Development 125, 4645-4654 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV9611

Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development

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Accepted 1 September; published on WWW 9 November 1998

SUMMARY

The sensory patches in the vertebrate inner ear are similar in function to the mechanosensory bristles of a fly, and consist of a similar set of cell types. If they are truly homologous structures, they should also develop by similar mechanisms. We examine the genesis of the neurons, hair cells and supporting cells that form the sensory patches in the inner ear of the chick. These all arise from the otic epithelium, and are produced normally even in otic epithelium cultured in isolation, confirming that their production is governed by mechanisms intrinsic to the epithelium. First, the neuronal sublineage becomes separate from the epithelial: between E2 and E3.5, neuroblasts delaminate from the otocyst. The neuroblasts then give rise to a mixture of neurons and neuroblasts, while the sensory epithelial cells diversify to form a mixture of hair cells and supporting cells. The epithelial patches where this occurs are marked from an early stage by uniform and maintained expression of the Notch ligand Serrate1. The Notch ligand Delta1 is also expressed, but transiently and in scattered cells: it is seen both early, during neuroblast segregation, where it appears to be in the nascent neuroblasts, and again later, in the ganglion and in differentiating sensory patches, where it appears to be in the nascent hair cells, disappearing as they mature. Delta-Notch-mediated lateral inhibition may thus act at each developmental branchpoint to drive neighbouring cells along different developmental pathways. Our findings indicate that the sensory patches of the vertebrate inner ear and the sensory bristles of a fly are generated by minor variations of the same basic developmental program, in which cell diversification driven by Delta-Notch and/or Serrate-Notch signalling plays a central part.

Key words: Notch1, Delta1, Serrate1, Chick, Ear, Bristle

INTRODUCTION

Peripheral sense organs can be grouped into two fundamentally different classes according to the developmental relationship between the peripheral sensory cells and the sensory neurons that innervate them. Organs of one class, typified by the mechanosensory structures of the skin and the spindle organs of muscles, are innervated by neurons generated at a remote central site in the embryo, such as the neural crest. Organs of the other class, by contrast, derive both their sensory neurons and their peripheral transduction apparatus from one and the same peripheral source. The inner ear is an organ of the latter type: its sensory neurons, as well as its sensory hair cells and supporting cells, derive from the otic placode (D'Amico-Martel and Noden, 1983; Hemond and Morest, 1991; Haddon and Lewis, 1996). In this basic feature the mechanosensory patches of the inner ear – the basilar papilla, sensing sound vibrations; the maculae, sensing linear acceleration and gravity; and the cristae, sensing rotation – resemble the mechanosensory bristles and other sensilla of insects, whose neurons and ancillary cells likewise have a common origin from peripheral ectoderm (Hartenstein and Posakony, 1989). This provides the basis on which to draw a developmental as well as a functional parallel between the system of neurons, hair cells and supporting cells that form a sensory patch in the ear, and the system of neuron, shaft cell, socket cell and sheath cell forming a sensory bristle on a fly (Fig. 1A,B; Lewis, 1991).

For both structures, the central developmental question is how these cell types are generated and organised in the correct spatial pattern (Fig. 1C) (Fekete, 1996; Goodyear and Richardson, 1997). If all the cells arise from a common source, how are they caused to become different? One mechanism for such cell diversification is lateral inhibition, whereby a cell that is becoming committed to a particular pathway of differentiation inhibits its immediate neighbours from doing likewise (Corwin et al., 1991; Lewis, 1991). In many developmental systems, including insect bristles, this type of interaction is mediated by Delta-Notch signalling, with the Notch protein acting as a transmembrane receptor in the cell receiving inhibition, and the Delta protein acting as a

transmembrane ligand in the cell delivering inhibition (Hartenstein and Posakony, 1990; Artavanis-Tsakonas et al., 1995; Lewis, 1996). We have previously reported the cloning of homologs of *Notch*, of *Delta* and of the *Delta*-related gene *Serrate* in the chick, and have shown that Delta-Notch-mediated lateral inhibition controls production of neurons in the vertebrate central nervous system as it does in the fly (Chitnis et al., 1995; Myat et al., 1996; Henrique et al., 1997; Haddon et al., 1998b). Here we examine how the neurons, hair cells and supporting cells of the chick inner ear are generated, with special reference to the role of these genes – *C-Notch1* (*N1*), *C-Delta1* (*Dl1*) and *C-Serrate1* (*Ser1*) – in their production. Our findings allow us to spell out a detailed system of developmental parallels between the sensory patches of the ear and the sensory bristles of insects.

MATERIALS AND METHODS

Embryos and staging

Fertile hens' eggs (Light Sussex × Rhode Island Red) were incubated in a humidified atmosphere at 38°C. The Hamburger-Hamilton tables were used for staging and to relate the stage number to the age in notional hours of incubation (Hamburger-Hamilton hours, or HHhours).

Descriptions of normal development are based on at least three embryos for each stage, for each antibody or in situ hybridisation probe.

Antibody labelling

Embryos were prepared for cryostat sectioning (10-15 μm) and immunostaining essentially as described by Strähle et al. (1994). Antibodies were: TuJ1 (Lee et al., 1990; Memberg and Hall, 1995); Islet1/2 antibody (originally designated Islet1; Tsuchida et al., 1994); BEN (Chedotal et al., 1996); 3A10 (Furley et al., 1990); and hair-cell antibody (HCA) (Bartolami et al., 1991). Ser1 polyclonal antiserum was raised against a 310 bp fragment of the Ser1 intracellular domain, as described by Varnum-Finney et al. (1998). FITC-labelled rabbit anti-mouse IgG (DAKO) and Texas-Red-conjugated goat anti-mouse IgG (Jackson) were used as secondary antibodies.

Sections were counterstained with the nuclear dye 7 amino-actinomycin D (7AAD; Molecular Probes; 50 μg/ml in PBS) and viewed either on a confocal microscope (BioRad MRC600) or on a conventional fluorescence microscope. In all cases controls were prepared, omitting either the primary or the secondary antibody.

Immunostaining for Ser1 or Islet1/2 was combined with in situ hybridisation for the expression of other genes as described by Henrique et al. (1997).

For whole-mount immunostaining of the basilar papilla, chick embryos were partially dissected in PBS, fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight, rinsed in PBS and further dissected to expose the basilar papilla. The tissue was immunostained with HCA as described by Bartolami et al. (1991) and then counterstained for actin using 2.5 mg/ml FITC-phalloidin (Sigma) in PBS at room temperature in the dark for 2 hours-overnight; specimens were rinsed and mounted in PBS and viewed with a BioRad MRC600 confocal microscope.

Culture technique

Otic placodes and otocysts were dissected from embryos at stages 8 to 25. The otic placode does not become visibly distinct until Stage 10 (36 HHhours); before this, we identified the prospective placode by position relative to hindbrain segments and somites. Each dissected fragment was transferred to 0.25% trypsin (Flow Laboratories) in PBS with 0.02% EDTA at 4°C (5-10 minutes for otic epithelium from 2-day embryos; 30-40 minutes for epithelium from 5-day embryos) and stripped of mesenchyme using tungsten needles. The epithelium was then flattened out on a feeder layer of mitomycin-C-treated fibroblasts (Freshney, 1993), or directly on laminin-coated glass. Cultures were incubated at 37°C, in a humidified 5% CO_2 atmosphere, in medium 199 (modified with Hanks salts) with 20% foetal calf serum, 2 mM glutamine, 1.1 g/l $NaHCO_3$, 2% chick embryo extract; no antibiotics were added. After 1-5 days, the cultures were fixed in 4% formaldehyde in PBS and stained with 3A10 or with HCA and Ser1 antisera; 7AAD was used as a counterstain.

