Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation

Nicolas Daudet and Julian Lewis*

Vertebrate Development Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
*Author for correspondence (e-mail: julian.lewis@cancer.org.uk)

Accepted 9 November 2004

Development 132, 541-551
Published by The Company of Biologists 2005
doi:10.1242/dev.01589

Summary

Lateral inhibition mediated by Notch is thought to generate the mosaic of hair cells and supporting cells in the inner ear, but the effects of the activated Notch protein itself have never been directly tested. We have explored the role of Notch signalling by transiently overexpressing activated Notch (N^{ICD}) in the chick otocyst. We saw two contrasting consequences, depending on the time and site of gene misexpression: (1) inhibition of hair-cell differentiation within a sensory patch; and (2) induction of ectopic sensory patches. We infer that Notch signalling has at least two functions during inner ear development. Initially, Notch activity can drive cells to adopt a prosensory character, defining future sensory patches. Subsequently, Notch signalling within each such patch mediates lateral inhibition, restricting the proportion of cells that differentiate as hair cells so as to generate the fine-grained mixture of hair cells and supporting cells.

Key words: Notch, Inner ear, Chick, Hair cells, Electroporation, Lateral inhibition, Prosensory determination

Introduction

The Notch signalling pathway is most famous for its role in lateral inhibition: cells embarking on a pathway of differentiation express a Notch ligand – Delta or Serrate – on their surface; this activates the Notch receptor in adjacent cells; and Notch activity in these neighbours inhibits them from embarking on the same pathway (Heitzler and Simpson, 1991). The developing vertebrate inner ear is one of the sites in which lateral inhibition seems to operate in this way. The inner ear is a closed epithelial structure, elaborately shaped and possessing in its walls a number of sensory patches that serve to detect sound (in the basilar papilla or organ of Corti) or acceleration and gravity (in the vestibular patches) (Fig. 1). Each of the sensory patches is a fine-grained mixture of mechanosensory hair cells and supporting cells, both derived from the same homogeneous population of sensory precursor cells (Fekete et al., 1998; Fekete and Wu, 2002). Lateral inhibition mediated by Notch signalling is thought to provide the mechanism for this cell diversification within the patch. Three types of evidence have led to this view.

First, similarities of function, developmental anatomy and genetic control suggest that the sensory patches in the vertebrate ear are in some sense homologous to the mechanosensory organs of a fly (Adam et al., 1998; Jarman, 2002; Muller and Littlewood-Evans, 2001), in which cell diversification is known to be governed by Notch-mediated lateral inhibition (Guo et al., 1996; Hartenstein and Posakony, 1990; zur Lage and Jarman, 1999).

Second, the components of the Notch pathway are expressed in a strongly suggestive pattern (Fig. 1) in the ear – the transmembrane receptor Notch ubiquitously (at least up to the time of hair-cell specification), the transmembrane ligands Delta1 and Serrate2 (Ser2, also known as Jagged2) in the nascent hair cells (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999; Zine et al., 2000), and members of the Hairy/Enhancer of Split (Hes) family of transcription factors, whose transcription is dependent on Notch activation, in the cells that are in process of becoming supporting cells (Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001). This is consistent with the idea that the nascent hair cells, by expressing Notch ligands, activate Notch in their neighbours, thereby preventing the neighbours from becoming hair cells too.

Third, defective Notch signalling in the ear of zebrafish (Haddon et al., 1998; Riley et al., 1999) or mammals (Kiernan et al., 2001; Lanford et al., 1999; Tsai et al., 2001; Zheng et al., 2000; Zine et al., 2001; Zine et al., 2000) results in excess production of hair cells. This is most strikingly exemplified in the zebrafish *mind bomb* mutant (Jiang et al., 1996; Schier et al., 1996), in which Delta proteins fail to be ubiquitinated in the normal way and are consequently unable to activate Notch (Itoh et al., 2003); as a result, the cells of the inner ear sensory patches all differentiate as hair cells, with none remaining as supporting cells (Haddon et al., 1998; Haddon et al., 1999). This matches exactly the predictions of the classic lateral inhibition model.

There are, however, gaps in this evidence and reasons to think that the full story cannot be so simple. The effects of loss of Delta1 in the mouse or chick ear have not been reported, and homozygous *Jagged2* mouse mutants show only a mild excess of hair cells (Lanford et al., 1999; Zhang et al., 2000).

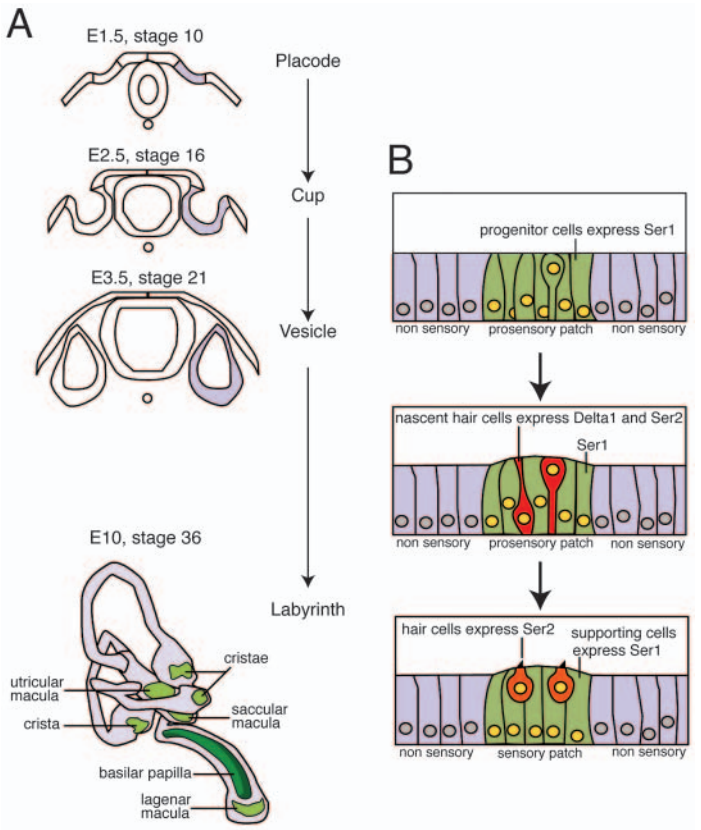


Fig. 1. Schematic diagram of development of the chick inner ear and its sensory patches. (A) The inner ear of a bird originates by invagination of the otic placode and remodelling of the resulting otic vesicle to form the labyrinth. The mature structure contains seven vestibular sensory patches, involved in perception of gravity and acceleration: three cristae (in the ampullae of the semicircular canals) and four maculae, those of the utricle, the saccule, the lagena, and the macula neglecta (which we neglect). The basilar papilla, an elongated sensory region extending along the cochlear duct, serves auditory function. (B) The patterns of Notch ligand expression in a sensory patch before, during and after hair-cell differentiation. One of the earliest genes to be expressed in prospective sensory patches codes for the Notch ligand Ser1. Within the Ser1 domains, nascent hair cells expressing Delta1 can be detected from E3.5 in vestibular regions, and from E5 in the cochlear duct. Delta1 expression foreshadows the differentiation of hair cells, which become identifiable by morphological and other molecular criteria about 24 hours later. Notch1 itself is expressed in sensory as well as non-sensory regions of the developing otocyst.

Misexpression of Delta1 by means of a retroviral vector in the chick ear failed to produce the expected disturbances of hair-cell production (Eddison et al., 2000). Moreover, while the nascent hair cells express Delta1 and Ser2/Jagged2, the precursor cells and supporting cells, more puzzlingly, also express a Notch ligand, Ser1/Jagged1, and do so strongly and persistently (Fig. 1). The loss of one copy of *Jagged1* in heterozygous *Slalom* (Tsai et al., 2001) and *Headturner* (Kiernan et al., 2001) mutant mice, instead of causing overproduction of hair cells, results in a mild reduction in their number in the cochlea, accompanied by variable loss of the anterior and/or posterior cristae and semicircular canals. The timing of gene expression in the normal embryo also hints that Notch signalling does something more than the simple theory proposes. Notch expression marks out the otic placode from surrounding ectoderm before it has even begun to invaginate to form an otic vesicle, and expression of Ser1/Jagged1 becomes visible soon after this, in a pattern that gradually resolves into a set of discrete domains marking the sites of the future vestibular and auditory sensory patches (Adam et al., 1998). This suggests that Notch activity has some additional role at early stages.

