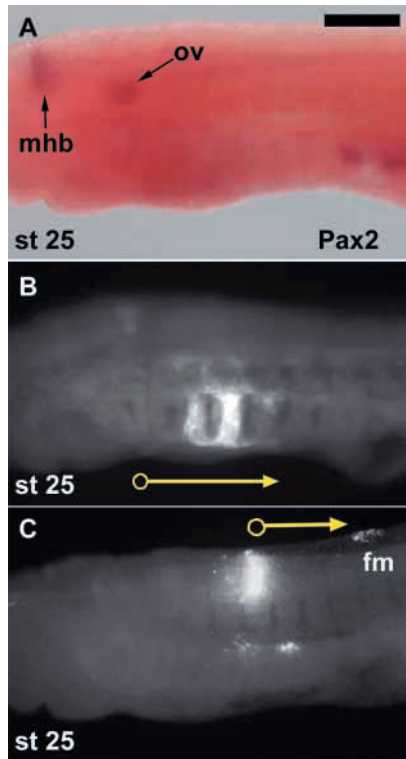
Fig. 6. Neural crest cells labeled at stage 22 and fixed for observation at stage 25. (A) *Pax2* expression at stage 25. Expression at the mid-hindbrain boundary (mhb) and the otic vesicle (ov) is used to indicate the position of the DiI-labeled cells; (B) Embryo labeled at the hindbrain region near the otic vesicle. At stage 25, labeled cells are found throughout mid-branchial arches; (C) Embryo labeled at the caudal hindbrain region. Cells have migrated ventrally to populate the dorsalmost portion of the caudal branchial arches. Cells have also migrated from the site of origin caudally into the dorsal fin. fm, fin mesenchyme; mhb, mid-hindbrain boundary; ov, otic vesicle; yellow circle, initial position of DiI; yellow arrow, direction and distance of migration of labeled cells along the rostrocaudal axis. Scale bar: 200 µm.

cranial ganglia may be a character state specific to gnathostomes.

DISCUSSION

Despite the critical position of agnathans in vertebrate evolution, relatively little is known about the contribution of cranial neural crest to the branchial arches and cranial skeleton (Newth, 1951; Newth, 1956; Langille and Hall, 1988; Horigome et al., 1999). Our current knowledge of cranial neural crest cell derivatives in the lamprey derives from a series of grafting experiments (Newth, 1951; Newth, 1956) and a set of extirpation experiments that demonstrated the contribution of cranial neural crest to trabeculae and branchial arch cartilage (Langille and Hall, 1988). Scanning electron microscopy has also been used to examine neural crest development in *Lampetra japonica* (Horigome et al., 1999). Although some preliminary vital dye labeling has been performed in the Japanese lamprey (Horigome et al., 1999; Shigetani et al.,
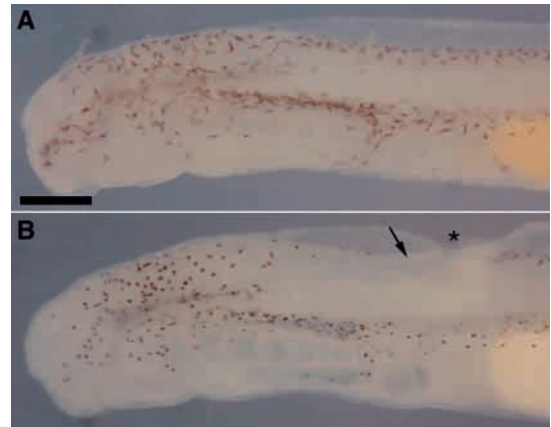

Fig. 7. Ablation of neural crest causes a reduction in pigment and loss of the dorsal fin. (A) Control stage 26 embryo. (B) Stage 26 embryo after neural crest ablation from hindbrain at stage 21. Pigment cells and dorsal fin are missing from the region dorsal to the site of ablation. Fewer pigment cells are seen ventrally when compared with the control (A). Pigment cells in the operated embryo are smaller and without the stellate morphology seen in pigment cells of the control embryo. arrow, area of reduced pigmentation; *, region missing dorsal fin. Scale bar: 250 µm.

2002), embryos were examined only externally such that only superficial migration could be observed.

