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Notch signaling during cell fate determination in the inner ear

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Abstract

In the inner ear, Notch signaling has been proposed to specify the sensory regions, as well as regulate the differentiation of hair cells and supporting cell within those regions. In addition, Notch plays an important role in otic neurogenesis, by determining which cells differentiate as neurons, sensory cells and non-sensory cells. Here, I review the evidence for the complex and myriad roles Notch participates in during inner ear development. A particular challenge for those studying ear development and Notch is to decipher how activation of a single pathway can lead to different outcomes within the ear, which may include changes in the intrinsic properties of the cell, Notch modulation, and potential non-canonical pathways.

Keywords

Notch; inner ear; hair cell; cochlea; otic vesicle; Jagged

1. Introduction

The inner ear is an intricate organ designed to transmit both auditory and balance information. The development of the inner ear involves dramatic morphogenetic and patterning events which mold its shape from a simple thickened epithelium or placode, to a complex membranous labyrinth complete with semicircular canals, a coiled cochlea, and six sensory areas innervated by afferent neurons that also derive from the placode. In mammals there is one auditory organ (the organ of Corti or oC) and five vestibular regions required for detecting both linear (maculae) and rotational movements (cristae of the semicircular canals). Each sensory organ is composed of two basic cell types, the sensory hair cell and its associated supporting cells arranged in a highly organized mosaic in which each hair cell is surrounded by supporting cells. Based on this arrangement and its similarity to sensory bristle patterning observed on the body of *Drosophila melanogaster*, it was proposed that patterning of the sensory regions could be achieved through cell-cell interactions mediated by the Notch signaling pathway [1, 2]. Studies since that time have largely confirmed this hypothesis as well as uncovered new roles for Notch during inner ear development. Here, I will review the evidence for the different roles of Notch in the inner ear.

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Notch receptors are single pass transmembrane proteins that are central components of a core signaling pathway that is highly conserved across all metazoan species. Because the ligands are also membrane bound, Notch mediates juxtacrine signaling (signaling between neighboring cells). Notch regulates the development of most organs and tissues, and the adult maintenance of stem cells and tissues. Mutations in the core pathway can lead to developmental disorders, adult diseases, and cancer [3]. In the inner ear, evidence has accumulated that Notch plays multiple roles during otic development including the generation of essential cell types including hair cells, supporting cells and neurons.

1.1. Canonical Notch signaling

The Notch receptor is activated by membrane-bound ligands that are part of the DSL family (Delta-like/Jagged in mammals, delta/serrate in *Drosophila melanogaster*, Lag-2 in *Caenorhabditis elegans*). In mammals, there are five canonical ligands, Jagged (Jag)1–2 and Delta-like (Dll)1, 3 and 4 and four different receptors (Notch1–4). The Notch receptor is first processed into its mature form via cleavage by a furin-like protease at S1 in the trans-Golgi to generate a non-covalently associated heterodimer at the cell surface. Activation of Notch involves two subsequent proteolytic cleavage events that are initiated by ligand binding (Fig. 1). Although not entirely understood, activation of Notch also requires that the ligands are ubiquitinated by the E3 ubiquitin ligases Mindbomb or Neuralized to promote endocytosis of the ligand. Binding of the ligand to the Notch receptor allows for the S2 cleavage by the ADAM family of metalloproteases. This cleavage releases the Notch extracellular domain (NECD) and promotes the cleavage of the intracellular portion of the Notch receptor by the γ -secretase complex at S3. This produces the active form of Notch, the Notch intracellular domain (NICD), which can translocate to the nucleus and interact with the core effector of the canonical pathway, CSL (CBF1/RBPJ κ /mammal; Suppressor of Hairless/fly; and Lag-1/nematode). In the absence of NICD, CSL acts as a transcriptional repressor. In the presence of NICD and other co-activators such as MAML, CSL is converted to a transcriptional activator (Fig. 1) [4–6].