In the present study, we have tested the effects of overactivating the Notch pathway in the embryonic chick inner ear, by using in-ovo electroporation of plasmid DNA to force transient expression of an activated form of Notch. We observed two consequences – one that we expected, and another that we did not: (1) within vestibular sensory patches, hair cell differentiation is inhibited; and (2) in parts of the inner ear that are normally non-sensory, ectopic sensory patches develop. These results indicate that Notch signalling has two quite different functions during inner ear development: initially, Notch activity in a group of cells makes them competent to form a sensory patch, conferring on them a *prosensory* character; subsequently, Notch-mediated lateral inhibition within such a patch governs the establishment of fine-grained patterns of differentiation of hair cells and supporting cells.

Materials and methods

Plasmids

The LZRS-pBMN-IRES-GFP plasmid (hereafter referred to as IRES-GFP) has been described elsewhere (le Roux et al., 2003). The DNA coding for amino acids 399-1194 of the published chicken Notch-1 intracellular domain (N^{ICD}) partial sequence (GI 5360173) was PCR amplified from embryonic day (E) 12 chicken brain cDNA and cloned into TOPO-II vector (Clontech, UK). An *Eco*RI-N^{ICD} fragment was then excised and ligated into the *Eco*RI site upstream of IRES-GFP to generate N^{ICD}-IRES-GFP. An N-terminal hemagglutinin (HA) tagged version of the N^{ICD} was generated by PCR from the original TOPO-II-N^{ICD} plasmid, then sequenced and cloned into IRES-GFP to generate HA-N^{ICD}-IRES-GFP. Plasmid DNA solutions were prepared using a plasmid purification kit (Qiagen, UK) and diluted for electroporation to 1 μg/μl in Tris-HCl buffer pH 8.0 tinted with Fast Green for visualisation.

Electroporation of embryonic chick inner ear

Fertile White Leghorn eggs were incubated at 38°C. Embryos were staged according to Hamburger-Hamilton (HH) tables. Micro-electroporation of the inner ear was performed at the otic cup stage (stages HH 13-17) as described in Momose et al. (Momose et al., 1999) with minor modifications, using a TSS-10 square-wave pulse generator (Intracel, UK) to generate three 100-millisecond bursts of 30 Hz, 7 volt square-wave electric pulses. After electroporation, eggs were sealed with tape and returned to incubation. The numbers of embryos analysed in detail for each condition were as follows: N^{ICD}-IRES-GFP, 124; HA-N^{ICD}-IRES-GFP, 98; IRES-GFP, 41. Following electroporation with either construct, GFP protein was successfully detected by immunostaining in the inner ear of approximately 70% of analysed specimens.

Embryos electroporated with N^{ICD}-IRES-GFP or HA-N^{ICD}-IRES-GFP were returned to the incubator for 1 (n=26), 2 (n=36), 3 (n=36), 4 (n=21), 5 (n=21), 6 (n=13), 7 (n=59), 8 (n=4) or 10 (n=6) days, then processed for further analysis. IRES-GFP controls were similarly analysed after 5 (n=11), 7 (n=25) or 10 (n=5) days.

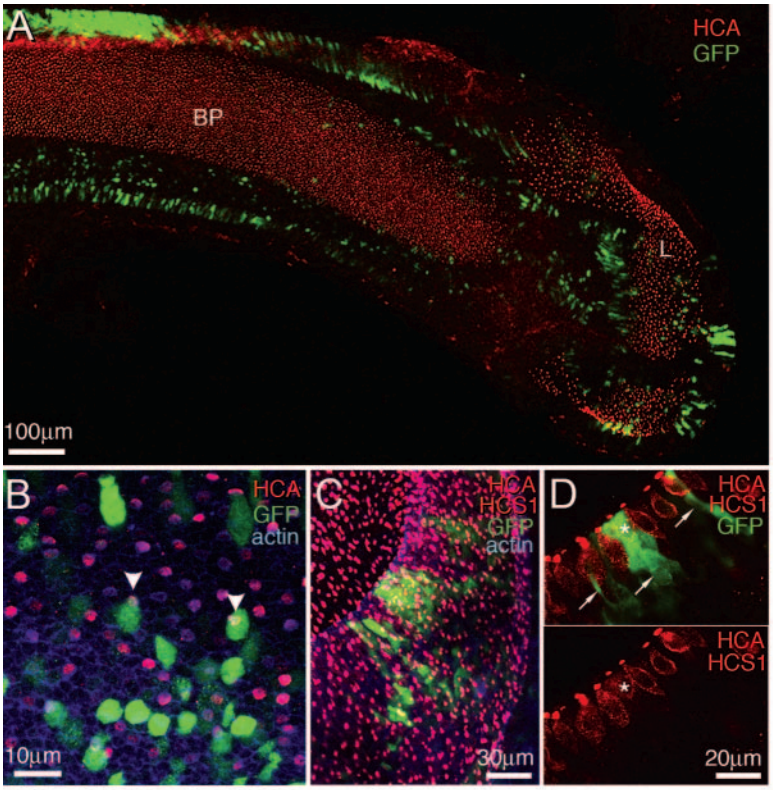


Fig. 2. Expression of GFP in the chick inner ear after electroporation of control IRES-GFP plasmid DNA. (A) Low magnification view of the cochlear duct of an E9 chick embryo electroporated at stage HH 17 (E2.5). In the basilar papilla and the lagenar macula, each red dot corresponds to the stereociliary bundle of one hair cell. (B) High magnification view of a more proximal region of the BP of the same specimen. Scattered GFP-positive cells (green) include both hair cells (with rounded GFP-positive cell body and HCA-positive apical surface, arrowheads) and supporting cells (elongated cell bodies and absence of HCA staining). (C,D) Utricular macula of an E8 chick embryo electroporated at stage HH 13 (E2). Several groups of GFP-positive cells are present within this sensory patch. (D) Transverse view of this epithelium at higher magnification. Some of the GFP-positive cells are hair cells (asterisk), with HCA-positive hair bundles and HCS-1-positive cytoplasm; others are supporting cells (arrows), with elongated cell bodies extending below the hair cell layer. All images other than (D) are projections of confocal optical sections of immunostained whole mounts; (D) is a single optical section. Counterstain for actin (blue) in B and C, combined with the red HCA fluorescence, makes hair bundles appear purple. BP, basilar papilla; L, lagenar macula.

Immunocytochemistry

Embryos were decapitated and their heads immersed in 4% paraformaldehyde in PBS at 4°C for 2 to 12 hours. For whole-mount immunostaining, the membranous part of the inner ear was dissected out from the surrounding cartilage and incubated for 1 hour in PBS containing 0.3% Triton X100 and 10% goat serum. All subsequent incubations and rinses were performed in PBS with 0.1% Triton X100 (PBT). Incubations with primary and secondary antibodies were carried out in PBT for 2 hours at room temperature or overnight at 4°C. Antibodies and reagents used were: rabbit serum anti-GFP (Molecular Probes, 1/2000), chicken IgY anti-GFP (Upstate, UK; 1/200; this antibody was used for Ser1 and GFP double-immunostaining), rabbit serum anti-chicken Ser1 (1/50), mouse anti-HA (Covance, UK; 1/500), mouse monoclonal IgG1 anti-HCA (Bartolami et al., 1991) (1/100), mouse monoclonal IgG2a anti-β-III tubulin (TuJ1, Covance Research, Cambridge Bioscience Ltd, UK; 1/1000), mouse monoclonal IgG2a anti-HCS-1 (J. E. Finley and J. T. Corwin, unpublished) (Gale et al., 2000) (1/100), Alexa A488-, A594-, and A633-conjugated secondary antibodies (Molecular Probes, The Netherlands; 1/500 dilution), Cy2-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch, PA; 1/200), Alexa 633-conjugated phalloidin (Molecular Probes; 1/100). Specimens were mounted in Slowfade (Molecular Probes) and observed under a Zeiss LSM510 confocal microscope. For cryosectioning, embryo heads were fixed as described above, then immersed in a graded series of sucrose-PBS (5-10-20%), embedded in 1.7% agar with 5% sucrose, frozen at –20°C, and sectioned at 15 μm thickness on a Reichert-Jung cryomicrotome.

We used immunofluorescence, as opposed to intrinsic GFP fluorescence, for the detection of GFP in all the experiments described in detail in the Results.

Hair cell counts

The fraction of GFP-positive cells differentiating into hair cells was estimated at E8.5-9.0 in vestibular sensory epithelium transfected at E2 with either IRES-GFP (n=4 specimens) or N^{ICD}-IRES-GFP (n=8 specimens). For each specimen, the number of GFP-positive hair cells (expressing the Hair Cell Antigen) and the total numbers of GFP-positive cells were counted within at least two 1000 μm^2 regions in vestibular sensory patches. The regions for counting were selected at random subject to the requirement that each should contain at least 20 GFP-positive cells and should be located in the central region of a vestibular sensory patch. The values obtained for each condition were pooled, and used to calculate the percentage of GFP-positive cells differentiating into hair cells.

