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Original Article

The cardiac neural crest in *Ambystoma mexicanum*

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ABSTRACT To establish whether a region of the cranial neural crest contributes cells to the developing heart of *Ambystoma mexicanum* (axolotl), as it does in many other vertebrates, we constructed a fate map for the neural crest in late neurula stage (stage 19-20) embryos. The fluorescent vital dye, DiI, was used as the lineage label. The various regions of the cranial neural folds were identified in relation to such landmarks as the developing forebrain, midbrain and hindbrain, and the appearance and extent of emerging somites. Labelled cells originating in the rhombencephalic region were found in the aortic arches and in the truncus arteriosus, and occasionally in the walls of the conus arteriosus. Cells were also found in the third and fourth branchial arches. Labelled neural crest from the adjacent anterior trunk region appeared neither in the heart nor the visceral skeleton, whereas those from the mesencephalic region contributed to the first hypobranchial cartilage and to the first three branchial arches, but not to the heart. No labelled cells from any of the regions were seen in the ventricle or auricle.

KEY WORDS: *cardiac neural crest, heart, development, axolotl, lineage labelling*

Introduction

Traditionally, the neural crest has been divided into two main regions, cranial and trunk, based largely on the developmental capabilities of each region (reviewed by LeDouarin, 1982; Hall and Hörstadius, 1988; Bronner-Fraser, 1995). In the head, neural crest forms much of the skeleton, and contributes to the muscles and vasculature. In the trunk, these tissues are derived primarily from mesoderm.

In urodeles, most studies on the cranial crest have concentrated on its contribution to the visceral skeleton. One of the earliest accounts was that of Landacre (1921) for *Ambystoma jeffersonianum (Plethodon glutinosis)*. Landacre relied on differences in cell size, pigmentation and number of yolk granules to distinguish neural crest from other tissues in histological sections. He noted that the dorsal portion of the migrating cranial neural crest gave rise to portions of several cranial ganglia, while the ventrally-migrating tongues moved into the mandibular and branchial arches.

By extirpating portions of the neural crest in *Ambystoma maculatum (punctatum)*, Stone (1922, 1926) was able to confirm that the cranial neural crest forms most of the visceral skeleton. However, because of the regulatory capacity of the amphibian embryo, extirpation lacks precision for fate mapping. Thus, Hörstadius and Sellman (1946) turned to vital staining to establish the origin of individual skeletal elements at the open neural plate stage (st. 16) in *Ambystoma mexicanum* embryos. Their results were later strengthened by those of Chibon (1966), obtained for another urodele, *Pleurodeles waltl,* at a similar developmental stage.

The discovery that the neural crest contributes to the cardiovascular system is more recent. First discovered in the chick, the "cardiac" crest (LeLièvre and LeDouarin, 1975; Kirby *et al.*, 1983) is located between cranial and trunk crest, extending from the otic placode to somite 3 (for reviews see Kirby and Bockman, 1984; Kirby and Waldo, 1995). Cardiac neural crest cells first migrate into pharyngeal arches 3, 4, and 6, where they participate in the formation of the aortic arch arteries (Bockman *et al.*, 1989; Miyagawa *et al.*, 1989). Some cells migrate from the pharyngeal arches into the outflow tract where they appear in the aorticopulmonary septum and populate the truncal folds (Bockman *et al.*, 1989). Those in the septum undergo apoptosis and are gradually replaced by cardiomyocytes (Poelmann *et al.*, 1998). The largest population of neural crest cells in the outflow tract is derived from the region of the neural fold that populates pharyngeal arch 4 (Phillips *et al.*, 1987).

In the rat, Fukiishi and Morriss-Kay (1992) observed that labelled cells between occipital somites 1 and 2, or 3 and 4, migrated within and dorsal to the third and fourth pharyngeal arches, respectively, and into the outflow tract of the heart.

