

Jagged 1 regulates the restriction of Sox2 expression in the developing chicken inner ear: a mechanism for sensory organ specification

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SUMMARY

Hair cells of the inner ear sensory organs originate from progenitor cells located at specific domains of the otic vesicle: the prosensory patches. Notch signalling is necessary for sensory development and loss of function of the Notch ligand jagged 1 (*Jag1*, also known as serrate 1) results in impaired sensory organs. However, the underlying mechanism of Notch function is unknown. Our results show that in the chicken otic vesicle, the *Sox2* expression domain initially contains the nascent patches of *Jag1* expression but, later on, *Sox2* is only maintained in the *Jag1*-positive domains. Ectopic human *JAG1* (*hJag1*) is able to induce *Sox2* expression and enlarged sensory organs. The competence to respond to *hJag1*, however, is confined to the regions that expressed *Sox2* early in development, suggesting that *hJag1* maintains *Sox2* expression rather than inducing it de novo. The effect is non-cell-autonomous and requires Notch signalling. *hJag1* activates Notch, induces *Hes/Hey* genes and endogenous *Jag1* in a non-cell-autonomous manner, which is consistent with lateral induction. The effects of *hJag1* are mimicked by *Jag2* but not by *Dl1*. *Sox2* is sufficient to activate the *Atoh1* enhancer and to ectopically induce sensory cell fate outside neurosensory-competent domains. We suggest that the prosensory function of *Jag1* resides in its ability to generate discrete domains of Notch activity that maintain *Sox2* expression within restricted areas of an extended neurosensory-competent domain. This provides a mechanism to couple patterning and cell fate specification during the development of sensory organs.

KEY WORDS: Sensory development, Sensory progenitor, Hair cell, Notch signalling, SoxB1, Chicken, Serrate 1

INTRODUCTION

Sensory organs of the inner ear originate from the otic placode and develop from a neurosensory-competent domain, with a stereotyped temporal and spatial pattern (Alsina et al., 2009; Bell et al., 2008; Fritzsche et al., 2006; Raft et al., 2007; Satoh and Fekete, 2005). Sensory patches emerge at specific locations and they can be identified by the expression of prosensory genes that foreshadows and accompanies sensory development (Cole et al., 2000; Neves et al., 2007; Oh et al., 1996; Wu and Oh, 1996). Yet, the molecular mechanisms that couple patterning and cell fate in the sensory patches are unknown.

Notch signalling is important for sensory development, but its mechanism of action is far from simple. Notch activity is required for the specification of sensory organs and for the determination of hair cells (Brooker et al., 2006; Daudet et al., 2007). The latter function results from the well-known mechanism of lateral inhibition, whereby Notch ligands are expressed in hair cells and signal to neighbouring cells to prevent their differentiation (Brooker et al., 2006; Daudet and Lewis, 2005; Haddon et al., 1999; Kiernan et al., 2005a; Lanford et al., 1999). However, the role played by Notch in the specification of sensory fate is not well understood. Early blockade of Notch signalling results in the loss of sensory

domains, and the overexpression of the intracellular domain of Notch (NICD) in the otic vesicle induces ectopic sensory patches. This has suggested that the specification of the sensory patches requires Notch signalling operating through lateral induction (Daudet et al., 2007; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010; Pan et al., 2010). The Notch ligand jagged 1 [*Jag1*, also known as serrate 1 (*Ser1*)] is expressed in the prosensory patches (Adam et al., 1998; Cole et al., 2000; Morrison et al., 1999) and is a good candidate to drive the early prosensory function of Notch. In mice, the loss of function of *Jag1* results in the disruption of the sensory epithelium and the loss of hair cells (Brooker et al., 2006; Kiernan et al., 2001; Kiernan et al., 2006; Pan et al., 2010; Tsai et al., 2001). Critical steps of this model are still missing, including whether *Jag1* is sufficient for prosensory specification and what links Notch activity to sensory fate.

Sox2 is a high mobility group (HMG) box domain transcription factor that belongs to the B1 subfamily of Sox proteins (Uchikawa et al., 1999). In the developing inner ear, *Sox2* is expressed in neurogenic and sensory progenitors, being downregulated in differentiated neurons and hair cells (Neves et al., 2007). *Sox2* is necessary for sensory fate specification in the inner ear and *Sox2* mutant mice show impaired sensory development with a reduced number of hair cells (Kiernan et al., 2005b). *Sox2*-deficient and *Jag1*-deficient mice show similar phenotypes, and *Sox2* is reduced in *Jag1* mutants or after pharmacological blockade of Notch. This suggests a common and hierarchical role of these genes in the specification of sensory fate (Dabdoub et al., 2008; Daudet et al., 2007; Kiernan et al., 2006). However, it is not known whether *Jag1* is able to induce *Sox2* expression and, if so, what the consequences are for cell fate.

This work addressed the role played by *Jag1* in patterning and cell fate specification of the sensory organs and how this is related to the function of *Sox2*. The expression patterns of these two genes

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and the effects of their gain of function were examined. The results show that the prosensory function of *Jag1* relies on its ability to maintain *Sox2* expression within restricted domains of the otic epithelium. This occurs through a mechanism of Notch-mediated lateral induction and allows sensory patches to retain *Sox2* function, which provides the competence to develop as sensory cells. By this mechanism, patterning and cell fate determination are coupled so as to generate the sensory organs at the correct time, size and location.

MATERIALS AND METHODS

Chicken (*Gallus gallus*) embryos

Fertilised hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for the designated times and embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). Embryos were dissected in cold phosphate-buffered saline (PBS, pH 7) and fixed overnight in 4% paraformaldehyde in PBS at 4°C.

Immunohistochemistry (IHC) in sections

Embryos were sectioned and processed according to Neves et al. (Neves et al., 2007). Blocking solution was 10% horse serum (Gibco) in PBST (PBS containing 0.1% Tween 20). Primary antibodies were as follows: goat polyclonal anti-*Sox2* (Santa Cruz, Y-17; 1:400); rabbit polyclonal anti-*Jag1* (Santa Cruz, H-114; 1:50); mouse monoclonal anti-Tuj1 (Babco, 1:400); mouse monoclonal anti-Islet1 [Developmental Studies Hybridoma Bank (DSHB), 39.4D5; 1:400]; rabbit polyclonal anti-MyoVIIa (Proteus BioSciences; 1:400); mouse monoclonal anti-MyoVIIa (DSHB, 138-1; 1:300); rabbit polyclonal anti-GFP (Clontech; 1:400); and mouse monoclonal anti-GFP (Invitrogen; 1:100). Secondary antibodies were Alexa Fluor 488-, 555- or 594-conjugated anti-goat, anti-mouse or anti-rabbit (Molecular Probes, Invitrogen; 1:400). Sections were counterstained with DAPI (100 ng/ml, Molecular Probes) and mounted in Mowiol media (Calbiochem). Images were obtained by conventional fluorescence microscopy (Leica DMRB fluorescence microscope fitted with a Leica DC300F CCD camera) or confocal microscopy (Leica DM IRBE confocal microscope with a Leica IM50 v4.0 camera). Three-dimensional reconstructions and volume calculations were made from serial 20-µm sections using BioVis3D software.

In situ hybridisation (ISH) in whole-mount embryos

Embryos were processed according to Wilkinson and Nieto (Wilkinson and Nieto, 1993) using the automated system from InsituPro VS (Intavis, Bioanalytical Systems). Probes were: *Sox2*, *Jag1*, *Notch1*, *Hairy1*, *Hes5.1* and *Hey1* (Fior and Henrique, 2008; Henrique et al., 1995; Palmeirim et al., 1997; Rex et al., 1997).

Quantitative real-time PCR (qRT-PCR)

Otic vesicles were dissected out and total RNA isolated using the RNeasy Mini Kit (Qiagen). For each retrotranscription, 15 ng of purified mRNA was used to synthesise cDNA with Superscript III DNA polymerase (Invitrogen) and random primers (Invitrogen). Real-time PCR was carried out using SYBR Green Master Mix (Roche), 1 µl retrotranscribed cDNA and specific primer sets for each gene (Invitrogen) (see Table S1 in the supplementary material) in a LightCycler 480 (Roche). *Gapdh* was used as calibrator gene. For each relative quantification, three lots of five to ten otic vesicles generated in three independent experiments were used. Each of these samples was retrotranscribed three times and each retrotranscription was used as template for each pair of primers in a triplicate PCR reaction. Expression levels of each gene were normalised to *Gapdh* and then referred to the levels in control otic vesicles, which were arbitrarily set to 1.