Rostrocaudal migration of branchial neural crest in lamprey

The present results, which are summarized in Fig. 9, demonstrate that cranial neural crest cells originating from the lamprey midbrain and rostral hindbrain contribute cells to the first and second branchial arches, the mandibular and hyoid arches of gnathostomes. This is similar to the migration described through DiI labeling experiments in urodeles (Epperlein et al., 2000) and anurans (Collazo et al., 1993), with the exception of the degree of caudal migration observed from injections into the rostral to mid-region of the hindbrain. Important differences between lamprey and gnathostomes are particularly notable in caudal regions of the hindbrain, where lamprey neural crest cells migrate extensively in both rostral and caudal directions. In the chick and mouse, limited migration of neural crest cells from individual rhombomeres to the adjacent rostral or caudal rhombomere has been noted (Birgbauer et al., 1995). By contrast, this is greatly exaggerated in the lamprey. Similarly, we find extensive migration of lamprey neural crest cells originating from the hindbrain to more caudal positions in the ventral pharyngeal region.

A second difference between agnathans and gnathostomes is that the lamprey caudal branchial neural crest cell population is initially unsegmented and moves in a sheet-like fashion. In vertebrates studied to date, cranial neural crest cell migration has been described as occurring in three streams, referred to as mandibular, hyoid and branchial. We find evidence for streams of neural crest cells moving into the first and second arches, analogous to the streams within the mandibular and hyoid arches of gnathostomes, but the emigration of cells into the presumptive pharyngeal region occurs as a sheet of cells that fill the entire arch-forming region. Cells within this sheet
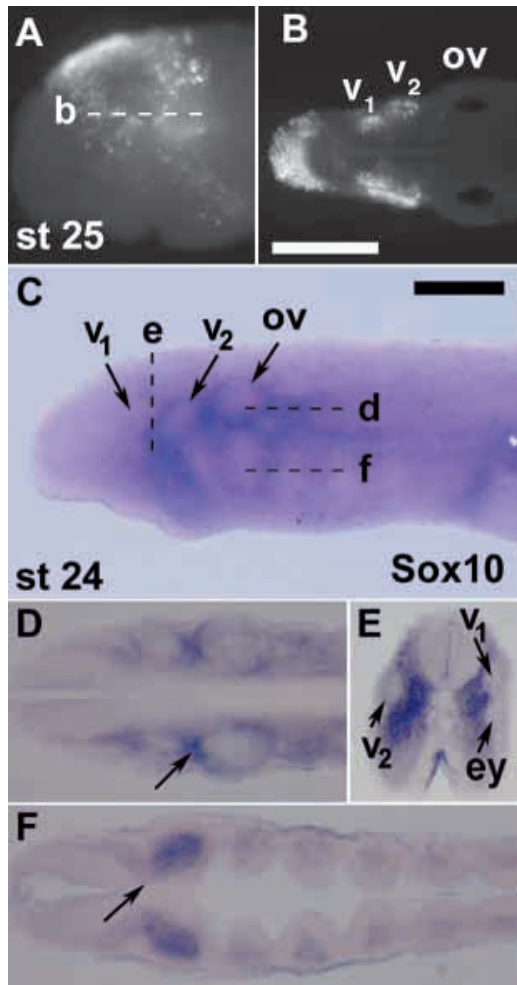
Fig. 8. (A,B) DiI-labeling of trigeminal ganglia. Ophthalmic (V$_1$) and maxillomandibular (V$_2$) lobes of the trigeminal ganglion contain DiI-labeled cells only when DiI also labeled cells of the ectoderm. (B) Horizontal section through plane b shown in A shows labeled cells in the trigeminal ganglion (V$_1$,V$_2$) rostral to the otic vesicle. (C-F) Sox10 immunopositive cells excluded from cranial ganglia. (C) Lateral view of stage 24 embryo immunolabeled with Sox10 antibody. Immunopositive cells are seen in the rostral pharyngeal arches and dorsally surrounding V$_1$ and V$_2$ lobes of the trigeminal ganglion, the eye and the otic vesicle (ov). d and f indicate horizontal planes of section shown in D and F, respectively. e indicates plane of transverse section shown in E taken from a stage 24 companion embryo. Sox10 immunopositive cells are seen between the otic vesicle and V$_2$ of the trigeminal ganglion (arrow in D) and lie medial to, but are excluded from, the placode-derived ganglia V$_1$, V$_2$ and eye (ey) in E. (F) Immunopositive cells are found in the mandibular arch (arrow) and weakly throughout the mesenchyme of the caudal branchial arches. Scale bars: in B, 200 µm for A,B; in C, 200 µm for C-F.

