NOTCH SIGNALING IN THE DEVELOPMENT OF THE INNER EAR

MARK KEITH LANG EDDISON

UNIVERSITY COLLEGE LONDON
AND
IMPERIAL CANCER RESEARCH FUND, LONDON

ICRF supervisor: Dr. Julian H Lewis

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Relative and absolute,
These the two truths are declared to be.
The absolute is not within the reach of intellect,
For intellect is grounded in the relative.

Shantideva
8th Century
Abstract

The sensory patches of the inner ear consist of two types of cell: sensory hair cells and supporting cells. The pattern is such that supporting cells surround each hair cell and no two hair cells touch each other. The aim of this study was to uncover the genetic mechanisms that control the differentiation and patterning of these two cell types.

The alternating pattern of hair cells and support cells has led to the suggestion that their differentiation is co-ordinately regulated by cell-cell interactions involving the Notch signaling pathway. The key players in this pathway are Delta, a ligand, and Notch, its receptor, mediating a process known as lateral inhibition - a mechanism which forces neighbouring cells of an initially equivalent group to become different. The findings in this study show that two Notch ligands Delta1 and Serrate2 are expressed in the nascent hair cells and are thought to deliver lateral inhibition to their neighbours, which become supporting cells. Intriguingly, the supporting cells also express a Notch ligand, Serrate1.

To functionally test the role of the Notch signaling pathway in the developing chick inner ear, retroviral vectors were used to misexpress components of the Notch signaling pathway. It is shown that a simple lateral inhibition model based on feedback regulation of the Notch ligands is inadequate to explain the generation and patterning of the sensory hair cells. The Notch ligand Serrate1 is regulated by lateral induction and not lateral inhibition; commitment to become a hair cell is not simply controlled by levels of expression of the Notch ligand Delta1, Serrate1, and Serrate2 in the neighbours of the nascent hair cell. At least one factor, Numb, capable of blocking reception of Notch signaling is concentrated in hair cells.
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Any merit of this work I dedicate to the benefit of all beings
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Chapter 1

Introduction

1.1 Cell diversification during development

A central question that pervades developmental biology is how different cell types arise from a group of initially equivalent cells. Although intracellular events can play a role in the determination of cell fate, generally speaking a cell’s fate is controlled by signals received from its environment. A key form of cell-cell signaling required for proper cell fate determination is the Notch signaling pathway, which has been shown over the last few years to be as important in vertebrates as it is in *Drosophila*. In this study I explore the role Notch signaling has during the development of the inner ear, a remarkably complex organ but one whose specialised sensory epithelium consists of relatively few cell types. It therefore provides a relatively simple system to investigate the rules that govern cell differentiation in vertebrates.

1.2 The development of the chick inner ear

In the chick, the inner ear develops from a thickened epithelial placode next to the hindbrain at the level of rhombomeres 5 and 6. This placode invaginates into a cup shape before pinching off from the surface head ectoderm and forming a hollow epithelial ball, called the otic vesicle. Over the next few days, the vesicle undergoes a remarkable morphological and functional transformation converting it into a complex membranous labyrinth. Within this epithelial sheet arise distinct sets of cells constituting the sensory patches that perform the variety of functions of the mature inner ear.
The sensory patches can be divided into two groups, the vestibular patches responsible for the detection of acceleration and gravity, and the more ventrally located patch, responsible for hearing. The vestibular patches consist of three cristae, and the maculae of the utricle, saccule and lagena (at the distal tip of the cochlea). The cristae are located at the bases of the three semicircular canals, and are involved in the detection of angular acceleration. The utricular, saccular, and lagenar macula function to sense gravity and linear acceleration. The ventral auditory patch, called the basilar papilla, is situated in the banana-shaped cochlea (Figure 1.1).