In situ hybridisation

In situ hybridisation was performed with DIG-labelled RNA probes and an alkaline-phosphatase-coupled anti-DIG antibody, which was then detected with NBT/BCIP or with Fast Red to give a fluorescent product, as described by Henrique et al. (1997). For whole-mount embryos, we followed the protocol of Henrique et al. (1995) and for cryostat sections, that of Strähle et al. (1994) with minor modifications. Details of the *Dl1*, *Ser1* and *N1* probes are given in Henrique et al. (1995) and Myat et al. (1996); the *Lunatic fringe* probe was as described by Laufer et al. (1997).

RESULTS

Fig. 2 gives an outline of inner ear development in the chick. The otic placode is first visible at 1.5 days of incubation (E1.5) as a thickening in the head ectoderm next to the hindbrain at the level of rhombomeres 5 and 6. The placode invaginates to form a cup, which closes and pinches off from the head ectoderm to become a pear-shaped otocyst. Over the following four or five days complex shape changes take place, converting the otocyst

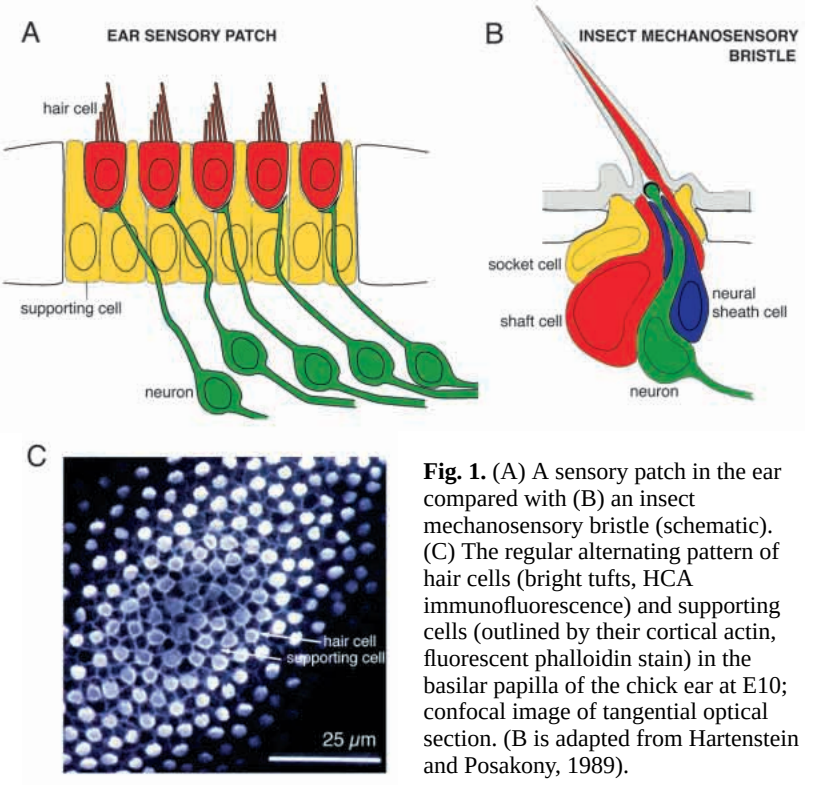

Fig. 1. (A) A sensory patch in the ear compared with (B) an insect mechanosensory bristle (schematic). (C) The regular alternating pattern of hair cells (bright tufts, HCA immunofluorescence) and supporting cells (outlined by their cortical actin, fluorescent phalloidin stain) in the basilar papilla of the chick ear at E10; confocal image of tangential optical section. (B is adapted from Hartenstein and Posakony, 1989).

into a membranous labyrinth with semicircular canals and utricle dorsally and saccule and banana-shaped cochlea ventrally. Neurogenesis occurs at the beginning of this period (E2–E3.5), hair-cell differentiation towards its end (from E5 onwards).

Expression of *N1* and *Dl1* in the otic placode foreshadows neurogenesis

By early stage 11 (40 HHhours), expression of *Notch1* marks out an ectodermal patch that includes the whole otic placode; within the patch, expression is uniform (Fig. 3A). By the end of stage 11 (about 3 hours later), a few scattered cells expressing *Dl1* begin to be seen in the anterior part of the placode. The number of cells expressing *Dl1* increases rapidly, but these remain confined to the anterior half of the placode (becoming anteroventral in the cup) and continue to be only a scattered subset of the cell population in that region (Fig. 3B).

To follow neurogenesis and to see how it relates to *Dl1* expression, we have used four antibodies: Islet1/2, TuJ1 and BEN, which detect neuronal antigens whose expression is reported to begin early in neuronal differentiation, before neurofilaments are seen in the cell, and 3A10, which binds to a neurofilament-associated epitope (see Materials and Methods).

In the otic epithelium, we first see immunostaining with Islet1/2, TuJ1 and BEN at stage 12/13 (48-49 HHhours), 6-7 hours after *Dl1* expression begins. By stage 14/15 (52 HHhours), staining with Islet1/2, TuJ1 and BEN is concentrated in the anteroventral part of the otic cup. Islet1/2, as a nuclear marker, gives the most precise indication of the behaviour of individual cells: within the anteroventral domain, the cells expressing Islet1/2 antigen form a scattered subset of the epithelial population (Fig. 3C). The cells expressing Islet1/2 and TuJ1 are concentrated basally in the epithelium, and some can be seen straddling the basal lamina, as though in the act of delaminating (Fig. 3D,E,F).

Doubly stained sections show that the Islet1/2 domain coincides with the *Dl1* expression domain, but that the individual cells in this region never express both markers simultaneously (Fig. 3H,I). By analogy with the embryonic central nervous system (Chitnis et al., 1995; Henrique et al., 1995; Myat et al., 1996), this suggests that the *Dl1*-expressing cells in the ear epithelium are neuronal precursors, expressing Dl1 transiently before switching on expression of markers of neuronal differentiation.

At stage 14-15 (52 HHhours), we begin to see a few cells expressing Islet1/2, TuJ1 and BEN antigens that have escaped to form the first rudiment of the cochleovestibular ganglion, pressed close against the anteroventral otic epithelium. On the basis of these observations, we can identify the anteroventral patch of expression of *Dl1*, Islet1/2, TuJ1 and BEN in the otic epithelium as the site of neurogenesis. A similar pattern of labelling persists in this neurogenic patch for about 36 hours, up to stage 21-22 (84-90 HHhours), and throughout this period there is a continuing exodus of cells expressing Islet1/2, TuJ1 and BEN from the otic epithelium into the developing ganglion.