Electroporation and vectors

HH12–14 embryos were electroporated into the right otic cup. The platinum cathode electrode was placed next to the right otic cup and the anode electrode placed parallel to it, on the other side of the embryo. The desired vector (1 µg/µl) mixed with pCIG vector (0.75 µg/µl) and Fast Green (0.4 µg/µl) was injected onto the otic vesicle by gentle air pressure through a fine micropipette. Square pulses (eight pulses of 10 V, 50 Hz,

250 mseconds) were generated by a CUY-21 square wave electroporator (BEX, Tokiwasaensu, Japan). Vectors used for electroporation were: pIRES2-EGFP-c*Sox2*, pCMV-c*Sox2*, pCMV-c*Sox2*ΔHMG, pCIG-h*Jag1*, pDsRed and 12xCSL-DsRed, pCIG-h*Jag2* and pCMV-c*Delta1*.

In vitro culture of otic vesicles

Electroporated and control otic vesicles were dissected from electroporated embryos, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37°C in a water-saturated atmosphere containing 5% CO₂ as described (Pujades et al., 2006). Additions were 1% foetal bovine serum (FBS) (Bio Whittaker Europe) and DAPT (Calbiochem) at 20 µM.

Atoh1 enhancer activity assays

293T cells (~40,000 cells per well in 12-well plates) were transiently transfected with Atoh1-BGZA or Atoh1-BG-EGFP (1.5 µg) alone or together with pCMV-c*Sox2* (0.1 µg) using a standard calcium phosphate precipitation method. Following transfection, cells were cultured in DMEM with 10% FBS for 36 hours. Cells were then harvested and β-galactosidase (β-gal) activity was determined. For each experimental group, three independent transfections were analysed in triplicate activity assays.

Statistics

Results are shown as averages ± s.e.m. of three independent experiments and Student's *t*-test was applied to assess statistical significance.

RESULTS

Sox2 expression becomes restricted to Jag1 domains during prosensory patch formation

Sox2 and *Jag1* are expressed in the prosensory patches of the developing inner ear of mouse and chick embryos (Adam et al., 1998; Cole et al., 2000; Hume et al., 2007; Mak et al., 2009; Morrison et al., 1999; Neves et al., 2007). The experiments that follow show that, before prosensory specification, the *Sox2* expression domain is broader than that of *Jag1*, but as prosensory patches develop *Sox2* persists only within the *Jag1*-positive domains. At embryonic day (E) 3, *Sox2* was expressed throughout the otic vesicle, but at lower levels laterally (Fig. 1A–C). The most dorsal aspect of the otic vesicle was devoid of *Sox2* expression (not shown, see diagram to the right in Fig. 1). *Jag1* was expressed within the *Sox2* domain but restricted to the anterior and posterior poles of the otic vesicle, connected through a domain of weaker expression that extended medially and ventrally. By E4, both *Sox2* and *Jag1* were detected in all the prospective sensory domains of the otocyst (Adam et al., 1998; Cole et al., 2000; Neves et al., 2007). *Sox2* and *Jag1* expression was restricted to the cristae (Fig. 1D), but in the prospective maculae and basilar papilla *Sox2* expression still remained broader than that of *Jag1* (Fig. 1E,F and diagram). Later in development (E7), the expression of *Sox2* and *Jag1* became confined to all sensory organs (Fig. 1G–I), *Sox2* expression always extending a few cell diameters beyond the *Jag1*-positive domain. In summary, *Jag1* was initially expressed within a larger *Sox2* domain, but as development proceeded *Sox2* expression was lost outside the *Jag1*-positive domains. This process followed a dorsal-to-ventral temporal sequence, mirroring the order of differentiation of the sensory organs (Bell et al., 2008; Wu and Oh, 1996). Taken together, this suggests that one function of *Jag1* might be to maintain *Sox2* expression in the prosensory patches.

hJag1 induces Sox2 expression outside the prosensory domains

In order to analyse the prosensory function of *Jag1*, we used a gain-of-function approach by means of the electroporation of full-length human jagged 1 (*hJag1*). The plasmid was able to drive the

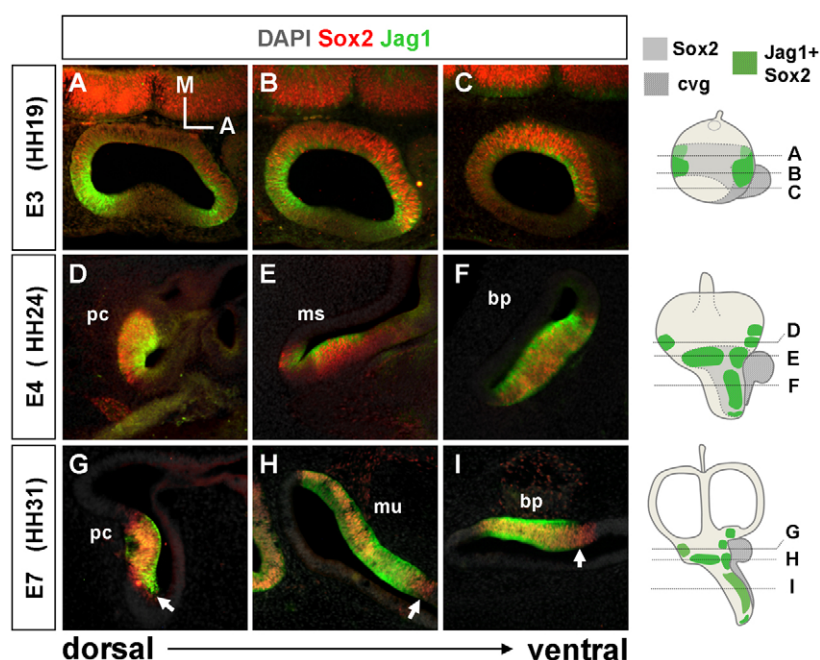


Fig. 1. Sox2 and Jag1 co-expression during chicken otic neurosensory development. Coronal sections of (A–C) E3, (D–F) E4 and (G–I) E7 otic vesicles immunostained for Sox2 (red) and Jag1 (green). Sections are shown in dorsal to ventral order from left to right. Arrows (G–I) point to the edges of the patches where Sox2 expression extends a few cell diameters outside the Jag1 domain. Diagrams on the right depict otocysts at the indicated stages and Sox2 (grey) and Jag1 plus Sox2 (hatched green) expression domains. Grey dashed lines indicate the cochlear vestibular ganglion. The level of sections shown in A–I is illustrated. bp, basilar papilla; ms, macula sacculi; mu, macula utriculi; pc, posterior crista; A, anterior; M, medial.

expression of hJag1 protein transiently, with the maximal value reached 20 hours after transfection (see Fig. S1A in the supplementary material). Embryos were electroporated before prosensory specification at E2 (HH12–14), were allowed to develop in ovo for different periods and then selected by GFP expression. GFP protein stability allowed us to use it as a tracer of electroporated cells and their progeny even after transgene expression had been shut down. The results are shown in Fig. 2A, where the diagrams on the left depict the location of sensory patches and the level of the sections.

The ectopic expression of *hJag1* resulted in the expression of Sox2 outside the prosensory patches (Fig. 2). This effect was not always visible 1 day after *hJag1* transfection (Fig. 2Aa–b'; $n=8$ embryos, bar chart), but it was clearly present after 2 days (Fig. 2Ac–d' and bar chart). During normal development, the cristae are singled out at the anterior and posterior poles of the otocyst (see the two Sox2-positive patches in Fig. 2Ac and diagram on the left). Electroporated otocysts (Fig. 2Ad,d'), however, exhibited Sox2 expression in both medial (arrows) and lateral (arrowheads) aspects of the otic wall, which corresponded to the domains of expression of the transgene ($n=11/12$ ectopic Sox2 domains induced by *hJag1*). The ectopic expression of chicken *Sox2* (*cSox2*) did not result in changes in the expression of Jag1 (see Fig. S1B in the supplementary material).

Confocal microscopy of the extended Sox2 domains revealed that the effect of *hJag1* was both cell-autonomous and non-cell-autonomous (Fig. 2Ae). Almost all of the electroporated cells expressed Sox2 (yellow, $97\pm1\%$), but not all Sox2-positive cells were double labelled (red, $77.3\pm12.5\%$; $n=3$). No cells co-expressed GFP and Sox2 after pCIG (control) electroporation (Fig. 2Af,f'; $n=3$). In parallel experiments, otic vesicles were analysed by qRT-PCR, which confirmed that *Sox2* mRNA levels increased 2 days after *hJag1* electroporation (Fig. 2A, bar chart).