Despite their distinctly different modalities all these sensory patches have essentially the same structure and consist of the same two epithelial cell types (with some sub-specialization within each category). Each patch consists of an epithelial array of supporting cells and mechanosensory hair cells, the sensory transducers. The hair cells are innervated by neurons that have themselves originated by delamination from the otic epithelium (reviewed in Fekete, 1996; Torres and Giráldez, 1998; Fritzch et al., 1998; Fekete 1999). The hair cells and supporting cells are arranged in a fine-grained alternating array such that supporting cells surround each hair cell and no two hair cells touch each other. In the mature sensory patch, the supporting cells rest on the basal lamina, while the hair cells rest on the supporting cells; and thin projections from the supporting cells, extending to the apical surface of the epithelium, separate each hair cell from neighbouring hair cells (Figure 1.2).

The patterned array of hair cells and supporting cells has lead to the suggestion that these two cell types are generated through competitive cell-cell interactions. Thus initially equivalent cells in the presumptive sensory patch compete for the hair cell fate, and a cell that adopts the hair cell fate delivers an inhibitory signal to its neighbouring cells preventing them from adopting a similar fate and forcing them to become
Figure 1.1: The gross structure of the chick inner ear at E10 (stage 36) and position of its sensory patches. The membranous labyrinth has been revealed by injection of white paint. Dorsal is top, anterior is to the left. The three semicircular canals (sc), utricle (u), saccule (s) and the anterior (ac), lateral (lc), and posterior cristae (pc) along with the lagenar macula (l) at the tip of the cochlea, constitute the vestibular apparatus. In the banana-shaped cochlea (ventral) is the basilar papilla (bp), responsible for the detection of sound (prepared by R. Massey and J. Lewis, (e)=endolymphatic duct).
Figure 1.2: Sensory cells in the vertebrate inner ear are arranged in a characteristic fine-grained pattern.

A) The regular alternating pattern of hair cells and supporting cells in the chick basilar papilla at E10. The hair cells are stained with hair cell antibody (white blobs) and the supporting cells outlined by their cortical actin, stained with fluorescent phalloidin (prepared and photographed by A.Myat).

B) A schematic representation of the sensory patch. Hair cells, here shown in blue, are surrounded by supporting cells and innervated by neurons of the stato-acoustic ganglion, which originate from the epithelium. Each sensory hair cell has a stereociliary bundle projecting from its apical surface into the ear lumen. This pattern of sensory hair cells in the ear suggests that it is generated by lateral inhibition, singling out a subset of cells from within a field of initially equivalent cells (from C.Haddon, 1997).
supporting cells. This mechanism for generating the fine-grained pattern of supporting cells and hair cells has been termed lateral inhibition (Corwin, 1991; Lewis, 1991).

1.3 Lateral inhibition is mediated by the proteins Notch and Delta

The concept of lateral inhibition was first described in the development of the fruit fly, Drosophila melanogaster, and is known to involve the genes Notch and Delta, where Notch acts as the receptor of intracellular signals, and Delta acts as the ligand (reviewed in Muskavitch, 1994). Both Delta and Notch were originally classified as 'neurogenic' genes: loss-of-function mutations in either gene gave rise to abnormalities of neuroectodermal cell lineages. Homozygous mutants in Delta and Notch resulted in the expansion (hyperplasia) of the embryonic nervous system, reduction in the embryonic epidermis and embryonic lethality (Lehmann et al., 1983). In the fly Notch also has an alternative ligand to Delta, encoded by the closely related gene Serrate (Fleming et al., 1990). Unlike Delta, Serrate loss of function does not cause a neurogenic phenotype. Delta and Serrate have different expression patterns and appear to regulate different developmental decisions (Thomas et al., 1991, Kooh et al 1993). However in some instances they are functionally interchangeable (Gu et al., 1995; Zeng et al., 1998).

Over the years, it has been discovered that the Notch pathway is central to many developmental decisions in the fruit fly; in fact hardly any tissue is not affected by Notch. Moreover the Notch signaling pathway has been conserved during the evolution of multicellular animals. Homologues of Notch called lin-12 and glp-1 have been found in the nematode (Yochem et al, 1988; Yochem and Greenwald, 1989), and several vertebrate Notch genes have also been identified: for example, in chick, mouse, zebrafish and man (Ellisen et al., 1991; Amo et al., 1992; Bierkamp & Campos-Ortega,
1993; Franco del Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993; Lardelli et al., 1994; Larsson et al., 1994; Henrique et al., 1995; Myat, 1995; Westin and Lardelli, 1997).