2. Lateral inhibition I: Hair cells vs. Supporting Cells

A role for Notch signaling in the inner ear was first proposed more than 20 years ago [1]. Based largely on the similarities of the hair cell/supporting cell mosaic to the sensory bristles on the fly's body [2], Lewis proposed that Notch signaling could generate the sensory mosaic via the classical patterning mechanism known as lateral inhibition. In its simplest sense, the idea of lateral inhibition is that a cell differentiating along a certain path can inhibit its neighbors from adopting the same cell fate [7], thereby creating an alternating pattern of cell fates. This process was well studied in the *Drosophila* epidermis, where it was demonstrated that Notch signaling mediates lateral inhibition to generate the bristle patterns on the fly integument. The mechanosensory bristles, called macrochaetes, are derived from a sensory organ precursor (SOP). SOPs are initially selected from 'proneural' clusters of cells that all have the capacity to differentiate as SOPs via their proneural gene expression. The proneural genes include certain members of the basic helix-loop-helix (bHLH) family of transcription factors, such as members of the *Achaete Scute Complex* (AS-C) and *atonal* (*ato*). Because all the cells within the proneural clusters express the proneural gene and have the capacity to become SOPs, these cells can be considered part of an 'equivalence group'. The proneural genes upregulate Notch ligand expression, activate Notch, which then acts to restrict the number of cells that can adopt an SOP fate through inhibition of the proneural genes in all but one of the cells [8]. This inhibition is achieved through a negative feedback system, in which cells expressing activated Notch downregulate the proneural gene and Notch ligand. Thus, one cell begins to emerge that has higher levels proneural gene and Notch ligand and surrounding cells have high levels of activated Notch. This effect, which initially may be small, is then amplified because cells having higher levels of activated

Notch express lower levels of the proneural gene and less Notch ligand, rendering these cells less able to signal back. Eventually, the cell expressing high levels of proneural genes and Notch ligand differentiates as an SOP, whereas the surrounding cells that have high levels of active Notch differentiate into the secondary cell fate (Fig. 2A).

Support for the hypothesis that Notch signaling mediates cell fate decisions during hair cell and supporting cell differentiation first came from expression studies in the chick, mouse and zebrafish, which demonstrated that Notch ligands are expressed in nascent differentiating hair cells, whereas the Notch receptor expression was more widespread [9–13]. Within the cochlea in particular, expression of *Dll1* and *Jag2* ligands were initially only observed in a single row medially at E14.5, presumably corresponding to the inner hair cells, and then expression expanded laterally into the outer hair cell rows, reflecting the pattern of differentiation [11, 13]. Expression of the Notch ligands in developing hair cells is transient, lasting only a few days, indicating that these ligands are required to initiate correct differentiation but not necessary to maintain it [9, 10]. These expression patterns largely recapitulate what would be expected if Notch mediates lateral inhibition in the sensory regions, similar to the *Drosophila* mechanosensory bristles. One noted difference however, is that in most instances the ligands are not initially expressed uniformly, as in *Drosophila* proneural clusters, suggesting the feedback mechanism in which a single cell is selected amongst the equivalence group may not be necessary. This raises the question as to how ligand expression is initially restricted to developing hair cells.

Perturbational studies in chick, zebrafish, and mouse clearly demonstrate a role for Notch-mediated lateral inhibition in determining hair cell/supporting cell fates. Similar to *Drosophila*, the predicted outcome for disrupting Notch signaling would be an increase in the default cell fate (hair cells) at the expense of the alternative cell fate (supporting cells), known from *Drosophila* studies as a ‘neurogenic’ phenotype (Fig. 2A). Such phenotypes have been demonstrated in mouse and zebrafish after disruptions are made to Notch signaling components [10, 12–15]. Similarly it is predicted that overexpression of activated Notch would impair hair cell formation and lead to an overproduction of supporting cells. This was demonstrated in the chick by electroporation of activated Notch1 (NICD1) into the sensory regions of the ear, resulting in patches of supporting cells within the sensory domains where NICD1 was expressed [16].

In the mouse, deletion of single Notch ligands initially led to a confusing phenotype [13, 14]. For example, individual deletion of *Jag2* or *Dll1* function results in an increase in the number of hair cell rows that form in the cochlea [13, 14]. Although these disruptions to Notch signaling led to an increase in hair cells, the mosaic nature of the epithelium was maintained and supporting cells were not lost, indicating that perhaps lateral inhibition was not the patterning mechanism. However, mutants that have reductions in both *Dll1* and *Jag2* gene dosage display clear loss of supporting cells within the oC and a more robust increase in the number of hair cells within the oC, although these mutants still display increases in the number of rows (Fig. 2Aiii) [15]. One possible explanation for the extra rows is that, if it is assumed that the prosensory region is actually larger than the oC region (and *Sox2* expression suggests that it is [17]), then cells at the margins of the oC may be more sensitive to loss of Notch signaling given that they effectively receive less inhibition than supporting cells directly within the oC (Fig. 3). As demonstrated in Fig. 3, supporting cells within the oC receive Notch inhibitory signals from all surrounding hair cells, but the ones at the borders of the oC receive inhibitory signaling from only one side, and thus may be more sensitive to changes in Notch dosage. Therefore, the predicted result of a mild disruption in Notch signaling would be that supporting cell/prosensory cells at the border would covert to hair cells, giving the appearance of extra rows. With severe disruptions in Notch signaling, supporting cells within the oC proper would convert to hair cells, giving a more traditional

