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Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear

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Notch signalling is well-known to mediate lateral inhibition in inner ear sensory patches, so as to generate a balanced mixture of sensory hair cells and supporting cells. Recently, however, we have found that ectopic Notch activity at an early stage can induce the formation of ectopic sensory patches. This suggests that Notch activity may have two different functions in normal ear development, acting first to promote the formation of the prosensory patches, and then later to regulate hair-cell production within the patches. The Notch ligand Serrate1 (Jag1 in mouse and humans) is expressed in the patches from an early stage and may provide Notch activation during the prosensory phase. Here, we test whether Notch signalling is actually required for prosensory patch development. When we block Notch activation in the chick embryo using the gamma-secretase inhibitor DAPT, we see a complete loss of prosensory epithelial cells in the anterior otocyst, where they are diverted into a neuroblast fate via failure of Delta1-dependent lateral inhibition. The cells of the posterior prosensory patch remain epithelial, but expression of *Sox2* and *Bmp4* is drastically reduced. Expression of Serrate1 here is initially almost normal, but subsequently regresses. The patches of sensory hair cells that eventually develop are few and small. We suggest that, in normal development, factors other than Notch activity initiate Serrate1 expression. Serrate1, by activating Notch, then drives the expression of *Sox2* and *Bmp4*, as well as expression of the *Serrate1* gene itself. The positive feedback maintains Notch activation and thereby preserves and perhaps extends the prosensory state, leading eventually to the development of normal sensory patches.

KEY WORDS: Notch signalling, Serrate, Delta, Chick embryo, Inner ear, Otic placode, Neuroblasts, Hair cell, Lateral inhibition, Lateral induction

INTRODUCTION

The vertebrate inner ear is composed of interconnected fluid-filled cavities, which are lined with an epithelium containing several distinct patches of sensory hair cells, responsible for the perception of sound, acceleration and gravity. The whole of this epithelium is derived from the otic placode, a thickening of the head ectoderm. In birds and mammals, the placode invaginates to create the otic cup, which closes to form a hollow vesicle, the otocyst. Over the next few days, the otocyst grows and develops a complex shape, while groups of cells at specific sites in its epithelial lining differentiate to form the sensory patches. Sensory patch development is itself a long process, beginning with the expression of genes that mark precursor regions, called prosensory patches, where hair cells will later arise. Recent studies have identified some of the major signalling pathways regulating this pattern of events, as well as transcription factors that are crucial for the execution of the developmental program (for reviews, see Fekete and Wu, 2002; Riley and Phillips, 2003; Barald and Kelley, 2004). The transcription factor Sox2, for example, is required for sensory patch formation (Kiernan et al., 2005b); Bmp4 is also expressed in the prosensory patches and has been reported to act as a diffusible signal that helps to regulate their extent (Li et al., 2005; Pujades et al., 2006). A crucial part is played by Notch signalling, and it is the role of this pathway that mainly concerns us in this paper.

Many experiments have shown that lateral inhibition mediated by Notch controls final cell fate choices in the developing ear. In this process, cells that become committed to a given pathway of differentiation inhibit their neighbours from doing likewise: the committed cells express Notch ligand(s) (of the Delta or Serrate/Jagged family) and deliver inhibition by activating Notch in their neighbours (Heitzler and Simpson, 1991). A hallmark of lateral inhibition is the negative regulation of Notch-ligand expression by Notch activity, creating a feedback loop that generates a fine-grained mixture of cells expressing either high or low levels of Notch ligand (Collier et al., 1996; Lewis, 1998). Lateral inhibition of this type, dependent on Delta1 and serrate 2/Jag2, regulates the choice between hair-cell and supporting-cell fates within sensory patches (Adam et al., 1998; Haddon et al., 1998; Lanford et al., 1999; Morrison et al., 1999; Riley et al., 1999; Eddison et al., 2000; Zine et al., 2000; Zine et al., 2001; Daudet and Lewis, 2005; Kiernan et al., 2005a; Brooker et al., 2006); and lateral inhibition mediated by Delta1 is also suspected to regulate the production of otic neuroblasts, which delaminate from the anterior part of the otic cup at an earlier stage (Adam et al., 1998; Haddon et al., 1998).

There are, however, hints that Notch signalling is also important in a different way – in the prosensory stage. Notch1 itself is expressed throughout the whole of the early otic epithelium, whereas one of its ligands, Serrate1 (Jag1 in the mouse) appears to be a marker of prosensory patches long before hair cells and supporting cells begin to differentiate (Adam et al., 1998; Morrison et al., 1999; Cole et al., 2000). Within these regions, Serrate1 is expressed in all of the cells uniformly, not in the pepper-and-salt pattern characteristic of lateral inhibition. Moreover, we have found that early ectopic expression of N^{ICD}, the activated form of Notch, in the chick otocyst can trigger the differentiation of ectopic sensory patches (Daudet and Lewis, 2005). Additionally, when *Jag1* is knocked out conditionally in the mouse, some of the sensory patches

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are completely lost, whereas others show a severe reduction in size and in hair-cell number (Brooker et al., 2006; Kiernan et al., 2006). All these findings suggest that an early phase of Notch activation, dependent upon Serrate1/Jag1, is required to define or maintain the prosensory patches and to enable them to attain their proper final size. To test this, we have examined the consequences of blocking Notch signalling in the inner ear of the early chick embryo with the γ-secretase inhibitor DAPT (Dovey et al., 2001), which prevents the release of the intracellular, active fragment of Notch (De Strooper et al., 1999; Mumm and Kopan, 2000). If Notch activation is indeed required for the establishment or maintenance of the prosensory state, this should result in a failure of sensory patch formation. We find in fact that the sensory patches in DAPT-treated inner ears are drastically reduced in number and size, although not lost completely, and we are able to clarify the chain of cause and effect leading to this result.

MATERIALS AND METHODS

Culture of chick embryos and DAPT treatments

White Leghorn and Brown chicken embryos were incubated at 38°C and staged according to the Hamburger-Hamilton (HH) tables (Hamburger and Hamilton, 1992). For culture ex ovo, embryos were collected at stage HH10 or stage HH11-12 and grown in Dulbecco's modified Eagle (DME) medium for 4-48 hours in roller tubes at 38°C as described previously (Connolly et al., 1995). We added to the medium either DAPT (Calbiochem) dissolved in dimethylsulfoxide (DMSO) (experimental embryos), or DMSO alone (control embryos). The final concentration of DAPT in the former case was 20-100 μM, and of DMSO in both cases 0.1% (0.013 M). For the culture of chick otocysts in vitro, we dissected otocysts from stage HH16-19 chick embryos and maintained them free-floating for 2-5 days in 24-well plates containing 500 μl of DME medium. Control specimens were grown in DME with 0.1% DMSO, and a final concentration of 20 μM DAPT was used in DAPT-treated samples. The cultures were supplemented with fresh medium every 2 days. At the end of the culture period, the otocysts were fixed for 2 hours at room temperature in 4% paraformaldehyde then processed for whole-mount in situ hybridisation (ISH) or immunocytochemistry.

