The three-sided romance of the lateral line: glia love axons love precursors love glia
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Summary
The lateral line system of fish and amphibians is closely related to the inner ear in terms of evolution, morphology and physiology. Several recent papers have shed new light on the postembryonic development of this system, and have revealed an unexpected triangular relationship where migrating sensory precursors guide axons, axons guide glia and glia, in turn, control the formation of sensory organs. They have also revealed the crucial importance of controlled cell migration not only for patterning the system, but also for determining polarity (and therefore directional sensitivity) of the mechanosensory hair cells. The remarkable accessibility of the lateral line system may allow a detailed analysis of cell migration and polarization, and may help us better understand the complex interactions between sensory precursor cells, neurons and glia during development.

Introduction: the lateral line and its development
The lateral line organ of fish and amphibians is a sensory system devoted to the perception and analysis of changes in the water flow along the body surface. It mediates a sense of touch at a distance. The individual sense organs, called neuromasts, have a core of mechanosensory hair cells surrounded by support cells and mantle cells. Neuromasts are present on the head, body and tail in species-specific patterns (Fig. 1); they may be found either on the surface or in canals that run under the skin. Neuromasts are innervated by sensory neurons whose cell bodies are clustered in cephalic ganglia. They are also innervated by efferent neurons that control the sensitivity of the system.

The lateral line is unique among sensory systems in that the individual sensory organs are derived from cephalic placodes that undergo stereotyped migrations and deposit clusters of undifferentiated cells, the prospective neuromasts or proneur-

Embryonic development of the lateral line in zebrafish
The lateral line system has been extensively described in the adults of many fish species. As in amphibians, it comprises two parts: the neuromasts on the head (anterior lateral-line system, ALL), which are innervated by neurons clustered in a pre-otic ganglion, and the neuromasts on the body and tail (posterior lateral line system, PLL), which are innervated by a postotic ganglion. Surprisingly, however, the development of this system remained unstudied for a long time, until Metcalfe(6) confirmed in the zebrafish the basic features first identified in amphibians. He showed that a PLL primordium...
can first be detected posterior to the otic vesicle at 20 haf (hours after fertilization), migrates caudad along the horizontal myoseptum, which separates dorsal from ventral somitic muscles, and reaches the tip of the tail at about 40 haf. During this journey, the PLL primordium deposits five proneuromasts, L1–L5, and splits into another two or three terminal proneuromasts upon reaching the tip of the tail. Each proneuroma differentiates after its deposition and becomes a mature neuromast in a few hours. Metcalfe(7) also showed that, as in amphibians, sensory growth cones comigrate with the primordium. By five days, the number of PLL neuromasts has increased from 7–8 to 11–13.(8)

One major question about PLL development was the nature of the system used to guide the PLL primordium during its long journey. It has now been demonstrated that a trail of cells along the migratory pathway expresses the chemokine SDF1, and that the migrating cells express its receptor CXCR4. Inactivating either the ligand or its receptor results in the inability of the cells to migrate along the proper pathway, and often in a complete lack of migration.(9,10) In all cases, the afferent axons extend to the primordium(9,11,12) supporting Harrison’s contention that the sensory fibers are towed by the migrating primordium.

The SDF1–CXCR4 interaction has also been implicated in the long-range migration of germ cells in fish(13,14) and in mouse and chicken(15,16) as well as in the caudal migration of efferent PLL neurons and other motor neurons in the hindbrain(17) and is therefore a major determinant of directional migratory processes. Intriguingly the various migration events that depend on SDF1–CXCR4 are aligned along the anteroposterior axis, suggesting that this system may be preferentially associated with migrations along this axis, much as the slit/robo and netrin/DCC systems are usually associated with guidance along the mediolateral axis, and the reelin system with guidance along the apicobasal axis of the central nervous system.

The intimate association of the SDF1–CXCR4 system with long-range migration also provides an easy explanation for the finding that CXCR4 expression directs the migration of several types of cancer cells towards tissues that express SDF1, and is responsible for the ensuing formation of tissue-specific metastases.(18)

