Cell migration in the postembryonic development of the fish lateral line

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SUMMARY

We examine at the cellular level the postembryonic development of the posterior lateral line in the zebrafish. We show that the first wave of secondary neuromasts is laid down by a migrating primordium, primII. This primordium originates from a cephalic region much like the primordium that formed the primary line during embryogenesis. PrimII contributes to both the lateral and the dorsal branches of the posterior lateral line. Once they are deposited by the primordium, the differentiating neuromasts induce the specialisation of overlying epidermal cells into a pore-forming annulus, and the entire structure begins to migrate ventrally across the epithelium. Thus the final two-dimensional pattern depends on the combination of two orthogonal processes: anteroposterior waves of neuromast formation and dorsoventral migration of individual neuromasts. Finally, we examine how general these migratory processes can be by describing two fish species with very different adult patterns, *Astyanax fasciatus* (Mexican blind cavefish) and *Oryzias latipes* (medaka). We show that their primary patterns are nearly identical to that observed in zebrafish embryos, and that their postembryonic growth relies on the same combination of migratory processes that we documented in the case of the zebrafish.

Key words: Neuromast migration, Secondary primordium, Pattern formation, *Danio rerio*, *Oryzias latipes*, *Astyanax fasciatus*, Evolution of pattern

INTRODUCTION

The postembryonic growth of an organism differs from the early stages of embryonic organisation in many aspects. The axes are defined, as well as the basic structure of the organism, and the task is not to build new parts of the body de novo; rather, postembryonic growth has to do with the orderly expansion of existing structures, such that coherence and functionality is maintained. The constraint for maintained functionality is particularly demanding in the case of sensory systems, where growth requires not only the continuous addition of cells of the appropriate types in adequate numbers and patterns but also the establishment of central projections that fit the somatotopic map established during embryogenesis (Alexandre and Ghysen, 1999; Gompel et al., 2001a).

The differences between the processes related to embryogenesis and those related to growth raise the question of whether embryonic development and postembryonic growth rely on the same mechanisms. One system that may be appropriate to investigate this question is the fish lateral line. The mechanosensory lateral line comprises superficial sense organs, the neuromasts, which are arranged on the head, body and tail in species-specific patterns. The number of neuromasts expands greatly during postembryonic growth, yet how this expansion is achieved has not been studied to date.

In zebrafish, the embryonic pattern is rather simple and comprises only about 20 neuromasts. A major component of this pattern is the 'lateral' branch of the posterior lateral line

(L-PLL), which runs from head to tail along the horizontal myoseptum and comprises seven to eight neuromasts at the end of embryogenesis. This line is formed by a migrating primordium that originates from the post-otic region and moves caudalwards during the second day of embryogenesis, leaving in its wake seven to eight groups of cells, each of which will become a neuromast (Metcalfe et al., 1985).

The adult PLL is much more complex, comprising several hundreds of neuromasts arranged in small rows of up to 20 or more neuromasts (stitches). This increase in number is accompanied by a transformation of the pattern, as the lines of stitches that are present on adult zebrafish (Metcalfe, 1989) do not correspond in any obvious way to the line of neuromasts that runs along the horizontal myoseptum in the late embryo. Thus the lateral line is both amplified in size and modified in pattern during postembryonic growth.

On the basis of the classical experiments of Stone on the formation of additional ('accessory') neuromasts during normal development (Stone, 1933) and following tail regeneration (Stone, 1937) in salamanders, it was generally assumed that the postembryonic growth of the fish lateral line would occur by budding from the primary neuromasts laid down during embryogenesis (W. K. Metcalfe, PhD thesis, University of Oregon, 1983) (Sahly et al., 1999). A recent description of the postembryonic growth of the zebrafish L-PLL has led to the proposal that, although budding is probably involved in stitch formation, two other processes are involved in the transformation of the embryonic system into the adult

system (Ledent, 2002). The first process is a reiteration of the embryonic course, with new primordia being generated to provide for additional neuromasts interspersed between those of the embryonic (primary) system. The second process is a migration of the differentiated neuromasts to more ventral positions.

Here we describe both processes at the cellular level, and we show that the same two processes are involved in the transformation of an embryonic pattern that appears highly conserved among teleost fishes into the widely different adult patterns present in *Astyanax fasciatus* and in *Oryzias latipes*.

MATERIALS AND METHODS

Fish

Blind and eyed *Astyanax* were obtained from a local pet shop, and wild-type medakas were obtained from Marc Vandeputte, INRA UR544, Jouy-en-Josas (France).

Labelling of the primordium cells with caged fluorescein

Zygotes at the 2-8 cells stage were injected with a 2% solution of dextran 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged fluorescein 10,000 MW (Molecular Probes D-3310, Eugene, OR). Caged fluorescein was dissolved in a 0.5 M KCl, 0.25% neutral red solution that had been filtered at 0.2 μm, and embryos were left to develop in tank water in the dark. At the desired time, embryos were anaesthetised in tricaine (0.5 mM MS222, Sigma A-5040, St Louis, MO) (Westerfield, 1995), mounted in 4% methylcellulose and examined to determine the position of the putative secondary primordium cells under Nomarski optics, with an orange filter in the diascopy light path to reduce background fluorescence. Uncaging was performed by exposing the desired region to UV light for 500 milliseconds, under a Zeiss 63× long working distance water immersion objective. This level of UV-irradiation has no detectable adverse effect on primordium migration or neuromast differentiation. The diameter of the UV beam was determined by the size of the episcopy field diaphragm. Using the 63× objective, and the diaphragm in the most closed position, a beam of 35 μm was obtained.

Vital labelling of peridermal clones with dextran-rhodamine

Labelled clones were obtained in the progeny of a single cell iontophoretically injected with 3% dextran-tetramethylrhodamine (3000 MW, anionic, Molecular Probes D-3307) in 0.1 M KCl tissue culture water (Sigma W-3500) at blastula stage (1000 cells) as described in Gompel et al. (Gompel et al., 2001a). Successfully injected embryos were maintained in Petri dishes and screened for labelled epidermal subclones at 24 hours after fertilisation (haf). Embryos of interest were raised and fed starting at day 4.

Lateral line labelling with 4-di-2-Asp

Neuromast hair cells of embryos, larvae, juveniles or adults were labelled by incubating the fish in 5 mM 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (4-di-2-Asp, Sigma D-3418) (Collazo et al., 1994) in tank water for five minutes. Labelled fish were rinsed in fresh water and anaesthetised with tricaine for observation. Trans-synaptic labelling of the sensory fibres can be obtained by incubating the fish in higher concentrations of 4-di-2-Asp (125 mM) and rinsing them for 3 hours in fresh water (Alexandre and Ghysen, 1999).

Time-lapse analysis

Time-lapse micrography was performed on larvae anaesthetised in tricaine and mounted in agar as described in *The Zebrafish Book* (Westerfield, 1995). Images were taken on a Zeiss Axioplan microscope equipped with a DAGE MTI camera controlled by NIH Image, at a rate of one frame every minute for primordium migration, or every 1.3 minutes for the analysis of the D0 cell cluster. The positions of each cell was followed on the time lapses and reported on a few chosen frames. The final images were assembled using Adobe Photoshop 3.0.

In situ hybridisation

In situ hybridisation was performed as described by C. Thisse and B. Thisse (http://zfin.org/zf_info/zfbook/chapt9/9.82.html).

