Notch signalling: the multitask manager of inner ear development and regeneration

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Abstract

Notch signalling is a major regulator of cell fate decisions and tissue patterning in metazoans. It is most famous for its role in lateral inhibition, whereby Notch mediates competitive interactions between cells to limit adoption of a given developmental fate. However, it can also function by lateral induction, a cooperative mode of action that was originally described during the patterning of the Drosophila wing disc and creates boundaries or domains of cells of the same character. In this chapter, we introduce these two signalling modes and explain how they contribute to distinct aspects of the development and regeneration of the vertebrate inner ear, the organ responsible for the perception of sound and head movements. We discuss some of the factors that influence the context-specific outcomes of Notch signalling in the inner ear, and the ongoing efforts to target this pathway for the treatment of hearing loss and vestibular dysfunction.

1. Introduction

A great diversity of fate decisions and cellular processes are regulated by Notch signalling, due to context-dependent differences in its transcriptional targets and the multitude of factors that influence the spatial pattern and dynamics of Notch activity. The development of the inner ear provides a great illustration of this versatility. Notch controls several key cell determination and patterning events during the differentiation of the neurosensory cells of the inner ear, through different ligands and modes of signalling. It is critical for the formation of the mechanosensory 'hair' cells that populate the sensory organs of the inner ear and are essential for hearing and our perception of balance. This has prompted considerable interest in the potential manipulation of Notch activity to stimulate HC regeneration in the damaged ear, a topic discussed at the end of this review.

2. The Notch signalling pathway

We provide below a very brief overview of the mechanisms of Notch signalling, lateral inhibition, and lateral induction. For additional molecular and biochemical details, or non-canonical modes of action of Notch, we refer the reader to other reviews (Bray, 2006; Henrique and Schweisguth, 2019; Yamamoto et al., 2010).

Basic components of the canonical Notch pathway

Notch receptors (**Figure 1**) are transmembrane proteins, composed of an extracellular domain with multiple EGF-like repeats and an intracellular domain (NICD). The Notch receptors are activated by transmembrane ligands belonging to the Delta/Serrate/Lag-2 (DSL) family. Binding of the DSL ligands to the Notch extracellular domain, however, is not sufficient for receptor activation. Their internalization in the 'signal-sending' cell, which is regulated by the E3-ubiquitin ligases of the Mindbomb and Neuralized families, is required to expose two proteolytic sites near the Notch transmembrane domain. These are then cleaved by two metalloproteases [protease A-disintegrin and metalloprotease-10 (ADAM10) and tumour necrosis alpha converting enzyme (TACE)] and the γ-secretase enzyme. The NICD is then released in the 'signal-receiving' cell, whilst the Notch extracellular domain is endocytosed with the ligand in the signal-sending cell. Following its cleavage, NICD is translocated to the nucleus where it forms a transcriptional complex with CSL (CBF1/SuH/Lag-1) and

Mastermind-like (MAML) to activate the expression of Notch target genes. The CSL protein can also function as a transcriptional repressor in the absence of NICD, keeping some of the Notch targets silenced. The classic direct targets and effectors of Notch belong to the *Hairy-and-Enhancer of Split (Hes)* and *Hes-related* gene family, which encode basic Helix-Loop-Helix (bHLH) transcriptional repressors. They antagonize the expression and activity of other genes, in particular the proneural bHLH factors.

Two contrasting modes of signalling: lateral inhibition and lateral induction

Importantly, Notch activity can feedback positively or negatively on the expression of the DSL ligands. This produces two distinct modes of signalling, lateral inhibition and lateral induction, which have different outcomes in terms of cell differentiation and patterning for interacting cells.

	Lateral inhibition	Lateral induction
Starting conditions	Equivalence group	Context-dependent
Target of Notch activity	Proneural factors	Context-dependent
Regulation of DSL expression by Notch activity	Negative	Positive
Cellular outcome	Alternate fates	Same fate

In lateral inhibition (**Figure 2A**), interacting cells compete for the adoption of the primary fate, defined by the expression of a proneural bHLH factor that i) promotes DSL ligand expression and ii) is repressed by Notch activity. Starting from a condition where all cells are (in theory) equal in their developmental potential, random variations in the expression of the proneural factor lead some cells to elevate their DSL levels – these become better signal-sending cells. The signal-receiving cells, on the other hand, reduce their levels of proneural factor and DSL expression; they are consequently diverted from the primary fate. The outcome of lateral inhibition is a diversification of cell fates, creating a branching point within a cell lineage, or a salt-and-pepper mosaic of two cell types within an epithelium. Its failure results in the overproduction of cells adopting the primary fate. Lateral inhibition is a very common and evolutionary conserved mechanism regulating

for example cell diversification in the sensory organs of drosophila, neurogenesis, the formation of secretory cells in the gut, or the production of HCs in the inner ear.

In lateral induction (**Figure 2B**), interacting cells cooperate to maintain Notch activity and adopt the same fate. Lateral induction is not as common as lateral inhibition, but it has been well studied in the Drosophila wing disc (see later) and it regulates vascular smooth muscle differentiation (Manderfield et al., 2012), neural crest induction (Cornell and Eisen, 2005), lens fiber differentiation (Saravanamuthu et al., 2009), interactions between the epidermis and the dermis (Ambler and Watt, 2010), and prosensory specification in the inner ear.

Cells with an edge: factors improving lateral inhibition

A critical parameter for efficient lateral inhibition is the strength of the intercellular feedback loop repressing the DSL ligand in the signal-receiving cells. However, signal-sending cells can use several tricks to improve their chances of delivering a 'loud and clear' message to their neighbours. For example, in drosophila proneural clusters (**Figure 2A**), Neuralized is restricted to the nascent sensory organ precursor (SOP), which enables these to deliver efficient lateral inhibition to neighbouring cells (Yamamoto et al., 2010). In the following rounds of division, lateral inhibition is also biased by the asymmetric inheritance of Neuralized and the endocytotic protein Numb, which reduces cell-surface levels of Notch receptors in one of the daughter cells (Couturier et al., 2013). The DSL ligands can also bind to Notch receptors in 'cis' (within the signal-sending cell) to prevent their activation (del Álamo et al., 2011). Finally, the expression of dominant-negative HLH (*emc* in Drosophila, *Inhibitor-of-Differentiation* or *Id* genes in vertebrates) can restrict the activity of the proneural bHLH genes to specific cells, which gain a strong competitive advantage in the race for adoption of the primary fate (Troost et al., 2015).

Hence, more than the expression levels of a DSL ligand, Notch receptor, or proneural gene, it is the *activity levels* of these components that determine, in a context-dependent manner, the outcomes of lateral inhibition.

Cells at the edge: lateral induction and the making of tissue boundaries

The best characterized example of lateral induction occurs during the formation of the dorso-ventral lineage boundary of the Drosophila wing (imaginal) disc (**Figure 2B**), which in the adult gives rise to the peripheral wing margin. The DV boundary acts as a cellular fence, which prevents cells belonging to the dorsal 'compartment' of the wing disc from mixing with those of the ventral compartment during tissue growth (reviewed in Dahmann et al., 2011). Notch signalling is necessary for the formation of the boundary. Each compartment expresses uniformly a DSL ligand (Serrate in the dorsal compartment, Delta in the ventral one) and their expression is stimulated by Notch activity (de Celis and Bray, 1997). But although the Notch receptor is present throughout the wing disc, Notch activity is strongly elicited along the DV boundary only, in a 1 to 3 cell wide domain where expression of the ligands becomes also elevated (de Celis et al., 1996). This restricted activation is due to the action of Fringe, a glycosyltransferase present in the dorsal compartment that modifies the extracellular domain of the Notch receptor to make it less sensitive to Serrate, but promotes Delta/Notch interactions in both cis and trans-signalling (LeBon et al., 2014; Rauskolb et al., 1999). Consequently, Delta (in ventral cells) can strongly activate Notch in the (dorsal) Fringe-expressing cells – creating a longitudinal band of Notch activity between the dorsal and ventral compartments.

Hence, the differing modes of regulation of DSL ligands and the modification of Notch receptors can radically transform the tissue-level outcomes of Notch signalling. This versatility is also manifest in the inner ear, where Notch signalling is truly multi-tasking and managing through different ligands and modes of action some of the key aspects of its development.