**Postembryonic development of the zebrafish PLL: primII and cell polarity**

If the embryonic development of the PLL has been clarified over the past few years, its postembryonic development remained somewhat of a mystery. Remember, the number of PLL neuromasts increases from 7–8 to 11–13 over the few days that follow the end of migration, and reaches about 30 (one neuromast for each somite) after 2–3 weeks (Fig. 2). From where do the additional neuromasts originate? Are they “accessory” neuromasts derived by budding from primary neuromasts deposited by the primordium, as described by Stone in amphibians? Or do they have a different origin? A detailed analysis of larval growth led to the suggestion that additional neuromasts are deposited by an additional primordium, primII.(19) The characterization of primII revealed that it deposits 4–5 neuromasts interspersed between the first and third “primary” neuromasts, L1 and L3, and is also at the origin of a line of dorsal neuromasts that develops at the same time.(20) The neuromasts derived from primII are usually two somites apart, implying that there must be yet another mechanism to fill the remaining somitic positions.
The story of primII took on an additional and unexpected meaning when Lopez-Schier and Hudspeth realized that the neuromasts deposited by this primordium have their hair cells polarized perpendicular to the hair cells deposited by primI. As in the inner ear, the lateral-line hair cells have an intrinsic polarity in the organization of the apical surface, where the kinocilium is off-center and the tuft of stereocilia increases in size towards the kinocilium. This structural anisotropy parallels a functional polarization whereby bending the cilia towards the kinocilium excites the hair cell, while bending in the other direction hyperpolarizes the cell.

Once deposited, the neuromasts tend to migrate ventrally, although the extent of this migration depends on the antero-posterior position. Lopez-Schier and Hudspeth noticed, however, that the proneuromasts deposited by primII begin to migrate ventrally before they differentiate, while those deposited by primI also migrate ventrally but only after they have differentiated. They propose that the direction of migration just preceding differentiation determines the polarization of the ciliary apparatus. This conclusion is supported by the finding that, when primI follows incorrect paths due to a defect in the SDF1/CXCR4 guidance system, the ciliary bundles are accordingly disoriented relative to the fish axes, but remain properly oriented relative to the direction of the last stretch of migration.

The interneuromastic cells

While primI is responsible for the first set of 7–8 neuromasts that are deposited during embryogenesis, and primII is responsible for inserting 4–5 additional neuromasts with perpendicular polarity, we are still far from the stage where 25–30 neuromasts are present, one for each somite. A first hint at the origin of late neuromasts came from an analysis of the interneuromastic cells.

Much as in the salamander, the fish primordium leaves in its wake a stream of interneuromastic cells that are easily detected when the primordium cells are labeled, for example by fluorescein uncageing. Interneuromastic cells are also marked by the expression of genes that are expressed in the lateral-line placodes, such as eya1 or claudin b. Interneuromastic cells were first thought to contribute to the glial population that ensheathes the nerve fibers, together with neural-crest-derived glia. Interneuromastic and crest-derived glial cells can be discriminated, however, based on the distinct expression of placodal markers and of foxd3, an early marker of crest-derived peripheral glia. The two types of cells are closely apposed, and even seem to establish intimate contact.

A careful analysis of the fate of interneuromastic cells derived from primI revealed that they tend to coalesce and to undergo cell proliferation over the next few days. The resulting clusters then differentiate into neuromasts, leading to the progressive completion of the pattern. primII also deposits a stream of interneuromastic cells, and it is plausible, but not demonstrated, that these cells are capable of generating additional neuromasts. It appears, therefore, that each primordium deposits a small number (5–8) of primary neuromasts and, in between the primary neuromasts, it deposits a stream of interneuromastic cells that are able to coalesce and proliferate and form additional neuromasts. In agreement with these authors, we propose to use the term “intercalary” for those neuromasts that develop from interneuromastic cells, and which differ therefore both from the primary and from the accessory neuromasts previously defined in amphibians. This nomenclature would avoid potential confusion associated with “secondary”, since this word has been used to describe both the neuromasts deposited by primII and those that arise from the interneuromastic cells.

**Figure 2.** Four stages in the development of the posterior lateral-line system of the zebrafish, modified from Ledent. At 2 days, five neuromasts (L1–L5) and two or three terminal neuromasts (ter) have been deposited by the migrating primordium primI. The embryo is about 3.5 mm long. At 5–6 days, the time when the larva is beginning to feed, the PLL line comprises 11–13 neuromasts: those that were deposited by primI, and a second set (LII.1–LII.4) that is being deposited by a second primordium, primII. At this age, the larva is about 5 mm long. At 2–3 weeks, the line of 11–13 neuromasts is progressively completed by intercalation of additional neuromasts, until there is a neuromast on every intersomitic border. The larva is about 7 mm long. At 2 months, each neuromast has given rise to a cluster of closely associated accessory neuromasts, thereby forming a “stitch”. The figure shows eight consecutive stitches that arose from eight consecutive neuromasts as presented in the panel above. The juvenile is about 25 mm long.
A developmental role for the glia?