Whole-mount in-situ hybridisation

Whole-mount in-situ hybridisation was performed as described in Ariza-McNaughton and Krumlauf (Ariza-McNaughton and Krumlauf, 2002) with minor modifications, but the prehybridisation treatment with proteinase K was omitted. Immunodetection of DIG-labelled RNA probes was performed using an anti-DIG peroxidase antibody (diluted 1:100) and either the TSA-FITC or the TSA-Cy3 amplification systems (Perkin Elmer).

For the timecourse analysis of GFP expression by in-situ hybridisation, embryos were electroporated with HA-N^{ICD}-IRES-GFP and then fixed at 1 (n=5), 2 (n=4), 3 (n=4), 4 (n=4), 5 (n=4) or 7 (n=8) days post-transfection.

Results

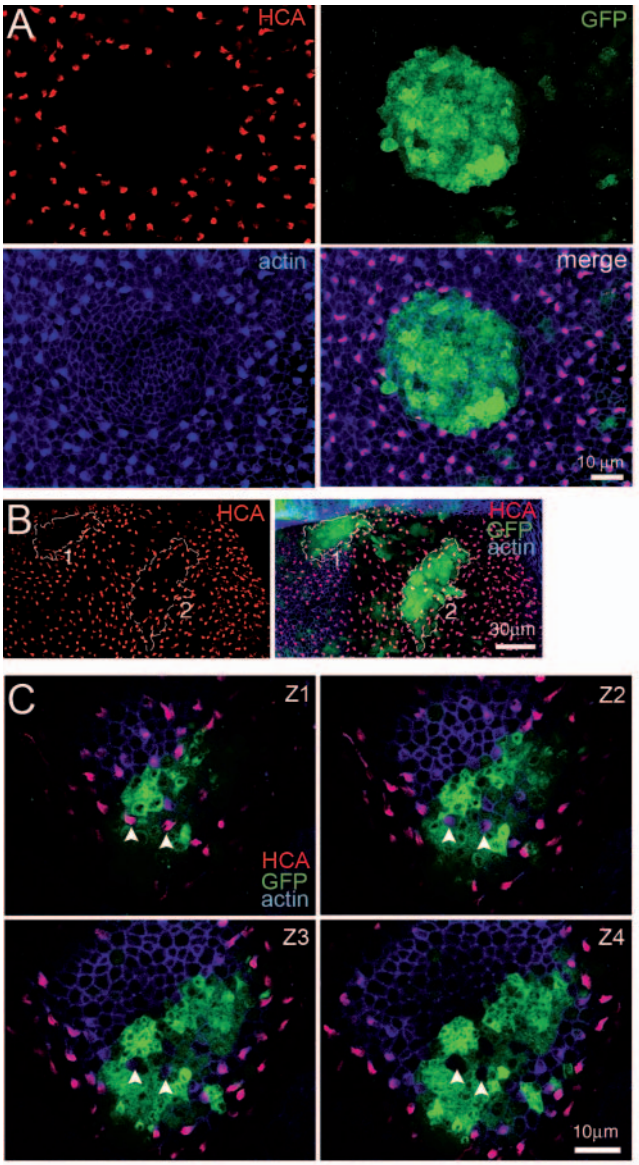
Electroporation gives controlled timing of transgene expression in the chick otocyst

The chick embryo offers special opportunities for testing gene function by mosaic gene misexpression. One method, used in several previous studies of inner ear development, is to infect cells with a retrovirus such as RCAS (Eddison et al., 2000; Morgan and Fekete, 1996; Stevens et al., 2003) carrying the desired transgene. However, new virus particles are then constantly produced by the infected tissue, spreading the infection so that one does not know for any given cell at what time expression of the transgene started.

An alternative approach is to incorporate the transgene into plasmid DNA and introduce this into the cells in ovo by electroporation (Muramatsu et al., 1997; Nakamura and

Funahashi, 2001). In this case, the time of onset of transgene expression is precisely determined by the time of electroporation (beginning within 2 hours), and subsequent transgene expression is limited in duration because the plasmid is soon lost from the transfected cells.

We used this technique to transfect cells of the embryonic chick inner ear at 2 to 3 days of incubation (stages HH 13-17) – the period in which the otic placode invaginates to form first a cup and then a closed vesicle. At these stages, the otic cup can be easily filled with a DNA solution and electroporated efficiently without compromising embryo survival. To assess the efficiency and timecourse of transgene expression, we used a control plasmid containing an IRES-GFP sequence under a constitutive RSV promoter. More than 100 embryos were examined both alive and after sectioning at varying times after electroporation. GFP fluorescence was directly visible in the otocyst between 24 and 72 hours after electroporation, but was very much decreased or absent in specimens examined 96 hours after transfection, suggesting that transcription from the original plasmid had probably ceased by this time. However, even small amounts of GFP protein could be detected by immunochemistry, and we were able to find GFP-positive cells with this method up to at least 10 days after electroporation. We could thus recognise transfected cells and their progeny and assess their fate between E7 and 10, when hair-cell differentiation is advanced and the hair cells are easily distinguished by immunostaining for Hair cell antigen (HCA), a receptor-like protein tyrosine phosphatase present in early differentiating hair bundles (Bartolami et al., 1991; Goodyear et al., 1995; Goodyear et al., 2003), and/or the HCS-1 antigen, an unidentified cytoplasmic protein specific to hair cells (Gale et al., 2000). At sites of transfection within vestibular sensory patches and in the basilar papilla, both hair cells and supporting cells were GFP-positive. The regular pattern of differentiation of these two cell types was not altered by transfection with the IRES-GFP vector (Fig. 2).



Forced Notch activation within sensory patches represses hair cell differentiation

To test how the direct activation of Notch would affect hair-cell differentiation, we inserted into the IRES-GFP plasmid a sequence coding for the intracellular domain of chicken Notch1 (N^{ICD}), the protein fragment that is released into the cytosol as a signal of receptor activation (Schroeter et al., 1998). Expression of N^{ICD} mimics the effect of activation of Notch in the expressing cell (Austin et al., 1995). We electroporated embryos with the N^{ICD}-IRES-GFP construct at E2-2.5 (stage HH13-17) and fixed them at E7-10 for whole-mount immunocytochemistry.

In 38 specimens (out of 59 analysed in eight separate experiments), groups of GFP-positive cells were detected within inner ear sensory patches. In 17 out of these 38 specimens, such GFP-positive cells were seen within vestibular patches and were then generally clustered together (contrasting with results of transfection with the control IRES-GFP construct, where the cells were more dispersed). These GFP-positive clusters displayed a greatly reduced density or complete absence of hair cells (Fig. 3A) compared with surrounding non-transfected tissue. For cases in which hair cells were seen within such GFP-positive regions (see region 2 in Fig. 3B), careful examination of *z*-series of optical sections

Fig. 3. Notch activity within a sensory patch acts cell-autonomously to inhibit hair-cell differentiation. (A) Whole-mount immunostaining in the utricle of an E9 embryo electroporated at stage HH 17 (E2.5) with the N^{ICD}-IRES-GFP construct. In this specimen, GFP-positive cells are clustered and form a patch completely devoid of hair cells. Around the GFP-positive region, hair cells differentiate normally. (B) Whole-mount immunostaining in the saccule of an E9 embryo electroporated at stage HH 17 (E2.5) with N^{ICD}-IRES-GFP construct. In this specimen, GFP-positive cells are mixed with GFP-negative (untransfected) cells. Two different GFP-positive regions are outlined (1, 2). In both regions, hair cell density is diminished compared with adjacent regions. In region 2, however, some HCA-positive cells (hair cells) can be seen in the central part of the transfected patch. (C) Careful examination of region 2 at higher magnification, and in *z*-series of optical sections (Z1 to Z4, from the apical surface of the epithelium to deeper planes) reveals that these HCA positive cells are all GFP-negative (arrowheads); only the cells transfected with the N^{ICD}-IRES-GFP construct are inhibited from differentiating as hair cells.

revealed that these hair cells were either GFP-negative, or displayed much less GFP staining than their immediate neighbours (Fig. 3C). In fact, whereas in control specimens transfected with IRES-GFP alone 16.7% of GFP-positive cells (37 out of 222, range 7 to 35% for individual fields; n=8) differentiated into hair cells, only 1.2% of GFP-positive cells (19 out of 1554; range 0% to 5% for individual fields; n=19) did so in specimens transfected with N^{ICD}-IRES-GFP (see Materials and methods). These results provide direct confirmation that Notch activation within a sensory-patch cell inhibits it from differentiating as a hair cell.