Notch ligands too have homologues in vertebrates: one Delta gene has been described so far in the chick (Henrique et al., 1995), whilst in the mouse three (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Shutter et al., 2000), and in zebrafish four Delta genes are known (Haddon et al., 1998b). In addition, vertebrates also possess homologues of the Drosophila Serrate gene (called Jagged in rodents and humans). In the chick, mouse and rat two Serrate genes have been discovered (Lindsell et al., 1995; Hayashi et al., 1996; Myat et al., 1996; Shawber et al., 1996a; Mitsiadis et al., 1997). In vertebrates, Notch malfunction has been shown to disrupt a wide variety of developmental processes including neurogenesis, somite formation, angiogenesis, and myogenesis (Shawber et al., 1996b; reviewed in Lewis 1998; Shima and Mailhos 2000).

1.4 The molecular mechanism of Notch signaling

Before discussing how lateral inhibition is mediated by Notch signaling, I will review the current thinking on how the Notch signal transduction pathway is thought to operate. What is described is no doubt an oversimplification of the reality, but it should give a general understanding of the remarkable way in which Notch activation can elicit a cellular response.

1.4.1 Structure of Notch and its ligands

Notch is a large single-pass transmembrane protein with an extracellular, ligand binding domain and a cytoplasmic domain required for signal transduction. In all
species the extracellular region consists of multiple epidermal growth factor (EGF)-like repeats and three copies of a Lin12/Notch/Glp motif. In the intracellular region Notch has a region of about 60 amino acids called the RAM 23 domain, six cdc10/Ankyrin repeats and a PEST-containing domain (reviewed in Weinmaster, 1997; Fleming, 1998) (Figure 1.3).

The Notch ligands Delta and Serrate are also single-pass transmembrane proteins with a series of extracellular EGF-like repeats; however, unlike Notch they all possess a conserved cysteine-rich motif referred to as the DSL domain (after Delta, Serrate and Lag-2) which is N-terminal to the EGF repeats. This DSL domain has been shown to be required for ligand function in invertebrates (Henderson et al., 1994; Muskavitch, 1994; Tax et al., 1994). Serrate, though structurally related to Delta, differs in that it is much larger than Delta, with more EGF repeats in the extracellular domain and a cysteine rich region between the EGF repeats and the transmembrane domain. Although the cytoplasmic domains of the Notch ligands do not share any significant homology, they are required for normal ligand functioning, as deletions of the cytoplasmic domain in either Delta or Serrate have been shown to block Notch signaling (Chitnis et al., 1995; Sun and Artavanis-Tsakonas; 1996, Henrique et al., 1997) (Figure 1.3).

1.4.2 Proteolytic processing of Notch

To function as a receptor, the Notch protein has to be cleaved by a variety of proteases, both before and after ligand binding (reviewed in Weinmaster, 2000) (Figure 1.4). The Notch receptor that appears at the cell membrane is produced by a proteolytic cleavage of the primary translational product of the Notch gene. This creates a heterodimer composed of a large amino-terminal fragment that is entirely extracellular, and a smaller carboxy-terminal fragment, which is largely intracellular. The functional
Figure 1.3: Schematic representation of the structure of *Drosophila* Notch, Delta and Serrate. The same overall arrangement of structural motifs in both ligand and receptor is conserved in other species.

i) The Notch receptor extracellular domain contains multiple EGF-like and 3 cysteine rich LIN-12-Notch repeats (LNR). Intracellularly, after the transmembrane domain are the RAM 23 domain involved in interaction with the CSL proteins, 6 ankyrin repeats, and a region rich in proline, glutamic acid, serine, and threonine (PEST).

ii) The Notch ligands Delta and Serrate, like the Notch receptor, have multiple EGF-like repeats in the extracellular domain. Serrate has more EGF-like repeats and inserts in some of them. Near the amino terminus is the DSL (Delta-Serrate-Lag2) domain, a conserved cysteine-rich motif. The Serrate ligands have an additional cysteine rich region (CRR) between the EGF-like repeats and the transmembrane domain. Cytoplasmic domains do not show significant sequence similarities or identified motifs (Adapted from Blair, 2000).
Figure 1.4: Schematic representation of the multiple proteolytic cleavages and associated proteases of Notch1.