Intercalary neuromasts arise from interneuromastic cells that were deposited by primI between 20 and 40 haf, yet they differentiate much later (1–3 weeks after fertilization). What, then, is responsible for this long delay in maturation? The answer is wholly unexpected: the control of the transition from 12 neuromasts to more than 30 (one for every somitic border) depends on—glial cells! This surprising result came from a different type of experiments, whereby the other two components of the interneuromastic environment, neurons and glia, were genetically or experimentally removed\(^{(23,25)}\) (Fig. 3). Either treatment has a most striking effect: it results in the precocious formation at 4–5 days of large numbers of additional neuromasts, and (when seen in fluorescence) converts every embryo into a tiny glowing jewel. Subtle abnormalities can already be detected at 2 days: clusters of interneuromastic cells show first signs of expansion, and experiments of BrdU incorporation demonstrate a much higher rate of cell proliferation than in untreated or wild-type embryos.\(^{(23)}\)

Since removing either sensory neurons or glia results in an identical overproduction of neuromasts, which of the two cell types is responsible? It has been shown that the axons can grow in the absence of glia (albeit with defasciculation abnormalities, Fig. 3A) but glia cannot migrate in the absence of axons.\(^{(27)}\) Since an identical phenotype is reached after removing the neurons (which effectively eliminates both axons and glia), or removing the glia alone, it follows that the determining factor is likely the absence of glial cells.

Could it be, then, that glia cells are responsible for preventing the precocious differentiation of interneuromastic cells into intercalary neuromasts? A very elegant way to test this

\[\text{Figure 3. Effect of removing glial or neuronal cells on PLL development. A: A hypersensitive (hps) mutant embryo stained at 5 days with anti-acetylated tubulin, a marker of neurites (red), and with FM1-43, a marker of hair cells (yellow). The absence of glial cells results in a dramatic increase in the number of neuromasts, but the effect on the nerve is limited to a mild defasciculation of the afferent fibers. Picture courtesy of D. Raible. B: neurogenin (ngn1) mutant embryo stained at 5 days with 4-Di-2-Asp, a marker of hair cells. The genetic removal of afferent neurons results in a massive increase in the number of neuromasts. Picture courtesy of H. Lopez-Schier. C: A wild-type embryo where the PLL ganglion was removed on one side by laser-mediated cell ablation at 1 day, and stained at 5 days with 4-Di-2-Asp (our unpublished experiments). The control side shows the expected pattern at this developmental stage: L1–L5, the neuromasts deposited by primI, and LII.1–LII.3, the first three neuromasts deposited by primII. The experimental side shows a continuous string of about 20 neuromasts, in agreement with the results generated by mutational or antisense inactivation of either neurons or glia.}\]
hypothesis would be to remove the glia by genetic means, and then to rescue the altered phenotype by injecting normal glial cells. The result of this experiment\(^{23}\) demonstrates that the local presence of wild-type glial cells is sufficient to prevent the precocious formation of intercalary neuromasts in a mutant embryo, thereby convincingly demonstrating the role of glial cells in controlling the emergence of late neuromasts. Figure 4, left column shows the sequence of events during normal development, with the sequence in fish that lack either neurons or glia schematized on the right of the figure.

The control of neuromast maturation by glial cells came as a surprise, since the peripheral glia are mostly thought of as accompanying peripheral neurites. It is possible, of course, that the prominent role of glial cells on lateral line development is a peculiarity of this organ, a highly derived mechanism that arose in the teleost lineage at the same time as the intercalary neuromasts themselves. Alternatively, it is also possible that glial cells have a much more general effect on sense organ development than hitherto realized, and that this aspect of glial function has simply been overlooked so far, possibly because of its complex interactions with nerve fibers. Indeed the realization that glial cells play multiple roles in the development and patterning of the central nervous system, from the guidance of migrating neurons in vertebrates to the formation of axon tracts in \emph{Drosophila}, is a relatively recent acquisition in neurobiology.

\section*{Accessory neuromasts}

Stone had already demonstrated in the amphibian PLL that two types of neuromasts are formed sequentially: “primary” neuromasts that are deposited by the migrating primordium, and “accessory” neuromasts that bud off from the primary neuromasts.\(^5\) He also demonstrated that the accessory neuromasts are generated by the support cells of the primary neuromasts.\(^4\) The capability of support cells to generate both support and hair cells is well documented,\(^{28}\) and accounts for the fact that hair cells are constantly renewed throughout life, with new hair cells being generated by division of support cells.\(^{29}\) It should be noted, however, that the cells that form new hair cells or accessory neuromasts may be stem-like cells that we cannot yet distinguish from the surrounding support cells.

Accessory neuromasts of amphibians remain closely associated to the primary neuromast from which they are derived and form small clusters called “stitches.”\(^4\) Stitches are also present on the adult zebrafish, and are likely formed by a similar process of budding. In the zebrafish, all neuromasts of a stitch seem to be innervated by the same neurons, further supporting the idea that they all derive from a single neuromast.\(^{19}\) Thus the formation of accessory neuromasts is unlikely to allow for the development of new perceptions, but may simply enhance the sensitivity of the system and compensate for the fact that, as the fish grows,
the overall density of neuromasts over the body surface is decreasing.