The ability of *hJag1* to induce Sox2 was spatially restricted in the otocyst. Sox2 was only induced within the domains that had expressed Sox2 during earlier stages of development. The forced expression of *hJag1* in the dorsal domain of the otocyst close to the origin of the endolymphatic sac was ineffective in inducing Sox2

expression (Fig. 2Ba–a"). Fate maps show that this domain derives from the dorsal and posterior otic cup (Abello et al., 2007; Bell et al., 2008; Brigande et al., 2000), which is devoid of neurosensory competence and does not express Sox2 (Neves et al., 2007) (Fig. 1). By contrast, as shown above (Fig. 2A), the forced expression of *hJag1* resulted in the expression of Sox2 in electroporated domains at the level of, and ventral to, the cristae, which derive from the Sox2-positive, neurosensory-competent domain. This is summarised in Fig. 2B (diagram to right), which illustrates the fraction of ectopic *hJag1* electroporations that were positive for Sox2 expression in the dorsal (A) or ventral (B) domains.

Between E3 and E4, the cristae become specified and the surrounding tissue downregulates Sox2 expression (Fig. 1A,D). We tested whether *hJag1* was able to induce Sox2 in the vicinity of the sensory patches once Sox2 expression had been restricted, i.e. from those domains that had lost Sox2 expression. We targeted *hJag1* electroporations to the presumptive cristae and surrounding domains at E3.5 and assayed for Sox2 (Fig. 2Bb–b"). In this case, *hJag1* did not induce Sox2 expression in the epithelium neighbouring the cristae ($n=0/6$ electroporated domains).

Taken together, these results suggest that *hJag1* is able to maintain the expression of Sox2 in those regions that initially expressed Sox2, but not to induce its expression de novo.

The effects of hJag1 on Sox2 expression require Notch activity

Jag1 is a ligand of the Notch receptor and Notch signalling is active in the prosensory patches (Murata et al., 2006). We tested whether *hJag1* results in Notch activation in the otic vesicle and whether Notch activity is required for the effects of *hJag1* on Sox2 expression.

hJag1 activated Notch in the otic epithelium, as shown by a fluorescent reporter assay of Notch activity in situ (Fig. 3A). Otic cups were co-electroporated with 12xCSL-DsRed (Hansson et al., 2006) and either NICD, *hJag1*-pCIG, or pCIG. Specimens were then sectioned and analysed for green and red fluorescence. Constitutively active Notch (NICD) activated DsRed expression from the reporter in all electroporated cells (Fig. 3Aa–a"), as did

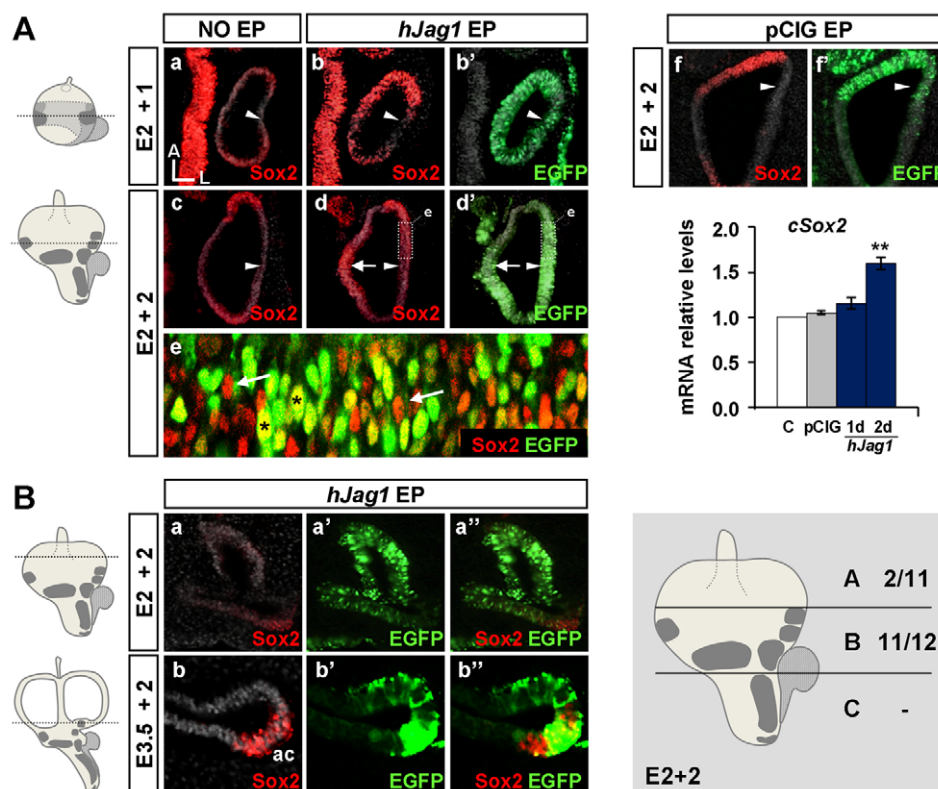


Fig. 2. *hJag1* induces Sox2 expression outside the prosensory patches. (A) (a-d') Coronal sections of otic vesicles from chicken embryos that were electroporated in E2 with *hJag1* and allowed to develop for 1 or 2 days as indicated. Sections were stained for Sox2 protein (red) and GFP (green). Electroporated domains are indicated by arrowheads (lateral) and arrows (medial). Diagrams on the left depict the location of sensory patches and the level of the sections. (e) Confocal image of an *hJag1* ectopic expression domain stained for Sox2 and GFP. Yellow indicates double-stained cells (asterisks) and red indicates Sox2-positive GFP-negative cells (arrows). (f, f') Control electroporation with pCIG. The bar chart shows the relative mRNA levels of Sox2 in otic vesicles transfected with control plasmids (pCIG, 1.050 ± 0.021) or with *hJag1* and allowed to develop for 1 day (1d, 1.2 ± 0.05) or 2 days (2d, 1.6 ± 0.07). C, untransfected otic vesicles. Differences at 2 days were significant (**, $P < 0.005$). Data indicate mean \pm s.e.m. A, anterior; L, lateral. (B) Electroporation of *hJag1* in (a-a') a dorsal domain at E2 or (b-b') in the vicinity of cristae of an E3.5 otic vesicle and allowed to develop for 2 days. The diagram on the right shows the proportion of dorsal (A) or ventral (B) *hJag1* electroporations that were positive for Sox2 expression after transfection in E2 followed by development for 2 days in ovo. C, ventral electroporations were not analysed.

hJag1 (Fig. 3Ab-b''), whereas the pCIG control did not (Fig. 3Ac', c''). Note that the endogenous levels of NICD are insufficient to activate the 12xCSL-DsRed reporter (Hansson et al., 2006).

Hes/Hey genes are direct transcriptional targets of the Notch signalling pathway and are expressed in the prosensory patches of the mouse and chick otocyst (Hayashi et al., 2008; Murata et al., 2009). We tested whether they are downstream effectors of the Jag1-mediated activation of Notch. The relative mRNA levels of four Hes/Hey genes were analysed by qRT-PCR 1 and 2 days after *hJag1* transfection (Fig. 3B). *Hairy1*, *Hey1* and *Hey2* mRNA levels were significantly increased after 1 day, *Hes5* remaining unaltered. This effect was transient, paralleling the profile of transgene expression. These results were confirmed by ISH for *Hairy1* and *Hey1* (data not shown).

To test the requirement of Notch signalling for the regulation of Sox2 by *hJag1*, we combined the in ovo electroporation of *hJag1* with the in vitro culture of explanted otic vesicles in the presence of DAPT (Fig. 3C). DAPT blocks Notch activation by inhibiting the γ -secretase activity required for the S3/S4 cleavage of NICD (Dovey et al., 2001; Geling et al., 2002). Otic vesicles were electroporated with *hJag1* and allowed to develop for 1 day. Otic vesicles were then isolated and cultured with either DAPT or

DMSO (carrier control) for an additional day, after which they were analysed by qRT-PCR for Sox2 expression (Fig. 3C, bar chart). The results show that Sox2 induction by *hJag1* was blocked by DAPT, indicating that it requires active Notch signalling.

In summary, *hJag1* activates Notch in the otic epithelium and this activation is required for *hJag1*-dependent Sox2 expression.

Jag1 operates through lateral induction

The results above show that the effects of *hJag1* are mediated by Notch signalling and that the transfection of *hJag1* results in coherent domains of Sox2 expression. The formation of such cooperative cell clusters has been associated with the mechanism of lateral induction mediated by Notch (Bray, 1998; de Celis and Bray, 1997; Lewis, 1998). By definition, lateral induction refers to the positive-feedback mechanism in which Notch activation in one cell induces the expression of the Notch-activating ligand in that cell (Bray, 1998).