The delaminating cells are neuroblasts rather than postmitotic neurons

3A10 staining is not seen in the ear until at least stage 17 (58 HHhours), 6 hours after delamination has begun, and it is confined to the ganglion. The 3A10-positive cells are identifiable as young bipolar neurons, with axons and dendrites

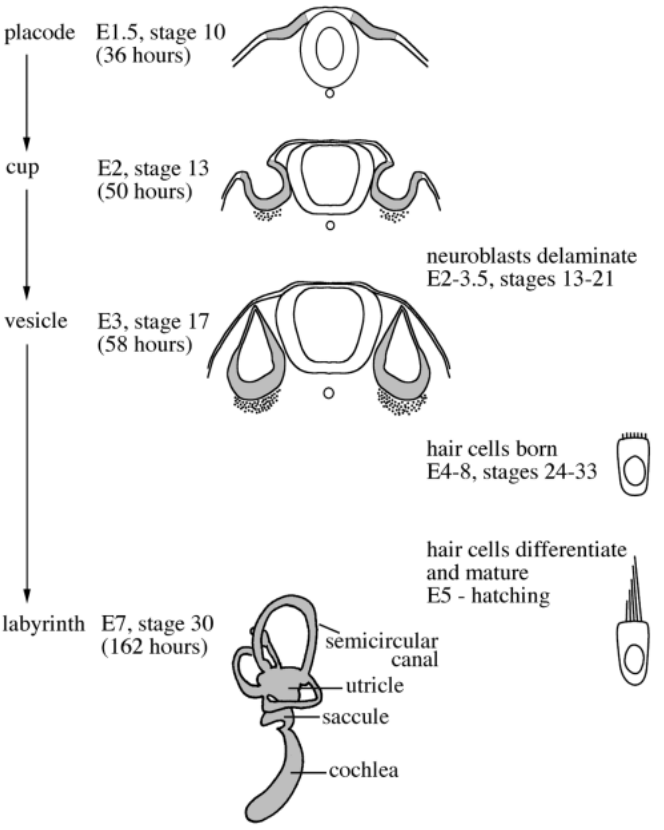

Fig. 2. Timetable of inner ear development in the chick.

(Fig. 3J). Over the subsequent days, the number of these neurons in the ganglion steadily increases.

From previous [^{3}H]thymidine studies of cell division in the ganglion (D'Amico-Martel, 1982), it is clear that the ganglion precursor cells delaminating from the otic epithelium are not postmitotic nascent neurons, but neuroblasts capable of dividing before they differentiate (see also Memberg, 1995). Our observations confirm this: Islet-positive cells can be seen in mitosis both in the neurogenic patch in the otic epithelium (Fig. 3D) and in the developing cochleovestibular ganglion (Fig. 3E), and this matches findings with BrdU labelling (data not shown).

BEN expression persists in the target epithelium as well as in the developing neurons

The Islet1/2 and TuJ1 antigens continue to be expressed in the ganglion, but in the otic epithelium cells expressing them have disappeared by stage 21-22 (84-90 HHhours), corresponding to the end of delamination of neuronal precursors from the otocyst (Fig. 3K).

BEN, however, behaves differently. In the ganglion, its expression is similar to that of Islet1/2 and TuJ1, but in the otic epithelium it persists after Islet1/2 and TuJ1 have disappeared, and marks the region that is invaded by processes from the ganglion cells, i.e. the presumptive sensory area (Fig. 3L). Thus BEN labels both the neurons and their peripheral target epithelium. Homophilic interactions mediated by BEN (a cell-surface adhesion molecule) may help the dendrites of the cochleovestibular neurons, as they grow back into the otic epithelium, to recognise the sensory patch in which they must make synapses (Chedotal et al., 1996).

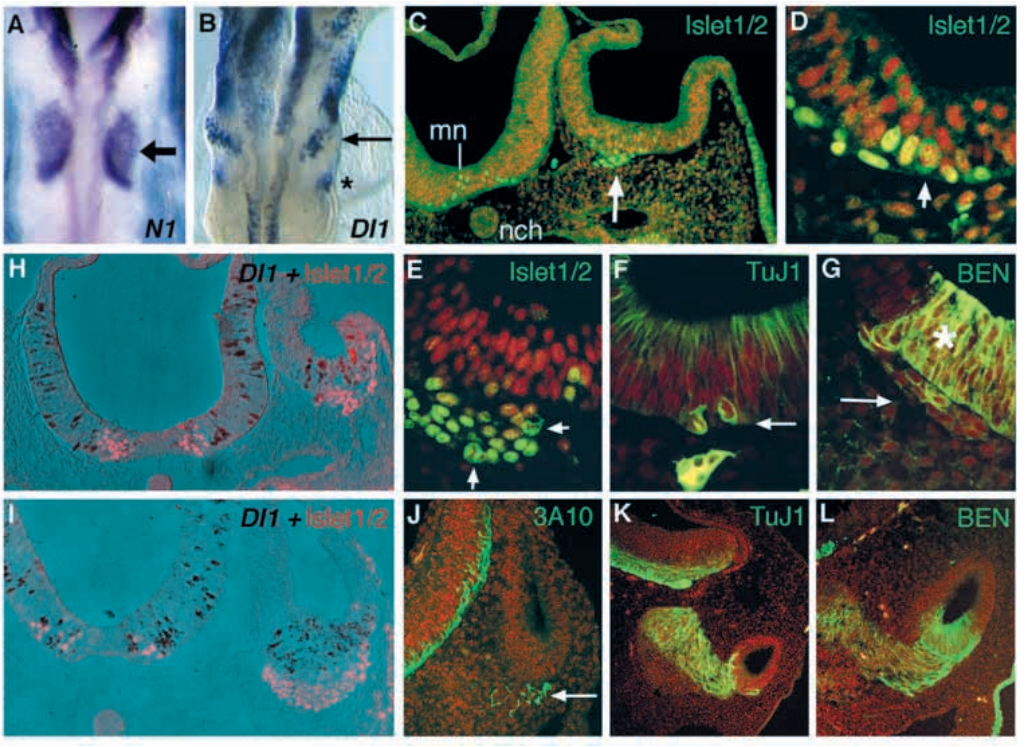

Fig. 3. Expression of *N1*, *Dl1* and neural markers in the ear rudiment during neurogenesis. *N1* and *Dl1* patterns are shown by in situ hybridisation, and Isl1/2, TuJ1, BEN and 3A10 by immunofluorescence; the red fluorescent nuclear dye 7AAD was used as counterstain in C-G and J-L. A and B are whole mounts, partially dissected; C-L are sections through the anterior, neurogenic region of the otic cup/otocyst, cut transverse to the main body axis. (A) Stage 11: *N1* is expressed throughout the placode (broad arrow). (B) Stage 12: *Dl1* is expressed in scattered cells in the anterior, neurogenic portion of the otic placode/cup (thin arrow). *Dl1*-expressing cells marked with an asterisk are glossopharyngeal, not otic. (C) Stage 14/15, Islet1/2 (green): arrow indicates delaminated neuronal precursors (rudiment of ganglion). nch, notochord; mn, motoneurons in neural tube. (D) Detail of specimen similar to C, showing Islet1/2-positive cells at the basal face of the epithelium, about to delaminate. Note that one of the Islet1/2-positive cells is in mitosis (arrow). (E) Similar to D, but at stage 18; arrows indicate mitotic cells in ganglion. (F) Stage 14/15, TuJ1 (green): note delaminating neuroblasts (arrow). (G) Stage 18, BEN (green): note stain in epithelial patch (asterisk) as well as in delaminated cells (arrow). (H) Stage 14/15, double-stained for *Dl1* expression (blue-black) and Islet1/2 (red, false colour). (I) Same as H, but at stage 17. The two markers are expressed in closely corresponding regions, but not in the same individual cells; this is as expected if *Dl1* is expressed transiently in the cells that are about to express Islet1/2 and delaminate. (J) Stage 18, 3A10 (green): some of the cells in the ganglion rudiment (arrow) have begun to differentiate as neurons. (K) Stage 21, TuJ1(green): note expression in ganglion cells extending processes into the otic epithelium at the site of the future sensory patch, but not in the epithelial cells themselves. (L) Stage 21, BEN: note expression both in the neurons and in the epithelial cells of the future sensory patch. (A is from Myat et al., 1996.)