lateral inhibition phenotype. However, it was interesting that even with severe disruptions in Notch the supporting cell losses were modest compared to the hair cell increases, raising the possibility that Notch also plays a role in proliferation. This possibility was examined in *Dll1/Jag2* mutants by labeling cells with BrdU during a period in which the oC was expected to be non-proliferative. No increases in the number of labeled hair cells was found, indicating the extra hair cells were not generated through ongoing proliferation [15], and likely arose through supporting cell to hair cell conversion. However, an increase in the number of labeled supporting cells was observed, suggesting that Notch may play a role, either directly or indirectly, in supporting cell proliferation as well as cell fate decisions.

2.1 Downstream targets of Notch during hair cell/supporting cell differentiation

In *Drosophila*, products of the *Hairy* and *Enhancer-of-split* genes are direct downstream targets of Notch/CSL and function to inhibit proneural gene expression. The Hes/Hey family of bHLH transcription factors are the vertebrate homologs of *Hairy* and *Enhancer-of-split* genes and appear to function in a similar manner [18]. Therefore, members of the Hes/Hey family are good candidates for effectors of lateral inhibition in the ear by inhibiting proneural genes (such as *Atoh1*) in differentiating supporting cells. In support of this, a number of studies have demonstrated that several Hes/Hey family members are expressed in the expected cell types (supporting cells), and *Hes/Hey* deletions lead to an overproduction of hair cells—as would be expected if they are functioning downstream of Notch [19–22]. However, several interesting aspects have emerged from these studies. First, different Hes family members are expressed in different domains of the cochlea [23] and seem to regulate different supporting cell populations. For example, inactivation of *Hes1* primarily leads to an overproduction of inner hair cells whereas deletion of *Hes5* causes an overproduction of outer hair cells [20]. However, when both genes are inactivated, a more robust increase in hair cells is observed in both domains, indicating there is some redundancy in their function [20]. Second, deletion of individual Hes members have relatively mild effects on hair cell production when compared to deletions in the core Notch pathway. Deletion of three Hes family genes (*Hes1*, *Hes5*, and *Hey1*) leads to a graded increase in numbers of hair cells that roughly corresponds to the number of Hes/Hey alleles that are inactivated. However, even in the triple mutant, relatively mild increases in hair cell production were observed [22]. For example, *Notch1* mutants have an almost 4-fold increase in hair cells [15], whereas triple *Hes1;Hes5;Hey1* mutants show less than a 2-fold increase in hair cells [22]. These data suggest that either (1) there are numerous Hes/Hey family members downstream of Notch that each exert small inhibitory effects, or (2) there are other factors downstream of Notch that play an important role as effectors in lateral inhibition.

3. Lateral Induction: Specifying the Sensory Progenitors

In addition to lateral inhibition, Notch also mediates lateral induction, which is important for the production of sensory progenitors in the inner ear. Although less well understood, the mechanism of inductive signaling differs intrinsically from lateral inhibition, in that it refers to the induction of a new cell type, as opposed to restriction of cell fate, and typically involves positive feedback rather than negative feedback (Fig. 2B) [2]. In *Drosophila*, this type of signaling occurs during wing development, in which Serrate/Jagged is expressed in dorsal cells and Delta is expressed in the ventral wing cells. Notch is expressed throughout the wing but expression of a Notch modulator (Fringe) restricts Notch activation to the wing margin, directly promoting the expression of *vestigial*, a transcriptional co-activator required for wing development and outgrowth [24, 25].

While there are marked differences in Jag1, Fringe and Dll1 expression patterns in the ear when compared to the *Drosophila* wing, the early and widespread expression of the Jag1 (Ser1) ligand suggests an inductive role for Jag1-Notch signaling in sensory formation. In

both chick and mouse, *Jag1* is expressed throughout the sensory patches from early points in development [9, 11, 26]. This contrasts with the salt and pepper expression pattern of *Dll1* and *Jag2* in the sensory regions just prior to differentiation [9, 11, 13], which is more consistent with lateral inhibition. Moreover, activation of Notch leads to upregulation of *Jag1* [2, 16, 27, 28] indicating positive rather than negative feedback, similar to the *Drosophila* wing margin.