It must be emphasised that patches of missing hair cells were detected in vestibular epithelia only. The GFP-positive cells within auditory epithelium (basilar papilla) were scattered and appeared to develop normally into hair cells and supporting cells with no special bias, and in only one case was a region devoid of hair cells observed. As we discuss later, this is most likely because hair-cell production in the auditory organ occurs 1 to 2 days later than in the vestibular patches (Adam et al., 1998; Bartolami et al., 1991; Katayama and Corwin, 1989), by which time N^{ICD} production from the original plasmid has ceased in most of the transfected cells.

Notch activity outside sensory patches can trigger ectopic hair cell formation

Unexpectedly, and in apparent contrast with the results just described, we found that in a large proportion of the specimens electroporated with N^{ICD}-IRES-GFP, groups of GFP-positive cells located outside the limits of sensory patches contained HCA-positive cells (24 out of 38 specimens analysed as whole-mounts showed such patches in vestibular regions, and 13 out of 38 in the cochlear duct). These ectopic hair cells were sometimes associated with abnormal outgrowths of the otic epithelium (see below); where there was no such deformity, they were often located in the vicinity of auditory and vestibular sensory patches, for example in regions such as the epithelium flanking the inferior edge of the basilar papilla (Fig. 4A,B) or at the external border of cristae (Fig. 4C,D). In some of the cases where small groups of cells exhibited high levels of GFP immunoreactivity (Fig. 4D; Fig. 5C) it was possible to confirm from the analysis of z-series of optical sections that the ectopic hair cells displaying HCA-positive stereociliary bundles were themselves GFP-positive. The ectopic sensory progenitor character of some of the N^{ICD}-IRES-GFP transfected cells was confirmed by their expression of the β-

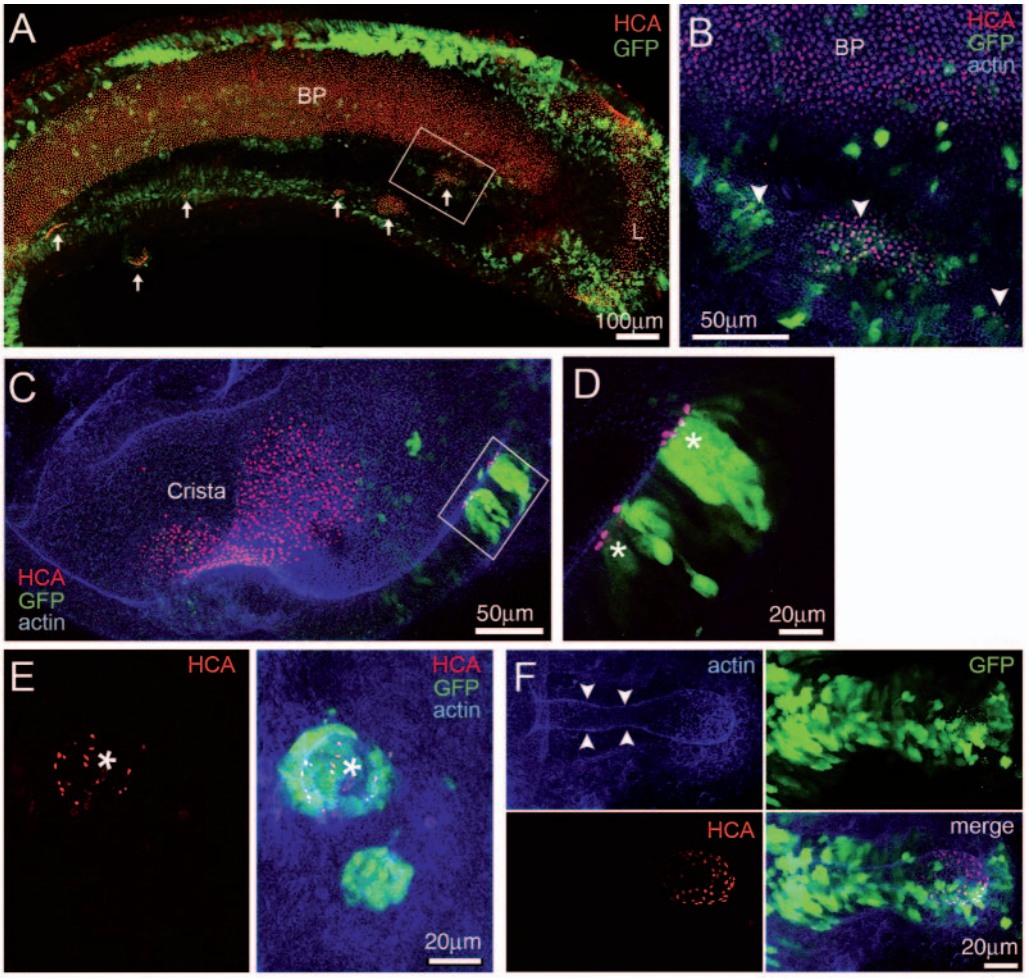


Fig. 4. Notch activity outside sensory patches can induce ectopic hair-cell differentiation. Whole-mount immunostaining in auditory and vestibular epithelia of E10 embryos electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP. (A) At low magnification, the overall pattern of HCA staining is normal in the basilar papilla and lagenar macula (compare with Fig. 2A), but several patches of ectopic hair cells (arrows) have differentiated in the region inferior to the basilar papilla. (B) Higher magnification view of clusters of ectopic hair cells neighbouring the distalmost region of the basilar papilla (white box in A). The ectopic hair cells lie within GFP-positive groups of cells (arrowheads). (C) Low magnification view of sensory epithelium of a crista and the surrounding non-sensory tissues. At some distance from the crista, clusters of GFP-positive cells are present (white box). (D) Higher magnification reveals that some members of each cluster of GFP-positive cells have differentiated as ectopic hair cells (asterisks). The optical section here is transverse to the ectopic sensory epithelium, and the hair bundles (HCA-positive) are on the apical surface. (E) Two compact groups of GFP-positive cells forming spherical or vesicular aggregates. One of these structures contains hair cells (asterisk); the other does not. (F) An ectopic sensory-organ-like structure located at the proximal end of the cochlear duct. GFP-positive cells form a tubular protrusion (arrowheads) ending in a hollow epithelial pouch containing hair cells (asterisk), whose HCA-positive hair bundles face the central lumen. BP, basilar papilla; L, lagenar macula.