Ser1 expression foreshadows the development of sensory patches in the inner ear epithelium

The epithelial sensory patches are defined by the presence of hair cells, which do not begin to differentiate overtly until E5, more than a day after neuroblast delamination has ended. Unexpectedly, expression of the Notch ligand Ser1 foreshadows this process, marking out what appear to be the prospective sensory patches long before hair cells differentiate. The patches of *Ser1* expression in the otic epithelium serve as landmarks that can be followed through from as early as stage 21 (E3.5) to at least stage 38 (E12).

Expression of *Ser1* in the ear rudiment begins at or before stage 11, as reported previously (Myat et al., 1996, Figs 2F-I); but the pattern is at first a rapidly changing one, with expression initially strongest in the peripheral parts of the otic placode/cup, resolving over a period of a day or two into a domain in the ventral part of the otic vesicle. By stage 21 (E3.5), the cochlea has begun to develop as a ventral downgrowth of the otic vesicle while the dorsal wall of the vesicle is thinning and beginning the shape changes that will create the semicircular canals (Bissonnette and Fekete, 1996). *Ser1* expression at this stage appears as a single continuous ventral domain, but its shape is becoming complex in parallel with the changing shape of the vesicle, with extensions corresponding to the future sensory patches (Fig. 4A,C).

By stage 26 (E5), hair cell differentiation is just beginning in the vestibular regions (Bartolami et al., 1991). *Ser1* is expressed in a set of well-defined discrete patches that evidently derive from the domain seen earlier and can be identified as the sites of the cristae, maculae and basilar papilla (Figs 4B,D). *Lunatic fringe*, a vertebrate homologue of *fringe* – a gene whose expression is linked to that of *Serrate* in the *Drosophila* wing disc – is also expressed in the developing inner ear (Laufer et al., 1997). We have compared its expression with that of *Ser1* at E5 and find that the two patterns overlap exactly (Fig. 4F).

Ser1 expression persists at least up to E12, both in the vestibular sensory patches, where hair-cell production continues indefinitely, and in the basilar papilla (the sensory patch of the cochlea), where hair-cell production ceases by E9/10 (Fig. 5). In both types of sensory patch, Ser1 appears to be expressed in all the cells, with no obvious change in expression when the hair cells differentiate. We cannot, however, exclude the possibility that Ser1 may be down-regulated in the hair cells themselves: these are closely apposed to and interdigitated with the supporting cells, which certainly do express the gene, and it is difficult to be sure whether apical staining is in the hair cells or in the apical processes of the supporting cells.

N1 is expressed widely in the otic epithelium, including all sites of expression of its ligands

As noted above, *N1* is already expressed throughout the otic placode at stage 11 (E1.5), and it continues to be expressed up to at least E12, in both sensory and non-sensory regions. Throughout this period, expression is strong in the whole of the ventral, thicker region of otic epithelium – the region that includes the sensory patches (Fig. 4C-E). Expression does, however, become somewhat less intense in the maturing sensory patches than in neighbouring regions (Fig. 4E). *N1* expression disappears only in the thin-walled dorsal regions that form the semicircular canals and neighbouring non-sensory parts of the utricle and saccule (Fig. 4C). Thus the receptor N1 is available at and adjacent to all sites of expression of the ligands Ser1 and, as we now discuss, Dl1 also.

Dl1 expression foreshadows hair-cell differentiation within the epithelial Ser1 domains

From E3.5 (stage 21) to at least E12, expression of *Dl1* in the otic epithelium is restricted to the prospective or actual sensory patches, as marked by *Ser1* expression; and within these patches, *Dl1* is restricted to a scattered subset of the cells. The timing of expression differs between patches, according to their different time courses of hair-cell production.

In the region of *Ser1* expression that corresponds to the future basilar papilla, expression of *Dl1* is absent or very weak at E3.5-E4 (Fig. 5A), but by E5 scattered cells expressing *Dl1* can be clearly seen (Fig. 5B). From E6 to E8, these continue to be visible, though with a varying distribution along the length of the basilar papilla (Fig. 5C). By E9, cells expressing *Dl1* have almost disappeared, with weak expression remaining only in the apical layer of the epithelium (Fig. 5D); this is the layer occupied by the bodies of the hair cells, which lose their connections to the basal lamina and move to the apical face of the epithelium as they differentiate (see Fig. 1A). By E12, expression of *Dl1* in the basilar papilla has disappeared completely (Fig. 5E).

Previous studies have shown that hair cells of the basilar papilla complete their last S-phase at a range of times between E5 and E8 (Katayama and Corwin, 1989) and begin to be identifiable by immunofluorescent staining for hair-cell antigen