3. Introduction to the inner ear

Aptly named by the early anatomists the 'labyrinth', the membranous part of the inner ear is composed of a complex 3D network of fluid-filled canals and chambers, lined up by specialized epithelial cells. The mammalian inner ear can be subdivided into a dorsal part containing the vestibular system, and a ventral part, the cochlea (**Figure 3**). The vestibular system has three semi-circular canals oriented along orthogonal axes and connected to three sensory organs, called cristae, which are sensitive to the angular rotation of the head. In addition, it contains the maculae of the utricle and the saccule, which act as gravity and acceleration sensors. The cochlea forms a coiled structure resembling a snail shell and hosts a sensory epithelium activated by

sound, called the organ of Corti (**Figure 3B-C**). The semi-circular canal system is highly conserved, but there are variations in the number and morphology of the other sensory organs across vertebrates. However, one universal feature is the 'salt-and-pepper' mosaic of mechanosensory HCs, interspaced from one another by SCs. The HCs are topped by an array of modified microvilli, or stereocilia, arranged in neat rows forming a staircase-like pattern. The stereociliary bundles are in contact with specialized extracellular gels or membranes and bath within a potassium-rich fluid called the endolymph. When inner ear fluids are displaced in response to changes in head position or to the vibrations of the middle ear ossicles, mechanotransduction channels located at the top of the stereocilia open, leading to an influx of potassium ions and cell depolarisation. This causes neurotransmitter release at the synaptic pole of HCs and the stimulation of the afferent neurites of the auditory and vestibular neurons, which relay this information to the central nervous system.

Inner ear development in a nutshell

We provide here a very brief summary of inner ear development in the mouse (**Figure 3**) and refer to other reviews for further details (Alsina and Whitfield, 2017; Basch et al., 2016a; Fritzsch and Beisel, 2001).

The majority of the cells that compose the inner ear (including the audio-vestibular neurons) derive from the otic placode, an epithelial thickening of the head ectoderm located on both sides of the embryonic hindbrain. The placode invaginates then pinches off the surface ectoderm to form the otic vesicle, or otocyst. As it grows, this simple sphere undergoes a drastic remodelling to give rise to various vestibular structures dorsally (endolymphatic duct, semi-circular canals) and the cochlear duct ventrally. This is accompanied by dynamic changes in the expression of molecular factors regulating the specification of otic progenitors. The precursors for the sensory organs are located within *prosensory* domains, which are produced sequentially by segregation from a large sensory-competent domain that extends along the ventro-medial wall of the otic vesicle. The prosensory cells then gradually exit the cell cycle and differentiate into HCs and SCs. The first HCs are formed in the vestibular patches. In the embryonic organ of Corti, the terminal mitosis of the prosensory cells proceeds from the distal end to the base of the cochlear duct (around E13-E15 in the mouse) but HC differentiation as well as other aspects of the maturation of the epithelium follow the opposite direction. The onset of function of the mouse cochlea occurs at approximately 2 weeks of age.

4. Roles of Notch during inner ear development

In 1991, as the roles of Notch and lateral inhibition were uncovered in the nervous system of Drosophila and vertebrates, Julian Lewis proposed that the same mechanism could control the production of the neurosensory cells of the inner ear (Lewis, 1991). Since then, the experimental evidence supporting this idea has accumulated, and new roles for Notch have been uncovered in prosensory specification and otic induction.

4.1 Lateral inhibition and the regulation of otic neurogenesis

Starting from approximately E8 and until E12 in the mouse, the precursor cells for the neurons of the cochleavestibular ganglion, or neuroblasts, delaminate from the antero-medial domain of the otic placode (then vesicle, see **Figure 3**). The neurogenic domain is included within a larger neuro-sensory competent domain, which gives rise to several of the sensory organs. After their delamination, neuroblasts proliferate and differentiate into two populations of neurons: the vestibular neurons, which are born first, and the auditory neurons (Koundakjian et al., 2007). The selection of neuroblasts is regulated by lateral inhibition (**Figure 4**). *Notch1* is present throughout the otic epithelium but Dll1 is restricted to the neurogenic patch, where it is expressed in a scattered manner (Alsina et al., 2004; Daudet et al., 2007). The neuroblasts express two proneural bHLH factors, Neurogenin1 (Neurog1) and NeuroD, which are respectively required for their initial specification and their delamination and survival (Kim et al., 2001; Liu et al., 2000, 1; Ma et al., 2000; Matei et al., 2005). Notch effectors and the modulator *Lunatic Fringe* (*Lnfg*) are also expressed there (Adam et al., 1998; Cole et al., 2000). The evidence that lateral inhibition controls neuroblast formation came first from the *mindbomb* zebrafish mutant, which shows excessive neuronal production throughout its nervous system and in the inner ear (Haddon et al., 1998). In the chick otocyst, the pharmacological inhibition of Notch activity with a gamma-secretase inhibitor (GSI) or through overexpression of a dominant-negative form of Mastermind also leads to excess neuronal differentiation and an increase in *Dl1* expression (Abelló et al., 2007; Daudet et al., 2007) as predicted by the standard model of lateral inhibition with feedback.

The factors that establish otic neural competence are still unclear but involve a complex interplay of diffusible signals emanating from the surrounding tissues and transcription factors (Gálvez et al., 2017; Raft and Groves, 2014). Some effectors of the Notch pathway could also play a part in this process. In fact, *cHairy1* (an

orthologue of *Hes1*) in the chick, and *her9* in the zebrafish (Radosevic et al., 2011), are expressed outside of the neurogenic domain. However, neither activation of canonical Notch nor a particular ligand has been firmly associated to their regulation. Instead, the transcription factor Tbx1 and retinoic acid, which promotes posterior identity in the otocyst, act upstream of *her9* in zebrafish. The inactivation of *her9* by morpholinos, similar to the absence of retinoic acid or *Tbx1* in the mouse (Raft, 2004, 1), leads to ectopic induction of neurogenesis in posterior regions of the otocyst (Radosevic et al., 2011, 1).

4.2 Lateral inhibition and hair cell fate decisions

Once prosensory cells exit the cell cycle, they differentiate into HCs or SCs and this decision is controlled by lateral inhibition (**Figure 5**). Lateral inhibition has been most studied in the organ of Corti, partly because defects in the number and organisation of inner HCs and outer HCs, organised respectively in one and three parallel rows, are very easy to spot. The basic rules of lateral inhibition appear nevertheless conserved across all inner ear sensory epithelia. The nascent HCs deliver lateral inhibition by expressing, in a transient manner, multiple DSL ligands: Dll1, Jag2 and Dll3 in the mouse (Hartman et al., 2007; Lanford et al., 1999; Morrison et al., 1999), DeltaA and DeltaB in the fish (Haddon et al., 1998; Riley et al., 1999), and at least Dll1 in the chick (Adam et al., 1998). In the signal-receiving cells, Notch activity represses the expression of *Atonal-homologue 1* (*Atoh1* in mammals, *cath1* in the chick, *atoh1a/b* in the fish) a bHLH proneural gene required for the formation of chordotonal organs in the fly, and HCs as well as other cell types (granule cells in the cerebellum, secretory cells of the gut lining, …) in vertebrates (Bermingham et al., 1999; Jarman and Groves, 2013). In zebrafish, *atoh1a* is expressed in a large territory before becoming restricted to the first (tether) HCs, suggesting that it defines a genuine equivalence group (Millimaki et al., 2007). In the mouse cochlea, however, *Atoh1* is highly expressed in nascent HCs but much harder to detect in the prosensory cells (Bermingham et al., 1999; Cai et al., 2013; Chen et al., 2002; Driver et al., 2013; Lanford et al., 2000; Woods et al., 2004; Yang et al., 2010), hinting at a different mode of regulation.