Immunostaining and in situ hybridisation

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 2-12 hours. For cryosectioning, they were then immersed in a graded series of sucrose-PBS solutions (5-10-20%), embedded in 1.7% agar with 5% sucrose, frozen at –20°C, and sectioned at 15 μm on a Reichert-Jung cryomicrotome.

For immunostaining, the fixed specimens (sections or whole mounts) were incubated for 1 hour in blocking solution (PBS, pH 7.4, containing 0.3% Triton X100 and 10% goat serum). All subsequent incubations and rinses were in PBS with 0.1% Triton X100 (PBT). Incubations with primary and secondary antibodies were in PBT for 2 hours at room temperature or overnight at 4°C. Antibodies and staining reagents used were: mouse monoclonal IgG2a anti-TuJ1 (Covance; 1/1000 dilution), mouse monoclonal IgG2b anti-Islet1 (39.4D5, Developmental Studies Hybridoma Databank, USA; 1/200), rabbit anti-cDelta1 (Henrique et al., 1997) and rabbit anti-cSerrate1 (Adam et al., 1998) (both at 1/100), Alexa-Fluor A488-, A594-, and A633-conjugated goat IgG secondary antibodies (Molecular Probes; 1/500), and Alexa-Fluor 633-conjugated phalloidin (Molecular Probes; 1/100). Specimens were mounted in Slowfade (Molecular Probes) with DAPI as a nuclear counterstain and were observed under a Zeiss LSM510 confocal microscope.

For ISH, digoxygenin (DIG)-labelled RNA antisense probes were prepared from plasmids containing fragments or complete cDNA of the following chicken genes: *Bmp4* (obtained from R. Johnson, UTMD Anderson Cancer Center, Houston, TX), *Delta1*, *cHes5.1*, *cHes5.2*, *cHes5.3* (obtained from D. Henrique, Universidade de Lisboa, Portugal), lunatic fringe (*Lnfg*; obtained from C. Tabin, Harvard Medical School, Boston, MA), *Notch1*, *Serrate1*, *Six1* (ChEST762g17, BBSRC chickEST database), *Six2* (ChEST70o11, BBSRC chickEST database), *Six4* (ChEST177e15, BBSRC chickEST database), and *Sox2*. For whole mounts, non-fluorescent ISH was performed as previously described (Ariza-McNaughton and Krumlauf, 2002) using anti-DIG alkaline-phosphatase antibody (1/2000; Roche) and NBT-BCIP (Roche).

For accurate comparison of the ISH results, the control and DAPT-treated embryos were from the same experimental batch and were processed for ISH under the same conditions. For any given gene, the ISH analysis was performed at least twice, and similar numbers of control and DAPT-treated embryos were processed in parallel. For the comparison of *Serrate1* expression in the inner ear of control and DAPT-treated embryos, high-magnification views of the otic region of 69 randomly selected embryos (30 controls; 39 DAPT-treated) were judged in a blind test by seven examiners, who were asked to score the intensity of *Serrate1* expression (strong/faint/absent) in the anterior and posterior regions of the otic cup.

For double fluorescent ISH on cryosections, the slides were air-dried for 30 minutes at room temperature; then 75 μl of hybridisation buffer containing a mixture of Delta1-DIG- and Serrate1-fluorescein isothiocyanate (FITC)-labelled RNA probes was added on top of the slides. The slides were incubated overnight at 65°C in a humidified chamber, then washed in the following solutions at 65°C: twice in 2× standard saline citrate (SSC)-50% formamide for 30 minutes; twice in 0.2×SSC-50% formamide for 30 minutes; and once in tris buffer saline pH 7.5 and 0.1% Triton-X100 (TBST) for 30 minutes. The DIG and FITC probes were then sequentially detected using anti-DIG and anti-FITC antibodies conjugated to horseradish peroxidase (HRP) (1/100; Roche) and tyramide labelled with cyanine 3 or fluorescein (TSA Plus fluorescence system; Perkin Elmer), following the manufacturer's instructions. Following revelation of the first probe, the slides were incubated for 20 minutes in Glycine-2N HCl to inactivate the HRP activity associated with the first antibody, and then washed in TBST before application of the second HRP antibody. The slides were mounted in Slowfade (Molecular Probes) and observed under a Zeiss LSM510 confocal microscope.

RESULTS

DAPT inhibits Notch activity in the inner ear of early chick embryos

The Notch pathway depends upon the activity of γ-secretases, which are required for the cleavage of the transmembrane Notch receptors and the release of the intracellular fragment of Notch that translocates to the nucleus to regulate gene transcription (De Strooper et al., 1999; Berezovska et al., 2000; Mumm et al., 2000). γ-secretase activity can be inhibited with small cell-permeant molecules such as DAPT, leading to a blockade of Notch signalling (Dovey et al., 2001; Geling et al., 2002). We have used this method to block Notch signalling in the embryonic chick inner ear.

To test whether such a blockade was effective, we dissected Hamburger-Hamilton (HH) (Hamburger and Hamilton, 1992) stage 12 [stage HH12; embryonic day (E)2] chick embryos free from the yolk and transferred them into roller tubes, in which we maintained them for 24 hours either in control or in DAPT-supplemented medium. At stage HH12, the otic placode had started to invaginate to form the otic cup. During the culture period, as in ovo, the cup then invaginated to form an otocyst, although with a delay, taking approximately 24 hours instead of 12. In embryos treated with DAPT, growth was slightly reduced, but the otic cups nevertheless closed in the same way.