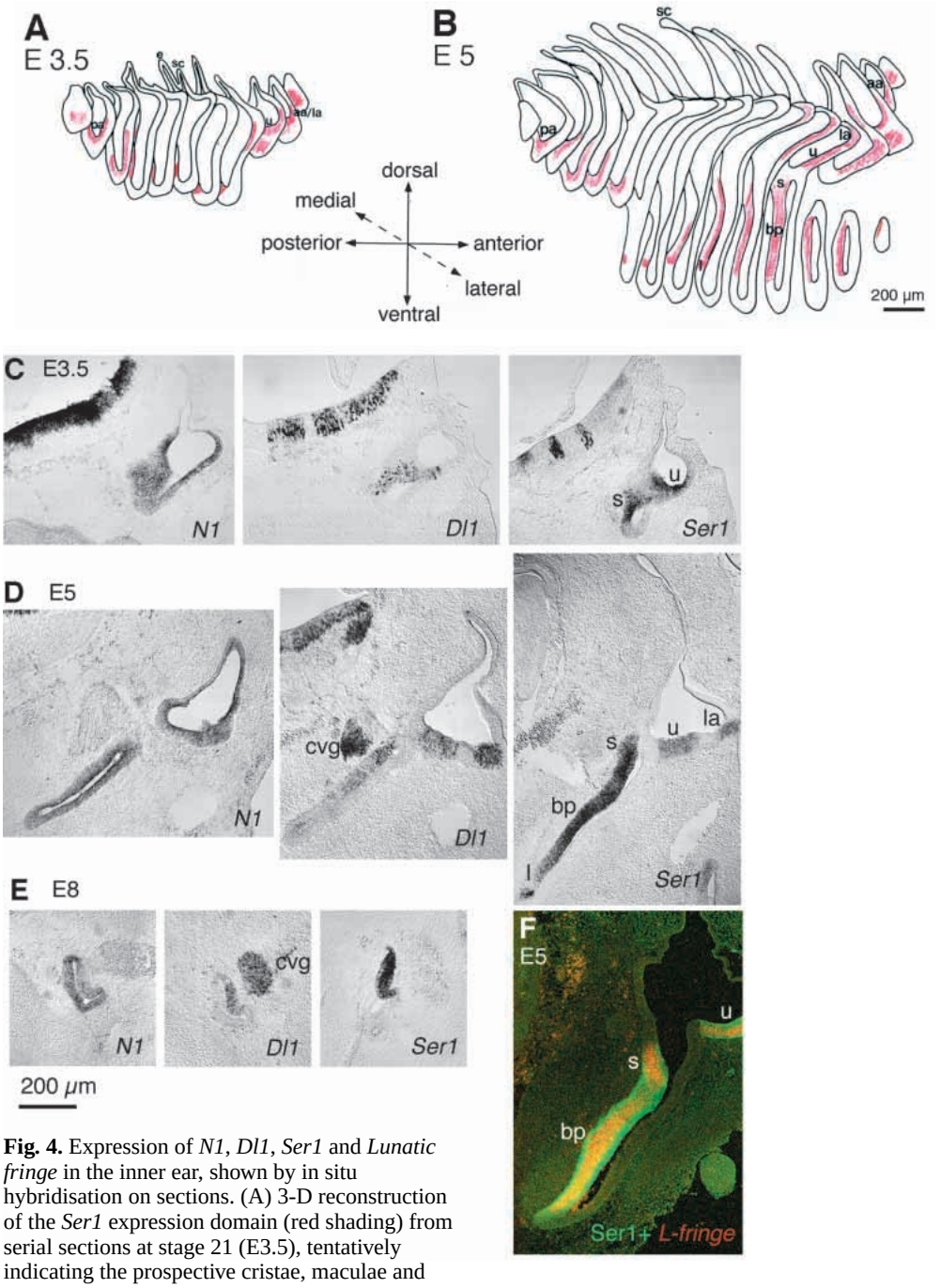

Fig. 4. Expression of *N1*, *Dl1*, *Ser1* and *Lunatic fringe* in the inner ear, shown by in situ hybridisation on sections. (A) 3-D reconstruction of the *Ser1* expression domain (red shading) from serial sections at stage 21 (E3.5), tentatively indicating the prospective cristae, maculae and basilar papilla. (B) Similar reconstruction of *Ser1* domains at stage 26 (E5). (C) *N1*, *Dl1* and *Ser1* at E3.5 (stage 21), in sections transverse to central body axis. (D) The same at E5 (stage 26); the cochlea appears in longitudinal section. (E) E8; the cochlea is shown in transverse section. (F) Double-stained section at E5, showing *Lunatic fringe* expression (red fluorescent in situ hybridisation signal, Fast Red technique) exactly overlapping Ser1 expression (green immunofluorescence). aa, la, pa, anterior, lateral and posterior ampullae (cristae) respectively; u, s, l, utricular, saccular and lagenar maculae; bp, basilar papilla; e, endolymphatic duct; sc, semicircular canal; cvg, cochleovestibular ganglion.

(HCA) at E6.25 (stage 29) (Bartolami et al., 1991). HCA-expressing cells are plentiful all along the length of the basilar papilla by E7.5, and the numbers continue to increase up to E9/10, after which hair-cell production ceases (Bartolami et al., 1991; Goodyear and Richardson, 1997). Comparison with the present observations indicates, therefore, that the cells expressing *Dl1* within the *Ser1* patch are nascent hair cells, and that these, like nascent neurons, express *Dl1* transiently, switching expression of the gene on as they become committed to their fate, about the time of their final mitosis, and switching it off again as they differentiate overtly, approximately one day later.

In the vestibular patches – the cristae and maculae – a similar relationship between *Dl1* expression and hair-cell differentiation is seen. Hair cells begin to be identifiable at or soon after E5 (stage 26) by immunofluorescent staining for HCA (Bartolami et al., 1991) and hair-cell production then continues throughout life (Kil et al., 1997). Correspondingly, scattered cells expressing *Dl1* are already visible in the prospective vestibular patches at stage 21 (E3.5), and they continue to be seen in these patches at all subsequent stages (Figs 4C-E, 5F-H).

In the cochleovestibular ganglion, meanwhile, we also see cells expressing *Dl1* (though in this site there is no expression of Ser1). At stage 21 (E3.5), the *Dl1*-expressing cells in the ganglion are few and sparsely scattered; from E5 up to at least E9, large numbers are visible (Figs 4E, 5C). Since the developing ganglion contains dividing neuroblasts up to E7 (D'Amico-Martel, 1982), it is possible that the *Dl1*-expressing cells are nascent or maturing neurons.

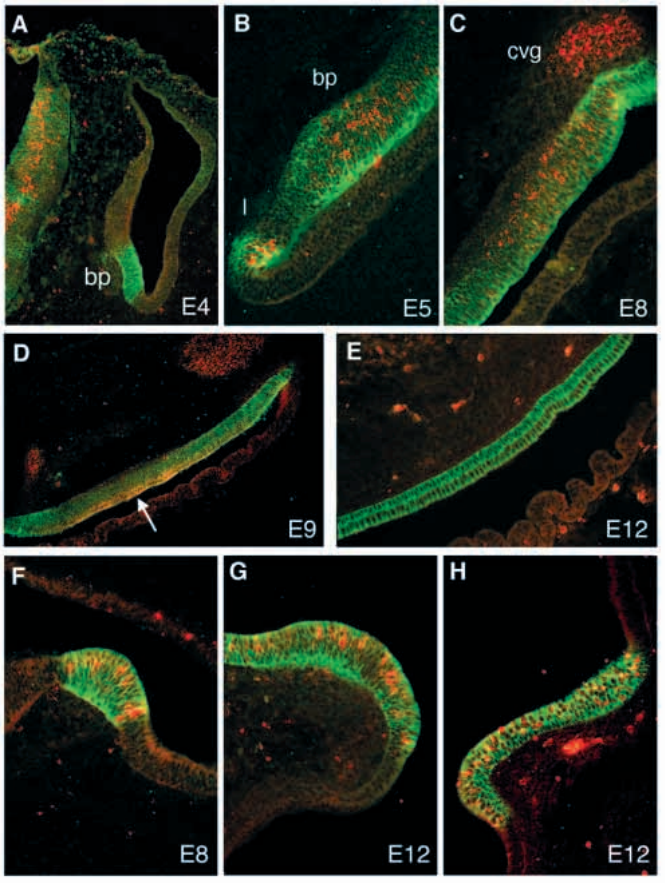

Fig. 5. Double-stained specimens showing expression of *Dl1* (in situ hybridisation, shown as red fluorescence using Fast Red detection), in relation to Ser1 (green immunofluorescence). (A) Prospective basilar papilla (bp) at E4 expresses Ser1 but not yet *Dl1*. (B) At E5: *Dl1*-expressing cells begin to be seen in basilar papilla (l, lagenar macula). (C) At E8: *Dl1*-expressing cells plentiful in basilar papilla; cochleovestibular ganglion (cvg) also expresses *Dl1*, but does not express Ser1. (D) At E9 in basilar papilla, last vestiges of *Dl1* expression are seen in hair-cell layer (arrow). (E) At E12 in basilar papilla, *Dl1* expression is no longer seen, but Ser1 persists. (F) Crista at E8. (G) Crista at E12. (H) Macula at E12.