Redundancies in the lateral inhibition machinery ensure robust cell fate decisions

In the *mindbomb* zebrafish, HCs are produced early and in excess at the expense of the SCs (Haddon et al., 1998). Perhaps not surprisingly, these HCs do not survive long without SCs and are rapidly eliminated from

the epithelium. This remains to date the most dramatic phenotype observed in any Notch mutant, presumably because the E3-ubiquitin ligase Mib is required for the activities of several DSL ligands (Itoh et al., 2003). An overproduction of HCs can also be elicited in organotypic cultures of embryonic organ of Corti by GSI treatment (Tang et al., 2006; Yamamoto et al., 2006), although high doses are required to achieve the strongest phenotype (Doetzlhofer et al., 2009). Whilst this confirms that Notch signalling is a key regulator of HC formation, it also indicates that multiple ligands or receptors must mediate lateral inhibition. There is in fact good evidence that the DSL ligands of HCs act in a cooperative and partly redundant manner: in the mouse organ of Corti of the Jag2 mutant, an additional row of IHCs is present but the OHCs are unaffected (Lanford et al., 1999). In the *Dll1* cKO (Brooker, 2006) or hypomorph mutant (Kiernan, 2005), both IHCs and OHCs are produced early and in excess, but this phenotype becomes much more dramatic in a compound *Dll1/Jag2* mutant, suggesting synergistic effects (Kiernan, 2005). The *Dll3* mutant does not exhibit any defect in HC numbers (Hartman et al., 2007), which suggests that this ligand is either not contributing to lateral inhibition, or that its absence is entirely compensated by Dll1 and Jag2. The *Notch1* cKO mouse has a phenotype that is as severe as the combined loss of Jag2 and Dll1 (Kiernan, 2005), suggesting that it is the main mediator of lateral inhibition. Its paralogues *Notch2* and *Notch3* are also expressed in the developing inner ear (Basch et al., 2011; Hao et al., 2012; Lindsell et al., 1996; Maass et al., 2015; Yamamoto et al., 2006) but their functions have not been tested. On the other hand, multiple Notch effectors of the *Hes/Hey* family are present in prosensory and SCs of the organ of Corti and these interact genetically: compound mutants for *Hes1*, *Hes5*, *Hey1* and *Hey2* have more severe phenotypes that single mutants, suggesting additive effects between Hes/Hey repressors (Benito-Gonzalez and Doetzlhofer, 2014; Li et al., 2008; Tateya et al., 2011; Zheng et al., 2000; Zine et al., 2001). Hence, the multiplicity of DSL ligands and Notch effectors makes the lateral inhibition of HC fate decisions a robust and relatively fail-safe mechanism, although some of its components (eg Dll1, Notch1) are clearly more critical than others to its operation.

Are hair cell fate decisions biased?

In drosophila, a number of factors can provide a positive or negative bias in the signal-sending or signal-receiving abilities of cells interacting by lateral inhibition, but are these at play during HC fate decisions? The

fact that there are strong differences in the expression levels of Atoh1 in prosensory cells (very low/absent) versus nascent HCs (very high) suggests that the latter might have a competitive advantage from the onset of lateral inhibition, but how this might be achieved is unknown. Numb, an endocytotic adaptor protein able to reduce the activity of the Notch receptor during asymmetric fate decisions in Drosophila (Couturier et al., 2013), does not appear to have such effect in the ear since its overexpression does not bias HC versus SC fate choices (Eddison et al., 2000; Eddison et al., 2015). The E3-ubiquitin ligase Mib, which is required for the internalization and activity of DSL ligands (Itoh et al., 2003), could in theory provide an advantage to some signal-sending cells but this has not been directly tested.

4.3 Lateral induction and prosensory specification

At the time the role of Jag2/Dll1 in the lateral inhibition of HC formation was uncovered, it became clear that an additional DSL ligand, Jag1, had a very distinct function. In fact, Jag1 is expressed long before HC differentiation by the prosensory cells, and later by the SCs, which contact one another – a first hint that its expression is not repressed by Notch activity (Adam et al., 1998; Eddison et al., 2000; Morrison et al., 1999). Furthermore, the *Jag1* mutants had a very distinct phenotype from the *Dll1* or *Jag2*-deficient mice: they exhibited a circling behaviour, due to the absence of their vestibular cristae, and an organ of Corti with more inner HCs but fewer outer HCs than normal (Kiernan et al., 2001; Tsai et al., 2001). This, along with experiments showing that early and transient overexpression of an active form of Notch can induce the formation of ectopic sensory territories and Jag1 expression in the chick inner ear (Daudet and Lewis, 2005), suggested that Jag1/Notch signalling regulates, by lateral induction, prosensory specification (**Figure 6**).

Notch activity promotes prosensory specification by maintaining Sox2 expression

Prosensory specification is the series of events leading to the formation of the prosensory cells, the population of otic progenitors competent to differentiate into HCs and SCs. Fate map experiments relying on the classic chick-quail grafting technique have shown that sensory organ progenitors are originally located within a large medial domain extending along the antero-posterior axis of the otic placode (Sánchez-Guardado et al., 2014). In the early otic vesicle, they are contained within a large ‘pan-sensory’ domain extending along the antero-posterior axis, which encompasses the anterior neurogenic patch and expresses among other

markers *FGF10*, *Sox2*, and *Jag1* (Adam et al., 1998; Alsina et al., 2004; Cole et al., 2000; Mann et al., 2017; Sánchez-Guardado et al., 2013). Next, distinct prosensory patches arise progressively by segregation from the pan-sensory domain - a mechanism that could have contributed to the multiplication and diversification of the inner ear sensory organs in the course of vertebrate evolution (Fritzsch and Beisel, 2001). Thus, the original pool of sensory-competent cells must be maintained and expanded throughout formation of the sensory organs. Lateral induction is in theory ideally suited to fulfil these roles: the positive feedback loop linking Notch and Jag1 expression could i) maintain Notch activity and prosensory character within interacting cells and ii) specify new prosensory cells by recruiting 'naïve' cells (Notch-OFF) into a Notch-ON state. By large, the experimental evidence supports this idea but indicates that Notch activity is not sufficient for prosensory specification. In fact, overexpression of NICD in the early chick (Daudet and Lewis, 2005; Neves et al., 2011) or mouse (Hartman et al., 2010; Liu et al., 2012a; Pan et al., 2010; Pan et al., 2013) otocyst does not convert all transfected cells into prosensory cells, and the capacity of Notch to induce ectopic sensory organs is restricted to a narrow developmental window (Liu et al., 2012a). Conversely, blocking Notch activity with GSI in cultures of chick otocysts strongly impairs, but does not completely prevent, sensory organ formation (Daudet et al., 2007). Likewise, the RBPJ(k) cKO mouse exhibits a severe atrophy of the vestibular organs but a partial formation of the cochlear prosensory domain (Basch et al., 2011; Yamamoto et al., 2011). Some studies, however, have reported that GSI or TACE inhibitor treatments can suppress prosensory specification in organotypic cultures of embryonic (E12-E13) organ of Corti (Hayashi et al., 2008; Munnamalai et al., 2012). These seemingly contradictory results may be explained by the differences in experimental approaches and the fact that a RBPJ(K)-null may not be equivalent to a Notch-OFF condition, due to the requirement for RBPJ(K)/CSL for the constitutive repression of some of the Notch target genes (Barolo et al., 2002): in the absence of RBPJ(K), the Notch targets that would normally be repressed by RBPJ(k) in a Notch-OFF condition might be expressed, mimicking in fact a Notch-ON situation. Nevertheless, the fact that the recurrent consequence of blocking Jag1/Notch activity at early stages of ear development is a strong reduction (as opposed to a complete absence) of prosensory territories strongly suggests that the primary function of lateral induction is to maintain prosensory specification, rather than inducing it 'de novo'.

The prosensory factors targeted by Notch activity are still unknown, but are likely to include Sox2, which is required for the formation of all inner ear sensory organs (Kiernan et al., 2005) and is positively regulated by Notch (Daudet et al., 2007; Pan et al., 2010). The expression of Sox2 is widespread in the early otocyst before becoming restricted to the anterior neurosensory competent domain and the prosensory patches (Neves et al., 2007; Neves et al., 2011; Steevens et al., 2017). Furthermore, recent lineage-tracing studies relying on an inducible Sox2-CreER mouse model have confirmed that the early Sox2-expressing cells include prospective prosensory cells but also a large proportion of cells that will eventually lose Sox2 and give rise to non-sensory territories (Gu et al., 2016, 2; Steevens et al., 2019). This reduction of Sox2 expression may be due to the localized dampening of lateral induction. In fact, Jag1 is down-regulated in between segregating sensory organs in the chick and zebrafish otocyst (Ma and Zhang, 2015; Mann et al., 2017) and if Notch activity is artificially increased, the cristae (as well as other sensory organs) fail to segregate (Mann et al., 2017). Thus, the spatial regulation of lateral induction could determine the number and size of sensory organs that segregate from the initial pan-sensory domain. This might explain why forcing Notch activity tends to "induce" ectopic sensory territories close to the endogenous sensory organs (see for example Hartman et al., 2010; Liu et al., 2012a): in these experiments, Notch over-activation could maintain prosensory character (and Sox2) in sensory-competent regions that would otherwise lose this character over time.