Evidence for an inductive role for *Jag1*-mediated Notch signaling came from *Jag1* knockouts, which result in inner ears with very little sensory development, including loss of all cristae; severely reduced utricular maculae; cochleae lacking hair cells and supporting cells in the basal regions; and reduced hair cell rows in the rest of the oC [14, 29]. Because loss of both hair cells and supporting cells was observed, these data indicated that *Jag1* plays an important role in the development of the sensory progenitors, which give rise to both cell types [30]. In support of this conclusion, markers of the sensory progenitors such as *Sox2* [17] and *p27^{Kip1}* [31] showed reduced expression, indicating that sensory progenitors are either not maintained or not specified in these mutants. As sensory markers are reduced or lost at otocyst stages in *Jag1* mutants prior to any differences observed in cell proliferation or cell death [27], *Jag1*-mediated Notch signaling is likely required for sensory progenitor specification.

Strong evidence for Notch's role in sensory progenitor specification came from overexpression studies in the mouse and chick demonstrating that ectopic activation of Notch can induce ectopic sensory regions to form in non-sensory regions of the ear [16, 27, 28]. In the chick, transfection of *NICD1* results in ectopic sensory regions (ESRs) composed of hair cells and supporting cells in both vestibular and auditory regions. In the mouse, constitutive or transient activation of *NICD1* also results in ectopic hair cell and supporting cell clusters in the vestibular and cochlea regions [27, 28]. In some cases *NICD1* activation led to an outpocketing in the epithelium, resembling an ectopic sensory organ, complete with sensory (*NICD*-expressing) and non-sensory (*NICD*-nonexpressing) regions [16, 27]. These results are consistent with the idea that sensory regions can direct the outgrowth of non-sensory regions [27, 32]. Taken together, both loss and gain of function results indicate that *Jag1*-mediated Notch signaling operates in a lateral inductive manner to specify sensory regions.

3.1. Conflicting results: Phenotype of *CSL/RBPJ κ* mutants

Although the phenotypes of both the *Jag1* loss of function alleles and *NICD* overexpression support a role for Notch in sensory specification, results from ear-specific deletions of the Notch canonical effector, *CSL/RBPJ κ* , call this interpretation into question. Although it would be expected that the *CSL/RBPJ κ* would largely phenocopy the *Jag1* mutants or show even more severe defects, one study demonstrates that prosensory markers such as *p27^{Kip1}* and *Sox2* were expressed throughout the early developing cochlea, indicating that sensory progenitors are established in the cochlea of these mutants [33], although many of the vestibular defects were similar. Furthermore, during differentiation in the cochlea there was increased cell death in the sensory domains, suggesting that sensory cells are lost through failed survival rather than a failure of specification [33]. A similar study using a different deletion paradigm showed a more severe phenotype in which hair cells are only present in the very apical regions of the cochlea. Examination of prosensory markers in this study showed an absence of prosensory markers in the basal regions of the cochlea, similar to the *Jag1* mutant, indicating sensory specification is partially impaired [34].

One intriguing possibility that may reconcile these different results is that the inductive role of *Jag1* in sensory specification is non-canonical and does not require *CSL/RBPJ κ* function. Supporting this possibility, in *Drosophila* eye development the proneural functions of Notch

that promote *ato* expression are CSL-independent, while the subsequent lateral inhibitory role of Notch in restricting the R8 cell fate is CSL-dependent [35]. Unfortunately the non-canonical pathways are not well understood, although there is mounting evidence for a strong interaction with the Wnt signaling pathway [36]. Interestingly, ectopic activation of β -catenin, an integral component of the Wnt pathway, results in ectopic sensory regions in the chick, similar to activation of the Notch pathway [37]. Further studies are required to determine whether there is a non-canonical role for Notch signaling in sensory specification, and whether this pathway involves interactions with the Wnt pathway.

However, given the overall phenotypic similarities of the *CSL/RBPJ κ* mutation and the *Jag1* mutants, it seems unlikely that these defects arise from different mechanisms. Thus, another possibility is that while Notch is the main effector of sensory specification, other signaling pathways also contribute. Based on loss or gain of function experiments that perturb sensory development, the possibilities include Wnt [37–39], Bmp [40, 41] and Fgf [42–44] signaling [45].

3.2. Downstream effectors of Notch in lateral induction

Based on their temporal and spatial domains of expression, members of the Hes-related family, Hey, including Hey1, Hey2, and HeyL are likely candidates for mediating downstream effects of Jag1-Notch signaling during sensory specification [46, 47]. *Hey1* in particular demonstrates a similar expression pattern to *Jag1* and other sensory markers at E10.5 in the mouse otocyst [46], and loss of Jag1 function leads to a much smaller *Hey1* domain of expression [27]. Both *Hey1* and *Hey2* are expressed in the prosensory region of the cochlear duct by E12.5, a time period that is consistent with sensory specification [47]. Notch inhibition via DAPT-treatment in cochlear cultures abolishes the prosensory domain of the cochlea in the base, and is accompanied by a downregulation of *Hey1* and 2. In the ear, loss of *Hey1* presents no obvious phenotype, although in combination with the *Hes1* and *Hes5* mutations hair cell numbers increase, indicating HEY1 plays a role in lateral inhibition [22]. As yet, there is no evidence that any of the HES-related genes play a role in the lateral induction/sensory specification functions of Notch, although firm conclusions about the role of the Hey genes will need to be tested by deleting combinations of the *Hey* knockouts in the ear, as these family members play redundant functions in other systems [48–50].