migrate extensively along the entire pharyngeal region in both rostral and caudal directions, and apparently are not excluded from any axial level. These variations in migratory pattern at the level of the hindbrain in different vertebrates may be the result of heterochronic differences in the development of the pharyngeal pouches, the endodermal outpocketings of the pharynx. These pouches contact the overlying ectoderm,

creating mechanical barriers to migration along the rostrocaudal axis that prevent further migration and cell mixing. This results in the sequestration of neural crest cells into individual arches. Interestingly, a similar pattern of neural crest migration followed by sequestration may occur in the amphibian. In *Rana*, the ventral migration of branchial neural crest cells occurs initially as a single stream that is subsequently partitioned into four subdivisions by the formation of the pharyngeal pouches (Stone, 1929). Thus, it is possible that rostrocaudal mixing of the branchial crest occurs before the pharyngeal pouches form. Other investigations of neural crest migration in amphibians have not reported the mixing of branchial crest streams (Hörstadius and Sellman, 1946; Epperlein et al., 2000). Nevertheless, the results of Stone (Stone, 1929) may suggest similarities in the migration of the branchial neural crest between agnathans and basal gnathostomes. Our results in the lamprey suggest that caudal hindbrain neural crest cells have not been specified to contribute to a particular branchial arch. Instead, neural crest cells originating from a single axial level can migrate in either rostral or caudal directions and the neural crest cells in this region become compartmentalized only after the endodermal outpockets have fused with the ectoderm to form the pharyngeal pouches.

Some rostrocaudal migration of cranial neural crest cells has been noted in other vertebrates, although not as marked as in the lamprey. When Schilling and Kimmel (Schilling and Kimmel, 1994) performed a series of single cell labeling experiments in zebrafish, they found that cranial neural crest cells contributed to the caudal gill arches. Cells originating from a single axial level contributed progeny in up to three adjacent arches. Furthermore, segmental restriction in their migration appeared later, and in a rostrocaudal manner. Their results suggest that similar mechanisms observed here in the lamprey may also exist in other vertebrates, but are less obvious or have been missed.

Although we find that cells originating at a particular axial level can populate different branchial arches, this does not necessarily rule out the possibility that one axial level may contribute more cells to one arch than others. In previous ablation studies, Langille and Hall (Langille and Hall, 1988) found a correlation between the removal of neural crest from a specific region and the subsequent loss of cartilage from associated branchial arches. However, they did not distinguish between a reduction and complete loss of cartilage.

Positional identity in the pharyngeal region along the anteroposterior axis

Our data suggest that neural crest cells in the caudal branchial region are sequestered by the formation of the pharyngeal pouches. Therefore, it is possible that pharyngeal identity occurs independent of neural crest cells and that positional information may be conferred to the branchial neural crest extrinsically only after the pouches have formed. Consistent with this, Veitch et al. (Veitch et al., 1999) showed in the chick that pharyngeal endoderm was patterned correctly in the absence of neural crest.

There is evidence that retinoic acid (RA) signaling is important for proper patterning of the pharyngeal endoderm in the mouse (Wendling et al., 2000), but not for the neural crest cells therein. In lamprey, Kuratani et al. (Kuratani et al., 1998b)

have demonstrated the importance of retinoic acid signaling in development of the pharynx. Treatment of lamprey embryos with all-trans RA resulted in the loss of anterior structures, including the pharynx (Kuratani et al., 1998b), similar to the effect of RA treatment in other vertebrates. Effects on the pharyngeal arches were concentration dependent, with rostral arches affected by lower levels of RA treatment. Thus, positional identity along the anteroposterior axis may depend on graded RA signaling. RA signaling has also been shown to affect pharyngeal development in the invertebrate chordates, amphioxus and ascidians (Escriva et al., 2002; Hinman and Degnan, 1998). Taken together, such results suggest that pharyngeal patterning in the lamprey probably occurs independently of neural crest cell entry and depends on RA signaling.