In amphibians, the possible contribution of the neural crest to the cardiovascular system has been largely unexplored. In *Xenopus*, Sadaghiani and Thiébaud (1987) observed that some neural crest

Abbreviations used in this paper: DiI, 1,1-dioctadecyl 3,3,3',3' tetramethylindocarbocyanine perchlorate; PBS, phosphate buffered saline; DMF, dimethylformamide.

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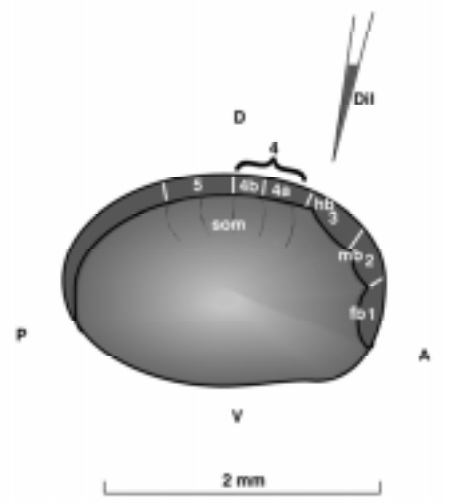

Fig. 1. Lateral view of a stage 20 axolotl embryo indicating the five adjacent zones demarcated along the neural fold. *Each region was defined using visible landmarks, such as indentations of the emerging neuromeres and the appearance of somites, as reference points for the different sections. Zones 1-3 correspond roughly to the fore-, mid- and hindbrain. Zone 4 lies over the first two somites. (Sections "a" and "b" are each one somite in length.) Zone 5 is also two somites in length. fb, forebrain; mb, midbrain; hb, hindbrain; som, somites; a, anterior; p, posterior; d, dorsal; v, ventral.*

cells from the posterior part of the rhombencephalon (the branchial crest) penetrated into the wall of the truncus arteriosus. However, they did not report whether vagal crest cells, located just posterior to the branchial arch segment, made any contribution to the developing cardiovascular system.

We have used the fluorescent vital dye DiI to construct a fate map for the cranial neural crest in late neurula (stage 19-20) axolotl embryos. We chose this stage over the open neural plate stage as it provides a more distinctive set of landmarks, including the neuromeres and the first four somites. We found that the cardiac neural crest is located in the region above somites 1 and 2. The cardiac crest would appear to follow a ventral migratory pathway to the rostral end of the endocardium where it contributes to the walls of the truncus arteriosus and the aortic arches.

Results

In order to establish the location of the cardiac neural crest in the axolotl, a fate map was constructed by labelling stage 19-20 (late neurula) with DiI. Either the right or left neural fold was injected in one of five zones (Fig. 1). Most embryos were allowed to develop until they reached feeding stage. By this stage, the cartilaginous visceral skeleton has completely formed, heart development has reached its juvenile stage, and secondary gill filaments are prominent. DiI labelling of the neural crest derivatives was bright and distinct, and was stable for at least two weeks.

Table 1 shows the number of embryos that were labelled in each zone and the frequency with which labelled cells were detected in the brain, heart and cranial skeleton. Other derivatives (e.g., pigment cells, mesenchyme) were also found. However, as our objective was to locate the cardiac crest and not to create a comprehensive catalogue of crest derivatives, these are not reported. As expected, the particular elements labelled were characteristic of a zone. In other words, neural crest and neural tube derivatives have a specific fate depending on their origin along the antero-posterior axis of the neural tube.

Identification of the cardiac crest

Only embryos that had been labelled in zone 4 (rhombencephalon) displayed fluorescently-labelled cells in the heart. By stage 37, zone 4 neural crest cells were found in the 4th branchial arch and had reached a position just anterior to the developing heart tube (not shown). By stage 44, the large aortic arches branching off from the truncus arteriosus were lined with labelled cells (Fig. 2D-F). Labelled cells were also found in the truncus arteriosus in 13 of 15 embryos (Table 1; Fig. 2B,D). In 3 of these embryos, the walls of the conus arteriosus also contained labelled cells (Fig. 2B). However, labelled cells were never seen in the more posteriorly-located ventricle and auricle.