Besides Sox2 and Notch, many transcription factors and signalling pathways are likely to promote or antagonize prosensory specification (Żak et al., 2015). For example, the LIM-homeodomain transcription factor Lmx1a, expressed in non-sensory domains, antagonizes Notch activity. In the *Lmx1a*-null mouse, cells normally contributing to non-sensory territories in between sensory organs adopt (or retain) a prosensory character (Mann et al., 2017), producing fused sensory organs (Koo et al., 2009; Nichols et al., 2008). Several mutant mice exhibit defects in sensory organ segregation or size and some of the underlying genes could impact on prosensory specification. The interactions between Notch signalling and Sox2 are most likely the tip of the iceberg, and much remains to be learnt about the genetic and epigenetic factors regulating prosensory specification.

Which Notch components mediate its prosensory function?

Jag1 is positively regulated by Notch activity and can activate its own expression 'in trans', which indicate that it functions by lateral induction (Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010; Neves et al., 2011; Pan et al., 2010). However, this does not mean that it is the only DSL ligand capable of regulating prosensory specification. In fact, Jag2 (Neves et al., 2011) or Dll1 (Mann et al., 2017) can induce ectopic sensory patches when overexpressed at early stages of chick inner ear development. Furthermore, the inner ear of *Dll1*-null mice exhibits a very small (or absent) saccule (Brooker, 2006; Kiernan, 2005), whilst it is the only sensory patch that seems to develop normally in the *Jag1*-cKO mice (Brooker, 2006; Kiernan et al., 2006). This suggests that Notch activity elicited by Dll1 during neurogenesis could contribute to the maintenance of the saccule progenitors. If several DSL can mediate the prosensory function of Notch, what about Notch receptors and their effectors? Receptors other than Notch1 must at least contribute to prosensory specification, since HCs do form (and in excess) in the *Notch1* cKO mouse. The identity of the Notch effectors is equally elusive. It was originally proposed that lateral induction may be mediated through Hey1 and Hey2 (also known as Hesr1/2), since they are expressed within the prosensory domain before HC formation in the organ of Corti (Hayashi et al., 2008) and the vestibular organs (Petrovic et al., 2015; Tateya et al., 2011). However, *Hey1/Hey2* double KO mice do not have any defect in prosensory specification or any reduction in HC numbers (Benito-Gonzalez and Doetzlhofer, 2014, 1). Prosensory specification is also unaffected in the triple KO for *Hes1*, *Hes5*, and *Hey1* (Tateya et al., 2011). Some Notch receptor(s) and effector(s) specifically involved in lateral induction may eventually be identified. However, the great level of functional redundancy seen during lateral inhibition is a strong hint that Notch could promote prosensory specification through multiple ligands, receptors, and effectors. It is also clear that some canonical Notch effectors are not solely regulated by Notch in the ear. For example, Hey1/2 expression are maintained in the cochlea of *RBPJK* cKO mice (Basch et al., 2011) and only partly reduced after pharmacological inhibition of Notch activity (Petrovic et al., 2015), whilst FGF, Hedgehog, BMP and Wnt signalling can also regulate their expression (Benito-Gonzalez and Doetzlhofer, 2014; Doetzlhofer et al., 2009; Munnamalai et al., 2012; Petrovic et al., 2015; Tateya et al., 2013). Although we do not know if these transcriptional effects are direct or not, a number of signalling pathways seem able to 'hijack' traditional Notch effectors to impact on HC fate decisions as well as prosensory specification.

4.4 Notch signalling during otic placode formation

Notch1, Dll1 and Jag1 and some Notch effectors (Hes1, cHes5.2) are expressed in the pre-otic ectoderm on both sides of the hindbrain before the placode itself is morphologically recognizable (Jayasena et al., 2008; Jayasena et al., 2008; Myat et al., 1996; Shida et al., 2015). At this stage, Wnt and FGF signals secreted by the neighbouring tissues (neural tube, mesoderm, endoderm) promote otic induction (reviewed in Chen and Streit, 2013), and Pax2-positive precursors for the otic and epibranchial placodes are still intermingled in the pre-otic territory (Groves and Bronner-Fraser, 2000; Streit, 2002). Although it is tempting to imagine that Notch signalling could regulate the specification of these different cell types, experimental manipulation of Notch activity produced conflicting results. In the mouse, sustained NICD overexpression in the Pax2-lineage leads to the expansion of an otic placode-like epithelium, which expresses the otic marker Pax8, but not others such as Pax2, Sox9 ,Gbx2, and Hmx3 (Jayasena et al., 2008). Since the placode is slightly reduced in size in a partial *Notch1* mutant, the authors proposed that Notch activity could promote otic placode formation, possibly by augmenting Wnt signalling (Jayasena et al., 2008). However, in chick embryos, overexpression of NICD inhibits otic placode formation whilst cells in which Notch is reduced (by expressing a dominant-negative form of Dll1 or CSL) tend to adopt an otic fate (Shida et al., 2015). These differences may be due to species-specific functions for Notch, or to the distinct experimental approaches used, since electroporation in the chick embryo would affect more cells than those of the Pax2-lineage. Another uncertainty is the mode of action (inhibition, induction, or else) of Notch in this context, since the consequences of blocking Notch activity on the expression of Dll1 and Jag1 have not been investigated. More work is therefore necessary to clarify the roles of Notch signalling during otic placode formation.

5. Managing and multitasking: specificity and integration of Notch functions in the developing inner ear

The diversity of functions fulfilled by Notch signalling in the inner ear is remarkable and more often than not, these overlap in space and time. How then are the effects of different ligands and various modes of signalling integrated and converted into distinct responses?

5.1. Competence states determine the context-specific effects of Notch signalling

What determines the specific cell identities adopted through lateral inhibition or induction is the intrinsic competence of the interacting cells, which can be defined as their capacity to adopt a particular fate (or express a set of transcription factors) when Notch is ON or OFF. The competence of the neurosensory progenitors changes during development. In mammals and birds, they produce neuroblasts first, then HC and SC. This transition is at least partly dependent on antagonistic cross-interactions between Neurog1 and Atoh1: in the *Neurog1*-null mouse, some of the prospective neuroblasts adopt a prosensory character, up-regulate Atoh1 (Raft et al., 2007), and HCs form earlier than expected (Matei et al., 2005). Among the other potential regulators of Atoh1 and Neurog1 are the Inhibitors-of-differentiation (Id1-4) HLH factors, which act as dominant-negative regulators of the proneural bHLH factors (reviewed in Jones, 2004). All Ids are present in the developing inner ear (Jones, 2006; Kamaid et al., 2010; Ozeki et al., 2007) and the overexpression of Id1-3 in ovo (Kamaid et al., 2010) or in organotypic cultures of mouse organ of Corti (Jones et al., 2006) can prevent HC differentiation, presumably by blocking Atoh1 activity. However, the absence of *Id1* and *Id3* in double KO mice does not produce obvious defects in prosensory specification or HC differentiation, at least in the cochlea (Jones, 2006), suggesting an important level of redundancy between *Id* genes. Another factor that probably regulates the competence state of neurosensory progenitors is Sox2: it is required for their formation and can directly regulate Atoh1 expression, but it can also antagonize its 'pro-HC' and 'proneural' effects when expressed at high levels (reviewed in Gálvez et al., 2017; Raft and Groves, 2014), which suggests dose-dependent effects very similar to those described during central neurogenesis (Pevny and Nicolis, 2010).

The epigenetic landscape: a regulator of neurosensory competence?

Interactions between transcription factors are critical for setting up a competence state, but their ability to access their genomic targets is dependent on chromatin structure. The competence of otic progenitors is therefore expected to be sensitive to epigenetic modifications such as post-translational modifications of histones or DNA methylation. Explorations of this complex level of regulation have started relatively recently, but there are already some indications of its importance (Doetzlhofer and Avraham, 2017). For example, de

novo mutations in the *Chd7* gene, which encodes a chromatin remodelling enzyme, cause the CHARGE syndrome, associated to congenital defects in inner ear morphology and function. During otic development, CHD7 expression becomes progressively restricted to the neurosensory regions (Hurd et al., 2010). The phenotype of *Chd7*-deficient mice is dose-dependent and complex, but it includes defects in neurogenesis and vestibular morphogenesis (absence of cristae and semi-circular canals) that are associated to a reduction of Sox2 and Jag1 expression (Hurd et al., 2012), consistent with abnormal neurosensory specification. Are there any epigenetic modifications specifically associated to the specification of the neurosensory lineage? A recent study compared the open chromatin landscape of FACS-sorted Sox2-positive and negative cells in the embryonic mouse cochlea and found that approximately 29,300 ATAC-Seq peaks were enriched in the prosensory cell population (Wilkerson et al., 2019), suggesting that may be the case. Further studies on more restricted populations of cells, or even single cells, will be needed to confirm these first insights and to determine if and how epigenetic marks influence the competence states of otic progenitors.