Neural crest and the formation of branchial arch cartilages

In vertebrates, a subpopulation of neural crest cells differentiates into a segmental set of cartilages that support the gill arches. Langille and Hall (Langille and Hall, 1988) have previously established that the neural crest is required for formation of branchial arch cartilages in the lamprey. This set of cartilage bars is located lateral to the gills in the lamprey pharyngeal walls, between the ectoderm and the underlying core of mesoderm. By contrast, in gnathostome fish, these cartilages are located medial to the mesoderm, between the mesodermal core of the arch and the endoderm. This observation suggests developmental differences in branchial arch cartilage formation between agnathans and gnathostomes that lead to differential localization of branchial arch cartilage condensations. Based on these topological differences, Kimmel et al. (Kimmel et al., 2001) have suggested that the arrangement of the cartilage bars is related to the migratory properties of the neural crest cells into this region. The 'outside-in' hypothesis proposes that the neural crest cells that invade the mandibular arch segment of the lamprey undergo both medial and lateral migration. By contrast, the neural crest cells that invade the caudal branchial arches were proposed to migrate only along a lateral pathway in the lamprey. In zebrafish, neural crest cells probably first migrate laterally beneath the ectoderm into the pharyngeal arch region, and then subsequently migrate medially to form a concentric ring about a core of mesoderm (Miller et al., 2000). Based on the restriction of Dlx gene expression (Neidert et al., 2001) to the lateral portion of the branchial arches in lamprey, Kimmel et al. (Kimmel et al., 2001) have suggested that a medial migration of neural crest may not occur in the lamprey and that this may explain the observation that the branchial arch cartilages in gnathostomes are located medial to the mesodermal core, while in agnathans, these cartilages are external, or lateral to the mesoderm. More recently, however, Meulemans and Bronner-Fraser (Meulemans and Bronner-Fraser, 2002) have shown that another neural crest marker, *AP2*, is located in a ring surrounding the mesodermal core of each pharyngeal arch. This calls into question the proposed 'outside-in' hypothesis. Furthermore, Damas (Damas, 1944) inferred the presence of neural crest cells medial to the mesodermal core in caudal branchial arches of the lamprey. These discrepancies could only be reconciled by using vital dye labeling to analyze the definitive distribution of neural crest cells.

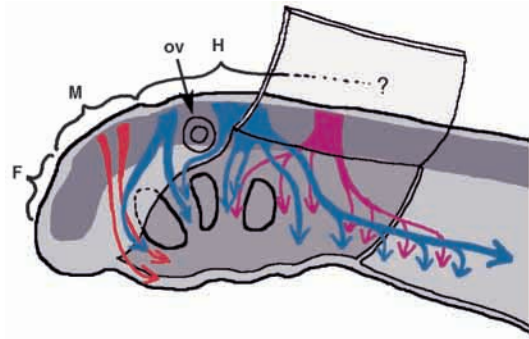Our DiI labeling data suggest that the localization of cranial

Fig. 9. Cranial neural crest cell migration in the lamprey. Orange arrows indicate migration from the midbrain region; blue arrows indicate migration from the rostral hindbrain (anterior to the otic vesicle) or middle hindbrain region; pink arrows indicate migration of cells from the caudalmost region of the hindbrain or the rostral neural tube. Neural crest cell migration from the midbrain and rostral hindbrain appear similar to other vertebrates. In the mid and caudal regions of the hindbrain and the rostral neural tube, migration appears to involve greater rostral to caudal movement of cells than has been described in other vertebrates. Rostral migration of neural crest is also seen in cells emigrating from the caudal hindbrain region. Although morphological markers and expression of a mid-hindbrain marker (*Pax2*) have allowed us to determine regions within the hindbrain, we are presently unable to determine the posterior limit of the hindbrain (indicated by ?), and the boundary between the hindbrain and the neural tube, because of the lack of sufficient markers for this region and the transient nature of rhombomeric constrictions.

neural crest cells in the lamprey does not differ from that seen in the branchial arches of zebrafish. We find that neural crest cells completely surround the mesodermal core in each of the branchial arches (Figs 3-5), indicating that medial migration within the caudal branchial arches is not a new feature of gnathostomes. This suggests that another mechanism is responsible for the different differentiation properties seen in formation of the branchial arch cartilages in agnathans and gnathostomes.