Dechorionation of medaka eggs

We used the natural medaka hatching enzyme (Yamamoto, 1975) prepared as follows (J. Wittbrodt, personal communication): embryos were taken as soon as the first of the clutch hatched, and washed in distilled water. After removing the liquid, the embryos were shock-frozen in liquid nitrogen and thawed three times. They were then homogenised in an Eppendorf tube with an Eppendorf pestle and centrifuged for 5 minutes. The supernatant was recovered and the pellet was re-extracted with the same volume of PBS, centrifuged, and the second supernatant was combined to the first. The solution was centrifuged again for 5 minutes to separate the fat, and the aqueous part was used as hatching enzyme solution. Embryos to be dechorionated were deposited in a small hole made in parafilm, covered with 2-3 μl of the solution and left to incubate in a humidified Petri dish for up to 4 hours at 28°C. The outer chorion was then easily removed with forceps and a tungsten needle.
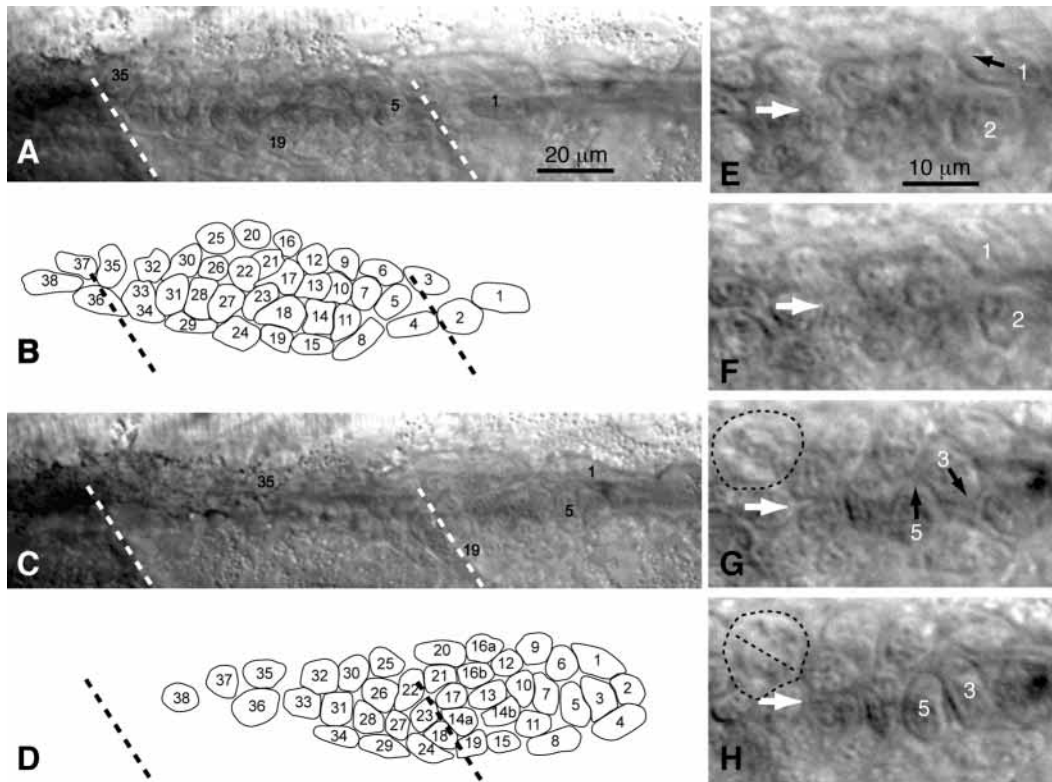
RESULTS

A later cluster of migrating cells

The primary L-PLL is complete at 42 haf. At this time the primary primordium, primI, has completed its journey to the tip of the tail and has formed the terminal neuromasts (Gompel et al., 2001b). Given the evidence for additional waves of neuromast deposition (Ledent, 2002), we examined larvae at 48 haf for the presence of a second migrating primordium. We consistently observed in the vicinity of L1, the anteriormost neuromast of the primary L-PLL, a cluster of subepidermal cells similar in size and shape to the cells of primI (Fig. 1A,C). We tentatively call this cluster primII. A similarly located cluster of primordium-like cells was already observed based on the expression of the gene *eya1* [figure 6J-K in Sahly et al. (Sahly et al., 1999)].
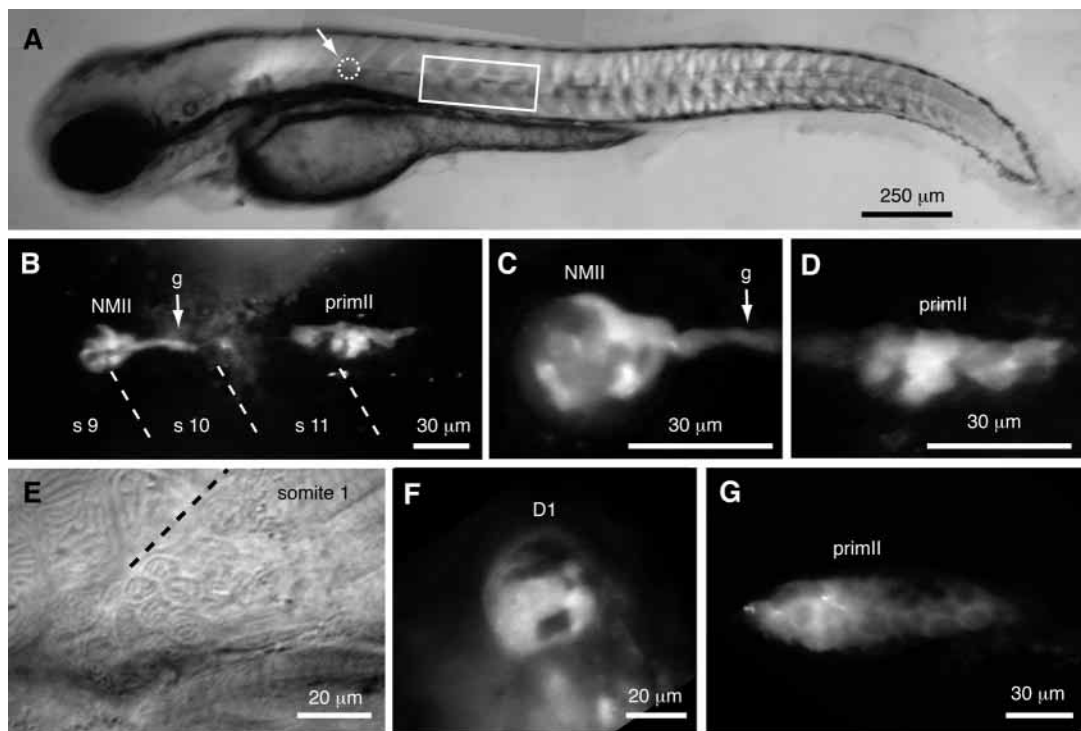
We relied on time-lapse micrography to examine whether this group of cells migrates towards the tail, as did primI on the previous day. We observed that the cells migrate caudalwards along the horizontal myoseptum, i.e. they follow the same path as the primI cells. In two cases where we followed the migration at the time these cells reach the position of the primary neuromast L1, we observed that they crawl along its dorsal side, as also illustrated in Fig. 4A. By the time the migrating cells reach the position of more posterior primary neuromasts, the latter have already migrated out of the way (see below). During their journey, the migrating cells largely keep their positions relative to each other (Fig. 1B,D), and they occasionally undergo mitosis, as was also the case for the primI cells, at a rate of about two per hour (Fig. 1G,H).