Unprocessed Notch is cleaved in the golgi by the Furin protease, at the site marked S1. This generates an amino terminal fragment that contains most of the extracellular domain, and a smaller carboxy fragment that is mainly cytoplasmic. Notch is presented at the cell surface in this form. Upon ligand binding the smaller fragment is cleaved in its extracellular domain marked S2. This releases extracellular Notch and allows for a final cleavage at S3. This final cleavage is dependent on Presenilin γ-secretase activity, and releases the Notch intracellular domain from the membrane. (Adapted from Weinmaster, 2000).
Notch heterodimer is stabilised through non-covalent interactions that are calcium dependent (Rand et al., 2000). This cleavage is brought about by a furin-like convertase during export to the cell surface (Logeat et al., 1998).

Ligand binding is thought to cause a conformational change that induces two further cleavages of the Notch receptor, both in the smaller fragment. The first of these severs the extracellular region of Notch, while the second releases the intracellular domain of Notch, allowing the transmission of the Notch signal. The cleavage of the extracellular region of Notch occurs close to the transmembrane domain and is achieved by an ADAM (a Disintegrin and Metalloprotease) protease called TACE (Brou et al., 2000). The cleaved extracellular domain of Notch is thought to be endocytosed by the ligand-expressing cell, and this endocytosis is critical to Notch receptor activation (Parks et al., 2000). The gene product of Kuzbanian (Kuz), another ADAM protease, has also been implicated in this Notch cleavage (Pan and Rubin, 1997), but its exact role is debatable. It has been reported that Notch processing can occur in Kuz- cells, but not in cells lacking TACE (Mumm et al., 2000), suggesting Kuz maybe functionally redundant in this cleavage. Moreover, Kuz also has been shown to cleave the Notch ligand Delta, and this cleavage has been shown to be necessary for signaling (Qi et al., 1999). It maybe therefore that the original report was wrong, and that the target of Kuz is not Notch but Delta.

The final cleavage of Notch, which requires the prior TACE cleavage, happens at a site within the transmembrane domain, near the cytoplasmic face. The current evidence suggests that this cleavage is performed by a presenilin-dependent \( \gamma \)-secretase or a \( \gamma \)-secretase activity of presenilin itself. Presenilins were first identified by mutations that cause early-onset Alzheimer’s disease, where they are involved in proteolysis of the amyloid precursor protein at an aberrant site, leading to the accumulation of the amyloid peptides associated with this disease. Null alleles of
Drosophila presenilin have the same neurogenic phenotypes as mutants with a loss in Notch activity (Struhl and Greenwald, 1999). Importantly, the production of the intracellular fragment of Notch from the membrane-bound form is decreased in cells deficient in presenilin1 (PS1) activity, but is rescued by overexpression of PS1 (De Stoop et al., 1999; Song et al., 1999; Ray et al., 1999). Hence the presenilins are thought to regulate or bring about this final cleavage, which releases the small intracellular Notch fragment that mediates the next step in the signal transduction pathway.

1.4.3 Intracellular transduction of Notch signal

According to the current model, Notch signaling activates transcriptional responses by a translocation of this small Notch fragment (Nintra) to the nucleus, where it directly interacts with transcription factors. However, evidence for a different model also exists, and there may be more than one way in which activated Notch can elicit a response in the cell (reviewed in Artavanis-Tsakonas et al., 1999).

Evidence for a direct role of Nintra in eliciting gene transcription came from the observation that activated forms of Notch show strong nuclear localisation (Kidd et al., 1989), and that Nintra contains two potential nuclear localisation sequences that flank the ankyrin repeats (Lieber et al., 1993; Rebay et al., 1993). However, immunocytochemical analysis has not detected Notch in the nuclei of developing animals (Ahmad et al., 1995). More recently it was argued persuasively that only small amounts of Nintra, below the limit of immunohistochemical detection, are required for in vivo Notch signaling (Schroeter et al., 1998; Struhl and Adachi, 1998). Using a sensitive reporter-based assay, nuclear access has been demonstrated to be important for Notch signal transduction, and by manipulating Nintra subcellular localisation with sequences
that targeted it to the membrane or nucleus, Notch activity was respectively either blocked or potentiated (Struhl and Adachi, 1998).