Cells from folds on both sides of the embryo contributed to the walls of both the left and right aortic arches. However, embryos labelled on the right side consistently displayed a larger number of labelled cells in the truncus arteriosus and the aortic arches than those labelled on the left side.

In the visceral skeleton, label was restricted to chondrocytes in the fourth, third, and occasionally the second branchial arches (Fig. 2B).

In an attempt to narrow the boundaries of the cardiac neural crest, zone 4 was further divided into two sections (4a and 4b), each one somite in length (Fig. 1). However, no differences were detected in the labelling patterns of cells from the two areas. Both

TABLE 1

NUMBER OF EMBRYOS WITH LABELLING IN VARIOUS ELEMENTS

Zone labelled	**1**	**2**	**3**	**4**	**5**
No. Examined	6	10	8	15	8
		Brain and Spinal Cord			
Ant. Prosencephalon	**6**	1	0	0	0
Post. Prosencephalon	2	**10**	4	0	0
Mesencephalon	0	3	**8**	0	0
Rhombencephalon	0	0	0	**15**	0
Spinal cord	0	0	0	0	**8**
		Cranial Skeleton			
Anterior Meckel's	0	**10**	3	0	0
Palatoquadrates	0	**10**	2	0	0
Trabeculae[1]	0	**10**	4	0	0
Posterior Meckel's	0	**8**	**7**	0	0
Hyoid[2]	0	**9**	**6**	0	0
BB/HB[3]	0	2	**6**	1	0
Branchial arch 1	0	1	**8**	1	0
2	0	0	**8**	**8**	0
3	0	0	**8**	**15**	0
4	0	0	2	**15**	0
		Heart			
Truncus &conus	0	0	1	**13**	0
Ventricle & atrium	0	0	0	0	0

[1] Includes cornu and anterior trabeculae

[2] Includes ceratohyal and hypohyal

[3] BB/HB: Basibranchials and Hypobranchials

The numbers in bold indicate that label appeared in the element in >50% of the embryos labelled in that zone.

areas contributed to the same elements of the cardiac outflow tract and to the posterior branchial arches. Therefore, in the premigratory neural crest over somites 1 and 2, there appears to be no clear antero-posterior separation between the presumptive chondrogenic and cardiovascular subpopulations.

Other zones

Zone 1

Embryos that had been labelled in the most rostral right or left neural fold displayed fluorescently-labelled cells in the anterior forebrain region at feeding stage. This zone did not contribute to the cranial skeleton or to the cardiovascular system.

Zone 2

Cells from zone 2 were found in the posterior forebrain region, and were most highly concentrated at the forebrain-midbrain border. Labelled cells were scattered throughout the prosencephalic region, but were concentrated more dorsally and posteriorly in the telencephalon. The mesencephalon contained very few labelled cells. With respect to the cranial skeleton, label was found in Meckel's (Fig. 3A), the ceratohyal (Fig. 3C) and the palatoquadrate cartilages. Label was also obtained in the cornu and anterior trabeculae, but not in the posterior elements such as the posterior trabeculae or the basal plate (Fig. 3C).

Zone 3

In the brain, labelled cells from zone 3 were found mainly in the mesencephalon. Cells of presumptive neural fold origin were found in the first, second and third branchial arches, but few were seen in the fourth arch (Fig. 3E). Zone 3 cells also contributed to the ceratohyal, first hypobranchial, and the posterior Meckel's cartilages, and occasionally to the other elements.

Zone 5

To ensure that the boundaries established for the cardiac neural crest did not extend past zone 4, we labelled an area, defined as zone 5, which extended over somites 3 and 4. This marks the beginning of the trunk neural crest (Fig. 1). As expected, label was found in the spinal cord, but not in the cardiac region nor in any skeletal elements.