Besides their similarity, there are marked differences between the primI and primII groups. The primI cells migrate at a rate of about 1.5-1.7 somites/hour (Gompel et al., 2001b), whereas primII cells migrate at only about 0.2 somites/hour

Fig. 1. Time-lapse video microscopy of primII. The cells pictured in A,C are outlined and numbered in B,D. Cells 1, 5, 19 and 35 have also been numbered in A,C to illustrate the change in the overall morphology of the primordium during its journey. The broken lines indicate intersomitic boundaries. The time lapse covered 2 hours 30 minutes between frames A and B, at a rate of one frame per minute. (E-H) Irregularity in the progression of primII cells. (E,F) Although most cells move to the tail (white arrows), cell 1 near the front of the primordium moves backwards for a short while (black arrow). This cell then resumed its caudal progression. (G,H) The cells labelled with black arrows (cells 3 and 5 in B,D) move sideways rather than along the main path (white arrows). Time separation between E and F, and G and H was 10 minutes. A cell at the metaphase/early telophase of mitosis is seen in G,H (outline). Anterior is towards the left.

Fig. 2. Labelling primII cells. Zygotes were injected with caged-fluorescein and allowed to develop for 48 hours, and the primII cells were irradiated with a brief UV pulse while on somite 6 (A, arrowed circle). On the next day, fluorescein was detected in one secondary neuromast located at the junction between somites 9 and 10, as well as in the primordium which had moved caudally to somite 12 (B, corresponding to the framed region in A). The neuromast (NMII) and primordium (primII) are shown at a higher magnification in C,D. g, glial cell. (E-G) Uncaging the D0 group, which stays on the first somite for many hours (E), appears on the next day in the presence of fluorescent cells in D1, the first neuromast of the dorsal line (F), as well as in primII (G). Anterior is towards the left.
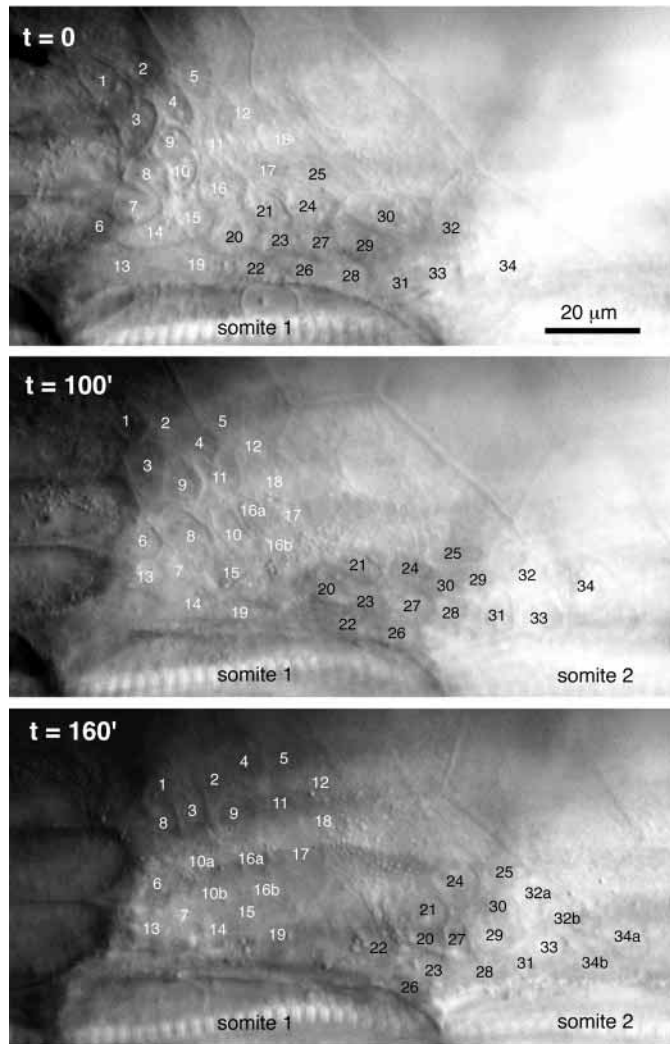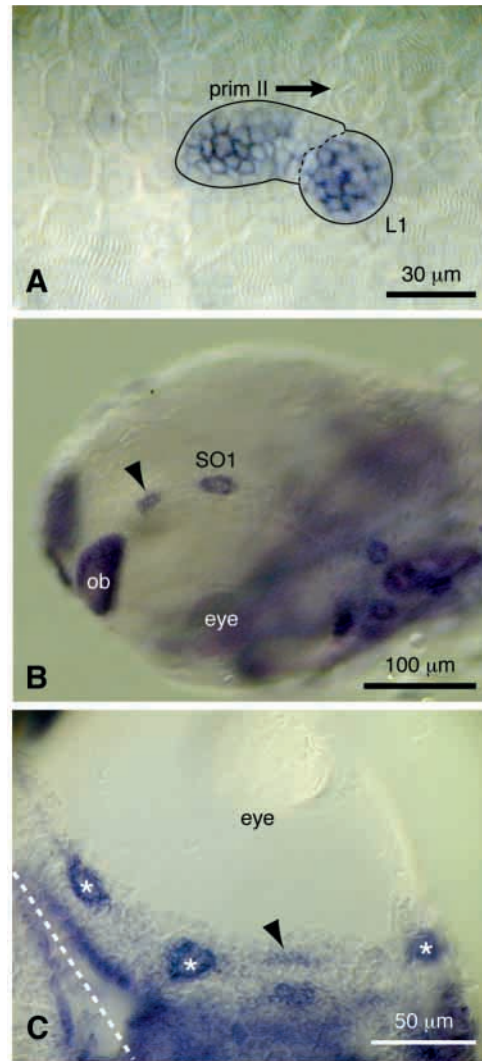
Fig. 3. Video time lapse illustrating the splitting of D0, a large group of primordium-like cells that stays for many hours on somite 1, into primII (black-numbered cells) and D1 (white-numbered cells). Sister cells resulting from a mitosis are named a and b (e.g. 16a and 16b). Anterior is towards the left.

Fig. 4. Expression of the gene CB701 in primII (A) as well as on secondary primordia of the ALL system (B,C). At 48 haf, primII migrating towards the tail (arrow) has reached the primary neuromast L1 (A). The expression in the primordium is more intense at the trailing edge, much as it was in primI (Gompel et al., 2001b), and presumably defines the cells that will form the first secondary neuromast. (B) A secondary primordium (arrowhead) of the supra-orbital branch of the ALL system, between the first neuromast of this line (SO1) and the olfactory bulb (ob). (C) A secondary primordium (arrowhead) of the infra-orbital branch as seen in a near ventral view of the head, with the ventral midline shown as the broken line. Asterisks indicate neuromasts of the infra-orbital line. Anterior is towards the left.

(Fig. 1A-D). In the case of primI, the movement of all cells is somewhat jerky but otherwise very ordered. The movement of the cells in primII is much more chaotic, and cells can occasionally move backwards for a while (Fig. 1E-F, black arrow in E) or translocate between other cells (Fig. 1G,H, black arrows in G). Finally, primI comprises about 100 cells, whereas primII contains fewer than half this number (Fig. 1B,D).

The shape of the primordium differs in the somitic and in the intersomitic regions (Fig. 1B,D). Cells seem to go faster after they have crossed an intersomitic region, as illustrated by the relative position of cells 1 and 5 in Fig. 1A,C, and to slow down as they approach the next intersomitic region. This variation is consistent with the observation that the slowing down of proneuromasts, as they are deposited by the migrating primordium, invariably leads to a final arrest at intersomitic boundaries.