At the end of the 24-hour culture period, we fixed the embryos and examined the expression of known Notch target genes of the Hairy and Enhancer-of-Split (Hes) family: *cHes5.1*, *cHes5.2* and *cHes5.3*. These genes encode basic helix-loop-helix proteins of the Orange subtype (bHLH-O) that repress of the expression of other bHLH proneural proteins (reviewed by Bertrand et al., 2002). In mouse and chick embryos, *Hes5* genes are direct effectors of Notch activity during neurogenesis (de la Pompa et al., 1997; Kageyama and Ohtsuka, 1999; Hatakeyama et al., 2004; Fior and Henrique,

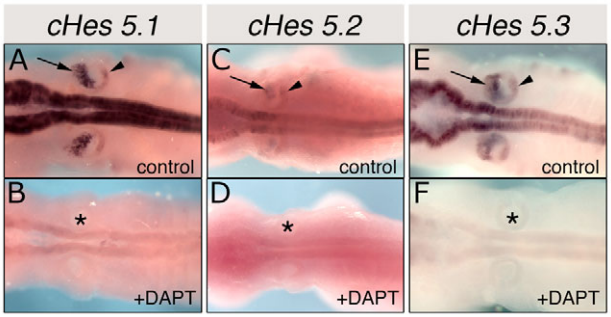

Fig. 1. Expression of Notch target genes of the cHes5 family is repressed by DAPT treatment. (**A-F**) Stage HH12 embryos cultured for 24 hours in control (A,C,E) or DAPT-containing (B,D,F) medium and processed for whole-mount in situ hybridisation for *cHes5.1*, *cHes5.2* or *cHes5.3*; dorsal views, with anterior on the left. In control specimens, all cHes5 genes are expressed in the neural tube, and in the anterior (arrows) and posterior (arrowheads) domains of the otic cup, although the expression of *cHes5.2* (C) appears fainter and more restricted than that of *cHes5.1* (A) and *cHes5.3* (E). After 24 hours in DAPT medium, the cHes5 genes are no longer detected in the neural tube or in the otic cup (asterisks in B,D,F). For each Hes gene analysed, the DAPT and control embryos shown were from the same experimental batch.

2005). In embryos grown for 24 hours in control medium, we found strong expression of c*Hes5.1* and c*Hes5.3* in the neural tube and in the anterior and posterior region of the otic cup (Fig. 1A,F). The c*Hes5.2* gene was also expressed in the inner ear and neural tube (Fig. 1C), but at lower levels and in a more restricted domain. In embryos grown in medium containing 100 μM DAPT, the expression of all three *cHes5* genes was dramatically reduced or absent in the neural tube, and was totally abolished in the otic epithelium, in all of the embryos (more than 20) analysed (Fig. 1B,D,E). This validated the use of DAPT as a blocker of Notch activity for our experiments.

Delta1 regulates the production of otic neuroblasts by lateral inhibition

Two separate regions of the early otocyst – one near its anterior pole, the other near its posterior pole – are normally destined to form sensory patches. The anterior region, and only the anterior region, gives rise to neuroblasts as well as to hair cells (Hemond and Morest, 1991; Adam et al., 1998; Alsina et al., 2004). Neuroblast production begins early, in the otic cup, well before the onset of hair-cell differentiation, and correlates with early expression of *Delta1* in the anterior region from late stage HH11 (Adam et al., 1998), which is also marked by the expression of all three *Hes5* genes and of the proneural gene *Ngn1* (Ma et al., 1998; Ma et al., 2000; Andermann et al., 2002; Alsina et al., 2004). Accordingly, we found that the blockade of Notch signalling had drastic effects on neurogenesis and on the fate of the anterior prospective sensory cells – effects rather different from those seen in the posterior region, where neurogenesis was not a developmental option.

During normal development of the anterior patch, the neuroblasts express *Delta1* transiently while still in the epithelium, where they are scattered in a pepper-and-salt fashion among other cells that express Hes genes (Adam et al., 1998) (and data not shown). The neuroblasts then delaminate from the epithelium to give rise to neurons of the cochleovestibular ganglion, expressing, among other markers, Islet1, *NeuroD* and *NeuroM* (Adam et al., 1998; Liu et al., 2000; Kim et al., 2001; Alsina et al., 2004). These gene-expression patterns are similar to those seen in the developing central nervous system (CNS) and suggest that lateral inhibition regulates neurogenesis in the ear as it does in the CNS (de la Pompa et al., 1997; Kageyama and Ohtsuka, 1999; Fior and Henrique, 2005). This suggestion is supported by evidence from mutants: a twofold increase in the number of otic neurons is observed in the inner ear of the *mind bomb* (*mib*) zebrafish mutant, in which Notch signalling is defective (Haddon et al., 1998). As a further test of the role of lateral inhibition in the production of otic neurons, we examined the expression of Delta1 and of the neuronal markers TuJ1 and Islet1 in the otic cup of stage HH12 embryos treated for 24 hours with DAPT. We observed a strong upregulation of *Delta1* expression at the mRNA (Fig. 2A,B) and protein levels (Fig. 2C-E). Expression of *Delta1* was no longer restricted to a scattered subset of the cells in the neurogenic region of epithelium, but was more or less universal within that region, although still not seen outside it (Fig. 2B). The size of this neurogenic epithelial patch (containing the *Delta1*-expressing cells) was, however, severely reduced (compare Fig. 2A with 2B), and the reduction in the epithelial population was accompanied by a large number of Islet1-positive otic neurons in the mesenchyme underlying the otic cup (Fig. 2D). Evidently, cells that would ordinarily have remained epithelial had instead become neuroblasts and delaminated – an expected consequence of a loss of lateral inhibition. Strikingly, in approximately half of the specimens examined, neuroblasts expressing Islet1, TuJ1 and Delta1 were also found in the lumen of the otic cup (Fig. 2E), suggesting that the normal epithelial architecture was disrupted in this part of the otic cup (as it was in the neural tube; data not shown). To confirm the role of lateral inhibition in the regulation of neuroblast production, we next analysed *Delta1* expression in the otic cup of stage HH11 embryos (14 somites) maintained for 4 hours in control medium, or in medium supplemented with DAPT. This developmental stage corresponds to the normal onset of expression of *Delta1* in the otic cup of the chick embryo (data not shown) (Adam et al., 1998), and we hypothesized that a short DAPT treatment at this stage should not be long enough to result in a compacted neurogenic patch, as seen after a 24 hours of treatment. Indeed, we found that the extent of the neurogenic patch was comparable in control and stage-matched DAPT-treated embryos (compare Fig. 2F with 2H). However, the total number of cells expressing *Delta1* was increased almost threefold in DAPT-treated embryos (mean=49.5; s.d.=12.5; n=3 embryos) as compared with control embryos (mean=17.3; s.d.=11.9; n=3 embryos). Furthermore, *Delta1*-expressing cells were clearly separated from one another in controls (Fig. 2G), whereas, in DAPT-treated embryos, clusters of contiguous *Delta1*-expressing cells were seen delaminating from the otic cup (Fig. 2I). Interestingly, similar treatment with DAPT at stage HH10 (ten somites), before the onset of the expression of *Delta1* in ovo, did not result in a precocious appearance of *Delta1*-expressing cells in the otic cup (data not shown). This suggests that Notch signalling does not control the timing of the initiation of neuroblast formation in the neurogenic patch. Altogether, these results confirm that lateral inhibition mediated by Delta1-Notch signalling operates in the anterior part of the otic epithelium to limit the proportion of cells that become neuroblasts. When the signalling fails, practically all cells in that region become committed to the neural fate, and few or none remain epithelial. In the posterior region of the ear rudiment, meanwhile, there is no expression of the *Delta1* at this early stage, and no such loss of cells from the epithelium resulted from the DAPT treatment.