Discussion

The objective of these experiments was to create a fate map of the cranial neural crest for the stage 20 (late neurula) axolotl embryo, in order to locate the cardiac neural crest. Previous fate maps of the axolotl neural crest were created for earlier stages (Hörstadius and Sellman, 1946; Chibon, 1966), and did not identify a contribution to the heart. Because we labelled the converging neural folds, which include part of the future neural tube, as well as the premigratory neural crest, our results are not restricted to the fate of neural crest cells alone. However, we did not catalogue contributions to smaller neuronal components such as cranial ganglia.

Zone 4, extending from the cranial margin of somite 1 to the posterior end of somite 2, was identified as the cardiac neural crest region. It also contributes cells to the posterior branchial arches and the hindbrain. The neural crest cells migrate ventrally from this position toward branchial arches 3 and 4 (Landacre, 1921; Graveson *et al.*, 1995) and the anterior portion of the heart. By stage 44, the cardiac neural crest cells are found in the truncus arteriosus and the outflow tract, and in the arteries leading to the posterior branchial arches. The function of the cardiac neural crest and its position along the neural fold in the axolotl embryo correspond well with the location and role of the cardiac neural crest in the chick (Kirby and Waldo, 1990, 1995) and in the rat (Fukiishi and Morris-Kay, 1992).

With respect to the cartilaginous elements, none were found to originate from zone 1, in agreement with the findings of Chibon (1966) and Hörstadius and Sellman (1946). Cells from zone 2