Deposition of secondary neuromasts

We examined whether the primII cells observed in 48 haf larvae contribute secondary neuromasts to the L-PLL. The slow rate of migration of these cells would require very long time lapses, of the order of 10-20 hours, to document the deposition of a proneuromast. We relied instead on the activation of a caged fluorochrome.

We injected zygotes at the 2-8 cells stage with caged fluorescein, let them develop for 48 hours and activated the primII cells with a short pulse of UV-light (Fig. 2A). We observed the larvae on the next day, when the earliest secondary neuromast can first be detected. We consistently found fluorescent cells both in this neuromast and in the
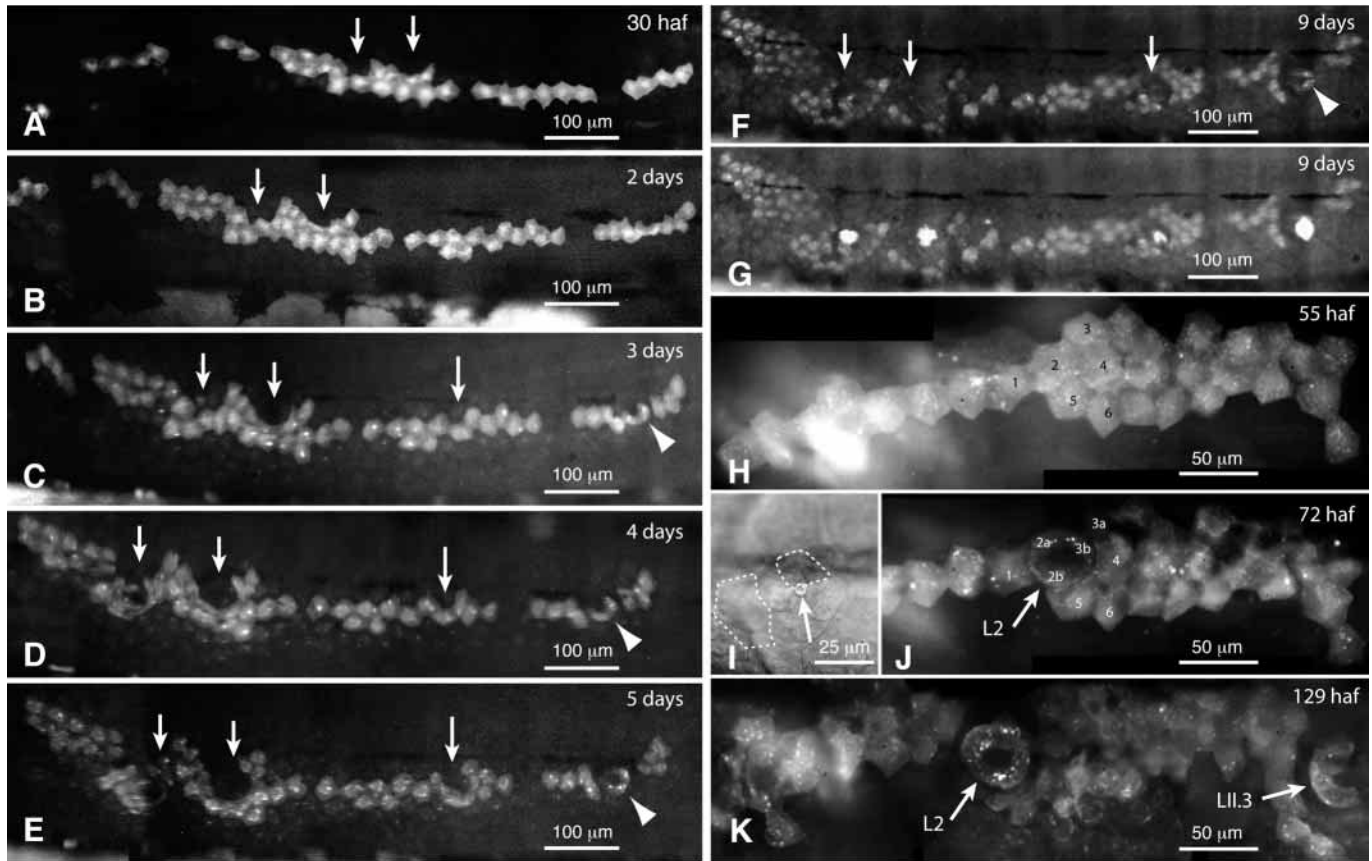
Fig. 5. Neuromast migration. (A) A labelled clone of epidermal cells extends anteroposteriorly at the level of the horizontal myoseptum, where proneuromasts are deposited. Each labelled cell can be identified on the basis of its shape and position relative to neighbours, and traced for several days. Neuromasts (arrows) have differentiated at 48 haf just dorsal to the clone and are seen to move ventrally through the clone in B-F. Note that some cells in the clone are recruited as pore cells by one of the neuromasts (arrowheads in C-F). (G) 4-Di-2-Asp labelling reveals the presence of the neuromast hair cells. This view was combined to that of the clone and superimposed on a bright field view showing the line of melanocytes along the horizontal myoseptum. The recruitment of epidermal pore cells is shown in more detail in H-K. A clone similar to that in A-G was examined at higher magnification. The progeny of cells marked 2 and 3 in H will contribute to the annulus-like pore of neuromast L2 at 72 haf (J). The morphology of these epidermal cells changes from polygonal to crescent shape, thereby defining the pore through which the hairs will protrude (I). Later on, other cells of the same clone were recruited to form the pore of the third secondary neuromast (K). Anterior is towards the left.

migrating primII cells (Fig. 2B-D). We also observed fluorescence ensheathing the nerve (Fig. 2C), suggesting that glial cells are derived from the primordium.

The secondary neuromasts that differentiate from day 3 to day 7 are regularly spaced, with a distance of two or rarely three somites between consecutive organs. Thus, at six days after fertilisation, four to five secondary neuromasts are deposited between L1 and L3 at fairly constant positions: between somites 9 and 10 for the anteriormost neuromast ($n=27/39$), 11 and 12 for the next one ($n=26/39$) and 13-14 ($n=16/39$) or 14-15 ($n=14/39$) for the third one. The imprecision in neuromast position is cumulative from anterior to posterior, as in the case of the primary neuromasts (Gompel et al., 2001b). This suggests that the mechanism responsible for the deposition by primII is similar to that used by primI (Itoh and Chitnis, 2001).

Our results leave no doubt that primII deposits cells on every second (or third) somite, as we never saw labelled cells at intervening positions ($n=39$). Yet in older larvae, neuromasts are found on every intersomitic border (Metcalfe, 1989). We believe,

therefore, that the formation of the intervening neuromasts probably involves yet another primordium (or more).

We do not know what the fate of the secondary primordium is after the deposition of the earliest four to five secondary neuromasts. The activated fluorescence decreases with time, whereas background fluorescence increases, and observations become impossible after 6 days. In addition, the secondary primordium appears to get smaller and smaller as it migrates, and Nomarski optics become increasingly unsatisfactory as the fish grows.