The early expression of Serrate1 in the otic cup is independent of Notch activity

For a first indication of the effect of DAPT on the development of prosensory patches in the otic epithelium, we examined the expression of *Serrate1*. In the chick embryo, the otic placode forms immediately anterior to the first somite and becomes morphologically discernible at around the ten-somite stage (stage HH10) (Bancroft and Bellairs, 1977). *Notch1* is detected in the otic placode cells from stage HH10 (Groves and Bronner-Fraser, 2000), but *Serrate1* is not, and only becomes apparent in the posterior rim of the otic cup approximately 7 hours later (stage HH11, 13 somites) (Myat et al., 1996; Cole et al., 2000). Subsequently, *Serrate1* is expressed in both the anterior and the posterior prosensory regions, and appears to be a marker of the prosensory state (Myat et al., 1996; Adam et al., 1998; Cole et al., 2000). In the anterior region, the domains of *Serrate1* and *Delta1* expression are roughly coextensive; however, whereas *Delta1* expression is seen in scattered cells, expression of *Serrate1* is seen in contiguous cells and is more uniform, implying that it is not regulated by lateral inhibition. When the neuroblasts delaminate, the cells in the neurogenic neighbourhood that remain epithelial continue to express *Serrate1*, so that *Serrate1*-positive patches persist at both ends of the otocyst (Adam et al., 1998; Cole et al., 2000; Fekete and Wu, 2002).

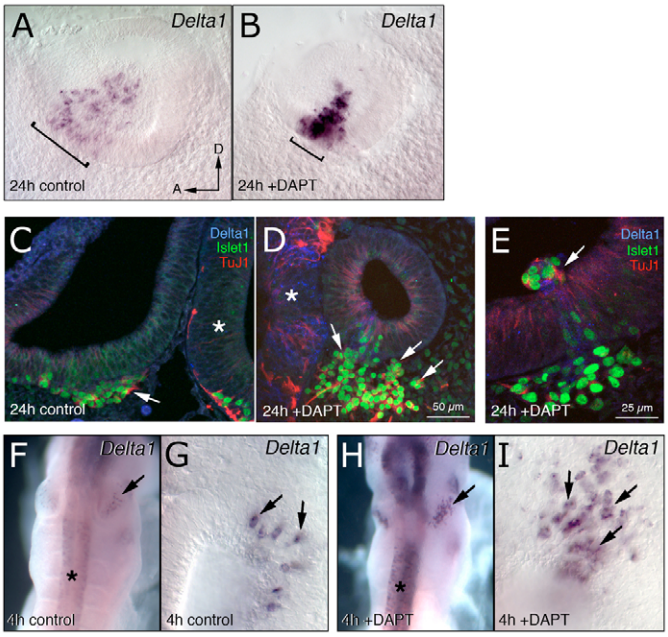

Fig. 2. Delta1 regulates neuroblast production by lateral inhibition. (**A**,**B**) Whole-mount view of dissected ears from stage HH12 embryos cultured for 24 hours in control (A) or DAPT-supplemented (B) medium then processed for *Delta1* in situ hybridisation. In control embryos, *Delta1* is expressed in scattered cells located in the anterior and medial region of the otic cup, corresponding to the delaminating neuroblasts. In DAPT-treated specimens, expression of *Delta1* is more intense in the anterior region of the otic cup, and cells expressing *Delta1* contact one another, but the size of the neurogenic patch is reduced (square brackets). (**C-E**) Transverse views of the otic cup of stage HH12 embryos cultured for 24 hours in control (C) or DAPT-supplemented (D,E) medium, and immunostained for three proteins: Delta1 (blue), Islet1 (green) and TuJ1 (red). In DAPT-treated embryos, an abnormally large number of Islet1-positive neuroblasts delaminate from the otic cup and accumulate in the underlying mesenchyme (arrows in D, compare with C). In control specimens (C), Delta1 protein is almost undetectable, whereas its expression is increased in the inner ear (D,E), as it is in the neural tube (asterisk in D), of DAPT-treated embryos. (E) In DAPT-treated embryos, Islet1- and TuJ1-positive neuroblasts are frequently found in the lumen of the otic cup (arrow). (**F-I**) Dorsal views (anterior is up) of stage HH11 embryos cultured for 4 hours in control (F,G) or DAPT-supplemented (H,I) medium then processed for whole-mount *Delta1* in situ hybridisation. A short DAPT treatment induces a strong upregulation of *Delta1* in the neural tube (asterisk) and in the otic cup (arrow in F,H). Closer examination of the anterior part of the otic cup shows that the delaminating neuroblasts are scattered in control embryos, but are more numerous and are frequently organised as cell clusters in DAPT-treated embryos (arrows in G,I). A, anterior; D, Dorsal.

The uniformity of *Serrate1* expression in cells within each putative prosensory patch suggests that Serrate1 in these sites might not only activate Notch, but also be positively regulated by this protein, creating a lateral-induction positive-feedback loop that could serve to maintain and extend the domain of Notch activation. Previous studies have indeed indicated that Notch activity promotes Serrate1 expression (Eddison et al., 2000; Daudet and Lewis, 2005), but the effect of a complete and broad inhibition of Notch signalling on Serrate1 expression has never been reported.

We compared *Serrate1* expression patterns by in situ hybridisation in the otic cup of a total of 70 embryos removed from the egg at stage HH12 and kept for 24 hours in either control (n=31) or DAPT-supplemented (n=39) medium. In the vast majority (96%) of control embryos, *Serrate1* expression was detected in both the anterior and posterior patches of the otic cup. In DAPT-treated embryos, the anterior and posterior patches were affected very differently. In the anterior (neurogenic) region, where *Delta1* expression was strongly upregulated, *Serrate1* expression was either completely lost (53%) or reduced (47%) (compare Fig. 3A with 3B). In confirmation of this finding, when we examined the Serrate1 protein distribution by immunostaining, we found that only a very small group of cells expressing Serrate1 protein remained at this site in DAPT-treated specimens (Fig. 3D,D′). We obtained similar results when we treated other embryos with DAPT a day later, by dissecting otocysts free from the rest of the embryo at stages HH16-17 and culturing them for 24 hours in control or DAPT-containing medium (data not shown). For both periods of treatment, the loss of Serrate1 expression in the anterior region was precisely what one would expect as a by-product of the conversion of almost all of the prosensory cells to a neuronal fate.