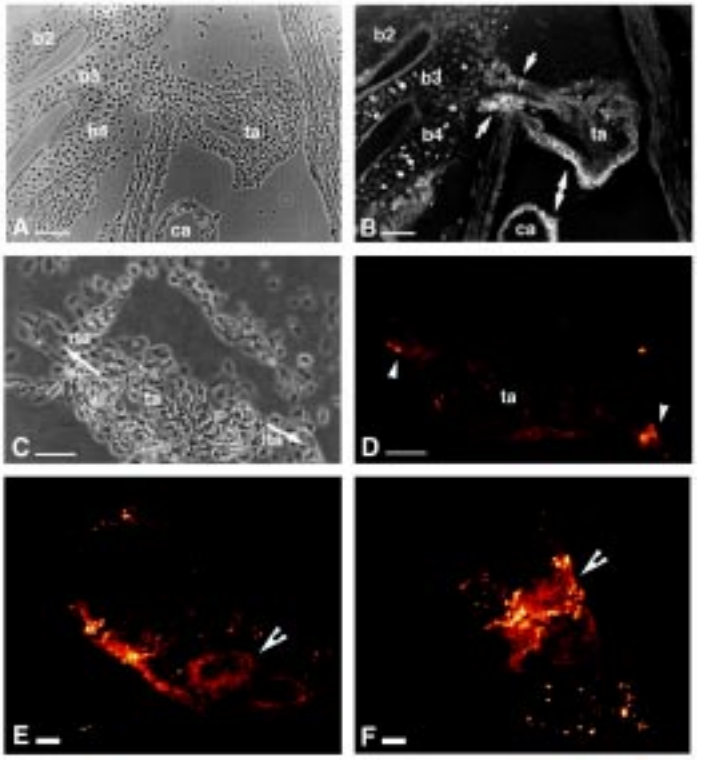

Fig. 2. Labelled neural fold cells from zone 4 contribute to branchial arches and anterior heart. (A) *Phase contrast image of a frontal section through the anterior portion of a stage 44-45 axolotl heart and gill filaments. The anterior of the embryo is at the top of the plate. In this ventral view, the location of the gill arches in relation to the truncus arteriosus (ta) of the heart is shown. b4, gill arch 4; b3, gill arch 3; b2, gill arch 2; ca, conus arteriosus.* **(B)** *Fluorescent image of the same section as seen in (A). Labelled cells from zone 4 line the walls of the branchial arches (small arrows). Other labelled cells (indicated with a double-headed arrow) are found in the walls of the truncus arteriosus (ta) and the conus arteriosus (ca). Labelled chondrocytes are also seen in the third (b3) and fourth (b4) branchial arches.* **(C)** *Phase-contrast image of a frontal section (ventral view) through the anterior portion of a stage 44-45 axolotl heart after zone 4 labelling. The truncus arteriosus (ta) is the region of the heart from which the major arch arteries branch. Aortic arch arteries on the right (rta) and left side (lta) of the truncus arteriosus are shown.* **(D)** *Confocal laser scanning image of the same section. Note the fluorescing cells (arrowheads) in the outflow arteries and in the lining of the truncus arteriosus (ta).* **(E,F)** *Magnified confocal images of the right (E) and left (F) aortic arch arteries (rta and lta in C). DiI-labelled cells such as the ones indicated by the arrow, appear to form part of the arterial wall. Bar, 100 μm in A, B, C, and D; 10 μm in E and F.*

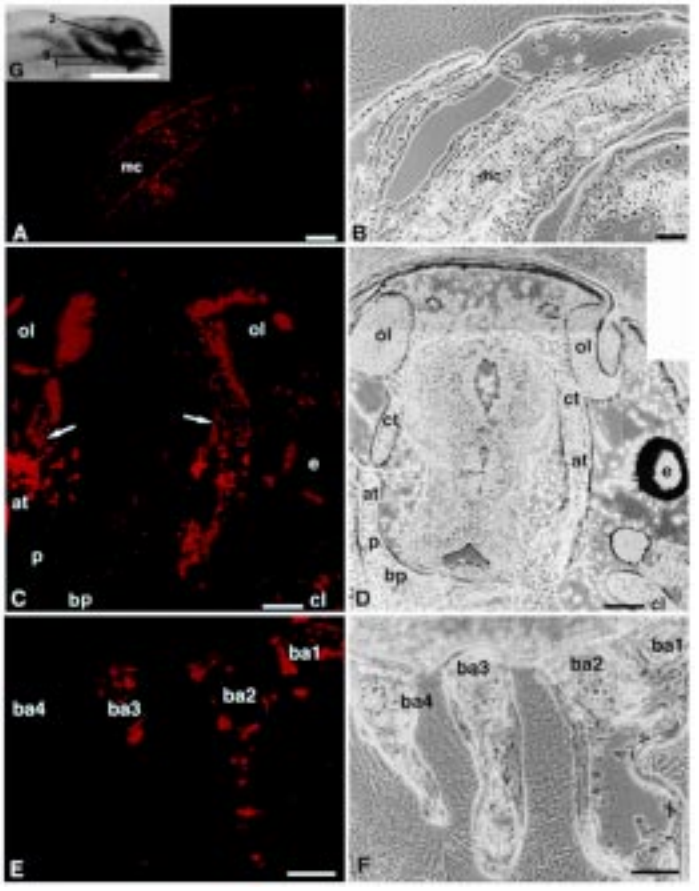

Fig. 3. Location of labelled cranial neural crest cells from zones 2 and 3. (A) *Confocal laser scanning image of a frontal section (ventral view) through the right half of Meckel's cartilage (mc) of a stage 44-45 axolotl embryo originally labelled in zone 2. Both chondrocytes and surrounding mesenchymal cells were labelled.* **(B)** *Phase contrast image of the same section. The plane of the section is indicated by line 3 in Figure 2G.* **(C)** *Fluorescent image of a frontal section through the neurocranium of a stage 44-45 axolotl embryo labelled in zone 2. Components of the cranial skeleton which contained fluorescently-labelled cells were the ceratohyal (cl), the cornu trabeculae (indicated by arrows) and the anterior trabeculae (at). No labelled cells were found in the posterior trabeculae (p) or the basal plate (bp). Areas around the olfactory vesicles (ol) and the eye (e) were also labelled.* **(D)** *Phase contrast image of the same section. ct, cornu trabeculae. The plane of the section is indicated by line 2 in Figure 2G.* **(E)** *Fluorescent image of a frontal section through the branchial arches of a stage 44-45 axolotl embryo labelled in zone 3. Labelled chondrocytes were found in the first branchial arch (ba1), the second branchial arch (ba2) and the third branchial arch (ba3). Hardly any labelled cells were found in the fourth branchial arch (ba4).* **(F)** *Phase contrast image of the same section. The plane of the section is indicated by line 1 in Figure 2G.* **(G)** *Photograph of the head of a stage 44-45 axolotl embryo, stained for cranial cartilage, indicating the plane of sections for Figure 2A-F. Bar, 100 μm in A, B, E, and F; 200 μm in C and D; 3 mm in G.*