We tested the ability of *hJag1* to induce the expression of endogenous *Jag1*. Otic vesicles were electroporated with *hJag1* and analysed for the expression of *Jag1* with chick-specific probes and primers by ISH and qRT-PCR. Transcript levels of endogenous *Jag1* significantly increased with respect to the control 1 day after

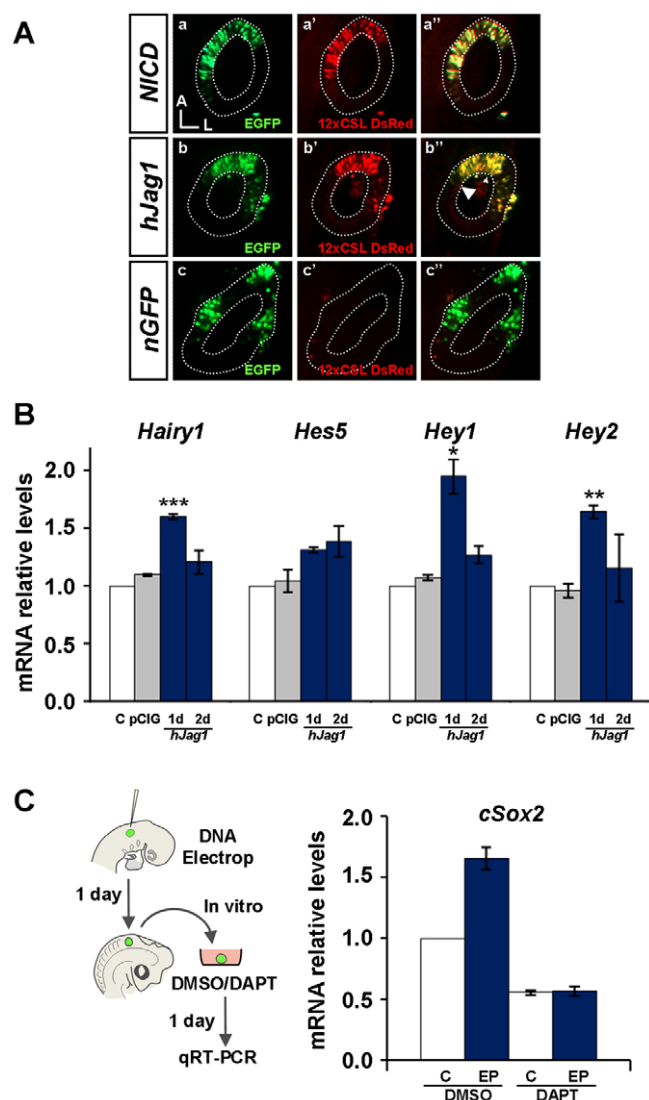


Fig. 3. *hJag1* activation of Notch is required for *Sox2* induction. (Aa-c") Coronal sections of chicken otic vesicles co-electroporated with 12xCSL-DsRed and either NICD, *hJag1* or pCIG, analysed for GFP and DsRed expression. A, anterior; L, lateral. (B) The relative mRNA levels of *Hairy1*, *Hes5*, *Hey1* and *Hey2* in otic vesicles transfected with control plasmids (pCIG; *Hairy1*, 1.1 ± 0.01 ; *Hes5*, 1.04 ± 0.10 ; *Hey1*, 1.07 ± 0.03 ; *Hey2*, 0.10 ± 0.06) or with *hJag1* for 1 day (1d; *Hairy1*, 1.60 ± 0.02 ; *Hes5*, 1.31 ± 0.02 ; *Hey1*, 1.9 ± 0.15 ; *Hey2*, 1.64 ± 0.06) or *hJag1* for 2 days (2d; *Hairy1*, 1.21 ± 0.01 ; *Hes5*, 1.38 ± 0.13 ; *Hey1*, 1.27 ± 0.07 ; *Hey2*, 1.16 ± 0.29). C, untransfected otic vesicles. *, $P < 0.05$ for *Hey1*; **, $P < 0.005$ for *Hey2*; ***, $P < 0.001$ for *Hairy1*. Differences were not significant after 2 days and not for *Hes5*. (C) (Left) Experimental design. Otic vesicles were electroporated in E2, isolated after 1 day, and cultured in vitro. (Right) The relative mRNA levels of *Sox2* in otic vesicles transfected with *hJag1* (EP) and cultured in DMSO carrier control (1.65 ± 0.09) or DAPT (0.57 ± 0.04). C, untransfected otic vesicles in control conditions (left) or with DAPT (right, 0.55 ± 0.01). Data indicate mean \pm s.e.m.

hJag1 transfection (Fig. 4A). This effect was transient and faded after 2 days, paralleling the temporal profile of transgene expression (see Fig. S1A in the supplementary material). *hJag1* did not induce *Notch1* or delta 1 (*Dl1*) transcription (Fig. 4A).

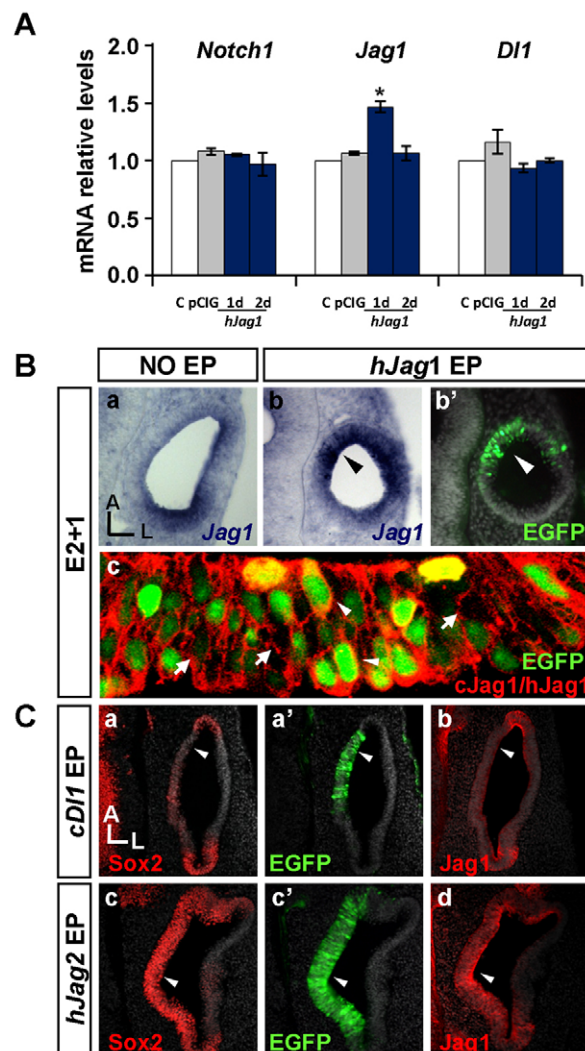


Fig. 4. *hJag1* regulates endogenous *Jag1*. (A) The relative mRNA levels of *Notch1*, *Jag1* and *Dl1* in chicken otic vesicles transfected with control plasmids (pCIG; *Notch1*, 1.08 ± 0.03 ; *Jag1*, 1.07 ± 0.02 ; *Dl1*, 1.16 ± 0.11) or with *hJag1* for 1 day (1d; *Notch1*, 1.05 ± 0.01 ; *Jag1*, 1.48 ± 0.05 ; *Dl1*, 0.94 ± 0.03) or 2 days (2d; *Notch1*, 0.97 ± 0.10 ; *Jag1*, 1.07 ± 0.06 ; *Dl1*, 1.00 ± 0.02). C, untransfected otic vesicles. *Jag1* mRNA levels significantly increased 1 day after *hJag1* electroporation (*, $P < 0.05$), but differences were not significant at 2 days and not for *Notch1* or *Dl1*. Data indicate mean \pm s.e.m. (B) (a-b") Coronal sections of otic vesicles that were electroporated with *hJag1* in E2 and allowed to develop for 1 day. Sections were processed for ISH with *Jag1* probe and immunolabelled for GFP (green). (c) Confocal image showing a detail of an ectopic *hJag1* domain immunostained for *Jag1* (red) and GFP (green). Yellow indicates double-stained cells (arrowheads) and red indicates *Jag1*-positive GFP-negative cells (arrows). (C) (a-b") Coronal sections of otic vesicles that were electroporated with *cDl1*-pCIG in E2 and allowed to develop for 2 days. Sections were immunostained for *Sox2* (red, a) and GFP (green, a'). The alternate section was processed for *Jag1* (red, b). Arrowheads indicate electroporated domains. (c-d) A similar experiment after *hJag2* transfection. A, anterior; L, lateral.