Origin of the secondary primordium

At 48 haf, primII is found next to L1, and the first secondary neuromast always appears posterior to L1. It might be, therefore, that primII originates from L1 (Sahly et al., 1999). To find out whether this is the case, or whether primII arises from a more anterior region, we tried to trace its origin in younger embryos. In 38±2 haf embryos, we observed a group of primII-like cells about three somites anterior to L1. We uncaged this putative primordium and observed fluorescent
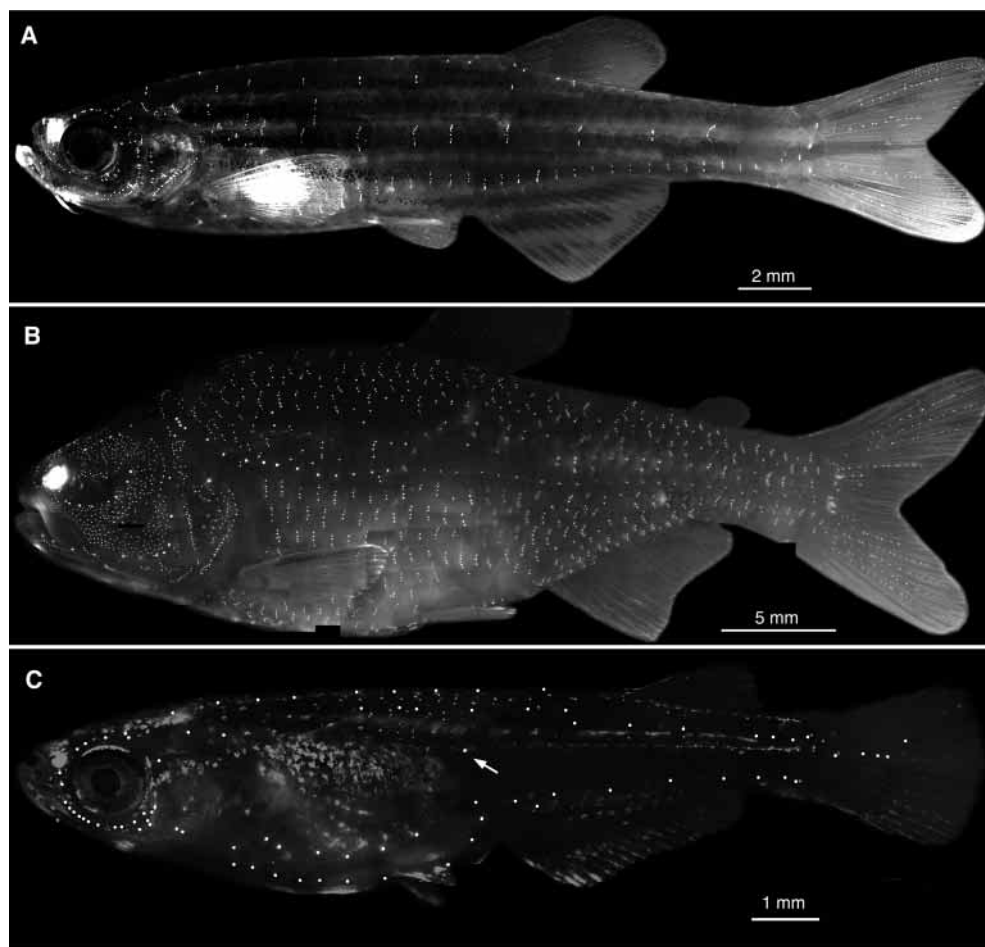
Fig. 6. The juvenile lateral line of *Danio* (A), *Astyanax* (B) and *Oryzias* (medaka; C). The general pattern is identical to that in the adults, except for the size of the stitches. In *Danio* adults the stitches comprise two or sometimes three parallel rows of neuromasts totalling up to 30 organs. (C) In the medaka juvenile shown here, stitch formation had not yet taken place; in the adults the stitches are much smaller than in *Danio* or *Astyanax* – two to three neuromasts at most. One untypical neuromast has remained close to the horizontal myoseptum (arrow), whereas the other organs of the L-PLL have migrated away from it. In medaka, where many superficial fluorescent cells obscure the pattern of neuromasts, we reduced the intensity of this fluorescence by a factor of two to make the lateral line pattern stand out. Anterior is towards the left.

cells in primII at 48 haf. Thus, primII originates from a region anterior to L1.

We then examined embryos at 34±2 haf. We had previously noticed the presence around that time of a large group of proneuromast-like cells on somite 1 (A. G., unpublished; Fig. 2E). We assumed that this group might correspond to the first neuromast of the dorsal line, D1, which differentiates about 10 hours later at a slightly more dorsal position. We had therefore dubbed this large group D0. Uncaging D0 usually resulted in the presence of fluorescent cells both in primII and in D1 (Fig. 2F,G). Out of 17 successful irradiations, we observed labelling of both primII and D1 in ten cases, and labelling of only D1 or primII in five and two cases, respectively.

We conclude that primII is not generated by budding from L1 but originates together with D1 from a common pool of cells, D0, which stays for several hours on somite 1.

Fate and origin of D0

We performed time-lapse analyses of D0 lasting for 2-4 hours. In most cases we observed chaotic displacements of the cells reminiscent of Brownian movement, but no net migration of the cluster. In one case, however, we could observe the onset of primII migration with subsequent splitting of D0 (Fig. 3). This time lapse revealed that, as in the case of neuromast deposition, neighbouring cells tend to remain together and the splitting does not involve any massive reorganisation or sorting out within the cluster.

We occasionally observed 34±2 haf embryos where the D0 cluster had not yet formed. In such cases, we often observed groups of cells very similar to those of D0, at intermediate positions between the PLL ganglion and somite 1. Time-lapse analysis of these cells revealed much local movement, but no coherent migration. We uncaged these cells and examined the distribution of labelled cells on the next day. Out of 15 successful experiments, we observed in all cases fluorescence in both D1 and primII on the next day.

We conclude that the common precursor to primII and D1 originates from a pre-somitic, presumably post-otic, region. We have not been able to define precisely the place of origin of this pool of precursor cells, either by time-lapse analysis or by uncaging. Neither do we know whether our failure to observe migration of precursor cells between the ganglion and somite 1 is due to a very slow speed of migration, or to long intervals of static behaviour. Our observation of a single D0 splitting event amidst many hours of time lapses documenting D0 stability makes us favour the latter explanation.