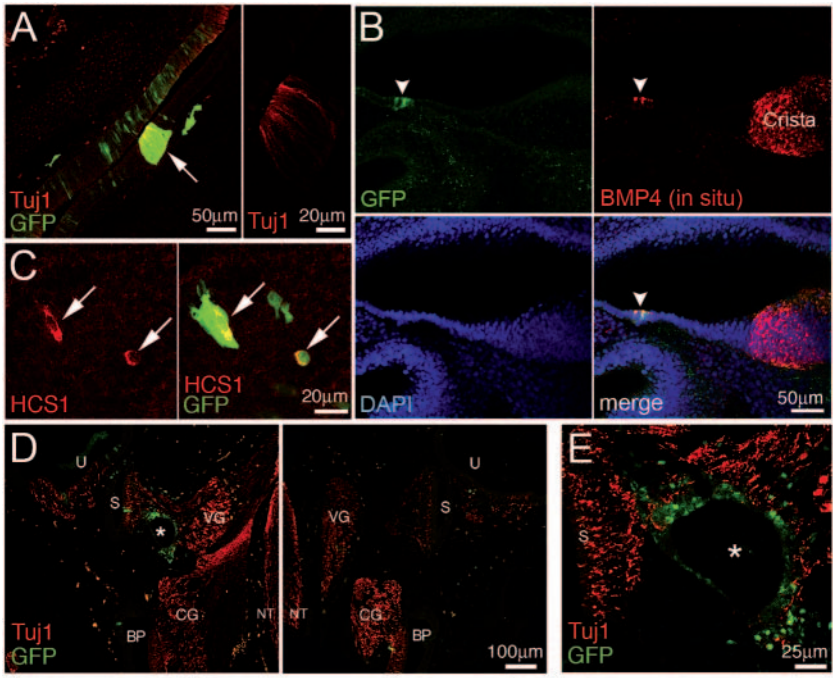


Fig. 5. Notch activity outside sensory patches can induce ectopic expression of multiple sensory-patch markers. (A) Cryosections of the cochlear duct from an E7 embryo electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP. In the cochlear duct at this stage, TuJ1 staining is a feature of sensory progenitors and nascent hair cells (Molea et al., 1999), as seen in the proximal (upper) part of the basilar papilla. A cluster of N^{ICD}-IRES-GFP-positive cells is present in the lateral wall of the cochlear duct (arrow), which is normally devoid of hair cells. At high magnification (right panel), the cells of this cluster are seen to express high levels of TuJ1. (B) Whole-mount in-situ hybridisation for Bmp4 (red) and immunostaining for GFP (green) in vestibular epithelia of E5 embryos electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP. The crista expresses Bmp4 in the normal fashion for a sensory patch, while a group of GFP-positive cells express Bmp4 ectopically (arrowheads). (C) Cryosections of the dorsal region of the posterior crista from an E7 embryo electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP. Some of the N^{ICD}-IRES-GFP-transfected cells have differentiated as ectopic hair cells, labelled for HCS-1 (arrows). These cells were also positive for HCA (not shown). (D,E) Immunostaining of GFP and β-III tubulin (TuJ1) in transverse paraffin sections of the inner ear from an E9 embryo electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP. Left and right panels in D show the electroporated and non-electroporated sides of the embryo, respectively; nerve cells and neurites penetrating sensory patches are strongly TuJ1-positive (dorsal is up). On the electroporated side, in addition to the utricle, saccule and cochlear duct/basilar papilla, an extra epithelial region with a central lumen can be seen (asterisk). At higher magnification (J), TuJ1-positive nerve fibres from the vestibular ganglion are seen to contact the GFP-positive cells in the ectopic end-organ-like structure. BP, basilar papilla; CG, cochlear ganglion; NT, neural tube; S, saccule; U, utricle; VG, vestibular ganglion.

III isoform of tubulin (Fig. 5A) and of *Bmp4* (Fig. 5B), two markers of prosensory regions of the otocyst (Molea et al., 1999; Wu and Oh, 1996). The hair-cell specific marker HCS-1 antigen (Gale et al., 2000) was also detected in some N^{ICD}-IRES-GFP transfected cells, supporting their identification as ectopic hair cells, as shown in Fig. 5C.

In many cases, ectopic hair cells were found within GFP-positive patches that were organised into structures with a finger-like or vesicular shape – apparently outpocketings from the otic epithelium (Fig. 4E,F; Fig. 5D,E). The GFP immunoreactivity was variable among the cells of these 'vesicles', and some of the cells appeared unlabelled. In most instances, the GFP-positive vesicles had a central lumen (Fig. 4F; Fig. 5E), and hair cells, where present, were correctly polarized, so that their stereociliary bundles lay at the lumenal surface (Fig. 4F). These structures resembling ectopic sensory organs were preferentially observed at the junction between the cochlear duct and the vestibular part of the inner ear, and nerve fibres originating from the vestibular ganglion appeared to connect with at least one such ectopic sensory patch (Fig. 5D,E).

Although ectopic hair cells were detected in approximately 60% of transfected specimens, only a small fraction of GFP-positive regions within each specimen contained ectopic hair cells. In fact, we even found cases where two transfected patches were in very close proximity (Fig. 4E), and yet ectopic hair cells could be detected in only one of those patches. As we discuss below, this variability might reflect variation in the time for which plasmid-directed gene expression persisted in the transfected cells.

Serrate-1 is upregulated in Notch ^{ICD} transfected cells

The results just described suggest that Notch activation in a group of cells in the early otocyst can drive them to adopt a prosensory character, so that they become competent to generate hair cells subsequently. From previous work, it appears that expression of the Notch ligand Ser1 is a marker of this state: the *Ser1* gene is expressed in the chicken otocyst from as early as E3.5 in a broad ventromedial domain spanning the future sensory regions, and as ear morphogenesis proceeds, this expression domain resolves into a number of discrete patches corresponding precisely to the regions in which hair cells will develop (Adam et al., 1998; Cole et al., 2000; Myat et al., 1996). We therefore looked to see whether Ser1 was expressed in our ectopic N^{ICD}-induced sensory-like patches.

In specimens transfected with N^{ICD}-IRES-GFP at E2 and examined 5 or 7 days later, we found that Ser1 was indeed ectopically expressed in GFP-positive cells located outside the normal sensory patches (Fig. 6B). As noted previously for the occurrence of ectopic hair cells, however, ectopic Ser1 expression was only visible in a small fraction of such GFP-positive cells within each specimen. The levels of Ser1 immunoreactivity of transfected cells located within the normal sensory patches were not affected – they remained high (data not shown). In specimens examined at earlier stages, preceding hair-cell differentiation, expression of Ser1 in ectopic locations was detectable from 48 hours post-electroporation in transfected cells displaying high GFP levels (Fig. 6A), but was not seen at earlier time points (12 and 24 hours, results not shown). These results indicate that Notch activity in a patch of

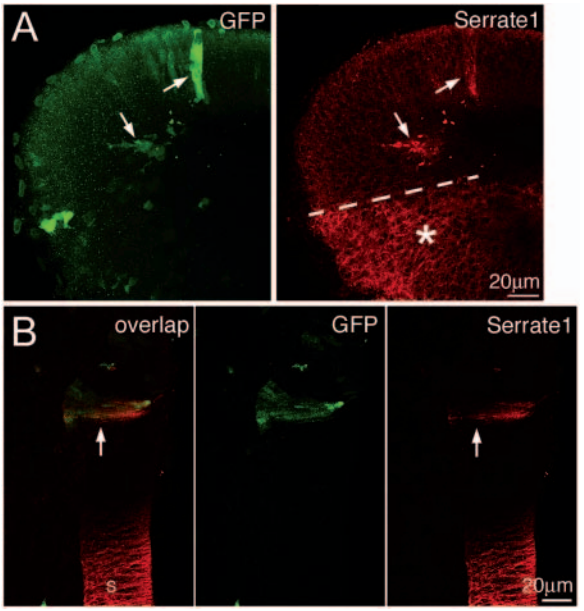


Fig. 6. Ectopic Ser1 expression in N^{ICD}-IRES-GFP transfected cells. Embryos electroporated at stage HH 13 (E2) with N^{ICD}-IRES-GFP construct. (A) Whole-mount immunostaining 48 hours post-electroporation. Ser1 is detected in its normal broad ventromedial domain of the otocyst, with a clear limit of expression (dashed line). It is also detected ectopically in N^{ICD}-IRES-GFP transfected cells (arrows). (B) Transverse cryosections 5 days post-electroporation. The saccular macula shows normal Ser1 expression, while ectopic Ser1 expression is seen in a cluster of GFP-positive transfected cells (arrow). S, saccular macula.

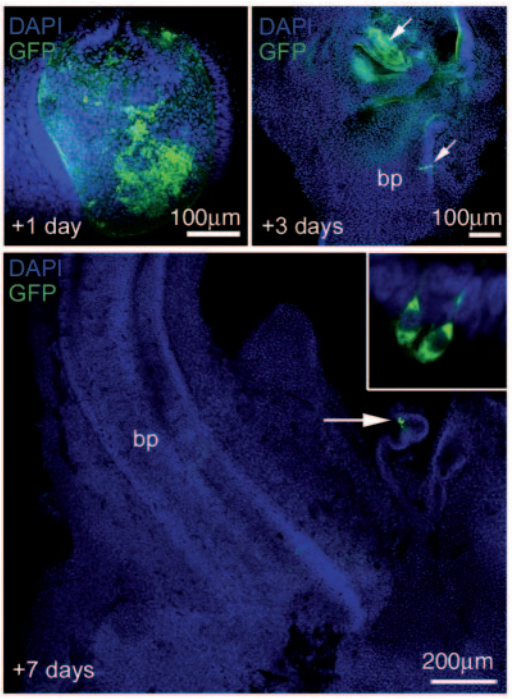


Fig. 7. In-situ hybridisation analysis of GFP mRNA persistence after electroporation of chick inner ear at stage HH 13 (E2) with HA-N^{ICD}-IRES-GFP construct. Representative micrographs of whole-mount specimens analysed at 24 hours, 3 days and 7 days post-electroporation. The number of cells expressing GFP mRNA decreases over time, and by 7 days post-electroporation scarcely any such cells are visible. The two cells positive for GFP mRNA in the 7-day specimen (shown enlarged in the insert) might be ones in which the plasmid has survived by integration into the host cell genome.

otic epithelium can induce Ser1 expression, one of the normal markers of future sensory-patch character.