The induction of endogenous *Jag1* by *hJag1* was further confirmed by ISH (Fig. 4Ba-b'; $n=3/3$) and by double immunostaining for GFP and *Jag1* (Fig. 4Bc). Immunostaining revealed that not all *Jag1*-positive cells were GFP positive. This

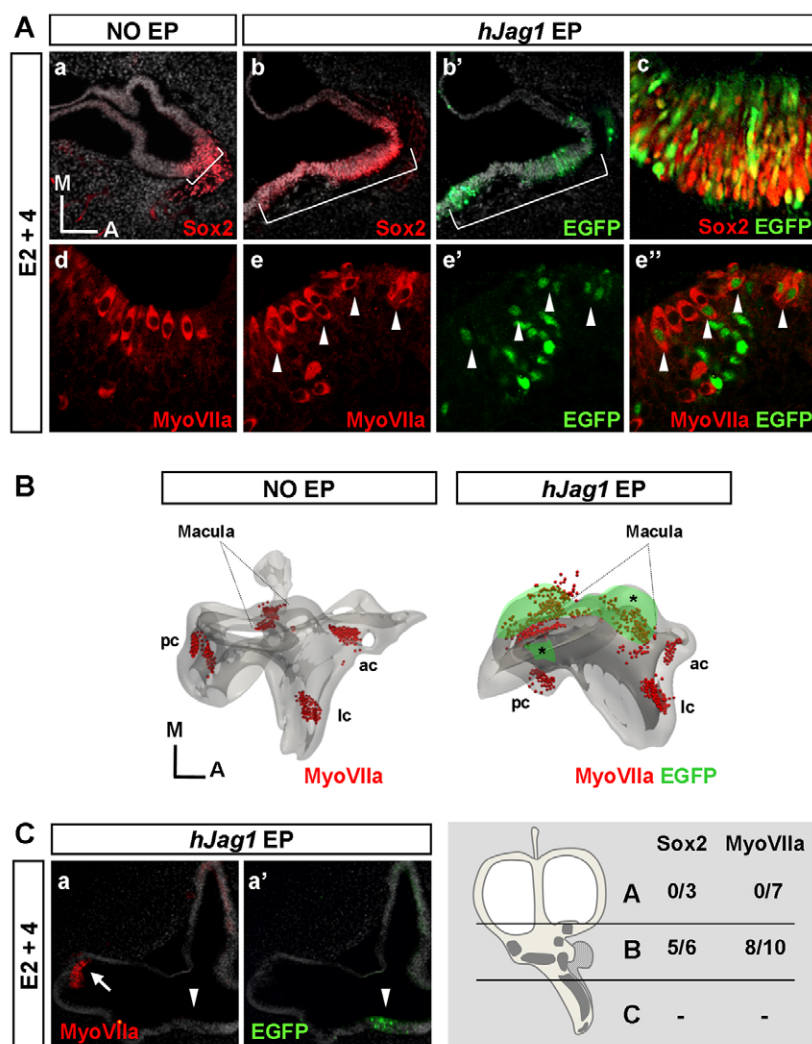


Fig. 5. *hJag1*-induced Sox2 domains result in enlarged sensory organs. (A) (a–b') Coronal sections of otocysts from chicken embryos that were electroporated in E2 with *hJag1* and allowed to develop for 4 days. Sections were stained for Sox2 protein (red) and GFP (green). Brackets indicate Sox2 domains in the anterior crista. (c) Detail of one enlarged Sox2-positive domain. (d–e'') Confocal analysis of macular otic epithelium in untransfected (d) and *hJag1*-transfected (e–e'') otocysts immunostained for MyoVIIa (red) and GFP (green). Arrowheads indicate double-labelled hair cells. (B) Three-dimensional reconstruction of otocysts transfected with *hJag1*. Embryos were electroporated in E2 and allowed to develop for 4 days in ovo. Reconstructions were from serial coronal sections double stained with GFP (green shadowed area) and MyoVIIa (red dots). Asterisks indicate GFP-positive MyoVIIa-negative cells outside the expanded sensory domain. (Ca, a') Coronal sections of otocysts that were electroporated in E2 with *hJag1* in the dorsal aspect of the otocyst and allowed to develop for 4 days. Sections were immunostained for MyoVIIa (red) and GFP (green). The diagram on the right shows the proportion of dorsal (A) or ventral (B) *hJag1* electroporations that generated extended patches (either Sox2-positive or MyoVIIa-positive); C, ventral electroporations were not analysed. ac, anterior crista; lc, lateral crista; pc, posterior crista; A, anterior; M, medial.

indicates that a fraction of the anti-Jag1 immunoreactivity was not driven by the *hJag1* transgene, but corresponded to the endogenous Jag1 expression induced in neighbouring cells.

A crucial question is that of the specificity of the different Notch ligands in the processes described above. Are other Notch ligands equally efficient in lateral induction and in promoting Sox2 expression? We electroporated chick *Dll1* (*cd11*) and *hJag2* under the same conditions as described above and analysed the expression of Jag1 and Sox2 (Fig. 4C). *cd11* was unable to mimic the effects of *hJag1* (Fig. 4Ca–b; $n=0/3$), whereas the forced expression of *hJag2* induced both Sox2 and Jag1 (Fig. 4Cc–d; $n=3/3$). This suggests that both lateral induction and Sox2 regulation are specific to the cellular response to Jag ligands.

In summary, the forced expression of *hJag1* was able to activate Notch, to induce Notch targets and to induce *Jag1* expression in a non-cell-autonomous manner, without affecting *Notch1* expression. This strongly supports the notion that *Jag1* operates by a mechanism of lateral induction that relies on a positive-feedback loop provided by ligand induction and receptor activation.

***hJag1*-induced Sox2 patches develop as sensory organs**

Since *hJag1* is able to extend Sox2 expression outside the prosensory patches, we examined whether this resulted in larger sensory organs. *hJag1*-transfected otic vesicles were analysed after

4 days of development in ovo, which is equivalent to E6, when nascent hair cells express differentiation markers such as MyoVIIa (Sahly et al., 1997) and supporting cells express Sox2 (Neves et al., 2007). Serial sections were used to reconstruct three-dimensional models of the otic vesicles to analyse the size and position of the sensory organs.

The expression of Sox2 induced by *hJag1* was maintained after 4 days (Fig. 5Aa–b'). The high-magnification image in Fig. 5Ac shows Sox2-positive cells located at the basal layer of the sensory patch corresponding to the supporting cells (Neves et al., 2007). Transfected cells also differentiated as hair cells, as revealed by MyoVIIa staining (Fig. 5Ad–e'', arrowheads).

Fig. 5B shows a dorsal view of a three-dimensional reconstruction of an otocyst that was electroporated in the region of the maculae (right), as compared with the corresponding contralateral control otocyst (left). The macular domain was expanded in the transfected otic vesicle and corresponded well to the GFP-positive region. The ability of *hJag1* to induce sensory cells was restricted to the regions of the otocyst located at, or ventral to, the level of the cristae and GFP-positive cells dorsal to those domains did not express MyoVIIa or Sox2 (Fig. 5B, asterisks). This is also illustrated in Fig. 5Ca, a', in which an ectopic *hJag1*-positive domain in the dorsal otocyst is not paralleled by MyoVIIa expression (arrowheads; arrow points to a normotopic crista). The results of several experiments are summarised in the

diagram in Fig. 5C. As above, *hJag1* overexpression was able to induce Sox2-positive or MyoVIIa-positive domains in the ventral, but not in the dorsal, aspect of the otocyst.

The volume of the otic vesicle increased by 20% in *hJag1*-electropotated otocysts, but the size of the cochlear vestibular ganglion (CVG) was unaffected, as was *Ngn1* expression (see Fig. S2 in the supplementary material).

Sox2 is sufficient to induce neurosensory fate in the otic epithelium

The data described above suggest that the prosensory function of Jag1 relies on Sox2 to specify the sensory fate. Therefore, the question arises as to whether Sox2 is sufficient to specify sensory fate in the absence of Notch activation. We electroporated E2 otic vesicles with *cSox2* and analysed hair cell differentiation. Electroporations outside sensory domains were selected by comparison with the untransfected contralateral otic vesicle (Fig. 6A–B', compare domains indicated with arrows). The ectopic expression of *Sox2* induced Islet1 and resulted in ectopic sensory and neurogenic patches ($n=14/16$ ectopic domains).