In the posterior region of the otocyst, by contrast, *Serrate1* expression persisted in all of the DAPT-treated specimens that were analysed (Fig. 3A,B). Although the size of the *Serrate1*-positive domain, as well as the intensity of *Serrate1* expression, appeared reduced in approximately half of the specimens relative to controls cultured without DAPT, the reduction was slight in comparison with the effect in the anterior region. Culture with DAPT for only 4 hours, instead of 24, starting at stage HH12, was enough to block expression of all of the *Hes5* genes and to cause a strong upregulation of *Delta1* (not shown) and of *Serrate1* expression in the neural tube (asterisk in Fig. 3F), demonstrating a blockade of Notch activity. Yet, in the posterior region of the otic cup, this DAPT treatment caused no detectable change of *Serrate1* expression as compared with the control cultures (Fig. 3E,F).

To test whether Notch activity was required in the early initiation of *Serrate1* expression, we next treated stage HH10 embryos (approximately 7 hours before *Serrate1* is normally expressed in the otic region; Fig. 3G) with DAPT for 24 hours, and compared *Serrate1* expression pattern to that of embryos

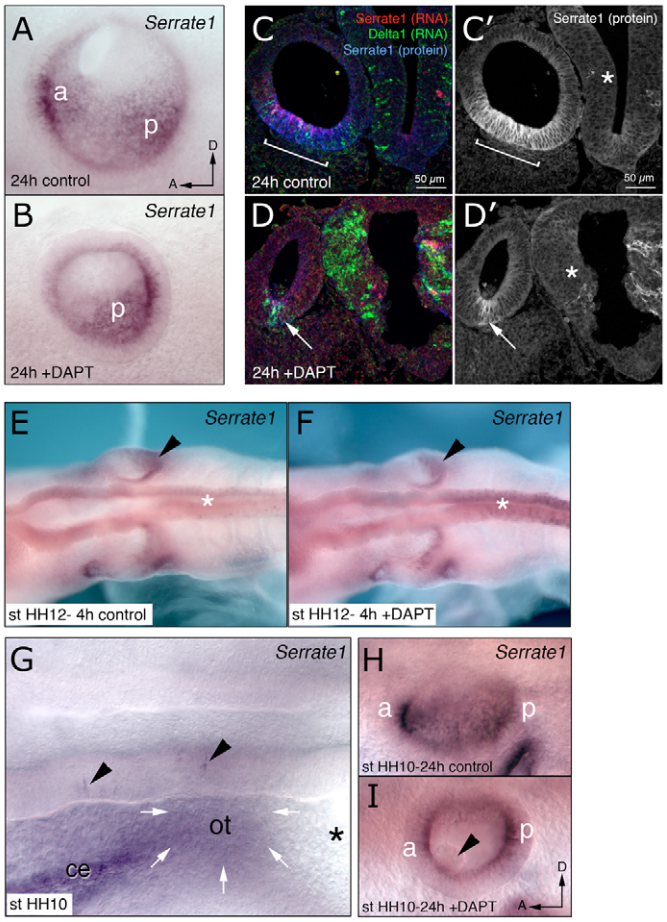

Fig. 3. Serrate1 is not regulated by lateral inhibition in the inner ear. (**A**,**B**) Whole-mount view of dissected ears from stage HH12 embryos cultured for 24 hours. In the control (A), *Serrate1* is most strongly expressed in two patches of cells located in the anterior (a) and posterior (p) regions of the otic cup. After 24 hours of DAPT treatment (B), *Serrate1* expression is absent or greatly reduced in the anterior region, but persists in the posterior region. (**C-D′**) Transverse sections of the neural tube and inner ear of stage HH12 embryos cultured for 24 hours in control (C) or DAPT-supplemented (D) medium; double in situ hybridisation for *Serrate1* (red) and *Delta1* (green) and immunostaining for Serrate1 protein (blue, and monochrome in C′,D′). *Delta1* expression is upregulated in both the inner ear and neural tube of DAPT-treated embryos (compare C and D). By contrast, in DAPT-treated embryos, both the intensity and the extent of Serrate1 expression are reduced in the inner ear (compare bracketed regions in C,C′ with arrowed regions in D,D′), although they are increased in the neural tube (asterisks in C′,D′). (**E**,**F**) Stage HH12 embryos cultured for 4 hours in control (E) or DAPT-supplemented (F) medium. After this brief DAPT treatment, *Serrate1* expression is upregulated in the neural tube (asterisks), but not in the posterior rim of the otic cup (arrowheads), where its levels of expression appear unchanged as compared to control specimens. (**G**) Dorsal view of a stage HH10 embryo processed for *Serrate1* whole-mount in situ hybridisation; anterior is to the left. *Serrate1* expression is not detected in the presumptive otic placode field (ot, arrows), which is located anterior to the first somite (asterisk). Notice the expression of *Serrate1* in scattered neurons within the neural tube (arrowheads) and in a region of the cephalic ectoderm (ce). (**H**,**I**) Whole-mount view of dissected ears from stage HH10 embryos cultured for 24 hours. In control otocysts (H), the anterior (a) and posterior (p) patches of *Serrate1* expression are present. In DAPT-treated specimens (I), *Serrate1* expression is absent or reduced in the anterior region, where abnormal extrusion of neuroblasts in the otocyst lumen can be seen (arrowhead; see also Fig. 2E). However, the posterior patch of *Serrate1* expression persists. A, anterior; a, anterior region; ce, cephalic ectoderm; D, dorsal; ot, presumptive otic placode field; p, posterior region.

maintained for 24 hours in control medium. We found that, in control embryos, *Serrate1* expression was present in both the anterior and posterior region of the otic cup after 24 hours of culture (Fig. 3H; n=12). In the corresponding stage HH10 embryos treated with DAPT for 24 hours, and as previously noted in our experiments on stage HH12 embryos, we found that the anterior patch of *Serrate1* expression was greatly reduced in size or absent. However, in all of the embryos analysed, the posterior patch of *Serrate1* expression remained (Fig. 3I; n=19). Taking all these data for the posterior patch together, we infer that, at these early developmental stages at least, Notch activity is not needed to drive *Serrate1* expression: some other factor induces it, thereby helping to give the prosensory patch its special character.

Blocking Notch activity downregulates expression of *Sox2* and *Bmp4*

From the foregoing, it appears that *Serrate1* expression lies upstream from Notch activation in the early prosensory regions. What lies downstream? Are other aspects of prosensory patch character more severely disrupted when Notch activation is blocked? To find out, we examined the effects of DAPT on ten other genes (*Bmp4*, *Sox2*, *Six1*, *Six2*, *Six4*, *Gbx2*, *Notch1*, *Wnt3a*, *Lfng* and *Soho1*) involved in sensory patch development, of which *Sox2* and *Bmp4* proved the most informative (Fig. 4).