In summary, the competence state of otic neurosensory progenitors is the product of the interactions between bHLH proneural genes, Sox2, their regulators, and the epigenetic factors impinging on their activities in a context-specific manner. What a cell does in response to Notch activity will change according to its competence state, which explains why blocking or activating Notch activity produces different outcomes at different stages of inner ear development.

5.2. Integration of Notch activity from multiple ligands and modes of signalling

Notch effectors are activated in a dose-dependent manner

There are several DSL ligands expressed in the inner ear, regulated in opposite ways, which raises the question of how their effects are integrated within signal-receiving cells. We do not know if there are any receptor(s) other than Notch1 associated to lateral inhibition and induction in the ear. Nevertheless, we can infer from other systems that even a single type of Notch receptor can be differentially activated by distinct DSL ligands. For example, biochemical assays have shown that Dll4 can bind 10 times more strongly than Dll1 to the Notch1 receptor (Andrawes et al., 2013); in cell lines carrying fluorescent reporters of Notch activity, Dll1 and Dll4 trigger respectively transient versus sustained activation of the Notch1 receptor (Nandagopal

et al., 2018). In the inner ear, Jag1 and Dll1 may also bind differently to Notch1. In fact, the amount of cleaved Notch1ICD increases drastically within sensory patches at the time of HC formation (Murata et al., 2006), suggesting that Jag1 is a relatively weak ligand for Notch1 compared to Dll1 or Jag2. Another observation that fits with this idea is that whilst Dll1 immunostaining is detected exclusively within intracellular vesicles, Jag1 tends to accumulate at the cell membrane of prosensory cells (Chrysostomou et al., 2012). This suggests that the internalization of Dll1 and therefore its trans-signalling effect is stronger than that of Jag1. The strength of Notch activation could next be translated into a dose-dependent expression of Hes/Hey effectors: in chick otocysts, the expression of *Hes5* is reduced faster than that of *Hey1* following GSI treatment, implying that *Hes5* requires higher levels of Notch activity for its induction (Petrovic et al., 2015). Comparable findings were obtained in the mouse organ of Corti: the pharmacological inhibition of Notch activity induces dose-dependent changes in the expression of its *Hes/Hey* effectors, and *Hey* genes are less sensitive than *Hes* genes to this blockade (Basch et al., 2016b; Doetzlhofer et al., 2009; Maass et al., 2015). It is therefore possible that inputs from different Notch receptors are simply added and converted into a dose-dependent combination of *Hes/Hey* genes. The subtype of NICD produced may not be critical, since substituting the Notch1ICD by the Notch2ICD in transgenic mice (on a mixed genetic background) leads to the formation of a normal-looking organ of Corti, although the hybrid receptor is not as efficient as the intact Notch1 receptor (Liu et al., 2015).

Ligand-receptor interactions are modulated by Fringe-dependent glycosylation

If this model for the integration of Notch activity is correct, it is at the DSL/Notch binding and activation steps that the effects of a given ligand/receptor pair are determined: the more ligand/receptor interactions, the stronger Notch activation, the more *Hes/Hey* genes are induced. Besides the amount of cell-surface ligand and receptor, factors modifying these interactions could potentially 'tune' a given Notch receptor to a specific ligand. Among these are Fringe proteins, which are expressed in the developing ear (Cole et al., 2000; Morsli et al., 1998). The initial analysis of a *Lnfg* mutant mouse cochlea showed no HC patterning defects, but surprisingly, the absence of *Lnfg* could partly rescue the overproduction of inner HCs elicited by *Jag2* absence (Zhang et al., 2000). The authors proposed that LFng could reduce Dll1/Notch activation and its absence could

perhaps restore normal levels of lateral inhibition in the absence of Jag2, although other scenarios are possible. In a recent study, Basch and colleagues (2016b) revisited the roles of LFng and its paralogue *Manic Fringe* (*MFng*) during cochlear development. They found that Lnfg is dynamically expressed from the medial to the lateral domain of the prosensory domain during its specification, then becomes enriched in SCs; Mfng is expressed later and is restricted to HCs. However, there is transient co-expression of Lfng and Mfng at the medial boundary of the organ of Corti and when both genes are inactivated, supernumerary inner HCs (and their SCs) are produced, resembling the *Jag2* mutant phenotype and other mutants with a partial loss of Notch function in the ear (Basch et al., 2016b), including the *Jag1* cKO (Brooker, 2006; Kiernan et al., 2006). This suggests that MFng and LFng increase Notch1 sensitivity to its ligands and are required for robust lateral inhibition in the inner HCs region, but further work will be needed to establish which particular ligand-receptor interactions are modulated by LFng/MFng. An interesting hypothesis raised by the authors is that Fringe could modulate cis-inhibition, which is the capacity of DSL ligands to inhibit the activation of Notch receptors 'in cis'. We do not know yet if there is any ligand acting in this way in the ear, but Dll1 seems to be a relatively poor cis-inhibitor, at least in the chick inner ear: its over-expression under the control of either a Notch-responsive or constitutive promoter can produce large patches of transfected cells without HCs (Chrysostomou et al., 2012). This result suggests that Dll1 elicits Notch activity *in trans* but does not prevent Notch activation *in cis* (otherwise more/all Dll1 expressing cells would be HCs), although further work is needed to validate this conclusion.

Making sense of Notch signalling in the ear: mathematical models to the rescue

As we progress in our description of the mechanisms of Notch signalling in the ear, we are faced with an increasingly complex and dynamic picture: multiple ligands regulated in a different way, acting *in trans* or *in cis*, activating one or more Notch receptors, regulating the expression of several *Hes/Hey* and proneural genes, themselves subjected to regulation by external factors and feeding back on ligand expression! Evidently, changing the activity of one component can generate phenotypes that are difficult to interpret. To understand the logic of Notch signalling at a system level, we need mathematical models. The first model of lateral inhibition by Collier and colleagues (1996) used differential equations to compute ligand expression

as a function of Notch activation. Their simulations showed that starting from random conditions, an alternated mosaic of (computer) cells with either high-Notch/low-ligand or low-Notch/high-ligand could be formed if there was a strong enough negative feedback loop between Notch activity and ligand expression in signal-receiving cells. In an extension of this model, Petrovic and colleagues (2014) studied the interplay between lateral inhibition and induction by introducing in the model two ligands, regulated in opposite manner. They found that the two modes of signalling can co-exist and produce a cellular mosaic only if i) lateral induction does not elicit too high levels of Notch activity and ii) the intercellular lateral inhibition feedback loop is reinforced by the positive autoregulation of the proneural gene that it controls. These predictions agree with the differential expression of Hes/Hey genes in response to Dll1 versus Jag1 in the ear (Petrovic et al., 2014; Petrovic et al., 2015) and suggest that the Atoh1 autoregulatory loop (Abdolazimi et al., 2016; Bermingham et al., 1999; Helms et al., 2000; Woods et al., 2004) is critical for robust lateral inhibition. Another recent study investigated the impact of apical surface area on the outcomes of lateral inhibition (Shaya et al., 2017). Using in silico simulations and experiments conducted in cell lines, the authors proposed that smaller cells were more likely to act as signal-sending cells during lateral inhibition. One observation supporting this conclusion was that immature HCs have smaller apical surfaces compared to that of SCs in the embryonic basilar papilla, although we do not know whether this is also the case for uncommitted HCs. Models are powerful discovery tools and their predictions, at times counter-intuitive, can open up new lines of enquiries: would manipulation of cell size be sufficient to bias HC fate decisions? What would happen if the auto-regulatory feedback loop controlling Atoh1 expression was altered, or if the strength of lateral induction or inhibition was artificially boosted? With the recent progress in genome editing technologies, these questions can now be tested experimentally in a wide range of model organisms.

5.3. Integration of Notch signalling with cell proliferation and rearrangements

Developing tissues change considerably in form through cell addition and changes in cell morphology and position. These can in turn impact on the intercellular signalling pathways dependent on diffusible or cell-surface molecules. In the mammalian cochlea, convergent-extension movements responsible for the elongation of the epithelium cause local cell rearrangements during cell differentiation (Yamamoto et al., 2009).