Fig. 6C,C' show a detail of one cluster of electroporated cells that delaminated from the otocyst and co-expressed GFP and Islet1. Ectopically delaminated GFP-positive cells also expressed Tuj1 (also known as Tubb3) (not shown). The volume of the CVG significantly increased in Sox2-transfected otocysts, as did the transcript levels of *Ngn1* (see Fig. S2 in the supplementary material). This suggests that Sox2 is sufficient to specify sensory fate in the otic epithelium. This was not modified by *hJag1* (see Fig. S2 in the supplementary material).

Domains transfected with *cSox2* also expressed MyoVIIa, indicating that they differentiated as hair cells ($n=14/16$ ectopic domains electroporated with Sox2; Fig. 6D–E'). In contrast to *hJag1*, after the forced expression of Sox2 all regions of the otocyst were able to generate MyoVIIa-positive ectopic patches (Fig. 6F, diagram). Even the dorsal-most electroporations, targeted at the endolymphatic duct, resulted in ectopic MyoVIIa-positive cells (Fig. 6E,E'). This suggests that Sox2 expression provides the competence to develop into sensory cells and that this is extended to the whole otocyst.

Atoh1 is a basic helix-loop-helix (bHLH) transcription factor that is expressed in sensory progenitors and behaves as a master gene for hair cell determination (Bermingham et al., 1999; Zheng and Gao, 2000). In order to further confirm the ability of Sox2 to induce sensory fate, we analysed its ability to activate *Atoh1* expression. We made use of a reporter construct that contains either the *lacZ* or *EGFP* gene under the control of the murine *Atoh1* regulatory region (Ebert et al., 2003; Helms et al., 2000; Timmer et al., 2001). This enhancer region resides ~3.4 kb 3' of the coding sequence and is sufficient to recapitulate the endogenous *Atoh1* expression pattern in several species. We transfected human 293T cells with these reporter constructs alone or together with *Sox2*, and analysed β -gal activity (Fig. 6G, bar chart) or EGFP expression (Fig. 6G, photomicrographs) 2 days after transfection. The results show that Sox2 induced a large increase in the activity of the *Atoh1* reporter, strongly suggesting that Sox2 is able to promote *Atoh1* expression.

DISCUSSION

The generation of prosensory patches remains one of the most intriguing questions in inner ear development. Its understanding requires deciphering how sensory potential is acquired and restricted to specific domains of the otic epithelium. The expression of *Jag1*

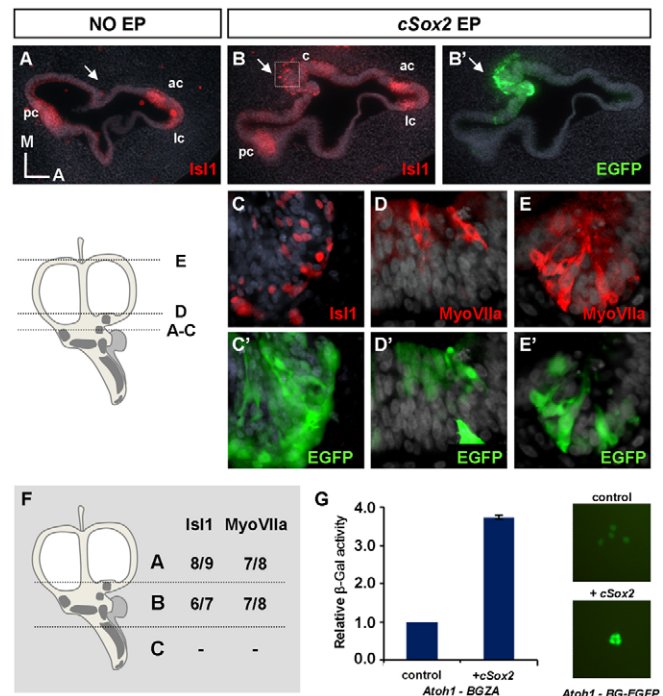


Fig. 6. Sox2 is sufficient to induce *Atoh1* and ectopic hair cells. (A–B') Coronal sections of chicken otocysts electroporated in E2 with *cSox2* and allowed to develop for 3 days. Sections were immunostained for Islet1 (red) and GFP (green). Arrows indicate ectopic domains. (C,C') Detail of an ectopic domain stained for Islet1 (red) and GFP (green). (D,D') Detail of an ectopic domain stained for MyoVIIa (red) and GFP (green). (E,E') Detail of an ectopic domain in the endolymphatic duct, stained for MyoVIIa (red) and GFP (green). The plane of sections in A–E is indicated to the left. (F) The proportion of dorsal (A) or ventral (B) *cSox2* electroporations that generated extended MyoVIIa-positive patches; C, ventral electroporations were not analysed. (G) (Left) Relative β -gal activity in 293T cells co-transfected with *Atoh1*-BGZA reporter and *cSox2* (right bar, 3.74 ± 0.05) with respect to *Atoh1*-BGZA transfection alone (left bar, normalised to 1). Differences are significant ($P < 0.05$). Data indicate mean \pm s.e.m. (Right) Photomicrographs of 293T cells transfected with the *Atoh1*-BG-EGFP reporter alone (top) or co-electroporated with Sox2 (bottom) and analysed for GFP expression by fluorescence microscopy.

and Sox2 foreshadows the emergence of the sensory organs from early developmental stages (Adam et al., 1998; Cole et al., 2000; Neves et al., 2007) and these genes are necessary for correct sensory organ development (Brooker et al., 2006; Kiernan et al., 2005b; Kiernan et al., 2006). The data presented in this work dissect the mechanism that links Jag1, Notch activation and Sox2 during sensory specification. We propose a model in which Jag1 restricts Sox2 expression to prosensory domains through a mechanism that involves Notch activation by lateral induction, while, in turn, Sox2 specifies sensory fate within those domains. This confines sensory competence to the prosensory patches, ensuring the development of sensory organs of the correct size and location.

Patterning and cell fate: sensory patch formation results from the restriction of Sox2 expression regulated by Jag1

One feature of the Jag1 and Sox2 expression patterns is that the Sox2 domain is initially broader than that of Jag1, but, as development proceeds, Sox2 persists only in association with Jag1.

This suggests that the restriction of Sox2 driven by Jag1 underlies the transition from a common and extended neurosensory-competent domain to discrete prosensory patches. Several studies have shown that *Jag1* and Notch signalling are indeed necessary for Sox2 expression in the otic epithelium (Dabdoub et al., 2008; Daudet et al., 2007; Kiernan et al., 2006). Our results indicate that *Jag1* is not sufficient to induce Sox2 expression de novo, but maintains its expression in those regions that already expressed Sox2 early in development.

The model proposed here suggests that all sensory organs develop from a common neurosensory domain, the singling out of sensory organs being an independent process that requires the local expression of *Jag1*. The neurosensory competence is dependent on Sox2 (Kiernan et al., 2005b) (this work) and is acquired early in development. The initiation of Sox2 expression and neurosensory competence in the inner ear do not depend on Notch, but on FGF signalling and Sox3 (Abello et al., 2010). It is only later in development that the initially broad and continuous Sox2 domain resolves into smaller and individual domains that correspond to prosensory patches. We propose that this patterning process depends on the activation of Notch by Jag1 and relies on the ability of Notch signalling to maintain Sox2 expression. This explains why ectopic activation of Notch is unable to generate ectopic hair cells in all domains of the otocyst (Daudet and Lewis, 2005; Hartman et al., 2010; Pan et al., 2010), but only within the neurosensory-competent domains of the otic placode.

Although the maintenance of *Jag1* expression in the sensory patches is Notch dependent, its onset is not (Daudet et al., 2007). Thus, the regulation of initiation of *Jag1* expression is a key event to complete an understanding of the patterning process. *Jag1* is an evolutionarily conserved target of canonical Wnt signalling (Katoh, 2006) and there is evidence that during inner ear development, Jag1 is induced by Wnt (Jayasena et al., 2008). Interestingly, the gain of function of Wnt results in ectopic and fused sensory patches (Sienknecht and Fekete, 2008; Stevens et al., 2003). This suggests that Wnt signalling might regulate the expression of *Jag1* in the prosensory patches.

Jag1 functions through lateral induction

The model discussed above requires Notch to be active in all the cells of the patch where Sox2 expression is to be maintained. This cannot result from lateral inhibition, which creates a salt and pepper pattern. On the contrary, it requires a mechanism in which Notch activation by a ligand results in the induction of that ligand and in consequence in a continuous domain of Notch activity. Such a positive-feedback mechanism is known as lateral induction (Bray, 1998; Lewis, 1998) and has been associated with the formation of morphological boundaries and spatial patterns in development (Baek et al., 2006; Cheng et al., 2004; de Celis and Bray, 1997).