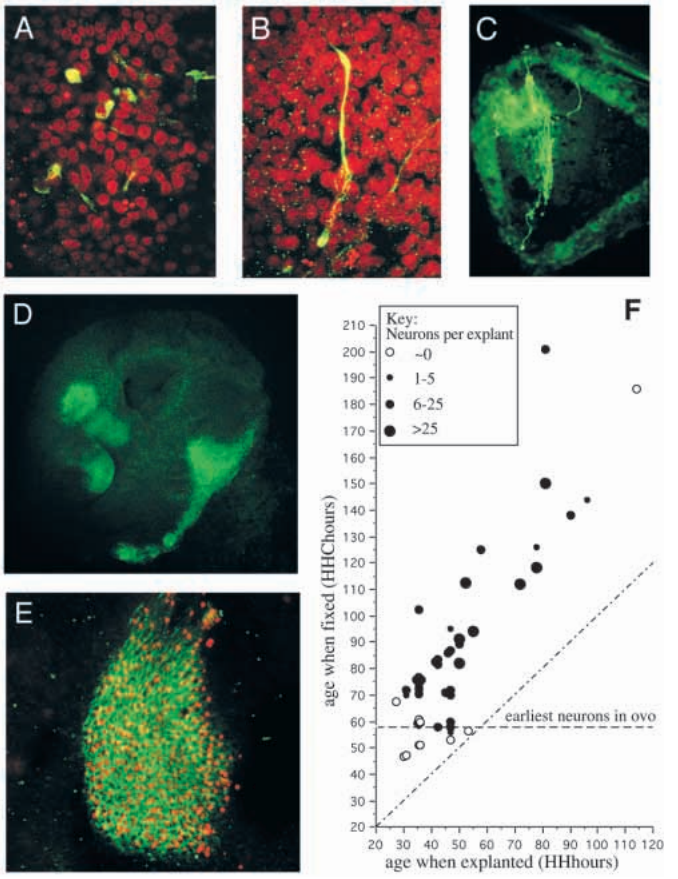

Fig. 6. Otic epithelium in culture. (A,B) Explants taken from stage 13 and cultured for 13 hours, then stained with 3A10 antibody (green) to show differentiating neurons and counterstained with 7AAD for nuclei. (C) Explant taken from stage 12 and cultured for 48 hours, then stained with 3A10. (D) Explant taken from stage 13 and cultured for 4 days, then stained with Ser1 antibody to show sensory patches. (E) Detail of explant similar to that in D, stained for Ser1 (green) and HCA (red): the Ser1 patches correspond to patches of hair cells. (A,B,D and E are confocal images). (F) Graph of neuron production in cultured otic epithelium explants. The results are plotted as a function of age (HHhours) of the embryo from which the explant was taken and the final age following culture (HHChours) when they were fixed. Distance above the diagonal dotted line represents time spent in culture. The approximate numbers of neurons in each explant as visualised with 3A10 antibody were scored, as indicated in the key. 3A10-positive cells are first seen in the cochleovestibular ganglion at Stage 17 (58 HHhours) and this is indicated in the graph. Each point represents one culture dish, containing several (1-12) explants. Cultures were scored '~0' if some or all explants produced no neurons and none produced more than 5.

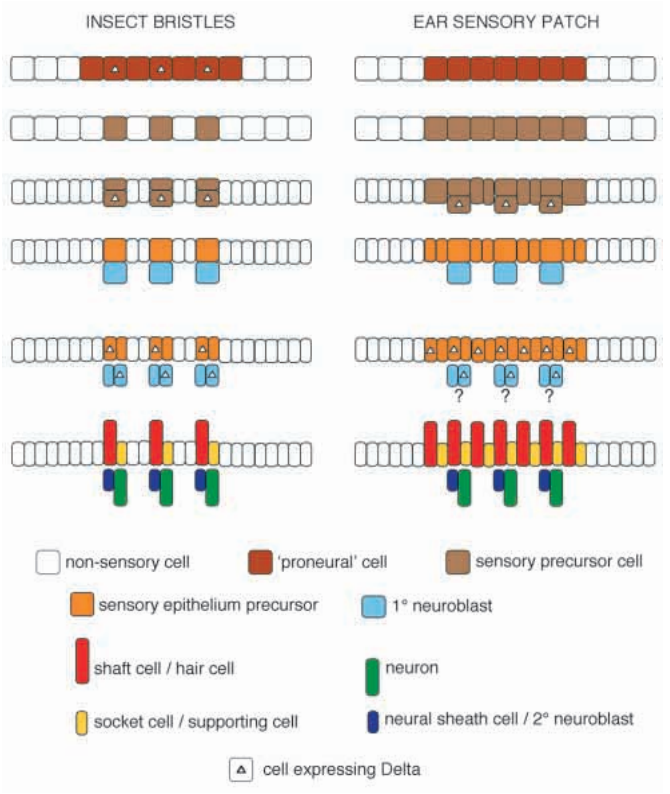

Fig. 7. Development of a sensory patch in the ear compared with development of an insect sensory bristle. The diagrams show the steps by which the constituent cell types become different from one another; at each step, cells expressing Delta, and presumably delivering lateral inhibition, are marked with a Δ. The schematic cross-sections are not intended to depict accurately the numbers of cells or division cycles. The actual ratio of hair cells to supporting cells in chick basilar papilla, for example, is between 1:3 and 1:7 (Goodyear and Richardson, 1997, see also Fig. 1C), rather than 1:1, and this is consistent with predictions for a system patterned by Delta-Notch signalling (Collier et al., 1996), which also give a 1:many ratio (depending on the geometry of cell packing). Question marks at lower right indicate that details of neuronal cell lineage in relation to *Dl1* expression are uncertain. Note also that in the ear, as in the case of non-innervated bristles in *Drosophila*, some regions of epithelium may generate hair cells and supporting cells but no neurons.

Otic epithelium isolated in vitro generates neurons and hair cells on schedule

In *Drosophila*, the mechanisms that generate the sensilla are presumed to be intrinsic to the epithelium from which these structures form. To study whether the same is true for the inner ear, we isolated otic epithelium and observed its differentiation in culture.

We first examined the genesis of neurons. We set up cultures of otic epithelium taken from embryos at a range of stages from Stage 9 (30 HHhours) to Stage 25 (114 HHhours). The explants were cultured for various lengths of time and then fixed and stained with 3A10 to reveal neurons.