Sox2 codes for a transcription factor belonging to the group B Sox (SRY related and HMG box) family, and it has recently been shown to be essential for inner ear development: in mice with a null mutation in the gene, no sensory patches develop (Kiernan et al., 2005b). In the chick, *Sox2* is at first expressed uniformly in the otic placode and cup but then becomes enriched in the developing sensory patches, where its expression is maintained until at least E12 (Uchikawa et al., 1999) (and data not shown). In embryos grown for 24 hours in control medium, we saw strong *Sox2* expression throughout the otic vesicle in 85% of the embryos analysed (n=40), with occasionally a relative enrichment in the anterior and posterior regions of the otic cup (Fig. 4A). In the DAPT-treated embryos, by contrast, *Sox2* expression was usually greatly diminished, and only 29% of the specimens showed strong expression (n=45; Fig. 4B).

Several BMP genes are expressed in the early inner ear and have been implicated in the formation of the sensory cristae and their semicircular canals (Chang et al., 1999; Gerlach et al., 2000), and in controlling the extent of other sensory patches (Li et al., 2005; Pujades et al., 2006). In particular, *Bmp4* expression is first detected in the posterior rim of the otic cup at stages HH12-15, becomes localised to the primordia of the anterior and posterior cristae from stage HH16-17, and subsequently is detected in or close to all the prospective sensory patches (Oh et al., 1996; Wu and Oh, 1996; Gerlach et al., 2000). The majority (74%) of embryos grown for 24 hours in control medium showed clear expression of *Bmp4* in the anterior and posterior domain of the otic cup (Fig. 4C), whereas the remaining ones showed only the posterior patch of expression. Only one control embryo (out of 19) failed to show expression of *Bmp4* in the otic epithelium. By contrast, in DAPT-treated embryos, *Bmp4* expression was undetectable in the otic cup of the majority (69%) of the specimens

examined (Fig. 4D; *n*=26), and limited to the posterior patch of most of the remaining embryos; only one embryo displayed both the anterior and the posterior patches of *Bmp4* expression. Hence, like *Sox2* expression, but even more strikingly, blocking Notch activity dramatically reduced *Bmp4* expression in both the posterior and anterior prosensory regions of the otic cup.

Sustained blockade of Notch activity inhibits Serrate1 expression and impairs sensory patch development and hair-cell production

In the roller-tube cultures used for the above experiments, the explanted embryos cannot be satisfactorily maintained for more than 48 hours. Therefore, this approach could not be used to study the impact of DAPT upon the production of hair cells, which begins after stage HH25 (E3.5-4). To overcome this problem, we dissected otocysts free from the rest of the embryo at stage HH16-17 (2.5 days) and transferred them to medium supplemented or not with DAPT (Fig. 5); here, they continued development for up to a further 5 days – sufficient time for the development of hair cells. We analysed the otocysts after 2 and 5 days in culture. We found that a 20 μM dose of DAPT was sufficient to abolish expression of c*Hes5.1* and c*Hes5.3* at both of these time points (data not shown). Expression of *Bmp4* was also affected. In control otocysts, several patches of *Bmp4* expression were usually present: after 2 days in culture, the mean number of patches of *Bmp4* expression detected per otocyst was 1.8 (*n*=18 otocysts); after 5 days, it was 2.5 (*n*=12 otocysts) (Fig. 5A,D). In the DAPT-treated otocysts, the patches of *Bmp4* expression were greatly reduced in number: after 2 days, there were, on average, only 0.5 patches per otocyst (*n*=31); and after 5 days there were 0.8 (*n*=17 otocysts) (Fig. 5B,C,E,F). Furthermore, the size of the remaining patches and the levels of *Bmp4* expression within them were drastically reduced as compared with the controls (arrowheads in Fig. 5B,E).

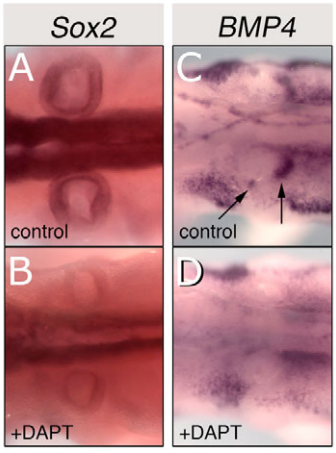

Fig. 4. Blocking Notch signalling reduces the expression of *Sox2* and *Bmp4*. (**A-D**) Whole-mount view of stage HH12 embryos cultured for 24 hours in control (A,C) or DAPT-supplemented (B,D) medium. All panels are dorsal views (anterior left). (A) *Sox2* is expressed throughout the early otic epithelium of control embryos, sometimes with an increased expression in the anterior and posterior regions of the otic cup. (B) In DAPT-treated embryos, *Sox2* expression is greatly decreased in the otic cup, as it is in the neural tube. (C) *Bmp4* is expressed in control embryos in the posterior rim of the otic cup and in a small patch in the anterior part (arrows). (D) After DAPT treatment, *Bmp4* expression in the otic epithelium is greatly diminished.

We next looked at Serrate1 protein expression after 5 days in culture and found similar results: in control otocysts (Fig. 5G), there were on average 2.5 distinct patches of Serrate1 expression per