Several studies have suggested that *Jag1* expression in the otic epithelium is maintained by lateral induction (Daudet et al., 2007; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010). However, there is no direct demonstration that the mechanism operates in a Jag1-dependent manner. Our results show that *hJag1* activates Notch signalling and induces endogenous *Jag1* expression in a non-cell-autonomous manner. Furthermore, we show that scattered ectopic transgene expression results in the generation of a coherent domain where all the cells express Jag1. Lateral induction and Sox2 expression are associated with Jagged-like ligand activity but not with Dll. Differential effects of Notch ligands on ear and neural development have been suggested previously, both in chick and mammals (Brooker et al., 2006;

Daudet et al., 2007; Eddison et al., 2000; Ramos et al., 2010). Since active Notch mimics the effects of Jag1 in the expansion of prosensory patches (Daudet and Lewis, 2005; Hartman et al., 2010; Pan et al., 2010), it is likely that the differences between the effects of Jag1 and Dll arise from their ability to interact with the receptor, and Fringe proteins are likely candidates to mediate this selectivity (Fortini, 2009; Zhang et al., 2000).

Sox2 and sensory fate specification

Sox2 is sufficient to specify neurosensory fate (i.e. sensory and neuronal precursors) in the otic epithelium. Other SoxB1 genes are also sufficient to specify neuronal fate in the chick otic epithelium (Abello et al., 2010), and, in the course of our study, Puligilla et al. (Puligilla et al., 2010) showed that Sox2 is able to induce neuronal fate in the developing mammalian cochlea. Our work shows that Sox2 is also able to induce sensory markers and to activate the *Atoh1* enhancer, i.e. to promote early steps in sensory commitment. This ability is independent of Notch activity, but it probably depends on other partners that provide context dependence (Kamachi et al., 2000). However, Sox2 is known to counteract Atoh1 function in the ear (Dabdoub et al., 2008). This seemingly contradictory function of Sox2 is reminiscent of that of SoxB1 genes in the neural tube (Bylund et al., 2003; Pevny and Placzek, 2005). Sox2 expression in otic progenitors would define a population of cells that is committed to the neurosensory fate but prevented from differentiation. This ensures expansion of the neural-competent population and the generation of different cell types.

Neuronal versus sensory specification in the inner ear

In the amniote inner ear, the generation of neurons and of hair cells proceed sequentially, neurons being specified prior to sensory cells (Bell et al., 2008; Raft et al., 2007). Sox2 is expressed in neuronal and sensory progenitors and it is sufficient to specify both cell fates (Neves et al., 2007) (this work). Thus, it is tempting to suggest that Sox2 expression defines this dual competence. It is conceivable that Sox2-positive progenitors would generate neurons as an early fate and sensory cells as a late fate. By facilitating the persistence of Sox2 and neurosensory competence, Jag1-dependent Notch activity might allow the expression of late fates only within restricted domains. Notch activity would also allow the expansion of the progenitor pool, so that all cell types could be formed at the correct times. As a consequence, Sox2 expression associated with Jag1 would predict sensory fate, whereas Jag1-independent Sox2 expression would predict neurogenesis, and this corresponds well with the expression of Sox2, Jag1 and Dll during neurosensory development (Abello et al., 2007; Adam et al., 1998; Neves et al., 2007).

In summary, the present work provides evidence for a link between Jag1 and Sox2 functions during sensory organ development in the chick inner ear. Our model (Fig. 7) proposes that sensory organ generation would result from two processes – patterning and cell fate specification – that are regulated independently. First, a broad Sox2-positive domain would be set by FGF signals and confer neurosensory competence to a subdomain of the otic placode. This region would go through the stage of neurogenesis as an early fate of neurosensory-competent progenitors. Wnt activity would drive Jag1 expression, which, in turn, would maintain local Sox2, whereas Sox2 is switched off in neighbouring regions. As a result, progenitor cells residing within the Jag1-Notch patches would allow the expression of late fates and become hair and supporting cells. The model provides a

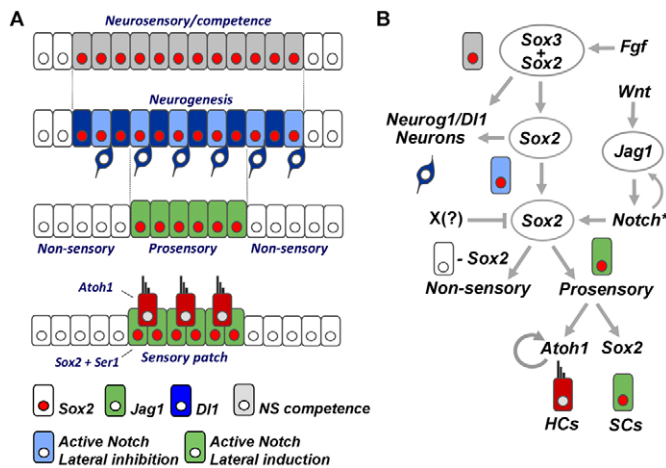


Fig. 7. Jag1 and Sox2 interaction couples patterning and cell fate during sensory organ generation. (A) Summary of the development of neurosensory elements in the chicken inner ear. Neurosensory competence is established early in the otic placode by SoxB1 genes. Neurogenesis starts as an early fate with the expression of neurogenic genes and Df1 in nascent neurons, with the corresponding activation of Notch in epithelial progenitors via lateral inhibition. An independent signal now sets the expression of Jag1 within certain domains of the neural-competent epithelium (green hatched cells). The expression of Jag1 maintains Notch and Sox2 within those domains while it is switched off in the neighbouring regions. Neurosensory progenitors now develop toward their late (sensory) fate. Neural competence remains, providing potential for regeneration in supporting cells. (B) Signals involved in the generation of prosensory domains. FGF regulates Sox2 and Sox3 expression in the otic epithelium, whereas the onset of Jag1 expression in the otic epithelium depends on Wnt signalling. Jag1-dependent Notch signalling regulates the maintenance of Jag1 and Sox2 expression in prosensory domains, but it is switched off in the surrounding areas by as yet unknown inhibitory factors (X). Other signalling pathways are likely to be important but are not included in the scheme for the sake of simplicity. Asterisk indicates activated Notch. HC, hair cell; NS, neurosensory; SC, supporting cell.

mechanism by which patterning and cell fate originate independently but are coupled by the link between Notch and Sox2. It also provides insight into some unexplained observations; for example, that ectopic active Notch1 expression does not always result in sensory determination or that sensory organs and their innervating neurons map to similar locations in the otic placode. However, further work is required to investigate some crucial aspects of this model, such as what sets the expression of Jag1 in the patches and what determines the timing of the generation of neurons and sensory cells.

Acknowledgements

We thank Cristina Pujades, Berta Alsina and Hernan Lopez-Schier for reading the manuscript and Marta Linares for excellent technical assistance; Elisa Marti (IBMB-CSIC, Barcelona, Spain) and Hisato Kondoh (GFSB-Osaka University, Japan) for kindly sharing their expression constructs with us; Paul Scotting (University of Nottingham, UK) and Domingos Henrique (IMM, Lisbon, Portugal) for sharing probes and for inspiring discussions; and Jane Johnson (CBN, University of Texas, USA) and Johan Holmberg (LICR, Karolinska Institute, Sweden) for kindly providing *Atoh1* and Notch reporter constructs, respectively. Islet1 and MyoVIIa monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). This work was supported by grant MICINN BFU-2008-00714 and PLE-2009-0098, Spain, and by a fellowship from FCT, SFRH/BD/25688/2005 to J.N.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.060657/-DC1>