Plasmid-directed N^{ICD} expression is transient in the vast majority of transfected cells

The foregoing data suggest that Notch activity has two distinct functions in the inner ear: at early stages, it may induce cells to adopt a prosensory character; later, it mediates lateral inhibition, limiting the number of cells within a sensory patch that are allowed to differentiate as hair cells. This interpretation fits the observations if we assume that N^{ICD} expression in the transfected cells is transient, lasting long enough to induce a prosensory state, but not so long as to block subsequent hair-cell production in every case. This would be consistent with previous electroporation studies in chick embryos, showing that transgene expression begins very rapidly (within 2 hours), peaks typically at 24 hours post-electroporation, and then declines because plasmid DNA is not stably integrated into the genome (Nakamura and Funahashi, 2001).

To see how long transcription of N^{ICD} in fact persisted after electroporation, we used in-situ hybridisation to investigate the expression of *GFP* mRNA in the inner ear at various times after electroporation of the N^{ICD}-IRES-GFP plasmid (Fig. 7). Because in this construct, *GFP* and N^{ICD} coding sequences are transcribed as a single unit, the presence of *GFP* mRNA gives a direct indication of the presence of N^{ICD} mRNA. We found that *GFP* mRNA is present in large patches of cells in the otocyst at 24 hours after electroporation, but at 3 days after electroporation it is already restricted to smaller patches. At 7 days post-electroporation only a few scattered cells continue to express *GFP* mRNA; it is possible that these rare cells are those in which the plasmid DNA has been integrated into the host cell genome. At the same stage, however, GFP protein could be detected in a large number of cells in specimens processed for GFP immunostaining (compare Fig. 7 with Fig. 4); as expected, the protein persists and can be detected by a sensitive method after the mRNA has disappeared. These data support the notion that in the vast majority of transfected cells, transcription from the plasmid is transient and ceases within 3 to 7 days after electroporation as a consequence of cell division and plasmid degradation and/or dilution in the progeny of transfected cells. Electroporation experiments in the neural tube using the same plasmid but with an HA-tagged form of N^{ICD} led to similar conclusions, with N^{ICD} protein detectable in transfected cells at 1 and 2 days after electroporation at E2 but absent by 4 days (data not shown).

Since our electroporations were done at E2 and hair cells start being produced in the vestibular sensory patches at E4-5 and in the basilar papilla at E5-7 (Bartolami et al., 1991; Katayama and Corwin, 1989), the implication is that exogenous N^{ICD} is likely to be present for long enough to inhibit hair-cell differentiation in the vestibular patches but not long enough to do so in the basilar papilla. We do not know precisely when the ectopic hair cells were born in the

transfected patches outside normal sensory regions, but as we only detected these hair cells at 5 or more days after electroporation, it seems likely that in some cases at least they were born after the exogenous N^{ICD} had disappeared.

Discussion

In this paper, we have described two seemingly contradictory effects of forced expression of activated Notch (N^{ICD}) in the ear. On the one hand, N^{ICD} expressed within vestibular sensory patches inhibits cells from differentiating as hair cells; this confirms predictions from previous studies. On the other hand, when expressed in regions that are normally non-sensory, N^{ICD} causes ectopic sensory patches containing hair cells to develop; this is novel.

We suggest that the two effects represent distinct actions of Notch at different stages of development. At early stages, Notch activity drives cells into a prosensory state. Later, as cell differentiation begins, Notch signalling mediates lateral inhibition, whereby nascent hair cells within the prosensory patch inhibit their neighbours from differentiating as hair cells. We have argued that we are able to see and distinguish the two effects because, with our technique of transgenesis by electroporation, the exogenous N^{ICD} is expressed transiently, beginning early enough to exert the first, inductive, effect, and lasting long enough to exert the second, inhibitory, effect in some sensory patches but not in others.

Lateral inhibition mediated by Notch controls differentiation of hair cells and supporting cells

Previous studies of Notch signalling in the ear have focused almost entirely on its role in controlling the choices cells make between hair-cell and supporting-cell fates. As explained in the Introduction, there is substantial evidence that Notch-mediated lateral inhibition is critical for this process of cell diversification within sensory patches, but there are also findings that conflict with the standard account of how the inhibition is regulated.

To check the basic assumptions of the lateral inhibition model, therefore, we decided to use electroporation to misexpress N^{ICD} directly. According to the model, this should act within the transfected cells to prevent them from differentiating as hair cells. That is precisely the result we have obtained in the vestibular sensory patches. In the basilar papilla we did not see such an effect; but the basilar papilla differentiates 1 to 2 days later than the vestibular patches, and by this time gene expression from the electroporated plasmid has faded away. Thus we conclude that Notch activity within sensory patch cells is sufficient to prevent them from differentiating as hair cells – certainly in vestibular regions, and probably also in auditory regions.

In normal development, therefore, it seems that nascent hair cells must somehow escape Notch activation. Even though they express Notch1 [initially at least – see Adam et al. (Adam et al., 1998)], and even though the Notch ligand Ser1 is expressed all over the surfaces of the adjacent supporting cells (Adam et al., 1998), this evidently fails to trigger production of N^{ICD} in the nascent hair cells. How can this be? It is possible that members of the Fringe family of glycosylases play a part here, as they are expressed in the sensory patches (Cole et al., 2000; Morsli et al., 1998; Zhang et al., 2000) and can modify Notch to lessen its susceptibility to activation by Serrate (Haines and Irvine, 2003; Hicks et al., 2000). However, the mouse *Lunatic fringe* knockout shows only very mild disturbances of hair-cell production (Zhang et al., 2000). Another possibility is that nascent hair cells contain a factor such as Numb that makes them immune to Notch activation (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996). This suggestion is consistent with observations of elevated Numb expression in hair cells (Eddison et al., 2000). Alternatively, the supporting cells may lack a factor that is needed to make Ser1 and any other Notch ligands they express functional, such as Mind bomb or some related protein. Mechanisms postulating that cell fate choices are governed by differences in the expression of co-factors of these types (Schweisguth, 2004) could explain why simply overexpressing Delta1 has little effect on the pattern of hair cell production (Eddison et al., 2000), even though signalling via Notch is critical.

An early phase of Notch activity promotes formation of prosensory patches

Our most novel finding is that Notch activity outside the normal sensory patches can induce production of hair cells. These ectopic sensory cells were unequivocally identified by the co-expression of two specific markers of hair cells, the HCA and HCS-1 antigens. Further, some isolated ectopic hair cells were clearly GFP-positive, suggesting that N^{ICD}-IRES-GFP acted cell-autonomously to drive cells along the sensory pathway. These findings seem at first to contradict those previously discussed, indicating that Notch activity represses hair-cell differentiation. As we have explained, however, the paradox is easily resolved if one postulates that Notch is acting in different ways at early and late steps of sensory patch formation – early to induce a prosensory state, and later to restrict hair-cell differentiation – and that misexpression of its active form (N^{ICD}) is only transient in our electroporation experiments. Although alternative interpretations are possible – invoking, for example, spatial variations in N^{ICD} activity – the explanation in terms of transient expression is well supported by our timecourse data.

If Notch activity is indeed normally required for cells to adopt a prosensory character, one would predict that an early blockade or reduction of Notch signalling should hinder the specification of prosensory patches. Two sets of observations give support to that prediction. Firstly, mice carrying mutations of the *Jagged1* gene frequently have, in addition to hair cell patterning defects in the organ of Corti, missing or reduced anterior and posterior semicircular-canal ampullae (Kiernan et al., 2001; Tsai et al., 2001). Secondly, the prediction tallies with the results of previous experiments in which we used an RCAS viral construct to force expression of a truncated form of Delta1 ($Delta1^{dn}$) that acts in Notch-expressing cells to make them refractory to Notch activation (Henrique et al., 1997). Instead of provoking overproduction of hair cells, as we originally expected, this resulted in many sites in which a patch of RCAS infection containing no hair cells directly abutted a sensory patch containing no RCAS-infected cells (Eddison et al., 2000). This is just what one would expect wherever the RCAS infection happened to occur within a region normally destined to be sensory: by blocking Notch activation and preventing adoption of a prosensory character at this site, it would have created a boundary of the type that was seen.

Notch receptors are expressed throughout the early otocyst, but presumably they are activated only in regions where Notch ligands are also present. From what we know of Ser1 expression, it seems that these regions in fact coincide with the future or actual sensory patches: as discussed above, Ser1 is expressed uniformly within prosensory patches well before the actual hair-cell and supporting-cell fate decisions occur. This implies that all sensory patch precursor cells experience some level of Notch activation during their developmental history. Our results argue that such early Notch activity has an essential role in the specification of sensory versus non-sensory regions of the otocyst.