The transduction of the intracellular Notch signal depends on DNA binding proteins of the CSL family. This family consists of CBF/RBPJk in mammals, Suppressor of Hairless (Su(H)) in Drosophila, and LAG-1 in C.elegans. These proteins bind both Nintra (at the RAM domain and ankyrin repeats) and enhancer regions of Notch target genes. The CSL protein, in combination with Nintra, forms a functional transcription factor to stimulate target gene expression in the nucleus (Jarriault et al., 1995; Hsieh et al., 1996). The activity of Su(H) can be antagonised, in Drosophila at least, through its interaction with the Hairless protein, a negative regulator of Notch (Brou et al., 1994). No Hairless homologue has been found in vertebrates.

Whether CSL binds Nintra at the cell surface, and then translocates to the nucleus, or sits in the nucleus waiting for Nintra to arrive, is debated. In cultured insect cells the Su(H) protein has been observed to translocate from the cytoplasm to the nucleus subsequent to Notch activation (Fortini and Artavanis-Tsakonas, 1994; Frise et al., 1996). However, in vivo, and in vertebrate cells generally, nuclear CSL staining does not correlate well with Notch activity. For example, RBP-Jk is predominately nuclear in mouse embryos and no changes in cellular localisation are observed in Notch1 knockout embryos (de la Pompa et al., 1997; Sakai et al., 1995).

The target genes of Notch activation depend on the developmental context. Perhaps the best studied are the hairy-like helix-loop-helix (bHLH) proteins of the Enhancer-of-Split complex (E(spl)-C) in Drosophila (Delidakis and Artavanis-Tsakonas 1992) and their homologues the Hes genes in mammals (Sasai et al., 1992; Ishibashi et al., 1995; Jarriault et al., 1995; Ohtsuka et al., 1999). In Drosophila, Su(H) has been shown to bind to regulatory sequences of the E(spl) genes and upregulate expression of their encoded bHLH proteins (Bailey and Posakony, 1995; Lecourtois and
Schweisguth, 1995). The E(spl)/Hes genes in turn act primarily as transcriptional repressors, mediating inhibitory effects on cell genes promoting commitment to a specialised fate, such as the genes of the achaete-scute complex (Oellers et al., 1994; Tata and Hartly, 1995; Nakao and Campos-Ortega, 1996; Nakamura et al., 2000).

Although Notch activity generally elicits a response through the CSL/Su(H) pathway, there is mounting evidence that in some developmental decisions Notch can act independently of Su(H) (Shawber et al., 1996b; Matsuno et al., 1997; Wang et al., 1997; Ligoxygakis, 1998; Zecchini et al., 1999; Nagel et al., 2000).

In summary Notch signaling in itself does not specify a particular cell fate; rather it is a general developmental tool, whose signal transduction machinery and developmental consequences are dependent on the context in which it is operating.

1.5 Notch signaling in Drosophila can direct cell fate by a variety of mechanisms

That Notch signaling can operate in many different developmental contexts is in part due to proteins that interact with the central components of the pathway to influence the outcome of a Notch cell-cell signaling event. These proteins can either have a positive or negative effect on Notch signaling, and act at any point in the pathway, from ligand binding to signal transduction and transcriptional activity of the Nintra-CSL protein complex (reviewed in Panin and Irvine, 1998). These modifiers have made Notch signaling incredibly versatile. Types of cell fate decision that are influenced by Notch can be classified as lateral inhibition, asymmetric cell division, and inductive signaling. These three types of Notch signaling are well illustrated in Drosophila, and similar mechanisms also appear to operate in vertebrates (reviewed in Jan and Jan, 1998; Bray, 1998; Lewis, 1998; Irvine, 1999).
1.5.1 Lateral inhibition

Lateral inhibition, in its purest form is a local contact-mediated cell-cell interaction through which a population of initially equivalent cells compete for one of two fates. Cells that win the competition and adopt the primary fate inhibit their neighbours, forcing them to adopt the alternative or secondary fate. In the absence of lateral inhibition all of the cells of the equivalence group adopt the primary fate. Initially all cells express both ligand and receptor. Therefore if the competition is to have winners and losers, there must be a feedback loop that regulates the expression of the inhibitory ligand, such that receptor activation as well as inhibiting commitment to the primary fate also downregulates ligand expression.