Another possibility is that the main effectors of Jag1-mediated Notch signaling in sensory specification are non-Hes related genes. Given that lateral induction produces new tissue types or organs, non-Hes related genes may be more likely candidates given that Hes genes function as repressors [18], and it seems unlikely that new tissues could be produced from repression alone. In support of this, Notch activation in the *Drosophila* wing margin results directly in the expression of the gene *vestigial*, which promotes wing development and outgrowth [51, 52]. A strong potential Notch target during sensory induction is the SRY transcription factor Sox2 [53]. Loss of function alleles of Sox2 phenocopy the Jag1-deficient phenotype, in that there is loss of both hair cells and supporting cells, and prosensory marker expression is absent at early time points [17]. Moreover, activation of Notch in non-sensory regions of the inner ear leads to a rapid upregulation of Sox2 [27, 54, 55]. There is also evidence that *CSL/RBPJ κ* directly regulates Sox2 in the nervous system [56]. In the chick, ectopic expression of Sox2 induces sensory formation in non-sensory regions of the ear, similar to Jag1/Notch [57]. Taken together, these data support the hypothesis that Notch activates sensory formation via Sox2.

Fgf signaling may also lie downstream of Notch activity. The importance of Fgf signaling in sensory formation was first revealed by deletion of the fibroblast growth factor receptor 1 (Fgfr1) in the ear [42]. The phenotype in the Fgfr1-deficient cochlea resembled the *Jag1* and *Sox2* loss of function mutations, in that there were areas of missing sensory cells and islands

of hair cells and supporting cells. Subsequent studies have identified the likely ligand for this receptor as Fgf20 [43, 44]. What is currently unclear is whether Fgf signaling is required for specification or differentiation. Huh and colleagues [44] suggest it is required for differentiation as Sox2-positive cells were observed in the regions between the hair cell-containing clumps, indicating these cells are specified but cannot further differentiate. This study further suggested Fgf20 is specifically required for the differentiation of the lateral compartment of the cochlea (which includes OHCs and Deiters' cells), as inner hair cell numbers are maintained in the Fgf20 knockout [44]. However, although not quantified, very few inner hair cells appear present in the conditional Fgfr1 knockout, indicating that Fgf signaling may be required for inner hair cell differentiation as well, although further analysis is required to establish whether this is the case. In vitro rescue experiments indicate that FGF signaling is downstream of Notch, as disruption of prosensory development by the Notch inhibitor γ -secretase can be partially rescued by exogenous application of Fgf20 [58]. Interestingly, exogenous application of Fgf20 also restores Sox2 expression indicating that Fgf can independently control Sox2 expression [58].

One interesting aspect of these studies is that, unlike the *Jag1* or *Sox2* mutations, deletions of *Fgf20* and *Fgfr1* do not affect the vestibular system, raising the question as to whether Fgf signaling is a general factor required for the differentiation of all sensory areas, or is a factor specifically required in the cochlea. Intriguingly, there is some evidence that other Fgf ligands, such as Fgf10 and Fgf3, may play a role in vestibular sensory area differentiation. However, given the roles of these ligands in other aspects of inner ear development, such as placode induction and semicircular canal formation, a more targeted approach will need to be taken to determine whether they play a role in vestibular sensory formation [59, 60].