The situation is even more dynamic in the vestibular organs, where proliferation and HC production occur simultaneously, and for an extended period of time (Burns et al., 2012; Goodyear et al., 1999). Thus, lateral inhibition operates in a changing cellular environment, which could explain why direct contacts between immature and more mature HCs are frequently observed in developing sensory epithelia (Chrysostomou et al., 2012; Goodyear and Richardson, 1997). How then are HCs ultimately positioned to their right place? One solution, originally supported by computational modelling (Podgorski et al., 2007), could be some form of differential adhesion that would result in HCs repelling one another whilst adhering more strongly to SC. Strong candidates to mediate this function belong to the nectin family of immunoglobulin-like adhesion molecules. In the organ of Corti, nectin1 is expressed in HCs and binds strongly to nectin3, present in SCs; their inactivation in KO mice results in patterning errors, in particular in the outer HC region, but without significant changes in the number of HCs or SCs produced (Fukuda et al., 2014; Togashi et al., 2011).

Notch signalling may conversely, through some of its transcriptional targets, impact on cell proliferation. The expression of p27Kip1, which triggers the cell cycle exit of prosensory cells, is for example down-regulated in the *Jag1* cKO, in which IHCs and their SCs are produced in excess (Brooker, 2006). Likewise, the absence of *Hey/Hes* genes can result in increased cell proliferation in the organ of Corti (Tateya et al., 2011), although the mechanistic link between Notch and the cell cycle has not been established.

6. Notch signalling and hair cell regeneration

Hair cells are vulnerable to ototoxic drugs, acoustic trauma, and they spontaneously die during ageing. In mammals, the vestibular organs have a limited capacity for HC replacement, but the auditory HCs (approximately 15,000 in humans) are only produced during development. Their disappearance leads to irreversible hearing loss, for which the only available treatments are hearing aids or cochlear implants (reviewed in Géléoc and Holt, 2014). In contrast, non-mammalian vertebrates can regenerate HCs after damage throughout life. In the avian basilar papilla, a classic model for regeneration studies, SCs are normally quiescent but these are in fact 'tissue stem cells' that can after tissue damage i) transdifferentiate directly into HCs and ii) re-enter the cell cycle to produce new HCs and new SCs (reviewed in Rubel et al., 2013; Stone and Cotanche, 2007). Notch signalling is re-activated in the damaged basilar papilla during HC regeneration: Atoh1, DSL

ligands, and Notch effectors are upregulated one day after HC loss (Cafaro et al., 2007; Daudet et al., 2009; Stone and Rubel, 1999). Furthermore, blocking Notch activity with GSI during regeneration causes an upregulation of Atoh1 and excess transdifferentiation of SCs into HCs *in vitro* (Daudet et al., 2009; Lewis et al., 2012). This suggests that lateral inhibition regulates the regeneration of HCs in a very similar manner to what it does during their embryonic formation. But importantly, GSI do not induce spontaneous regeneration in an undamaged epithelium, which implies that Notch activity does not maintain SCs quiescent (Daudet et al., 2009). The situation differs in the chicken utricle, where the continuous turn-over of HCs is associated to mosaic Dll1 expression (Stone and Rubel, 1999). There, GSI or ADAM-10 metalloprotease inhibitors increase HC regeneration after damage, but also cause SC proliferation in the intact tissue (Warchol et al., 2017). Interestingly, GSI also stimulate SC proliferation after HC damage within the neuromasts of the zebrafish lateral line, another model for HC regeneration studies (Ma et al., 2008). However, we do not know for certain if these effects are due to inhibition of Notch activity: GSI and ADAM-10 metalloprotease inhibitors can interfere with the processing of other transmembrane molecules that could potentially impact on cell proliferation. Further studies relying on genetic manipulation of Notch activity will be required to clarify this point.

The limited regenerative potential of mammalian vestibular organs is improved by Notch inhibition

The adult mammalian vestibular system has a limited capacity for spontaneous HC regeneration (Forge et al., 1993; Warchol et al., 1993), which is thought to rely on transdifferentiation mainly (Rubel et al., 2013). This was recently confirmed using a genetic method for ablating utricular HCs and tracing the SCs: there is a very slow addition of new HCs (about 2 per week) in the adult mouse utricle, and SCs can convert into new HCs after extensive tissue damage (Bucks et al., 2017). We do not know which Notch receptors are expressed in the adult vestibular organs, but the utricle and cristae express at least Jag1 (Oesterle et al., 2008; Wang et al., 2010) and *Hes1/5* (Hartman et al., 2009; Slowik and Bermingham-McDonogh, 2013). Furthermore, Atoh1 is upregulated following ototoxic damage and interfering with Notch activity can improve the regenerative response of mammalian vestibular organs in vivo and in vitro (Jung et al., 2013; Lin et al., 2011; Slowik and Bermingham-McDonogh, 2013; Wang et al., 2010). However the capacity of SCs to convert to HCs in response to Notch inhibition remains very low in adult compared to neonate vestibular organs (Collado et al., 2011),

including in organotypic cultures derived from human tissue (Taylor et al., 2018). Combining Notch inhibition with overexpression of Atoh1 is not sufficient to induce robust HC regeneration either (Lin et al., 2011; Taylor et al., 2018), suggesting that other signals restrict the competence of vestibular SCs to transdifferentiate into HCs.

Notch activity is turned off in the adult organ of Corti: implications for regenerative therapies

Any report of successful auditory HC regeneration is invariably met, for good reasons, with a mix of excitement and scepticism. Where do we stand today with respect to the possibility to use Atoh1 or Notch-targeted therapies for hearing loss? The bulk of the evidence indicates that the capacity of Atoh1 to trigger ectopic HC differentiation (Zheng and Gao, 2000) is limited to stages when SCs are still relatively immature (Kelly et al., 2012; Liu et al., 2012b, 1); in the adult cochlea, there is no conclusive evidence that Atoh1 overexpression can lead to the regeneration of functional HCs (reviewed in Richardson and Atkinson, 2015). Likewise, the initial report that intra-cochlear administration of GSI may stimulate HC regeneration in adult mice exposed to acoustic trauma (Mizutari et al., 2013) has not been replicated so far. A major caveat with regeneration studies is the variability of HC death induced by ototoxic drugs or acoustic trauma: if HCs are observed several weeks after damage, are these surviving HCs or regenerated ones? One way to answer this question is to analyse tissue at different times post-treatment. If regeneration occurs, one should find HCs with immature features (e.g. short stereociliary bundles; expression of Atoh1) soon after damage, just as in the vestibular organs. None of the studies claiming successful regeneration of auditory HCs (in the adult epithelium) has provided such evidence. Finally, the expression of Notch components decreases rapidly during the maturation of the organ of Corti, as does the capacity of SCs to convert into HCs in response to GSI treatment (Maass et al., 2015; Maass et al., 2016). Even if a very low level of Notch activity was present in the adult damaged cochlea, it may not necessarily regulate HC regeneration. If Notch has context-dependent transcriptional targets and functions in the course of ear development, why should it be different at adult stages? The uncertainties about the molecular targets and therapeutic effects of GSI in the adult organ of Corti are valid reasons to advocate caution with regard to the ongoing clinical trials in hearing loss sufferers. The recent boom in

private and public investment for regenerative inner ear therapies (reviewed in Schilder et al., 2019) is welcome, and justified by the major economical and societal impact of hearing loss, but developing a successful cure will depend on our capacity to recognize (and fill) our gaps in the basic knowledge of HC development and regeneration.

7. Conclusion

Notch signalling has diverse and intertwined functions in the development and regeneration of the inner ear. As is the case in other tissues, the context-specific outcomes of Notch signalling in the ear are determined by the mode of regulation and signalling abilities of the DSL ligands but also, and crucially, by the competence state of the signalling cells, which changes as cells progress along their differentiation route. Understanding the genetic and epigenetic mechanisms responsible for these transitions could improve the prospect of regenerative therapies for inner ear disorders. Besides exploring the molecular interactions between Notch and the other major signalling pathways (FGF, Wnt, Hedgehog, etc), we need a deeper understanding of their integration with the cellular and physiological aspects of inner ear development. Whilst this entails significant intellectual and experimental challenges, the ongoing progress in single-cell and functional genomics, imaging, inner ear organoid cultures and computational modelling are opening up new and exciting venues for inner ear (and Notch) *aficionados*.