**Intercalary neuromasts and Joseph Fourier**

Although the development of the PLL in teleosts closely resembles that in amphibians, it differs in one important respect: the embryonic PLL comprises a small number of neuromasts, typically less than 10, and is progressively completed by the addition of intercalary neuromasts. Some of the additional neuromasts derive from a second primordium, primII, but others arise through cell proliferation of the interneuromastic cells. What could be the biological significance of the delay imposed on the differentiation of intercalary neuromasts?

It does not seem very likely that the precocious expansion of the interneuromastic cells into neuromasts would be a heavy drain on the embryo’s resources and, indeed, a subgroup of the interneuromastic cells.(23) An alternative, map in the hindbrain may put constraints on the number of primary neuromasts is that the establishment of a somatotopic pattern until one neuromast is present for each somite. Given that the interneuromastic cells deposited by primI are largely sufficient to provide one neuromast on every somite, the primary neuromasts deposited by primI have a spacing of about 5 somites on average, those deposited by primII have a spacing of about 2 somites, and the intercalary neuromasts are spaced by 1 or 2 somites on average. If the information from the corresponding sets of neurons is kept separate, this would endow the fish with the capability to perform a Fourier transform of the complicated wave forms that are reflected by nearby objects, thereby vastly increasing the capacity to handle and analyse this information. Such a capacity would probably contribute to the amazing capabilities of blind cavefish, which can not only discriminate between minute landmark differences, but also connect such landmarks to build a spatial map of their large-scale environment.

**primI versus primII**

Given that the interneuromastic cells deposited by primI are largely sufficient to provide one neuromast on every somite, what is the reason for having a second primordium do part of the job? A first answer is, again, neuronal: having two sensory systems that detect water movements along two orthogonal directions could well present serious advantages e.g. for localizing prey or predator. It will be very interesting to determine if the neurons that innervate primI and primII neuromasts differ in projection or connectivity. It will also be interesting to determine if the intercalary neuromasts originating from primI, and those originating from primII, retain the same orientation preference shown by the primary neuromasts.

A second reason for the existence of primII originates in the presence of two PLL primordia in the amphibians, the toad *Xenopus* and the salamander *Ambystoma*, as well as in two basal fish species, the sturgeon *Acipenser*, and the gar *Lepisosteus*. Instead of being intercalated, the two primordia establish two complete lines that run side by side the entire length of the animal. The stitches of one line are oriented in the anteroposterior direction, while those of the other line are oriented in the dorsoventral direction. It seems likely, therefore, that the principle of two PLL primordia establishing two lines of neuromasts with perpendicular sensitivity was already established before the fish and tetrapod lineages diverged, and was maintained in the zebrafish (and presumably in other teleosts as well).

**One somite, one neuromast**

Interneuromastic cells are present all along the pathway of primI, including at positions where primII has already deposited neuromasts. Yet there is, in general, only one neuromast at every intersomitic border. There must be, therefore, a mechanism to restrict the formation of neuromasts to the somitic borders, and a further mechanism to prevent interneuromastic cells from escaping the glial repression at borders where one neuromast is already present.

The nature of the first mechanism, that which restricts the position of neuromasts to somitic borders, is not known. It must be noted, however, that this restriction is present in euteleosts but not in more basal fish species, and seems therefore evolutionarily associated to the mechanism that allowed the formation of the PLL in two steps—primary neuromasts quickly sampling the entire length, and progressive maturation of intercalary neuromasts leading to the completion of the pattern until one neuromast is present for each somite.

As for the mechanism that restricts the formation of neuromasts to one per somite, the expansion of interneuromastic cells appears to take place at somites where there preexists no other neuromast. This constraint seems to disappear when glial cells are absent, however. The presence of two neuromasts on the same somitic border, in glia-less larvae, suggests that the release from glial repression depends on some external cue that is segmentally organized. One possibility is that this release is induced by incoming afferent fibers, and that innervation is the factor that allows interneuromastic cells to form an intercalary neuromast.

The primary neuromasts are innervated by afferent fibers that accompany or closely follow the primordium, and the accessory neuromasts within one stitch seem to be innervated by branches of the fibers that innervate the neuromast that gave rise to the stitch. Nothing is known yet of the innervation of intercalary neuromasts, and even the origin of their afferent neurons is obscure. Grant et al. have noted that after...
extirpation of the PLL ganglion neurons quickly regenerate, and we have observed that most or all additional neuromasts generated after laser ablation of the ganglion are innervated (unpublished results). The high resolution of the lateral-line organ, and its unexpected degree of developmental complexity, may make this system a good place to unravel the interactions between developing neurons, glia and sensory precursors—a system that may yet have other surprises in store.

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References