Molecular reiteration

Our results so far suggest that the formation of additional neuromasts during larval growth is a reiteration of the embryonic process. In a previous study (Gompel et al., 2001b) we described a gene, CB701, which is expressed in the hair cells of the neuromasts and in the L-PLL primordium during embryogenesis. The expression of this gene in the primordium
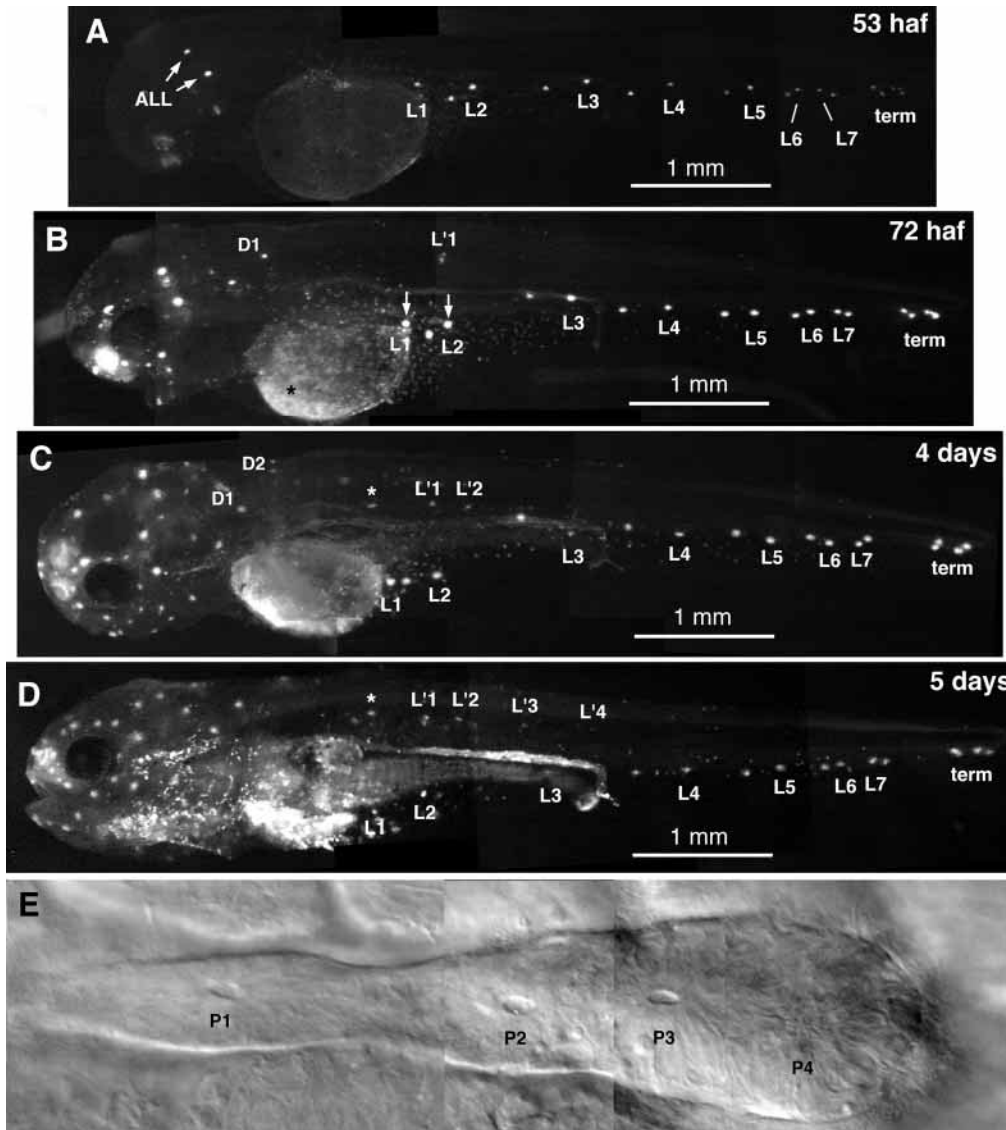
Fig. 7. Early development of the PLL in *Astyanax*. (A) At 53 haf, several neuromasts of the anterior lateral line (ALL) system have differentiated, as well as all the neuromasts of the L-PLL (L1-L7 and three terminal neuromasts). The white dots that alternate with the labelled neuromasts are the organs on the other side of the embryo. (B) At 72 haf, the anteriormost neuromasts L1 and L2 have migrated about half way to the ventral midline, whereas the first neuromast of the L′ and D line have formed. (C) At 4 days, additional neuromasts have formed on the D and L′ lines. This larva is slightly tilted so that the two D2 neuromasts can be seen side by side. (D) At 5 days, the L′ line has further extended. The asterisk labels a neuromast of the L′ line, which might belong to the same wave that produces L′1 to L′4, or be the first neuromast of a second L′ wave. (E) Nomarski micrograph of an *Astyanax* primordium showing its prepatterning. P1, P2 and P3 prefigure three neuromasts where the internal chamber through which the hairs will extend is already formed. A fourth pre-neuromast, P4, can be detected on the basis of the radial organisation of primordium cells. Anterior is towards the left.

is heterogeneous and defines the one or two next clusters that will be deposited. We found that this gene is also expressed in primII in a heterogeneous pattern, much as it was in primI (Fig. 4A), confirming that the mechanism leading to neuromast deposition is the same for primI and primII.

Using this molecular marker we could readily identify other secondary primordia along other lines, e.g. the supra-orbital (Fig. 4B) and infra-orbital (Fig. 4C) branches of the anterior lateral line. We conclude that the generation of additional neuromasts by additional primordia is a general feature of the postembryonic growth of the lateral line system. The gene *eya1* is also expressed both in the embryonic primordium and in primII (Sahly et al., 1999). Thus, primII appears to reiterate molecular features of primI as well as its cellular behaviours.

Neuromast migration

The L-PLL forms along the horizontal myoseptum. Progressively, however, the entire line drifts ventrally as the fish grows up, starting at day 4 (W. K. Metcalfe, PhD thesis, University of Oregon, 1983) (Ledent, 2002). At this time, the embryo is covered by an epithelium called periderm and made

of a single layer of large polygonal cells. As the sensory cilia of the hair cells extend from the neuromast to the outside through a hole in the periderm, the movement of the neuromasts cannot be explained by a simple process of subepithelial migration as in the case of primordium migration. Thus, the ventral displacement of differentiated neuromasts and the caudal migration of primordium cells probably involve different processes.

We wondered whether the ventral displacement of L-PLL neuromasts might reflect a general movement of the periderm towards the ventral midline (for example, as a result of unequal proliferation of the epidermal field), or whether the neuromasts can navigate through the peridermal field. We labelled clones of peridermal cells by injecting single blastula cells with dextran-rhodamine. Suitable clones generally arose from blastomeres near the margin.

We examined larvae at 24 haf and selected those where clones extend at the level of the horizontal myoseptum (Fig. 5A,H). We followed them daily. As expected, at 48 hours, neuromasts start to differentiate in the vicinity of the clones. In cases where the neuromasts differentiated just dorsal to the
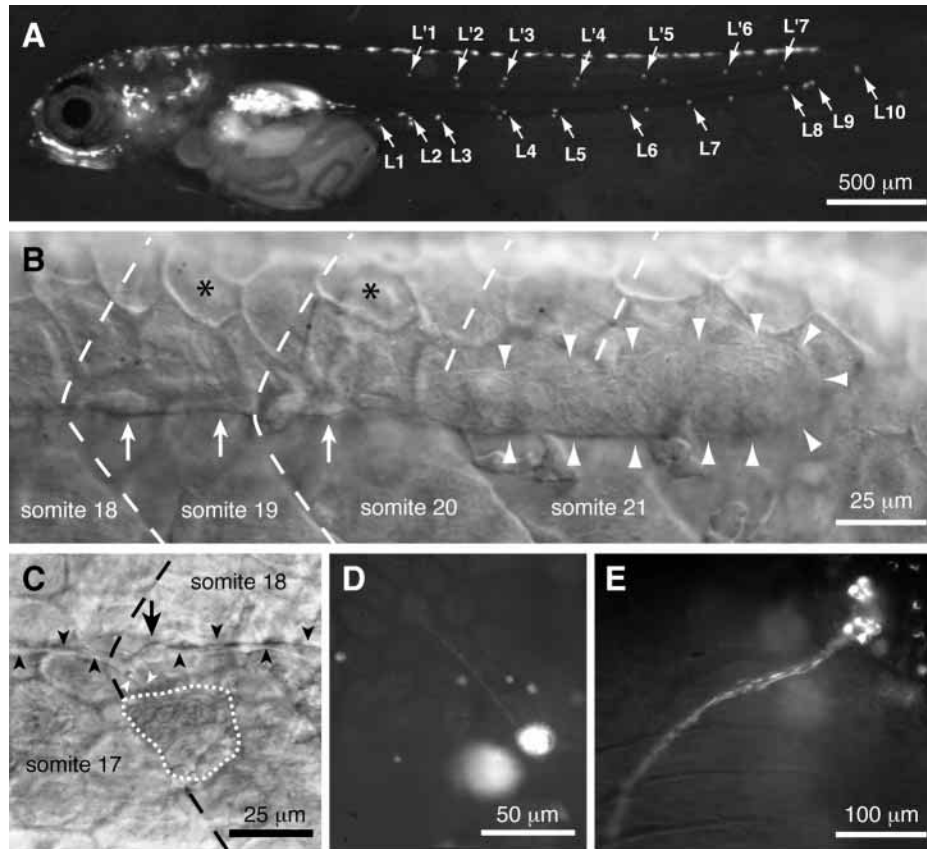
Fig. 8. Early development of the PLL in *Oryzias*. (A) Labelling with 4-di-2-Asp at 10-12 days of development reveals one lateral and one ventral line comprising ten neuromasts each at most. No labelling can be observed at earlier times. (B) At 3 days, a primordium (outlined by arrowheads) migrates from head to tail along the horizontal myoseptum, leaving a nerve (arrows) in its wake. (C) Soon after being deposited, and much before differentiation, pro-neuromasts (outlined in white) begin to migrate ventrally, towing their innervating fibres (white arrowheads). The nerve (black arrowheads) remains on the myoseptum. Black arrow indicates a glial cell. (D) At the time the neuromasts capture 4-di-2-Asp, trans-synaptic labelling of the axons reveals the path followed by the neuromasts when they migrated away from the horizontal myoseptum. (E) Some of the caudal neuromasts migrate dorsally rather than ventrally. These neuromasts are observed in juvenile fish, and we have no way of ascertaining whether they come from the L or L′-PLL line present in larvae (A), or whether they arise as a later set. Anterior is towards the left.