4. Lateral inhibition II: Neurons vs. Sensory Cells

In addition to sensory cell formation and differentiation, there is also evidence that Notch is involved in the production of the neural components of the ear. The neurons that innervate the hair cells of the ear arise from an anterior medial compartment of the otocyst that largely overlaps with an anterior sensory domain demarcated by *Jag1* and *Sox2* expression [26, 27]. In the mouse inner ear, neurogenesis begins about E9 and continues through about E14.5, with a peak of neuron production around E10 [61]. While hair cell production requires *Atoh1* [62], neurogenesis requires the basic helix-loop-helix transcription factor *Ngn1* [63]. Loss of function mutations suggest that Notch restricts the numbers of cells that can adopt a neural fate through lateral inhibition, similar to its later role in sensory cell differentiation [10, 14]. Fate mapping and lineage tracing experiments indicate that neurons and utricular/saccular sensory cells arise from the same region of the otocyst (anteriomedial) and can share a common progenitor [61, 64]. These results suggest that cells inhibited from adopting the neural fate by Notch signaling will instead adopt a sensory fate in the vestibular macula, or a non-sensory fate in the surrounding regions. Based on expression, the ligand that mediates these effects in mammals is *Dll1* [9]. *Dll1* expression is lost in *Ngn1* mutants, indicating that *Ngn1* promotes the expression of *Dll1*, whereas Notch activation leads to the downregulation of *Ngn1*, providing a negative feedback loop to instruct cell fate [11, 63]. Mutations in *Dll1* support these findings, as loss of *Dll1* led to an increased size of the ganglion, with severe effects on the development of the maculae [14]. These data suggest that, in the absence of *Dll1*-mediated Notch signaling, cells are diverted from an epithelial fate to a neuronal fate. This is an intriguing result, as in *Jag1* mutants, the maculae (particularly the saccular macula) are the least affected sensory organs [14, 29], indicating that *Dll1*-mediated Notch activation may play an important role in the specification of these organs.

5. Modulators of Notch Signaling in the Ear

Although the core signal transduction pathway is fairly straightforward, Notch signaling can be modified in a number of different ways, many of which we do not fully understand. Given that Notch plays numerous and complex roles in the ear, it is likely that the details of these modulators are important in our understanding as to how Notch signaling can lead to different outcomes during inner ear development. Here, I will review some of the newly emerging concepts in Notch modulation and the roles that they play during ear development.

5.1. Glycosylation of the Notch receptor

One major way that signaling can be modified is through *O*-glycosylation of the extracellular EGF domains of the Notch receptor [65, 66]. At least three different enzymes have been implicated in glycosylation of Notch, protein *O*-fucosyltransferase-1 (Pofut1), protein *O*-glucosyltransferase (Poglut/*Rumi*), and the Fringe proteins, which are β 1-3*N*-acetylglucosaminyltransferases. Pofut1 adds *O*-fucose to the Notch EGF repeats and is thought to be universally required for Notch signaling [67]. More recently, the gene *rumi* was identified in a *Drosophila* screen for modifiers of Notch signaling [68] and found to encode the Poglut protein which adds *O*-glucose to the EGF repeats of the Notch protein. The phenotype of the mouse *Rumi* deletion is quite severe, with embryonic lethality before E9.5, indicating it may have functions outside of Notch signaling [69]. The third enzyme family involved in Notch modulation is the Fringe family, which was identified a number of years ago in *Drosophila* because the wing defects resembled Notch mutations [70]. Identification of the protein revealed that it is a glycosyltransferase that extends the *O*-fucose glycans on the Notch receptors [71]. Functional analysis indicates that the role of Fringe proteins is to modulate the ligand/receptor interaction, such that it may enhance the effects of certain ligands, while inhibiting others [65, 72]. While there is only one Fringe protein in *Drosophila*, there are three Fringe proteins in mammals, including Lunatic Fringe (Lfng), Manic Fringe (Mfng) and Radial Fringe (Rfng).

Evidence of a role for these modulators during ear development is variable. For example, although the Pofut protein is thought to be universally required for Notch signaling, removal of this gene in the mouse leads only to a lateral inhibition-type phenotype in the ear (*i.e.*, more hair cells) [33]. As yet, inner ear phenotypes resulting from a deletion of *rumi* are unknown. The role of the Fringe proteins are particularly intriguing as *Lfng* is expressed early in the sensory/neural aspect of the otocyst, and later is expressed in the macular sensory organs and oC [26, 73], indicating it may play a role in different aspects of sensory/neural development. However, deletion of *Lfng* in the mouse displays a subtle phenotype in the ear that is only revealed when simultaneously deleting the *Jag2* gene [74], indicating it plays a minor role in lateral inhibition. Uncovering the full role for Fringe expression in the ear may not be revealed until deletions of several, or all three are achieved, although experiments to date have revealed little redundancy between Fringe proteins [75].