The phenotype of the *mind bomb* zebrafish has an interesting implication here. With its vast overproduction of hair cells at the expense of supporting cells, this mutant shows a failure of lateral inhibition without any failure of prosensory determination. The Mind bomb protein has been shown to act on Delta proteins and to be required for their activity as Notch ligands (Itoh et al., 2003); but there is no evidence that Mind bomb is required for the activity of Ser proteins. Combining the fish data with the chick data, it is therefore tempting to suggest that activation of Notch by Ser1 (SerA in zebrafish) is independent of Mind bomb, occurs early, and is specifically responsible for prosensory determination, while activation of Notch by Delta1 is dependent on Mind bomb, occurs later, and is specifically responsible for lateral inhibition. Such distinct actions are not likely to reflect signalling mediated by different Notch family members, as the chick appears to have only two Notch genes (judging from a TBLASTN search of the current release of the chick genome) and only one of these – Notch1 – is detectably expressed in the embryonic ear (Myat et al., 1996) (data not shown).

There is a precedent for the prosensory effect of Notch signalling. Indeed, studies in the *Drosophila* eye have revealed an exactly analogous twofold function for Notch signalling, first in driving cells into a proneural state and then, at a later stage, in mediating lateral inhibition to restrict the proportion of these cells that differentiate as neurons (Baker and Yu, 1997; Li and Baker, 2001). Moreover, experiments in *Drosophila* (Kurata et al., 2000) and in *Xenopus* (Onuma et al., 2002) have shown that ectopic expression of activated Notch can act at early stages to induce expression of members of the *Pax-Eya-Dach-Six* gene network, resulting in development of ectopic patches of eye tissue. Members of the same gene network, or their homologues, are expressed in a localized fashion in the otocyst and are implicated in the development of its sensory patches (Abdelhak et al., 1997; Kalatzis et al., 1998; Laclef et al., 2003; Li et al., 2003; Ozaki et al., 2003; Xu et al., 1999; Zheng et al., 2003). It will be interesting to see whether the ectopic sensory patches we observe in our experiments with N^{ICD} in the ear likewise reflect a role of N^{ICD} as inducer of the expression of these genes.

Other signalling pathways are likely to cooperate with Notch to specify prosensory regions of the otocyst

Only a fraction of N^{ICD}-transfected cells in our experiments gave rise to ectopic sensory patches containing hair cells. In part, this may reflect the variation that is certainly present in the efficiency of electroporation and in the initial number of plasmid copies per cell, affecting both the level and duration of expression of N^{ICD} in transfected cells. If N^{ICD} expression is too weak and brief, it may not suffice for the initial induction of a prosensory patch; if it is too high and prolonged, it may block the development of hair cells within it.

Regional differences in the competence of transfected cells to generate ectopic sensory patches may also be important. Indeed, we noted that ectopic patches tended to form more frequently in vestibular than in auditory regions, and most often in the neighbourhood of the normal sensory patches. Many genes encoding transcription factors or signalling molecules, including components of the FGF, BMP, Hedgehog and Wnt pathways, are expressed in various restricted domains of the early otocyst (Barald and Kelley, 2004; Fekete and Wu, 2002; Fritzsch and Beisel, 2003; Whitfield et al., 2002). It is thus likely that the cooperation of several signalling pathways is required for the establishment of prosensory patches. The detailed regulatory connections between these pathways and the expression and activation of Ser1 and Notch1 remain to be deciphered, but there seems to be a particularly close relationship with the Wnt signalling pathway. Stevens et al. (Stevens et al., 2003) activated this pathway ectopically in the chick ear, by infecting the early otocyst with an RCAS retrovirus that carried a constitutively active form of β-catenin. This, like the ectopic expression of N^{ICD}, resulted in development of ectopic sensory patches, although it also produced other abnormalities, including gross malformations and changes of hair-cell character, that we did not observe in our electroporation experiments. Thus the Wnt and Notch signalling pathways in the ear may be linked in some sort of regulatory cascade.

Notch activity stimulates expression of Ser1, creating a positive feedback loop

We have shown that ectopic N^{ICD} expression can induce ectopic Ser1 expression, and this agrees with our previous finding that Ser1 expression is downregulated in cells where the Notch pathway is blocked by a dominant-negative form of Su(H) (Eddison et al., 2000). Positive regulation of *Ser1* by Notch activity also provides an explanation of some striking features of the normal Ser1 expression pattern. Unlike Delta1, Ser1 is expressed strongly and uniformly by the cells in the prosensory patch, as though the cells are behaving cooperatively instead of delivering lateral inhibition to one another. As development proceeds, the boundary of the Ser1 expression domain becomes sharply defined, and the expression of Ser1 within this domain persists, continuing in the supporting cells even into adult life (Stone and Rubel, 1999), but disappearing from the hair cells, in which Notch activity is absent. All these features are to be expected, if Ser1 activates Notch and activated Notch stimulates Ser1 expression. As we have discussed elsewhere (Eddison et al., 2000), this positive feedback will give rise to lateral induction, as opposed to lateral inhibition: a cell expressing Ser1 will tend to make its neighbours also express Ser1, and the effect will be reciprocated. The positive feedback will tend to intensify and perpetuate the expression of Ser1 and to sharpen the boundaries between expressing and non-expressing regions. Against this background of Ser1 regulation, other influences must act to allow a subset of cells to escape Notch activation and differentiate as hair cells.

Conclusion

We have seen that Notch signalling in the ear involves two classes of ligands, regulated in opposite ways and important for different effects. Ser1 is positively regulated by N^{ICD}, is expressed in each prosensory or supporting cell, and, we suggest, acts on the neighbours of that cell to keep them in a prosensory or supporting-cell state – that is, to maintain the potential for future differentiation as a hair cell. Delta1 and Ser2 are negatively regulated by N^{ICD}, are expressed in each nascent hair cell, and act on its neighbours to prevent them from realizing their potential to differentiate as hair cells. Both classes of ligands activate Notch1, the only member of the Notch family that is expressed in the chick ear, as far as we can tell. The difference of function between the ligands reflects the difference in the way they are themselves regulated by N^{ICD}, and the difference in the times at which they are called into play.

This picture is self-consistent and fits the data, but it leaves several mysteries unresolved. How are the nascent hair cells singled out? How do they, and they alone, avoid producing N^{ICD}, thereby freeing themselves of inhibition? Why is there such a delay from establishment of the prosensory patch to the onset of hair-cell differentiation? Why do only a fraction of N^{ICD}-IRES-GFP transfected cells differentiate into ectopic sensory patches? And what mechanism dictates in the first place where Notch is to be activated so as to drive cells into a prosensory state? Our demonstration of the two-fold action of Notch highlights these questions and takes us a step closer to answering them. What we discover for the ear will surely help to illuminate the workings of the Notch pathway in other developing and adult tissues, where similarly multiple Notch ligands are frequently deployed in time-dependent interlocking patterns.

We thank Jeff Corwin and Guy Richardson for the generous gift of the HCS-1 and HCA antibodies respectively; Isabelle Le Roux and Domingos Henrique for reagents; Jenny Corrigan for cryostat sectioning; and Linda Ariza-McNaughton, Alex Davies and Gavin Wright for helpful advice and technical help. This work was supported by the Fondation pour la Recherche Médicale (N.D. postdoctoral fellowship) and Cancer Research UK.

References

Abdelhak, S., Kalatzis, V., Heilig, R., Compain, S., Samson, D., Vincent, C., Weil, D., Cruaud, C., Sahly, I., Leibovici, M. et al. (1997). A human homologue of the Drosophila eyes absent gene underlies branchio-oto-renal (BOR) syndrome and identifies a novel gene family. *Nat. Genet.* **15**, 157-164.

Adam, J., Myat, A., le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* **125**, 4645-4654.

Ariza-McNaughton, L. and Krumlauf, R. (2002). Non-radioactive in situ hybridization: simplified procedures for use in whole-mounts of mouse and chick embryos. *Int. Rev. Neurobiol.* **47**, 239-250.

Austin, C. P., Feldman, D. E., Ida, J. A., Jr and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637-3650.

Baker, N. E. and Yu, S. Y. (1997). Proneural function of neurogenic genes in the developing Drosophila eye. *Curr. Biol.* **7**, 122-132.

Barald, K. F. and Kelley, M. W. (2004). From placode to polarization: new tunes in inner ear development. *Development* **131**, 4119-4130.

Bartolami, S., Goodyear, R. and Richardson, G. (1991). Appearance and distribution of the 275 kD hair-cell antigen during development of the avian inner ear. *J. Comp. Neurol.* **314**, 777-788.

Cole, L. K., le Roux, I., Nunes, F., Laufer, E., Lewis, J. and Wu, D. K. (2000). Sensory organ generation in the chicken inner ear: contributions of bone morphogenetic protein 4, serrate1, and lunatic fringe. *J. Comp. Neurol.* **424**, 509-520.