clone, we observed that the labelled cells somehow give way to the moving neuromasts. Thus, the neuromasts appear as dark intrusions (Fig. 5B-E, arrows) indenting the labelled clone. Progressively, the neuromasts dive across the clone and eventually pass through it (Fig. 5F,G).

In some cases the labelled clone directly overlapped a differentiating neuromast (Fig. 5H). These cases revealed that neuromasts recruit overlying peridermal cells to form an annulus-like structure (Fig. 5I,J). As their shape changes from a polygon to a crescent, these cells form the pore through which the hairs will grow out (Fig. 5I). The displacement of the neuromasts involves a translocation of these specialised pore cells across the epithelial sheet (see also Fig. 5C-F, arrowhead). Usually three peridermal cells are recruited to form this anchoring buoy (Fig. 5J,K), and they begin their metamorphosis as soon as the hair cells begin to differentiate.

In salamander larvae, where the process of neuromast formation has been followed in great detail by Stone, it was observed that the differentiating neuromast protrudes and that the overlying ectoderm thins out and eventually gives way to the hair cell processes, thereby forming the pore through which the kinocilia emerge (Stone, 1933). Contrary to the situation in fish, however, there is no indication from Stone's data that ectodermal cells specialise and change their shape to form an annulus defining the pore, as we observed here.

We conclude from these observations that, in the zebrafish, differentiating neuromasts have the ability to induce a specialisation of the overlying cells into pore cells, and that these pore cells can navigate ventrally.

Embryogenesis and early growth of the lateral line in *Astyanax fasciatus*

In the previous sections we examined two of the processes that are involved in shaping the adult PLL of the zebrafish: reiteration of a modular process involving primordium migration and neuromast deposition along the anteroposterior axis, and ventral migration of differentiated neuromasts.

To see how general these processes may be, we examined the early steps of PLL formation in two fish species with very different adult PLL patterns: *Astyanax fasciatus* and *Oryzias latipes* (medaka).

Astyanax fasciatus is one of the very few fish species that have adapted to the total darkness of cave life (Jeffery, 2001). Two fully interfertile forms exist: the surface form, which has normal eyes, and the cave form, which is blind as a result of multifactorial eye regression during larval development (Sadoglu, 1975). Both surface and cave forms have a highly developed PLL system (Schemmel, 1967) (Fig. 6B), which probably played an essential role in the capability of this fish to survive in complete darkness. Indeed, cave adaptation seems to have occurred independently in different regions of its habitat (Borowsky and Espinasa, 1997).

We examined the embryonic and early postembryonic development of the PLL in the blind (Fig. 7) and eyed (not shown) forms. We observed that the primary L-PLL is fully formed on the third day of development, much as it is in *Danio* (Fig. 7A). The pattern is highly similar to that observed in zebrafish embryos, with a total of seven to eight regularly spaced neuromasts aligned along the horizontal myoseptum

and two to three closely packed terminal neuromasts. At 72 haf, however, the anterior neuromasts have already migrated about halfway to the ventral midline, and the first neuromast of the L′ line has formed (Fig. 7B). By the end of the fifth day, all neuromasts of the L line have migrated down to the ventral midline, and four to five neuromasts of the L′ line are already present (Fig. 7D).

Our results indicate that the PLL of *Astyanax* is shaped by the same mechanisms that are observed in *Danio*: formation of successive waves of neuromasts by primordia that repeat the embryonic process and ventral migration of neuromasts. The major difference with *Danio* is that reiterations and migrations seem to occur in quicker succession, thereby generating the much more extensive system observed in the adults. This conclusion is reinforced by an analysis of the migrating primordium. The use of molecular probes has recently revealed in *Danio* that the primordium is prepatterned in a way that prefigures the deposition of the next two or three neuromasts (Itoh and Chitnis, 2001; Gompel et al., 2001b). In *Astyanax*, this prepatterning is already expressed in terms of cell differentiation, and the radial organisation of prospective neuromasts is easily detected within the primordium (Fig. 7E). Furthermore, up to four pre-neuromasts can be recognised. Thus, it appears that in *Astyanax* the major aspects of lateral line formation have been both conserved and accelerated relative to the situation in *Danio*.

Embryogenesis and early growth of the lateral line in *Oryzias latipes*

The Japanese medaka is one of the few fish species where the lateral line system has essentially no lateral component. Instead, most of the PLL neuromasts are present on the dorsal and ventral regions of the body (Yamamoto, 1975) (Fig. 6C). An additional reason to examine the development of the medaka pattern is that, although the characiform *Astyanax* and the cypriniform *Danio* are phylogenetically close relatives, both belonging to the relatively primitive ostariophysian lineage, *Oryzias* belongs to the more evolved neoteleost/acanthopterygian/atherinomorph lineage.

Analysis of early medaka larvae had revealed the presence of one midbody line and one ventral line comprising six and seven neuromasts, respectively (Ishikawa, 1994). We have confirmed this observation in 13 day-old larvae (Fig. 8A). However, earlier larvae show no labelling with 4-di-2-Asp. To see whether the ventral neuromasts might originate along the horizontal myoseptum, as they do in zebrafish embryos, we examined 72 haf larvae for the presence of a primary primordium. This stage corresponds to the early pharyngula period of the zebrafish (Kimmel et al., 1995), when the primary primordium has undertaken its migration. We observed a primordium very similar to that described in *Danio*, migrating along the horizontal myoseptum (Fig. 8B). We also observed that, 1 day later, the undifferentiated proneuromasts have already begun their ventral migration along the intersomitic border (Fig. 8C). At the time the hair cells differentiate and pick up the dye, the L-PLL neuromasts have already migrated all the way to the ventral midline, towing their innervating axons along the way (Fig. 8D).