Neurons were regularly seen (Fig. 6A-C). A total of 184 explants in 48 separate cultures were scored according to the number of neurons produced and the results plotted as a function of age of the embryo from which the explant was taken and of the time in culture (Fig. 6F). The graph shows that explants of otic epithelium isolated from stage 9/10 up to stage 21/22 are capable of generating neurons, without need of any signal from adjacent head tissues, and do so according to the normal in ovo timetable.

To see whether hair-cell production occurred normally in isolated otic epithelium, we studied explants from embryos at E2 (stage 13), 36 hours before the first hair cells can be identified (Bartolami et al., 1991). The cultures were maintained for up to 5 days, equivalent to E7, a stage when large numbers of vestibular and auditory hair cells have developed in vivo. After this period in vitro large numbers of hair cells were readily identifiable by their HCA expression. As in vivo, the hair cells were grouped in several discrete patches, which stained by immunofluorescence for Ser1 (Fig. 6D,E).

DISCUSSION

Before examining the detailed parallels with the development of insect sensory bristles, we must first consider how our present findings compare with and add to previous observations on ear development and Delta-Notch function in vertebrates.

Cochleovestibular neurons originate from neuroblasts in the otic epithelium

We have confirmed previous studies reporting that the ear's neurons are derived by delamination from the otic epithelium (D'Amico-Martel and Noden, 1983; Carney and Couve, 1989; Hemond and Morest, 1991; Haddon and Lewis, 1996). Quail/chick grafting has shown that while almost all the cochleovestibular neurons derive from the placode, the associated glial cells derive from the neural crest (D'Amico-Martel and Noden, 1983). Thus the cells that we see delaminating from the otic epithelium are specifically neuronal precursors. Moreover, as we have seen, they are capable of dividing before they differentiate: they are neuroblasts rather than postmitotic neurons. Cochleovestibular neurons are born from the neuroblasts in the ganglion over a prolonged period, up to E7 in chick (D'Amico-Martel, 1982) and in fish throughout life (Presson and Popper, 1990). The embryonic ganglion thus consists of a mixture of neurons and neuroblasts, with a common origin from neuroblasts in the otic epithelium.

The control machinery for neurogenesis is intrinsic to the otic epithelium

Our culture experiments show that neurons are generated from the otic epithelium even when it is isolated from its usual surroundings: the otic epithelium is programmed for neurogenesis from as early as stage 9 (31 HHhours), before the otic placode is even identifiable morphologically. No signal from the hindbrain or other neighbouring tissues is needed to trigger neurogenesis at the appropriate time, at least from stage 9 onwards; on this point, we contradict the speculative suggestion that neurogenesis is induced by a signal from the hindbrain (Represa et al., 1991). The mechanisms that regulate genesis of neuroblasts in the otic epithelium seem to be intrinsic to the epithelium itself.

Expression of *Dl1* in the early otic epithelium foreshadows expression of neuronal differentiation markers in the same way as in the embryonic CNS

What are the regulatory mechanisms for neurogenesis in the ear? We have found that *Dl1* is expressed in scattered cells in the neurogenic patch in the otic epithelium, in a pattern suggesting that *Dl1* is expressed transiently in the neuroblasts at or soon after their commitment, before they begin to express other neuronal markers. The relationship of *Dl1* expression to expression of the neuronal markers TuJ1, Islet1/2, BEN and 3A10 is very similar to that seen for nascent motoneurons in the spinal cord (Ericson et al., 1992; Tsuchida et al., 1994; Chitnis et al., 1995; Henrique et al., 1995; Memberg and Hall, 1995; Haddon et al., 1998b), where commitment to a neuronal fate is regulated by Delta-Notch signalling, delivering lateral inhibition from the nascent neural cells to their neighbours. Our data provide circumstantial evidence that the same is true in the ear. There is, however, one striking difference: for the motoneuron, expression of *Dl1*, Islet1/2, BEN and TuJ1 is associated with the cessation of cell division; for the otic neuroblast it is not (see Memberg and Hall, 1995, for other examples). We shall see that this has a parallel in *Drosophila* bristle development.

Hair cell determination is correlated with a second round of expression of *Dl1*

The differentiation of hair cells and supporting cells is anticipated by a second round of *Dl1* expression, occurring against a background of continuing widespread N1 expression, and we have shown that the *Dl1*-expressing cells are most probably nascent hair cells. This conclusion is supported by preliminary observations in transgenic mice carrying a *Dl1:lacZ* reporter gene, whose product is detected in differentiating hair cells (Alastair Morrison, personal communication).

Hair cells and supporting cells arise side by side, forming, in the basilar papilla at least, a regular alternating pattern (see Fig. 1C). The precision of this pattern is refined by cell movements through which the newly differentiated hair cells and supporting cells become rearranged (Goodyear and Richardson, 1997); but the pattern is already evident when the differentiated cells first appear. This suggests that the initial pattern of cell commitment is regulated by lateral inhibition, whereby each nascent hair cell inhibits its neighbours from becoming hair cells, with the result that they become supporting cells instead. A simple mathematical model based on the known properties of the Delta-Notch signalling mechanism has shown that lateral inhibition mediated by Delta-Notch signalling can indeed generate an alternating pattern of just the type observed (Collier et al., 1996).

The timing of *Dl1* expression is as expected if this mechanism is operating in the ear. The decision to differentiate as either a hair cell or a supporting cell is presumably taken at or after a cell's terminal mitosis, since there is evidence from several systems that a hair cell can have a supporting cell as its sister (Stone and Cotanche, 1994; Weisleder et al., 1995; Jones and Corwin, 1996); and we have seen that *Dl1* expression, present in scattered cells in the developing basilar papilla from E5 to E8/9, parallels the time-course of terminal mitoses of hair cells, which complete their final S-phase between E5 and E8 (Katayama and Corwin, 1989). A similar correlation between *Dl1* expression and hair-cell production is seen in the vestibular patches.

The accompanying paper (Haddon et al., 1998a) provides evidence from a zebrafish mutant, *mind bomb*, that Delta-Notch-mediated lateral inhibition does indeed control the hair-cell/supporting-cell decision in the ear.

Loss of Delta-Notch signalling is unlikely to be the trigger for cell proliferation during hair cell regeneration

In the basilar papilla of a bird, there is normally no post-embryonic cell division or hair-cell production. If hair cells are destroyed, however, even in the adult, the mitotically quiescent supporting cells re-enter the division cycle and new hair cells are generated (Cotanche et al., 1994). An obvious suggestion is that the process is controlled by a signal from hair cells that normally acts continuously, so long as hair cells are present, to inhibit supporting cells both from dividing and from differentiating into hair cells. If so, the inhibitory signal must be something other than Dl1, since we have shown that expression of this gene is transient, ceasing as the hair cells become fully differentiated. Delta-Notch signalling may, however, play a part in controlling cell fate once the regenerative response has been set in motion (Jennifer Stone, personal communication), just as it appears to be involved, according to our observations, in the continuing generation of hair cells from stem cells in the vestibular sensory patches.

Ser1 expression is an early and persistent marker of sensory patches

We have shown that *Ser1* is a very early marker for the prospective sensory patches, reflecting, perhaps, a 'prosensory' cell state (Kelley et al., 1993) analogous to the proneural state preceding neuronal commitment in neurogenic epithelium. BMP4 expression has been reported to be a similarly early marker of all sensory patches (Wu and Oh, 1996), although in our hands, as also for the mouse embryo (Morsli et al., 1998), the BMP4 domain does not exactly coincide with the *Ser1* domain or with the location of all the sensory patches (I. Le Roux, unpublished observations).