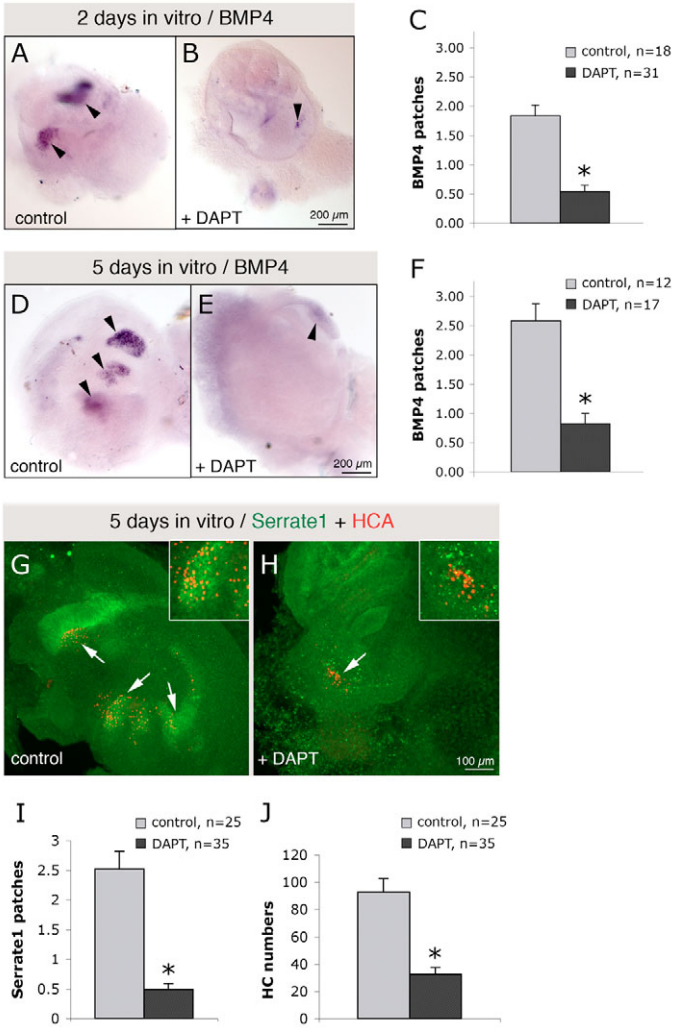

Fig. 5. Differentiation of sensory patches with the production of hair cells is inhibited by DAPT treatment initiated at an early stage. Organ cultures established at stage HH16-17 were maintained for 2-5 days in either control medium or in medium containing 20 μM DAPT. (**A-F**) *Bmp4* expression analysed after 2 or 5 days in vitro. In control specimens (A,D), typically two or three patches of strong *Bmp4* expression are observed (arrowheads); in DAPT-treated specimens (B,E), the size and mean number of *Bmp4*-positive patches are reduced and the intensity of *Bmp4* expression within them is greatly diminished. (C,F) Quantitative analysis of the mean number of patches of *Bmp4* expression in control versus DAPT-treated otocyst after 2 (C) or 5 (F) days in vitro. (**G**,**H**) Serrate1 and hair-cell antigen (HCA) immunostaining in control (G) and DAPT-treated (H) otocysts after 5 days in vitro. The micrographs are *z*-projections of confocal optical sections encompassing the entire thickness of the dissected otocysts. Serrate1 is expressed in several patches containing differentiated hair cells (arrows in G) in control otocysts. In DAPT-treated otocysts, Serrate1 expression is abolished or greatly reduced and hair cells are few, although densely clustered (arrow and inset in H). (**I**,**J**) Quantitation of the number of patches of Serrate1 expression (I) and of the number of hair cells (J) after 5 days of culture in control versus DAPT-supplemented medium. Error bars represent s.e.m. All the differences between control and DAPT-treated groups were statistically significant (*t*-test; $*P<10^{-6}$).

otocyst (*n*=25 otocysts), whereas, in the DAPT-treated otocysts (Fig. 5H), the mean number was only 0.5 (*n*=35 otocysts; Fig. 5I). Just as for *Bmp4* expression, the sizes of the remaining patches were severely reduced relative to the controls.

Lastly, in the same set of specimens, we used immunostaining for the hair-cell antigen (HCA), which labels the stereociliary bundles of hair cells (Bartolami et al., 1991), to compare the number of hair cells produced in control and DAPT-treated otocysts. If the only effect of a loss of Notch signalling were a loss of lateral inhibition during hair-cell differentiation, one would expect DAPT treatment to cause an increase in the number of hair cells. However, we found the opposite: the mean number of hair cells per otocyst after 5 days in culture was 92 in controls (*n*=25 otocysts), but only 32 (*n*=35 otocysts) in DAPT-treated otocysts (Fig. 5G,H,J).

Altogether, these data showed that a sustained blockade of Notch signalling initiated at stage HH16-17 causes a severe reduction in the number and size of sensory patches, and in the number of hair cells ultimately produced.

DISCUSSION

The Notch pathway has different roles in a variety of developing and adult tissues, creating boundaries of gene expression, mosaics of alternating cell types, or cyclic patterns of gene expression. Within the same tissue, Notch can have different functions, in different processes, and at different stages of development, as clearly exemplified in the inner ear. In this organ, the variety of functions can be explained in part by the presence of different Notch ligands, with differing modes of regulation and expression patterns. Hence, Delta1 is the main player in the lateral inhibition of neuroblast and hair-cell production, whereas Serrate1 seems to have a more specific role in the formation of prosensory domains (Brooker et al., 2006; Kiernan et al., 2006).

Our results clarify this picture in three main ways: (1) they confirm the role of Delta1-Notch signalling as mediator of lateral inhibition controlling otic neurogenesis; (2) they show that Notch activation is needed for the normal early expression of markers of prosensory patch character, but not for the early expression of Serrate1 itself; and (3) they show that Notch activation is necessary for the subsequent maintenance of Serrate1 expression and for the production of sensory patches of normal size. These findings lead to an account of how Notch signalling fits into the chain of cause and effect by which sensory patches develop, as we now explain.

In the anterior neurogenic patch, Notch-mediated lateral inhibition is needed to maintain a proportion of cells as epithelial prosensory precursors

In agreement with previous studies conducted in mouse and zebrafish (Haddon et al., 1998; Brooker et al., 2006), our results show very clearly that lateral inhibition regulates the production of otic neuroblasts. In the anterior region of the otic cup, scattered neuroblasts expressing Delta1 activate Notch in their neighbours, inducing the expression of the transcriptional repressors of the Hes5 family. The latter repress the expression of proneural bHLH genes such as *Ngn1*, specifying the neuroblast fate (Ma et al., 1998; Alsina et al., 2004). As predicted by the lateral inhibition model, blocking Notch signalling results in an upregulation of *Delta1* expression and in an over-production of otic neuroblasts. These defects resemble those seen in the inner ear of the zebrafish *mib* mutant, in which Notch signalling is defective because of the absence of the E3 ubiquitin ligase Mind bomb (Jiang et al., 1996; Haddon et al., 1998; Itoh et al., 2003).

The diversion of an excessive number of cells to become neuroblasts in the anterior region is accompanied by an almost complete disappearance of the anterior patch of epithelial cells expressing Serrate1. Meanwhile, expression of this marker is initiated and maintained in the posterior prosensory patch, for a while at least. This strongly suggests that all the prosensory cells in the anterior patch, but not the posterior patch, are competent to develop as neuroblasts and will do so if Notch signalling is defective.

Serrate1 expression is not regulated by lateral inhibition, and it is not detected in delaminating neuroblasts, but it could nevertheless activate Notch in the neurogenic patch and modulate neuroblast formation. Interestingly, lunatic fringe (*Lnfg*) is also expressed in the anterior neurogenic patch (Morsli et al., 1998; Cole et al., 2000), and several lines of evidence suggest that it could reduce the efficacy of Serrate1 as an activating Notch ligand (reviewed by Schweisguth, 2004). Mathematical modelling confirms that this, by bringing about a reduction in the background level of Notch activation, can enable Delta1-mediated lateral inhibition to operate, with some cells escaping lateral inhibition and others being subjected to it (J.L., unpublished). However, although there have been studies of the effects of *Lfng* knockout in the ear (Zhang et al., 2000), there is no published information as to whether this affects neuroblasts production. This remains an interesting question for the future.