References

- Abello, G., Khatri, S., Giraldez, F. and Alsina, B. (2007). Early regionalization of the otic placode and its regulation by the Notch signaling pathway. *Mech. Dev.* **124**, 631-645.
- Abello, G., Khatri, S., Radosevic, M., Scotting, P. J., Giraldez, F. and Alsina, B. (2010). Independent regulation of Sox3 and Lmx1b by FGF and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode. *Dev. Biol.* **339**, 166-178.
- 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.
- Alsina, B., Giraldez, F. and Pujades, C. (2009). Patterning and cell fate in ear development. *Int. J. Dev. Biol.* **53**, 1503-1513.
- Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-2476.
- Bell, D., Streit, A., Gorospe, I., Varela-Nieto, I., Alsina, B. and Giraldez, F. (2008). Spatial and temporal segregation of auditory and vestibular neurons in the otic placode. *Dev. Biol.* **322**, 109-120.
- Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.
- Bray, S. (1998). Notch signalling in *Drosophila*: three ways to use a pathway. *Semin. Cell Dev. Biol.* **9**, 591-597.
- Brigande, J. V., Kiernan, A. E., Gao, X., Iten, L. E. and Fekete, D. M. (2000). Molecular genetics of pattern formation in the inner ear: do compartment boundaries play a role? *Proc. Natl. Acad. Sci. USA* **97**, 11700-11706.
- Brooker, R., Hozumi, K. and Lewis, J. (2006). Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. *Development* **133**, 1277-1286.
- Bylund, M., Andersson, E., Novitsch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.
- Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev. Cell* **6**, 539-550.
- 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.
- Dabdoub, A., Puligilla, C., Jones, J. M., Fritzsch, B., Cheah, K. S., Pevny, L. H. and Kelley, M. W. (2008). Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc. Natl. Acad. Sci. USA* **105**, 18396-18401.
- Daudet, N. and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* **132**, 541-551.
- Daudet, N., Ariza-McNaughton, L. and Lewis, J. (2007). Notch signalling is needed to maintain, but not to initiate, the formation of prosensory patches in the chick inner ear. *Development* **134**, 2369-2378.
- de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**, 3241-3251.
- Dovey, H. F., John, V., Anderson, J. P., Chen, L. Z., de Saint Andrieu, P., Fang, L. Y., Freedman, S. B., Folmer, B., Goldbach, E., Holtszky, E. J. et al. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* **76**, 173-181.
- Ebert, P. J., Timmer, J. R., Nakada, Y., Helms, A. W., Parab, P. B., Liu, Y., Hunsaker, T. L. and Johnson, J. E. (2003). Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. *Development* **130**, 1949-1959.
- 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.
- Fior, R. and Henrique, D. (2008). 'Notch-Off': a perspective on the termination of Notch signalling. *Int. J. Dev. Biol.* **53**, 1379-1384.
- Fortini, M. E. (2009). Notch signaling: the core pathway and its posttranslational regulation. *Dev. Cell* **16**, 633-647.
- Fritzsch, B., Beisel, K. W. and Hansen, L. A. (2006). The molecular basis of neurosensory cell formation in ear development: a blueprint for hair cell and sensory neuron regeneration? *BioEssays* **28**, 1181-1193.

- Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep.* **3**, 688-694.
- 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.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *Dev. Dyn.* **195**, 231-272.
- Hansson, E. M., Teixeira, A. I., Gustafsson, M. V., Dohda, T., Chapman, G., Meletis, K., Muhr, J. and Lendahl, U. (2006). Recording Notch signaling in real time. *Dev. Neurosci.* **28**, 118-127.
- Hartman, B. H., Reh, T. A. and Bermingham-McDonogh, O. (2010). Notch signaling specifies prosensory domains via lateral induction in the developing mammalian inner ear. *Proc. Natl. Acad. Sci. USA* **107**, 15792-15797.
- Hayashi, T., Kokubo, H., Hartman, B. H., Ray, C. A., Reh, T. A. and Bermingham-McDonogh, O. (2008). Hesr1 and Hesr2 may act as early effectors of Notch signaling in the developing cochlea. *Dev. Biol.* **316**, 87-99.
- Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y. and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* **127**, 1185-1196.
- Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.
- Hume, C. R., Bratt, D. L. and Oesterle, E. C. (2007). Expression of LHX3 and SOX2 during mouse inner ear development. *Gene Expr. Patterns* **7**, 798-807.
- Jayasena, C. S., Ohyama, T., Segil, N. and Groves, A. K. (2008). Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. *Development* **135**, 2251-2261.
- Kamachi, Y., Uchikawa, M. and Kondoh, H. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet.* **16**, 182-187.
- Kato, M. (2006). Notch ligand, JAG1, is evolutionarily conserved target of canonical WNT signaling pathway in progenitor cells. *Int. J. Mol. Med.* **17**, 681-685.
- 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.
- Kiernan, A. E., Cordes, R., Kopan, R., Gossler, A. and Gridley, T. (2005a). The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. *Development* **132**, 4353-4362.
- Kiernan, A. E., Pelling, A. L., Leung, K. K., Tang, A. S., Bell, D. M., Tease, C., Lovell-Badge, R., Steel, K. P. and Cheah, K. S. (2005b). Sox2 is required for sensory organ development in the mammalian inner ear. *Nature* **434**, 1031-1035.
- Kiernan, A. E., Xu, J. and Gridley, T. (2006). The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. *PLoS Genet.* **2**, e4.
- 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.
- Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin. Cell Dev. Biol.* **9**, 583-589.
- Mak, A. C., Szeto, I. Y., Fritzsche, B. and Cheah, K. S. (2009). Differential and overlapping expression pattern of SOX2 and SOX9 in inner ear development. *Gene Expr. Patterns* **9**, 444-453.
- 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.
- Murata, J., Tokunaga, A., Okano, H. and Kubo, T. (2006). Mapping of notch activation during cochlear development in mice: implications for determination of prosensory domain and cell fate diversification. *J. Comp. Neurol.* **497**, 502-518.
- Murata, J., Ohtsuka, T., Tokunaga, A., Nishiike, S., Inohara, H., Okano, H. and Kageyama, R. (2009). Notch-Hes1 pathway contributes to the cochlear prosensory formation potentially through the transcriptional down-regulation of p27Kip1. *J. Neurosci. Res.* **87**, 3521-3534.
- Neves, J., Kamaid, A., Alsina, B. and Giraldez, F. (2007). Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick. *J. Comp. Neurol.* **503**, 487-500.
- Oh, S. H., Johnson, R. and Wu, D. K. (1996). Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J. Neurosci.* **16**, 6463-6475.
- Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **91**, 639-648.
- Pan, W., Jin, Y., Stanger, B. and Kiernan, A. E. (2010). Notch signaling is required for the generation of hair cells and supporting cells in the mammalian inner ear. *Proc. Natl. Acad. Sci. USA* **107**, 15798-15803.
- Pevny, L. and Placzek, M. (2005). SOX genes and neural progenitor identity. *Curr. Opin. Neurobiol.* **15**, 7-13.
- Pujades, C., Kamaid, A., Alsina, B. and Giraldez, F. (2006). BMP-signaling regulates the generation of hair-cells. *Dev. Biol.* **292**, 55-67.
- Puligilla, C., Dabdoub, A., Brenowitz, S. D. and Kelley, M. W. (2010). Sox2 induces neuronal formation in the developing mammalian cochlea. *J. Neurosci.* **30**, 714-722.
- Raft, S., Koundakjian, E. J., Quinones, H., Jayasena, C. S., Goodrich, L. V., Johnson, J. E., Segil, N. and Groves, A. K. (2007). Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development. *Development* **134**, 4405-4415.
- Ramos, C., Rocha, S., Gaspar, C. and Henrique, D. (2010). Two Notch ligands, Dll1 and Jag1, are differently restricted in their range of action to control neurogenesis in the mammalian spinal cord. *PLoS One* **5**, e15515.
- Rex, M., Orme, A., Uwanogho, D., Tointon, K., Wigmore, P. M., Sharpe, P. T. and Scotting, P. J. (1997). Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. *Dev. Dyn.* **209**, 323-332.
- Sahly, I., El-Amraoui, A., Abitbol, M., Petit, C. and Dufier, J. L. (1997). Expression of myosin VIIA during mouse embryogenesis. *Anat. Embryol. (Berl.)* **196**, 159-170.
- Satoh, T. and Fekete, D. M. (2005). Clonal analysis of the relationships between mechanosensory cells and the neurons that innervate them in the chicken ear. *Development* **132**, 1687-1697.
- Sienknecht, U. J. and Fekete, D. M. (2008). Comprehensive Wnt-related gene expression during cochlear duct development in chicken. *J. Comp. Neurol.* **510**, 378-395.
- 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.
- Timmer, J., Johnson, J. and Niswander, L. (2001). The use of in ovo electroporation for the rapid analysis of neural-specific murine enhancers. *Genesis* **29**, 123-132.
- 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.
- Uchikawa, M., Kamachi, Y. and Kondoh, H. (1999). Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. *Mech. Dev.* **84**, 103-120.
- Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.
- Wu, D. K. and Oh, S. H. (1996). Sensory organ generation in the chick inner ear. *J. Neurosci.* **16**, 6454-6462.
- 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. and Gao, W. Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580-586.