Tracing the domains of *Ser1* expression backwards through development gives an indication of how the pattern of sensory patches is set up. At E3.5 the saccular macula and the utricular macula appear to be one continuous ventral patch. The neurogenic region of the otocyst corresponds to the anterior part of this patch, i.e. the future utricular portion. The prospective anterior and lateral cristae appear initially continuous with the utricular macula, and the posterior crista, lagenar macula and basilar papilla appear initially continuous with the macula of the saccule (see also Fekete, 1996). This matches observations in fish embryos (Becerra and Anadón, 1993; Haddon and Lewis, 1996), where the corresponding maculae and cristae similarly appear to derive from one continuous primordial patch – a macula communis, whose form and location are closely similar to those of the *Ser1* expression patch in the E3.5 chick ear. Thus, despite their different final forms, the chick ear and the fish ear may be fundamentally alike in the way they organise their global pattern of sensory patches.

Our tissue culture experiments, as well as previous grafting experiments (Swanson et al., 1990) show, moreover, that the discrete sensory patches develop through mechanisms intrinsic to the otic epithelium, in agreement with results for neurogenesis at earlier stages. This contrasts with tissues such as the feather tracts

in the chick skin, which also depend on Delta-Notch signalling, but in which interactions between epidermis and mesenchyme play a crucial role (Crowe et al., 1998; Viallet et al., 1998).

The function of *Ser1* in the otic epithelium remains to be determined. The *Dl1* expression associated with hair-cell production is precisely restricted to the *Ser1* domains, suggesting that the two genes are functionally coupled in some way, and in these domains *Ser1* is coexpressed with *Lunatic fringe* (in agreement with observations of *Lunatic fringe* expression in the mouse ear; Morsli et al., 1998). This situation is reminiscent of the *Drosophila* wing disc, where Serrate, Fringe, Delta and Notch interact to define the special band of organiser tissue at the wing margin (Irvine and Vogt, 1997), in whose neighbourhood, incidentally, arrays of close-packed sensory bristles develop (Hartenstein and Posakony, 1989). By analogy with the wing disc it is possible, for example, that confrontation between cells that express *Ser1* and *Lunatic fringe* and cells that do not plays a part in organising development of the ear's sensory patches. Recent work has shown, however, that Serrate also acts in conjunction with Delta in the development of individual sensory bristles elsewhere on the insect's body (Zeng et al., 1998); so it may be wrong to look for specific parallels with the insect wing margin.

Sensory bristles in *Drosophila* and sensory patches in the ear are generated by essentially the same developmental program

Each insect sensory bristle is a functional unit formed from the progeny of a sensory mother cell (SMC): this cell is singled out from a proneural cluster in the insect epidermis, and (in the case of a standard mechanosensory bristle) divides twice to generate four different cells. At each division of the SMC and its progeny, Delta and Serrate, acting together in a quasi-redundant fashion as ligands for Notch, mediate lateral inhibition to force the sister cells to adopt different fates (Hartenstein and Posakony, 1990; Ghysen et al., 1993; Parks and Muskavitch, 1993; Jan and Jan, 1995; Zeng et al., 1998). In the first division, one daughter (the daughter delivering lateral inhibition) becomes committed as a neuroblast while the other becomes committed as a sensory epithelial precursor. In the second division, the daughters of the neuroblast become, respectively, a neuron (delivering lateral inhibition) and a neural sheath cell, while the daughters of the sensory epithelial precursor become, respectively, a bristle shaft cell (delivering lateral inhibition) and a bristle socket cell. Some SMCs follow variants of this program: in chemosensory bristles, for example, the neuroblast divides several times to generate 3-5 neurons (Nottebohm et al., 1994); in non-innervated bristles of the posterior wing margin, conversely, neurons are missing (Hartenstein and Posakony, 1989).

The sensory neuron of the bristle corresponds to the sensory neuron of the ear; the shaft cell, presumably, to the hair cell of the ear (see Tilney et al., 1995, for the structural similarities in cytoskeletal organisation); and the socket cell to the supporting cell. The neural sheath cell has no such obvious counterpart: the glial cells in the cochleovestibular ganglion derive from the neural crest, not the otic epithelium (D'Amico-Martel and Noden, 1983). One might, however, compare the neural sheath cell of the bristle to a second-generation neuroblast in the cochleovestibular ganglion – both of them are daughters of first-generation neuroblasts but have not differentiated into neurons.

Assuming these correspondences between the cell types, the correspondences in the developmental program can be inferred directly (Fig. 7). At the outset, however, there is a contrast. Whereas each bristle is typically isolated from the next by intervening epidermis, each sensory patch in the ear consists of a mass of contiguous hair cells and supporting cells, with no non-sensory cells between them. Thus it seems that the counterpart of the SMC is not a single isolated cell, but a cluster of contiguous sensory precursor cells (SPCs) that coexist instead of competing by lateral inhibition. (In *Drosophila*, SMCs likewise develop in contiguity, exceptionally, at the wing margin; Hartenstein and Posakony, 1989.) If we draw the ear/bristle parallel in this way, we can relate all the subsequent steps of sensory patch development to those of bristle development, with Delta/Serrate-Notch signalling acting repeatedly in a similar way in the two systems. The singling-out of neuroblasts in the ear corresponds to the determination of one of the two daughters of an SMC as a neuroblast. The production of neurons from neuroblasts in the cochleovestibular ganglion corresponds to the production of a neuron or of several neurons from the bristle neuroblast. And the genesis of hair cells and supporting cells from otic sensory epithelial precursors corresponds to the genesis of bristle shaft cells and supporting cells from sensory epithelial precursors in the insect epidermis. The two systems differ, it is true, in the numbers of cell divisions that occur at each step; but this is variable even between types of bristles in the fly.

In addition to the above systematic parallels in the developmental program, there are other facts that suggest a conserved process. For example, *Pax2*, along with its close relatives *Pax5* and *Pax8*, is strongly expressed in the early ear rudiment (Pfeffer et al., 1998) and is required for development of the cochlea (Torres et al., 1996); and its homolog in *Drosophila*, *Pax258*, is strongly expressed in the precursor cells of the sensory bristles (Czerny et al., 1997).

It seems clear that the mechanosensory organs of flies and vertebrates are fundamentally similar, not only in function and architecture, but also in the developmental programs that generate their precisely patterned arrays of cell types. The precise correspondence that we have identified should help in the search for other molecules that have a conserved role in the two systems.

We thank Anthony Frankfurter, Guy Richardson and Cliff Tabin for their gifts of TuJ1 antibody, HCA antibody and *Lunatic fringe* probe, and Jenny Corrigan for cryostat sectioning. Islet1/2, BEN and 3A10 were from the Developmental Studies Hybridoma Bank. The work was supported by the Imperial Cancer Research Fund, the Hearing Research Trust (Defeating Deafness), and the Howard Hughes Medical Institute (for DI-H) through the International Research Scholars Program.

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