formed Meckel's cartilage, the anterior portion of the trabeculae, part of the hyoid arch and the palatoquadrates. On Chibon's (1966) map, the corresponding area, defined as "30º-90º", reportedly formed both the anterior and posterior trabeculae and the basal plate as well as the palatoquadrates, Meckel's cartilage and the hyoid arches. The corresponding area on Hörstadius and Sellman's (1946) map, which includes their regions 3, 4 and part of 5, gave rise to only the trabeculae and the mandibular and hyoid arches. With the exception of the palatoquadrates, our results are in agreement with Hörstadius and Sellman (1946). We found no labelled cells from any zone in the posterior trabeculae or the basal plate, which supports Hörstadius and Sellman's (1946) contention that these structures were of endodermal origin.

Cells from zone 3 contributed mainly to the three anterior branchial arches and the hypobranchials (Fig. 4B). In contrast to the results of Chibon and Hörstadius, label was also found in the posterior portions of the hyoid arch and Meckel's cartilage. While this may reflect a more complete specification of the cartilage domains at a later developmental stage, we believe that the more likely explanation is that the fluorescent label DiI is simply a more sensitive lineage label than the vital dyes used in earlier work.

After labelling only one of the neural folds, fluorescence was found in both sides of the heart. However, the right side neural folds contributed more cells to the cardiac region than the left side neural folds (data not shown). This difference between adjacent lateral folds may be related to the asymmetry of the heart itself, and is also seen in chicks (Rosenquist *et al.*, 1989; Takamura *et al.*, 1990).

The work presented in this paper represents the beginning stage of an examination of the role and differentiation of cardiac neural crest in amphibians. Most of the heart is formed from the antero-ventral lateral plate mesoderm (Easton *et al.*, 1994). The pharyngeal endoderm not only plays an essential role in the specification of the heart-forming mesoderm, but also induces cranial neural crest to form cartilage (Graveson and Armstrong, 1987). However, this occurs while the mesoderm moves towards the ventral mid-line of the embryo during neurula stages (stages 14-20; Smith and Armstrong, 1990). Neural crest cell migration has not yet begun, and it is presently unknown whether it is specified before, during or after migration.

Although we have identified the presence and location of cardiac neural crest only in early cardiovascular development, our work provides a good foundation for further studies of cardiac neural crest in amphibians. The study of cardiac neural crest in birds and mammals has provided some insight into the pathogenesis of certain congenital abnormalities (e.g., DiGeorge Syndrome: Van Mierop and Kutsche, 1986). The examination of the cardiac neural crest in axolotls may provide similar insight into the mechanisms involved in axolotl mutations such as the cardiac *(c)* and premature death *(p)* mutation, and hopefully in mutations of other vertebrates as well.

Materials and Methods

Embryos

A. mexicanum embryos were obtained from spawnings performed at the University of Ottawa Axolotl Colony. The embryos were kept in modified 25% Holtfreter's solution (Asashima *et al.*, 1989) at 18-20°C. Developmental stages were determined using the staging tables of Bordzilovskaya *et al.* (1989). Stage 19-20, late neurula stage embryos (neural folds in contact throughout but not yet completely fused) were used for all experiments. Prior to microinjection, the jelly coat and vitelline membrane were removed from each embryo with sharpened watchmaker's forceps. Embryos were passed through one rinse of filter-sterilized 100% Steinberg's solution (Asashima *et al.*, 1989) with 50 mg/L gentamicin prior to being transferred to an operating dish containing the same medium. Operations were performed in 60 mm glass Petri dishes containing a 5:1 Permoplast-Paraplast mixture moulded to keep the embryos in place (Frost *et al.*, 1989).