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FIGURES

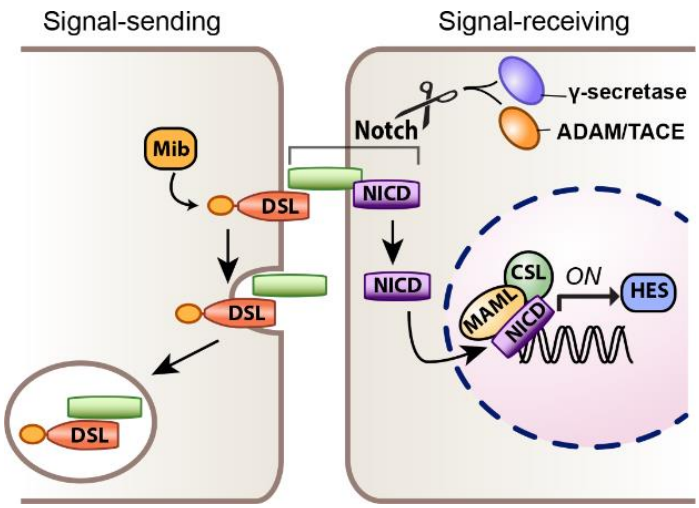

Figure 1: Notch signalling. The endocytosis of the DSL ligand, which depends on Mib/Neur activity, triggers the proteolytic cleavage of the Notch receptor in the signal-receiving cell. The Notch extracellular domain (in green) is internalized with the DSL ligand, whilst the NICD translocates to the nucleus and activates the expression of specific transcriptional target, such as those of the Hes family.

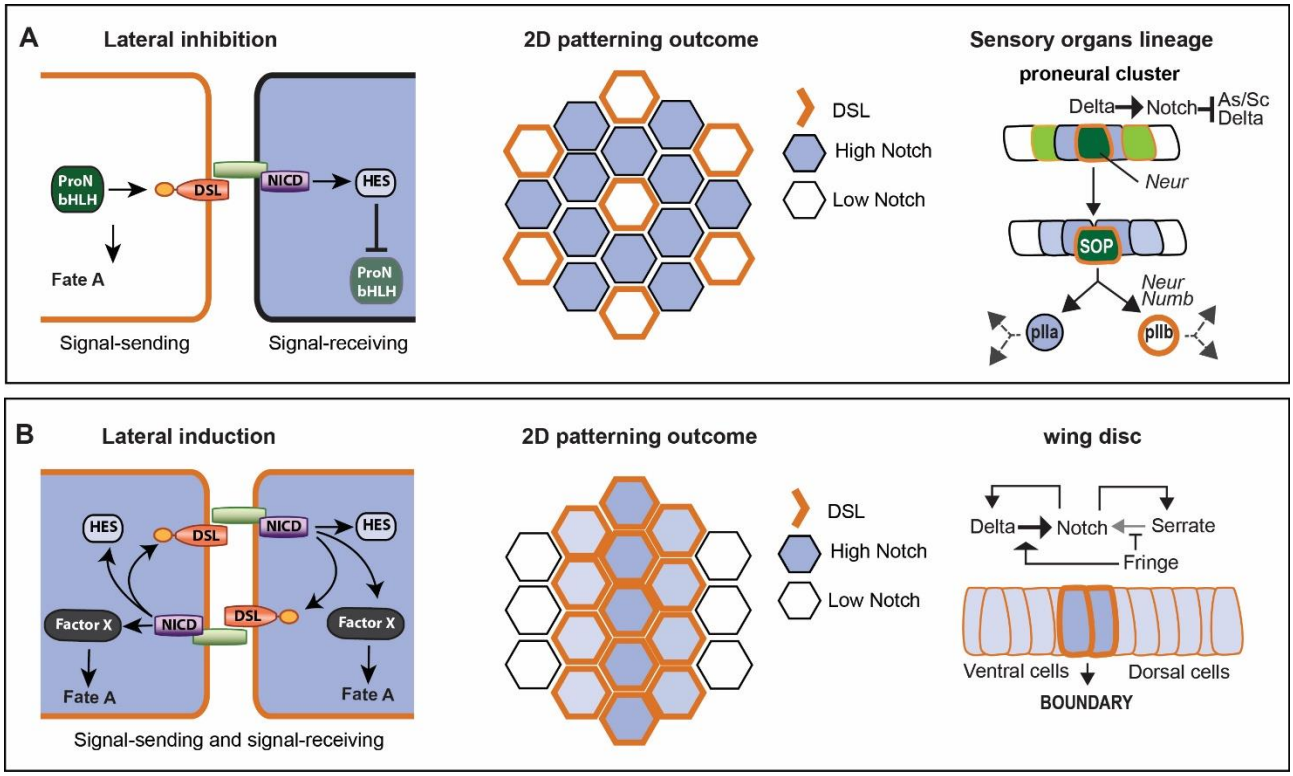

Figure 2: Two contrasting modes of Notch signalling. A. In lateral inhibition, a proneural bHLH factor induces DSL expression and its expression is repressed by Notch in the signal receiving cell. This results in a diversification of cell fates, or the formation of a salt-and-pepper cellular mosaic. In the proneural clusters giving rise to the adult mechanosensory bristles of drosophila, the proneural bHLH factor Achaete-Scute (As/Sc) is restricted by lateral inhibition to the sensory organ precursor cell (SOP). The SOP gains a strong advantage due to the expression of Neuralized (Neur). Lateral inhibition also operates in the progeny of the SOP and is biased by the differential distribution of Neur and Numb in one of the daughter cells after each round of cell division. **B.** In lateral induction, interacting cells are at the same time signal-receiving and signal-sending, and adopt the same character. In the drosophila wing disc, Delta and Serrate are regulated by lateral induction; Fringe, which is expressed in the dorsal compartment, enables strong Notch activation at the dorso-ventral boundary by reducing Serrate1/Notch and increasing Delta1/Notch signalling. In all diagrams, the arrows do not necessarily imply direct regulation or interaction.