5.2. Ligand endocytosis

Another type of modulation involves endocytosis in the signal-sending cell. The role of endocytosis has emerged because a number of mutations that affect the core endocytotic pathway also led to neurogenic phenotypes resembling Notch defects [76–78]. The requirement for endocytosis in the signal-sending cell remains unclear at present, but there are two main theories as to why this step may be important: (1) the ligand must be recycled via endocytosis into an activated form that can bind Notch, or (2) endocytosis provides a “pulling force” that allows the S2 cleavage region of the Notch protein to be accessed [6]. At present it is unclear which model is correct, and both events may be important for effective signaling. Ubiquitylation of the ligand by the ubiquitin ligases, Mindbomb (*Mib*) or

Neuralized (*Neur*) is a critical step in the endocytosis process [79]. In zebrafish, the *Mib* mutation has demonstrated that endocytosis is an important requirement for effective lateral inhibition in both the production of neurons and hair cells (see above [10]). Interestingly, the analysis of *Mib* to date has not revealed a requirement for endocytosis in lateral induction [10]. However, a number of different homologs of *Mib* and *Neur* exist in mammals, so further analysis is required to determine their roles in different aspects of Notch signaling in the ear.

5.3 Cis-inhibition: a role in the ear?

In addition to the canonical *trans* interaction between ligand and receptor between two cells, there is emerging evidence for a new type of ligand/receptor interaction, termed *cis*-inhibition [80–82]. This type of interaction is cell autonomous, as it involves the ligand and receptor on the same cell (Fig. 1). Although the details of the interaction are not yet known, the current model is that a Notch ligand will bind to the receptor on the same cell, thereby preventing interactions in *trans* and inhibiting NICD production. Because the interaction is essentially a titration effect, this type of signaling would be expected to be sensitive to gene dosage. *Cis*-inhibition was initially observed in the *Drosophila* wing margin [83–85]. Recent mathematical modeling and some experimental studies have suggested at least two roles for this type of ligand/receptor interaction [80]. In the case of lateral inhibition, this interaction would lead to fewer errors than those produced through the typical transcriptional feedback mechanism [86]. In the case of lateral induction, *cis*-inhibition could create a sharp boundary in response to graded ligand expression [87]. In the ear, *cis*-inhibition was recently examined during lateral inhibition in the chicken [88]. The study used two different methods to overexpress Dll1 to reveal whether a *cis*-inhibition mechanism was at work, or a more traditional transcriptional feedback mechanism. In the case of *cis*-inhibition, continuous Dll1 signaling would be expected to interfere with *trans* signaling, resulting in reduced Notch activation and increased numbers of hair cells. In the case of a traditional feedback mechanism, continuous Dll1 signaling would result in widespread NICD activation and reduced numbers of hair cells. The authors observed widespread NICD activation and fewer hair cells [88], arguing against a *cis*-inhibition mechanism during Notch-mediated lateral inhibition in sensory cell differentiation. However, the role of *cis*-inhibition has yet to be investigated during other aspects of Notch signaling, including lateral induction.

5.4 Notch-Wnt interactions in the inner ear

There is growing evidence and interest in the non-canonical pathways of Notch signaling. Although a number of reports suggest that non-canonical signaling may be at work in different contexts [36], the molecular details of this type of signaling are still unclear. The numerous interactions between Wnt and Notch pathways are an important exception, so much so that this pathway crosstalk has been termed ‘Wntch’ signaling [89]. These interactions differ based on context, and Notch appears to both synergize with Wnt signaling in some situations, while antagonizing it in others. In general, synergistic interactions tend to be canonical (CSL-dependent) while antagonistic interactions are generally non-canonical (CSL-independent) [36]. These interactions have been investigated during otic placode development. Results of this study demonstrated that Notch augments Wnt signaling in determining the size of the otic placode [90]. Although the details of how this is accomplished are not yet clear, the authors propose that Wnt is important in initiating Jag1 expression, thereby inducing Notch activation that positively feeds back upon the Wnt pathway [90]. Further studies are required to examine whether these two pathways also interact during sensory specification or differentiation events.

6. Conclusions

Notch signaling is a complex pathway that is used iteratively during inner ear development to determine the fate of key cell types, including neurons, hair cells and supporting cells (Fig. 4). Early in inner ear development, Notch augments the Wnt pathway to determine placode size. Subsequently, Notch mediates lateral inhibition in the anteromedial aspect of the otocyst to determine which cells will differentiate as neurons. Concurrently or slightly later, Notch mediates lateral induction via the Jag1 ligand to specify the sensory progenitors. Finally, within those sensory regions, Notch mediates lateral inhibition once again to determine which cells differentiate as hair cells and supporting cells. The challenge for the future is to determine how Notch mediates different outcomes in different developmental contexts. Although the core pathway is fairly straightforward, it is clear that Notch is modulated in many different ways. An emerging idea is that chromatin modification may be an important feature [91]. As our understanding of Notch modulation increases, so will our understanding of how the pathway functions in different contexts. Particularly important will be establishing the pathways downstream of Notch, especially those involved in lateral induction, which is currently much less well understood than its role in lateral inhibition.