Microinjection of DiI

A 0.3% stock solution of the vital dye, 1,1-dioctadecyl 3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes Inc.) was made up in 30% aqueous dimethylformamide (DMF). This stock solution was further diluted in a 0.3 M sucrose solution to a final concentration of 0.03% DiI. Borosilicate micropipets (Drummond Inc.) were pulled on a Vertical Pipette Puller, Model 700C (David Kopf Instruments) to give a tip diameter of 10 μm. The pipets were attached to a microinjection apparatus similar to that described by Stephens *et al.* (1981). DiI injections were made by inserting a micropipet into the neural fold and expelling approximately 50-100 nl of the pink dye solution.

The neural folds of each embryo were divided into five adjacent zones, as shown in Figure 1. Each embryo was labelled in either the right or left neural fold. To ensure that the dye had not diffused into adjacent zones, several embryos were fixed one hour after injection. These embryos were then embedded, cryosectioned and observed under epifluorescence. In all 8 cases, the dye remained confined to the original zone, and had not diffused into more anterior or posterior regions. The dye was also confined to the converging neural folds and presumptive neural tube, thus primarily targeting premigratory neural crest cells.

After injection, embryos were allowed to heal in the operating dish for approximately 1 h. Injected embryos were transferred to 24-well dishes (Falcon) lined with 1% Noble agar and containing sterile 100% Steinberg's solution with gentamicin. The next day they were transferred to 24-well dishes containing sterile 25% Holtfreter's solution with 0.1 mg/ml streptomycin. The embryos were allowed to develop in the dark for approximately two weeks, until they reached feeding stage.

Cryostat sections and microscopy

At stage 44-45, larvae were anaesthetised in 0.02% tricane methanesulfonate (MS-222, Sigma Chemical Co.), and then fixed in 4% paraformaldehyde/0.25% glutaraldehyde in PBS (phosphate buffered saline) for at least 12 h. They were then rinsed three times in 1 mg/ml sodium borohydride in PBS, three times in PBS, and soaked in 15% sucrose for 12 h at 4°C. They were embedded in 15% sucrose/7.5% gelatin (Sigma, 300 bloom) for 2 h at 37°C and then frozen in liquid nitrogen (Collazo *et al.*, 1993). Frozen blocks were stored at -80°C until sectioned. Embryos were sectioned at a thickness of 14-20 μm with a cryostat. Sections were mounted on slides with Geltol (Lipshaw Immunon) and covered with glass coverslips for viewing under epifluorescence on a Zeiss Axiophot microscope and a confocal laser scanning microscope. Each cartilaginous element, brain region or other organ was scored positive if five or more distinct cells in that element were labelled.

Photographs of the sections were taken using Ilford XP2 or Fuji ASA 400 color film. Digitized images were edited (i.e., montages and labels were created) with Adobe Photoshop 3.0 software.

Controls

To ensure that the DiI was not deleterious and that the fluorescence observed was from the vital dye, we compared the development of DiI-injected embryos to that of controls. Some control embryos were injected with 3% DMF in 0.3M sucrose (the carrier solution without the DiI). Uninjected embryos from the same spawnings were also observed.

Sections of embryos injected with DMF and sucrose were examined to ensure that any labelling observed was from DiI and not due to background fluorescence. At feeding stage (44-45) all larvae showed some background fluorescence in the liver and the gut, irrespective of the zone that was injected, but none elsewhere. If there were DiI-labelled cells in the gut or liver, they could not be distinguished reliably from background fluorescence and were, therefore, disregarded.

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