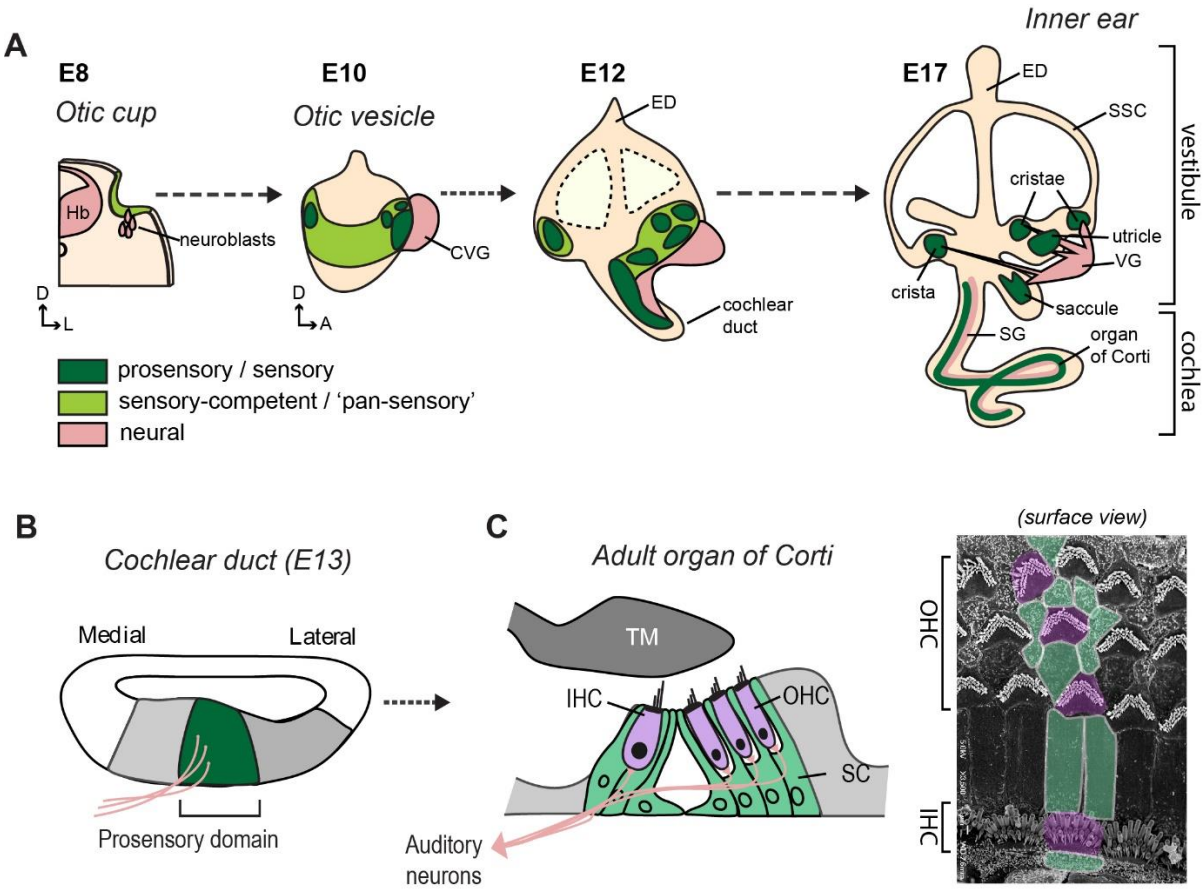

Figure 3: Development of the mouse inner ear. A. The otic cup invaginates in the underlying mesenchyme and closes into a vesicle, which then gives rise to the different structures of the inner ear. The neuroblasts delaminate from the anterior part of the otic placode/cup to form the CVG ganglion, from which the vestibular and auditory neurons innervating the HCs derive. The specification of the sensory organs is coupled to their progressive segregation from a large sensory-competent domain. By E17, the inner ear has reached an adult-like morphology. **B-C**. Transverse representation of the embryonic cochlear duct, with its prosensory domain, from which the adult SCs and HCs of the organ of Corti derive. The IHCs are the main sensory transducers and connected by the majority of nerve afferents, whilst the OHCs have electromotile properties essential for the sensitivity and frequency selectivity of the cochlea. Both types of HCs are interspaced by the cell bodies and apical surfaces of different types of specialized SCs. **D**. Scanning electron microscopy (courtesy of Andy Forge) view of the surface of the organ of Corti with is mosaic of HCs (purple) and SCs (green). The Abbreviations: E= embryonic day; Hb=hindbrain; CVG= cochlea-vestibular gangion; ED=endolymphatic duct; SSC=semi-circular canal; VG=vestibular ganglion; SG=spiral ganglion; SC=supporting cells; IHC= inner hair cells; OHC= outer hair cells; TM=tectorial membrane.

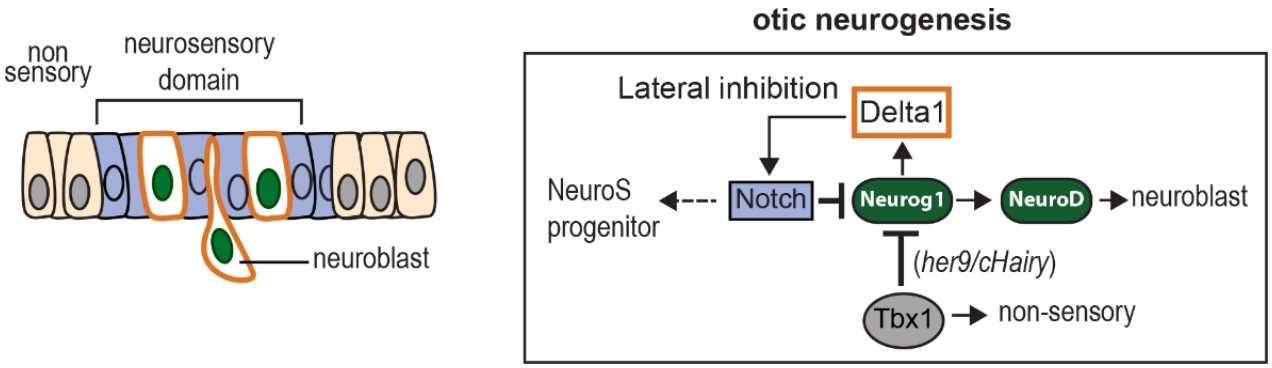

Figure 4: Lateral inhibition during otic neurogenesis. The neuroblasts delaminate from the neurosensory domain of the otic placode/vesicle and express Neurog1 and NeuroD. The signal-receiving cells remain as neurosensory competent progenitors. Tbx1 and some effectors of the Notch pathway antagonize Neurog1 outside of the neurosensory domain.

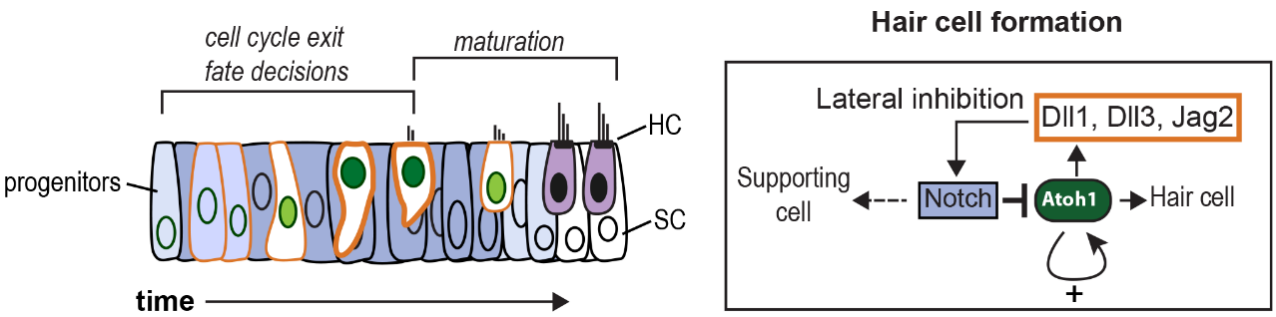

Figure 5: Lateral inhibition during hair cell formation. Left panel: schematic representation of the changes in the expression of Atoh1 (in green), DSL ligands (orange) and levels of Notch activity (blue) in the course of HC formation are represented. After cell cycle exit, nascent HCs up-regulate Atoh1 and several DSL, driving high Notch activity in prosensory domains. Following commitment to the HC fate, cells down-regulate Atoh1 and DSL expression; Notch activity decreases in the mature sensory epithelium. The right panel represents the basic regulatory circuit during the lateral inhibition of HC formation. The autoregulatory feedback loop controlling Atoh1 could lead to a rapid elevation of DSL expression levels, ensuring robust lateral inhibition.

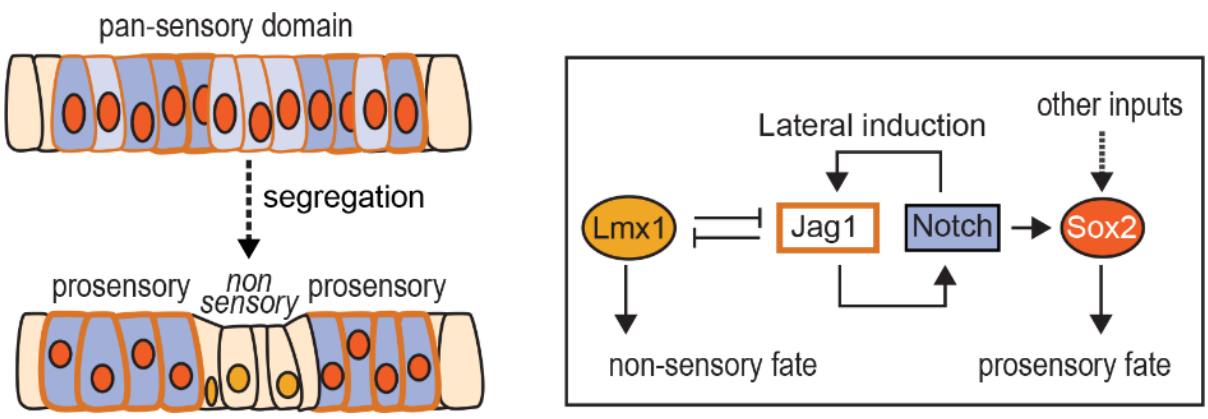

Figure 6: Lateral induction during prosensory specification and a hypothetical model for sensory organ segregation. Left panel: Jag1 and Sox2 are initially expressed in a continuous 'pan-sensory' domain, but their expression is downregulated in prospective non-sensory territories during sensory organ segregation. The upregulation of Lmx1a could contribute to a reduction of Notch activity and Sox2 expression during the separation of sensory organs. Right panel: hypothetic regulatory circuit linking Notch activity, Lmx1 and Sox2 expression. The arrows do not imply direct regulation or interaction.