We assume that a new primordium has by then generated the second wave of neuromasts along the horizontal myoseptum (L′ neuromasts, Fig. 8A), much as happened with the L′-PLL

line of *Danio* and *Astyanax*. Given the adult pattern, there is no doubt that this second line will migrate in turn and either contribute to the line already pioneered by the L neuromasts, or initiate the second ventral line which has been described by Yamamoto (Yamamoto, 1975). We also observed cases of L-PLL neuromasts that migrated dorsally (Fig. 8E), something that we never observed in zebrafish. The occasional organ that can be seen along the horizontal myoseptum in the adult (Fig. 6C, arrow) probably corresponds to a L-PLL neuromast that failed to migrate.

DISCUSSION

Postembryonic primordia

We have examined the origin of the earliest secondary neuromasts in the PLL system of the zebrafish. We show that the formation of secondary neuromasts reiterates the embryonic process, and that a secondary primordium originating from the presomitic region migrates caudalwards along the horizontal myoseptum and deposits clusters of cells at regular intervals of two to three somites. Each cluster later differentiates as a secondary neuromast. The spacing between deposited groups suggests that the secondary primordium documented here is responsible for only a subset of the secondary neuromasts, and that other primordia are required for completing the pattern. We can not rule out that some later-forming neuromasts originate from earlier ones, as is almost certainly the case during the formation of stitches.

Although we could not define the origin of the secondary primordia, we believe that they originate from the same region that gave rise to the primary system, and therefore that each additional wave of neuromasts recapitulates the embryonic process. We conclude that, in spite of their superficial differences, the embryonic development and the postembryonic expansion of the lateral line system are based on the same mechanism and presumably depend on the same developmental controls. A similar conclusion was documented in the case of the eye, which in fish can grow more than a millionfold after hatching: the cascade of gene activities and developmental decisions that are used to build the embryonic eye are constantly repeated in the marginal, expanding region of the growing eye (Harris and Perron, 1998).

An important question to ask is whether postembryonic neurones are produced at the same time as additional primordia are generated. Preliminary results indicate that the number of neurones in the PLL ganglion increases substantially after the primary line has been established (V. Chaar, personal communication), but the origin of these additional neurones has not been identified. Thus we do not know whether the reiteration of the embryonic process includes the formation of new sets of sensory neurones as well as the formation of new primordia.

Placodal origin of the glial cells

Although current literature still adheres to the idea that glial cells are neural crest derivatives (Le Douarin, 1982; Hall and Horstadius, 1988), we observed that the migrating primordium gives rise to cells spread along the lateral line nerve. This observation is consistent with the finding that at least part of the sheath cells surrounding cranial nerves VII and VIII, as

well as the satellite cells within the ganglia, are contributed by epidermal placodes in salamanders (Yntema, 1937). Lineage tracing analyses in fish and toads have failed to detect a crest contribution to lateral line glial cells, while demonstrating a placodal contribution (Collazo et al., 1994). Taken together, these and our data suggest that all or most of the lateral line glial cells are of placodal origin.

In contradiction with these lineage studies, it has been suggested recently that lateral line glial cells are of crest origin because the gene *fdk6* is expressed in pre-migratory crest cells, and later in lateral line Schwann cells and in the satellite cells of the ganglia (Kelsh et al., 2000). Because *fdk6* gene is not expressed by the migrating crest cells, however, its second wave of expression may correspond to a different cell population, rather than to a re-expression in the same cell population. Thus, although we consider that a placodal origin for lateral line glial cells is now well established, the possibility of a neural crest contribution cannot be ruled out.

Patterning of the PLL

The control of neuromast deposition along the anteroposterior axis allows for a one-dimensional patterning of the L-PLL. The control of neuromast migration through the epidermis allows for a completely independent control of pattern in the other dimension. In the zebrafish, the ventral migration of the neuromasts is not uniform along the anteroposterior axis. For example, the anterior most neuromasts do not migrate at all, resulting in a broken line that conspicuously avoids the region behind the pectoral fins. Thus the combination of two independent mechanisms for patterning along the two axes should allow for a remarkable degree of control of the final, two-dimensional pattern.

Another important factor in the control of neuromast migration is the time of origin of their primordium. For example, the set of L′-PLL neuromasts, which forms after the centralised L-PLL is complete, do not migrate at all in the zebrafish. The same is true for the D′-PLL neuromasts. In *Astyanax*, however, only the L-PLL neuromasts laid down by the primary primordium migrate ventrally during the early larval stages. The L′-PLL neuromasts deposited by the subsequent primordia remain aligned along the horizontal myoseptum until they form a complete line with one neuromast at every intersomitic border (data not shown). We have not determined whether the stitches that are present over most of the body surface at later times originate from neuromasts of this line, or whether new waves of neuromast formation occur after the L′-PLL line is complete. Finally, in *Oryzias*, all L-PLL and L′-PLL neuromasts end up migrating ventrally, thereby resulting in the conspicuous absence of lateral neuromasts in this species.

These examples suggest that the combination of anteroposterior primordium migration, dorsoventral neuromast migration, and their modulation in time, is sufficient in principle to generate any PLL pattern. This flexibility, which accounts well for the interspecific variability in PLL patterns, raises the question of whether there is a parallel flexibility in the somatotopy of the sensory projection. So far, however, only a one-dimensional somatotopy has been observed, with more posterior positions of sense organs being represented more dorsally in the hindbrain. Because the migration of differentiated (and innervated) neuromasts occurs only along the dorsoventral axis, it will have no effect on this organisation. Likewise, our observation that dorsal and lateral primordia may share a common origin has no implication for somatotopy as long as the latter deals only with the anteroposterior axis. Conversely, the remarkable variations of the final pattern along the dorsoventral axis may have been facilitated by the fact that changes in this direction have no bearing on the organisation of the sensory projection.

Evolution of PLL patterns: conservation of mechanisms explains both similarities and differences in pattern

Two examples of different patterns have been investigated in *Astyanax* and *Oryzias*. In both cases we found that the pattern laid down during embryogenesis is nearly identical to that in the zebrafish. Yet major phenotypic differences appear very early. For example, the primary neuromasts of *Oryzias* do not differentiate until day 10, long after they have been deposited and have migrated all the way to their final destination, whereas at the other extreme, the neuromasts of *Astyanax* begin to differentiate much before they are deposited by the migrating primordium. Thus, timing seems to play an essential role in the shaping of the lateral line system. Because heterochrony (change in the time of onset or speed of a given developmental process) is a major factor in the evolution of the developmental program, the prevalence of time in PLL design may make it highly susceptible to species-specific variation.

Given the similarities in generative mechanisms observed in the three species, it seems likely that the same mechanisms operate in all or most teleost fishes as well. The many PLL patterns that are observed in different fish species can indeed all be accounted for if dorsoventral neuromast migration can vary depending on position along the anteroposterior axis and on their time of origin. However, the generation of the PLL in amphibians (toads and salamanders) seems to operate along somewhat different principles. The complete pattern is developed progressively from head to tail, rather than developing piecemeal through a succession of generative waves (Stone in salamanders; Winklbauer in *Xenopus*; our personal observations on *Rana* tadpoles) (Stone, 1937; Winklbauer, 1989). Once formed, the neuromasts do not seem to migrate much.

A priori, however, either the amphibian or the teleost mechanism might be a derived character. F. Pichon has observed that in the primitive chondrostean fish *Acipenser*, the sturgeon, a complete line of neuromasts is progressively formed from head to tail, much as it is in the salamander (F. Pichon, personal communication). This would indicate that the modular process observed in the teleosts is a new (derived) feature. Its benefits are fairly obvious, as this saltatory system endows the larva with a primary, head-to-tail functional system at a very early stage of development.

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