Eddison, M., le Roux, I. and Lewis, J. (2000). Notch signaling in the development of the inner ear: lessons from Drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 11692-11699.

Fekete, D. M. and Wu, D. K. (2002). Revisiting cell fate specification in the inner ear. *Curr. Opin. Neurobiol.* **12**, 35-42.

Fekete, D. M., Muthukumar, S. and Karagogeos, D. (1998). Hair cells and supporting cells share a common progenitor in the avian inner ear. *J. Neurosci.* **18**, 7811-7821.

Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1996). The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc. Natl. Acad. Sci. USA* **93**, 11925-11932.

Fritzsch, B. and Beisel, K. W. (2003). Molecular conservation and novelties in vertebrate ear development. *Curr. Top. Dev. Biol.* **57**, 1-44.

Gale, J. E., Meyers, J. R. and Corwin, J. T. (2000). Solitary hair cells are distributed throughout the extramacular epithelium in the bullfrog's saccule. *J. Assoc. Res. Otolaryngol.* **1**, 172-182.

Goodyear, R., Holley, M. and Richardson, G. (1995). Hair and supporting-cell differentiation during the development of the avian inner ear. *J. Comp. Neurol.* **351**, 81-93.

Goodyear, R. J., Legan, P. K., Wright, M. B., Marcotti, W., Oganesian, A., Coats, S. A., Booth, C. J., Kros, C. J., Seifert, R. A., Bowen-Pope, D. F. et al. (2003). A receptor-like inositol lipid phosphatase is required for the maturation of developing cochlear hair bundles. *J. Neurosci.* **23**, 9208-9219.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Haddon, C., Jiang, Y. J., Smithers, L. and Lewis, J. (1998). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-4644.

Haddon, C., Mowbray, C., Whitfield, T., Jones, D., Gschmeissner, S. and Lewis, J. (1999). Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. *J. Neurocytol.* **28**, 837-850.

Haines, N. and Irvine, K. D. (2003). Glycosylation regulates Notch signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 786-797.

Hartenstein, V. and Posakony, J. W. (1990). A dual function of the Notch gene in Drosophila sensillum development. *Dev. Biol.* **142**, 13-30.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell* **64**, 1083-1092.

Henrique, D., Hirsinger, E., Adam, J., le Roux, I., Pourquie, O., Ish-Horowicz, D. and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr. Biol.* **7**, 661-670.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* **2**, 515-520.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Jarman, A. P. (2002). Studies of mechanosensation using the fly. *Hum. Mol. Genet.* **11**, 1215-1218.

Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M. et al. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development* **123**, 205-216.

Kalatzis, V., Sahly, I., El-Amraoui, A. and Petit, C. (1998). Eya1 expression in the developing ear and kidney: towards the understanding of the pathogenesis of Branchio-Oto-Renal (BOR) syndrome. *Dev. Dyn.* **213**, 486-499.

Katayama, A. and Corwin, J. T. (1989). Cell production in the chicken cochlea. *J. Comp. Neurol.* **281**, 129-135.

Kiernan, A. E., Ahituv, N., Fuchs, H., Balling, R., Avraham, K. B., Steel, K. P. and Hrabe de Angelis, M. (2001). The Notch ligand Jagged1 is required for inner ear sensory development. *Proc. Natl. Acad. Sci. USA* **98**, 3873-3878.

Kurata, S., Go, M. J., Artavanis-Tsakonas, S. and Gehring, W. J. (2000).

Notch signaling and the determination of appendage identity. *Proc. Natl. Acad. Sci. USA* **97**, 2117-2122.
Laclef, C., Souil, E., Demignon, J. and Maire, P. (2003). Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice. *Mech. Dev.* **120**, 669-679.
Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat. Genet.* **21**, 289-292.
Lanford, P. J., Shailam, R., Norton, C. R., Gridley, T. and Kelley, M. W. (2000). Expression of Math1 and HES5 in the cochleae of wildtype and Jag2 mutant mice. *J. Assoc. Res. Otolaryngol.* **1**, 161-171.
le Roux, I., Lewis, J. and Ish-Horowicz, D. (2003). Notch activity is required to maintain floorplate identity and to control neurogenesis in the chick hindbrain and spinal cord. *Int. J. Dev. Biol.* **47**, 263-272.
Li, Y. and Baker, N. E. (2001). Proneural enhancement by Notch overcomes Suppressor-of-Hairless repressor function in the developing Drosophila eye. *Curr. Biol.* **11**, 330-338.
Li, X., Oghi, K. A., Zhang, J., Krones, A., Bush, K. T., Glass, C. K., Nigam, S. K., Aggarwal, A. K., Maas, R., Rose, D. W. et al. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**, 247-254.
Molea, D., Stone, J. S. and Rubel, E. W. (1999). Class III beta-tubulin expression in sensory and nonsensory regions of the developing avian inner ear. *J. Comp. Neurol.* **406**, 183-198.
Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K. and Yasuda, K. (1999). Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev. Growth Differ.* **41**, 335-344.
Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol.* **51**, 185-218.
Morrison, A., Hodgetts, C., Gossler, A., Hrabe de Angelis, M. and Lewis, J. (1999). Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. *Mech. Dev.* **84**, 169-172.
Morsli, H., Choo, D., Ryan, A., Johnson, R. and Wu, D. K. (1998). Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci.* **18**, 3327-3335.
Muller, U. and Littlewood-Evans, A. (2001). Mechanisms that regulate mechanosensory hair cell differentiation. *Trends Cell Biol.* **11**, 334-342.
Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Commun.* **230**, 376-380.
Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.
Nakamura, H. and Funahashi, J. (2001). Introduction of DNA into chick embryos by in ovo electroporation. *Methods* **24**, 43-48.
Onuma, Y., Takahashi, S., Asashima, M., Kurata, S. and Gehring, W. J. (2002). Conservation of Pax 6 function and upstream activation by Notch signaling in eye development of frogs and flies. *Proc. Natl. Acad. Sci. USA* **99**, 2020-2025.
Ozaki, H., Nakamura, K., Funahashi, J. I., Ikeda, K., Yamada, G., Tokano, H., Okamura, H. O., Kitamura, K., Muto, S., Kotaki, H. et al. (2003). Six1 controls patterning of the mouse otic vesicle. *Development* **131**, 551-562.
Riley, B. B., Chiang, M., Farmer, L. and Heck, R. (1999). The deltaA gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by pax2.1. *Development* **126**, 5669-5678.
Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z. et al. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.
Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.
Schweisguth, F. (2004). Notch signaling activity. *Curr. Biol.* **14**, R129-R138.
Spana, E. P. and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17**, 21-26.
Stevens, C. B., Davies, A. L., Battista, S., Lewis, J. H. and Fekete, D. M. (2003). Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear. *Dev. Biol.* **261**, 149-164.
Stone, J. S. and Rubel, E. W. (1999). Delta1 expression during avian hair cell regeneration. *Development* **126**, 961-973.
Tsai, H., Hardisty, R. E., Rhodes, C., Kiernan, A. E., Roby, P., Tymowska-Lalanne, Z., Mburu, P., Rastan, S., Hunter, A. J., Brown, S. D. et al. (2001). The mouse slalom mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti. *Hum. Mol. Genet.* **10**, 507-512.
Whitfield, T. T., Riley, B. B., Chiang, M. Y. and Phillips, B. (2002). Development of the zebrafish inner ear. *Dev. Dyn.* **223**, 427-458.
Wu, D. K. and Oh, S. H. (1996). Sensory organ generation in the chick inner ear. *J. Neurosci.* **16**, 6454-6462.
Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.
Zhang, N., Martin, G. V., Kelley, M. W. and Gridley, T. (2000). A mutation in the Lunatic fringe gene suppresses the effects of a Jagged2 mutation on inner hair cell development in the cochlea. *Curr. Biol.* **10**, 659-662.
Zheng, J. L., Shou, J., Guillemot, F., Kageyama, R. and Gao, W. Q. (2000). Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* **127**, 4551-4560.
Zheng, W., Huang, L., Wei, Z. B., Silvius, D., Tang, B. and Xu, P. X. (2003). The role of Six1 in mammalian auditory system development. *Development* **130**, 3989-4000.
Zine, A., van de Water, T. R. and de Ribaupierre, F. (2000). Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* **127**, 3373-3383.
Zine, A., Aubert, A., Qiu, J., Therianos, S., Guillemot, F., Kageyama, R. and de Ribaupierre, F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* **21**, 4712-4720.
zur Lage, P. and Jarman, A. P. (1999). Antagonism of EGFR and notch signalling in the reiterative recruitment of Drosophila adult chordotonal sense organ precursors. *Development* **126**, 3149-3157.