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Highlights

- The role of Notch signaling in mediating hair cell and supporting cell differentiation in the inner ear
- The role of Notch signaling in specifying the sensory cell progenitors via lateral induction
- Potential downstream effectors of Notch involved in both lateral inhibition and lateral induction
- The function of Notch signaling in establishing the neural components of the ear
- Modulators of Notch signaling and their role during inner ear development

Figure 1.

Overview of Notch signaling. (i) Canonical Notch activation involving transactivation. Cleavage at the S1 site (not shown) processes the Notch protein (blue) into its mature form via cleavage by a furin-like protease in the trans-Golgi to generate a non-covalently associated heterodimer at the cell surface. DSL ligand (pink) on a the signal sending cell binds to the Notch receptor on the signal receiving cell and initiates the S2 cleavage event releasing the Notch Extracellular Domain (NECD). This cleavage event requires ligand endocytosis involving ubiquination mediated by E3 ubiquitin ligases Mindbomb (*Mib*) or Neuralized (*Neur*). The S3 cleavage event mediated by the γ -secretase complex liberates the Notch Intracellular Domain (NICD) from the membrane, allowing it to translocate to the nucleus where it, along with other co-activators, displace the co-repressors (orange) from CSL and convert it to a transcriptional activation complex on promoters of target genes. (ii) *Cis*-inhibition. In this type of Notch/ligand interaction, DSL ligand expressed on the same cell as the Notch receptor interact, interfering with transactivation.

Figure 2.

Overview of different types of Notch signaling in the ear and phenotypic consequences. **A.** Lateral Inhibition: (i) The signal sending cell has more Notch ligand (pink) and activates Notch (aNotch) in the signal receiving cell. aNotch inhibits proneural gene expression (Atoh1) and results in a downregulation of Notch ligand, thereby decreasing the effectiveness of the cell to signal back (negative feedback). This signaling proceeds until one cell (the signal sending cell) has high amounts of Notch ligand and very low levels of aNotch. Conversely, the signal receiving cell has low amounts of Notch ligand and high levels of aNotch. (ii) Ultimately, this leads the signal sending cell to differentiate as a hair cell, and the signal receiving cell to differentiate as a supporting cell. (iii) The phenotypic consequence of a Notch disruption of this type of signaling is an overproduction of hair cells at the expense of supporting cells. **B.** Lateral induction: In this type of signaling, ligand transactivation leads to upregulation of the ligand, resulting in equal signaling between cells, which both display high amounts of ligand and aNotch. This type of signaling leads to specification of the sensory progenitor cells (ii), although the molecular details of how this occurs are not yet known. (iii) The phenotypic consequences of disruptions in this type of

signaling lead to fewer sensory progenitors, resulting in reduction of both hair cells (HCs) and supporting cells. Scale bar=25 microns.

Figure 3.

Model of how graded disruptions in Notch signaling affect the cochlea during lateral inhibition. **A.** Normal arrangement of hair cells in the cochlea, with one row of inner hair cells and three rows of outer hair cells. Surrounding supporting cells are represented in light tan, and square darker tan cells represent surrounding non-sensory cells in the GER and LER. The light blue region represents the as yet unidentified signal that, in conjunction with Notch-mediated lateral inhibition, prevents the non-sensory cells from developing into hair cells. **B.** Mild disruptions in Notch signaling reduce inhibitory signaling. Because the cells in the non-sensory regions receive the least amount of inhibitory signaling (represented by 2 instead of 4 inhibitory signals in A) disruptions to Notch signaling affect these regions preferentially, leading to extra rows of hair cells in normally non-sensory regions. **C.** Severe reductions in Notch signaling affect lateral inhibition in both sensory and non-sensory regions, leading to a large increase in the number of hair cells and decrease in the number of supporting cells.

Figure 4.

Overview of the roles that Notch signaling plays in otic development. Developmental ages on the left represent stages in mouse otic development. In the model, Notch is depicted playing four key roles during ear development. Beginning at otocyst stages, Notch augments the Wnt pathway in defining the size of the otic placode. Next, otic progenitors (expressing Dll1) undergo lateral inhibition to define which cells will delaminate from the epithelium to become otic neurons. Third, Jag1-mediated lateral induction specifies the sensory progenitors, potentially via Sox2. Other signaling pathways, including Fgf, Wnt, and Bmp, may augment Notch in specification of some sensory regions (dashed lines). Alternatively, they may define the size of the sensory region, or play an important role in further

differentiating the cells into postmitotic precursors. In the final stage, Notch mediates lateral inhibition via the Dll1 and Jag2 ligands to determine which cells differentiate as hair cells and supporting cells.