The failure of lateral inhibition at the otic cup stage has dramatic effects upon tissue architecture as well as on cell differentiation in the anterior prosensory domain. The size of the otic cup of DAPT-treated embryos was reduced, and neuroblasts were frequently found in the lumen of the otic cup, indicating that the integrity of the epithelium or its apico-basal polarity was disrupted. In the neural tube, we found that the lining of the lumen was frequently disturbed in a similar way after DAPT treatment (data not shown), and such abnormalities are also seen in the spinal cord of *hes1*/*hes5* double-knockout mice (Hatakeyama et al., 2004). In both tissues, these defects are an expected consequence of the absence of non-neuronal cells required for the maintenance of tissue architecture.

These findings are consistent with studies in the conditional *Delta1*-knock-out (*Dll1-cko*) mice, in which the maculae of the saccule and utricle were lost or severely reduced in size (Brooker et al., 2006). As in the chick, this appeared to reflect an early over-production of neuroblasts at the expense of sensory progenitors (Brooker et al., 2006). This is consistent with recent lineage studies showing that otic neurons and sensory cells of the utricular macula can derive from a common ancestor in the otic cup (Satoh and Fekete, 2005).

Factors other than Notch signalling drive the initial pattern of Serrate1 expression in the early otocyst

In the posterior patch, at early stages, Serrate1 expression is largely maintained in the short-term despite the blockade of Notch activation by DAPT. Evidently, Serrate1 expression here is driven by factors other than Notch activity. These could include signals delivered by the Hedgehog (Riccomagno et al., 2002; Hammond et al., 2003; Koebernick et al., 2003; Bok et al., 2005), Wnt (Ladher et al., 2000; Stevens et al., 2003) or fibroblast growth factor (FGF) (Vendrell et al., 2000; Adamska et al., 2001; Leger and Brand, 2002; Wright and Mansour, 2003; Phillips et al., 2004; Ladher et al., 2005) pathways, all of which have been implicated in the induction and early patterning of the otocyst.

Effects of Notch signalling on prosensory development are mediated in part by Sox2 and Bmp4

Sox2 and *Bmp4* showed reduced expression in the posterior patch in the presence of DAPT. Because Serrate1 is the only Notch ligand detectably expressed at early stages in this site, it seems that it must normally be responsible for activating Notch so as to drive proper levels of expression of *Sox2* and *Bmp4* in this region.

Sox2 is absolutely required for the formation of all inner ear sensory patches and is the only gene with this property identified so far (Kiernan et al., 2005b). It is expressed in the common progenitors of hair cells and supporting cells and is needed for the expression of Math1 (Kiernan et al., 2005b), a bHLH transcription factor required for hair-cell differentiation (Bermingham et al., 1999). Mice in which the *Serrate1* orthologue *Jag1* has been knocked-out in the inner ear have severely reduced numbers of hair cells (Brooker et al., 2006; Kiernan et al., 2006), and this correlates with a loss of *Sox2* expression (Kiernan et al., 2006). Both in our experiments and in the mouse study, the expression of *Sox2* was reduced, but not completely abolished, implying that *Sox2* expression is partially but not entirely dependent on Notch signalling.

The blockade of Notch signalling caused a loss of *Bmp4* expression, and this effect was even more severe than the effect on *Sox2* expression. Conversely, as we showed previously, ectopic *Bmp4* (along with ectopic Serrate1) can be induced by N^{ICD} transfection (Daudet and Lewis, 2005). The function of *Bmp4* in the inner ear is not so well characterised as that of *Sox2*, but overexpression of the BMP antagonist noggin is known to result in defects in chick inner ear morphogenesis, including some truncations of the semi-circular canals (Chang et al., 1999; Gerlach et al., 2000). Differentiation of the cristae is also inhibited by noggin (Gerlach et al., 2000), and it has been reported that noggin reduces hair-cell production in long-term organotypic cultures of chick otocysts (Li et al., 2005). Similar defects are seen in *Jag1*-deficient mice (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006), hinting that loss of *Bmp4* expression could contribute, along with the reduction in *Sox2* expression, to the reduced production of sensory cells in our DAPT-treated otocysts.

This reduction in the size of sensory patches, and ultimately in hair-cell numbers, contrasts with the dramatic overproduction of hair cells seen in the inner ear of the *mib* zebrafish mutant (Haddon et al., 1998), in which Notch signalling is thought to be completely abolished (Itoh et al., 2003). However, interspecies differences in the sequence of events that underlies ear development can explain this discrepancy. In the zebrafish, neuroblasts delaminate from the otocyst between 22 and 36 hours post-fertilisation (hpf), but the specification of the first hair cells, the tether cells, starts from 14 hpf (Haddon and Lewis, 1996; Riley et al., 1997; Millimaki et al., 2007). Hence, in the fish, the prosensory patches are defined and begin their differentiation early (Millimaki et al., 2007), and failure of lateral inhibition entails that excess hair cells along with neuroblasts form when Notch signalling is blocked. In the chick, neuroblasts differentiate early but the sensory patches do not become competent to form hair cells until much later, and therefore the early blockade of Notch signalling does not directly affect hair-cell production.

Maintenance and expansion of prosensory patches depend on a Serrate1-Notch positive-feedback loop

In our long-term DAPT-treated cultures, expression of Serrate1 itself was markedly reduced. This suggests that, although Notch signalling is not required initially for the induction of Serrate1, it is required subsequently for its maintenance. Indeed, several studies have shown that Notch activation regulates Serrate1 positively in the ear (Eddison et al., 2000; Daudet and Lewis, 2005). In other words, Serrate1 is regulated by lateral induction, not lateral inhibition (reviewed by Lewis, 1998; Cornell and Eisen, 2005). The resulting positive feedback will tend to maintain high levels of both Notch activation and Serrate1 expression, and to propagate this condition from cell to cell, thereby maintaining and extending the prosensory domains.

Conclusion

We have argued that Notch signalling works in different ways at different stages in the development of sensory patches in the ear (Daudet and Lewis, 2005). Our new data show that, in this tissue, Serrate1 expression is switched on initially by factors other than Notch activity, but then requires Notch-dependent lateral induction for its maintenance. Delta1, by contrast, is regulated by lateral inhibition. Serrate1-Notch signalling maintains and extends prosensory patches, whereas Delta1-Notch signalling restricts the proportion of cells within them that are permitted to differentiate as neuroblasts or hair cells. We have established a chain of cause and effect that leads from Serrate1 induction via Notch activation to expression of *Sox2* and *Bmp4*, and thence ultimately to the differentiation of a normal sensory patch. The underlying molecular mechanisms, including those by which Notch activity governs the expression of Serrate1 and Delta1 in opposite ways, are important questions for the future.

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