Dlx genes also may play an important role in cartilage differentiation, as suggested by Kimmel et al. (Kimmel et al., 2001). We found pan-Dlx expression within neural crest cells in both medial and lateral positions in the caudal branchial arches. By contrast, Neidert et al. (Neidert et al., 2001) found that expression of four lamprey Dlx genes was confined to the lateral region in branchial arches. This finding may suggest a cartilage-specific Dlx domain in the lamprey branchial arches. There is evidence that expression in chondrocytes of the Type II collagen gene, *Col2a1*, is regulated in response to Dlx2, a downstream target of BMP2 signaling (Xu et al., 2001). However, virtually nothing is known of the developmental mechanisms governing cartilage formation in the lamprey. Recent studies have established that lamprey cranial cartilages are composed of a non-collagenous protein called lamprin, while caudal branchial arch and pericardial cartilages are composed of a different, as yet only partially characterized non-collagenous matrix protein (McBurney and Wright, 1996; McBurney et al., 1996; Morrison et al., 2000; Wright et al., 2001). Although the downstream targets involved in cartilage formation differ between agnathans and gnathostomes, speculation regarding the conservation of developmental

mechanisms that regulate formation of the branchial arch cartilages and the role of the neural crest awaits identification of key elements in this pathway within the lamprey.

Lack of neural crest contribution to cranial ganglia

Our data from DiI labeling and Sox10 immunoreactivity failed to detect a neural crest contribution to the cranial sensory ganglia, suggesting they may be entirely of placodal origin. The input of the neural crest into sensory ganglia would thus represent an important innovation of the neural crest in the gnathostome lineage. The previous evidence that neural crest contributes to cranial sensory ganglia in lamprey came from several studies showing HNK1 immunoreactivity in cells of fixed embryos (Hirata et al., 1997; Hirata et al., 1998; Morikawa et al., 2001). The HNK1 epitope serves as a good neural crest marker in some vertebrates, such as chick and reptiles, but is not a pan-species marker. Furthermore, it cannot distinguish neural crest from placodal cells. Interestingly, Hirata et al. (Hirata et al., 1997) found that cranial neural crest derivatives did not express the HNK-1 carbohydrate, with the exception of the cranial sensory ganglia. The only other evidence to suggest a neural crest contribution to cranial sensory ganglia came from extirpation experiments, but these gave contradictory results (Newth, 1951; Newth, 1956). We not only noted a lack of neural crest contribution to rostral cranial ganglia like the trigeminal ganglion, but also found no evidence of a neural crest contribution to the more caudal epibranchial ganglia. The latter are solely placode derived in birds. This may suggest that in the lamprey, all the cranial ganglia are placode derived, and that the contribution of the neural crest to the rostral cranial ganglia is a derived character in vertebrate evolution.

Conclusions

We have performed a detailed analysis of cranial neural crest migration in the lamprey, *Petromyzon marinus*. The results reveal important differences in the migratory pathways taken during embryogenesis and begin to resolve discrepancies in the literature regarding the mechanisms of cartilage development in the lamprey versus gnathostomes with respect to the contribution of the neural crest. Lamprey neural crest cells appear far less restricted in their movement than those in higher vertebrates, suggesting that constraints to migration may be a later innovation. By contrast, lamprey have fewer neural crest-derived cell types, perhaps suggesting a lower degree of differentiation potential. Important differences in neural crest derivatives exist between agnathans and jawed vertebrates in both the visceroskeletal elements and the peripheral nervous system (see Kimmel et al., 2001). Most notably, lamprey and hagfish, the two extant groups of agnathans, lack jaws. In addition, the lamprey is known to lack sympathetic chain ganglia but it is not clear if the lack of these structures represents the basal vertebrate condition, or if they were subsequently lost in the lamprey lineage. Because neural crest cells give rise to a wide range of derivatives, it is important to understand how their role in the vertebrate body plan has changed during the course of vertebrate evolution.

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