As previously discussed, in lateral inhibition mediated by the Delta-Notch cell-cell signaling pathway, Delta is the inhibitory ligand and Notch is the receptor: the feedback mechanism is one in which Notch activation in a given cell represses Delta expression in that same cell. In this model, then, initially all cells of the equivalence group express both Delta and Notch. Subtle differences in the levels of expression of Delta and Notch, due to random fluctuations, are then amplified through the feedback mechanism. Thus one cell having higher levels of Delta than its neighbours prevents them from adopting the primary fate and represses their transcription of Delta. In this way single isolated cells can adopt a fate different from that of their neighbours (Figure 1.5) (Simpson, 1990).

A well studied and often quoted example of lateral inhibition with feedback is the isolation and regular spacing of the sensory bristles in Drosophila. The back of the thorax of a fruit fly, called the notum, is covered in uniformly spaced sensory bristles, and each one derived from a single sensory organ precursor cell (SOP). Genetic analysis has shown that both Notch and Delta are required for correct SOP
Figure 1.5: Lateral inhibition with feedback: a simple model of how Delta-Notch signaling might single out cells from an equivalence group. A) A cell with slightly higher Delta due to random fluctuations in levels of Delta expression activates Notch on adjacent cells. The consequence of Notch activation is two-fold. Firstly, Notch activation down-regulates the expression of Delta in that cell, preventing it from inhibiting neighbouring cells. Secondly Notch activation prevents the cell from differentiating as the primary fate. Cells with high Delta, and thus low Notch activity, adopt the primary fate. B) Using the lateral inhibition model one can see how single cells are singled out from an initially equivalent group of cells. (Reproduced from Alberts et al., 1994)
specification. Loss of either at the time of SOP specification results in excessive numbers of SOPs, suggesting that Notch signaling acts to repress SOP fate (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991; Parks and Muskavitch, 1993). Heitzler and Simpson (1991) found that bristle formation is repressed in wildtype tissue bordering clones homozygous for hypomorphic Notch alleles. This can be interpreted as the result of a feedback loop between Notch and Delta, where cells with reduced Notch function increase in Delta activity and inhibit neighbours from adopting the SOP fate.

Transcriptional control of the feedback mechanism has been elucidated, and involves the achaete/scute (ac-sc) complex. The ac/sc genes encode basic helix-loop-helix (bHLH) proteins that are known to regulate neural development (Ghysen and Dambly-Chaudière, 1988). The initial expression domain of these genes defines the equivalence group, or proneural cluster from which the SOP differentiates. During SOP selection, expression of the ac-sc genes increases in the cell that adopts the SOP fate and is eventually limited to that cell. This limitation to the SOP cell is due to the increase in Notch activation in the presumptive epidermal cells surrounding the SOP. Notch activation via the expression of genes of the Enhancer of Split complex (Bailey and Posakony 1995; Leucourtois and Schweisguth, 1995) has been shown to inhibit ac-sc expression (Kunisch et al., 1994). Furthermore, the ac-sc complex has been shown to positively regulate the transcription of Delta (Kunisch et al., 1994, Haenlin et al, 1994). The consequence is a feedback loop that represses Delta expression upon Notch activation during SOP specification (Heitzler et al., 1996).

As eloquent as this may appear, several observations have been reported that argue against SOP selection via the simple lateral inhibition model (reviewed in Baker, 2000). These objections are discussed in subsequent chapters.
1.5.2 Asymmetric cell divisions

In the purest form of lateral inhibition, single cells in an equivalence group are singled out by random fluctuations in gene expression, for example Notch or Delta. But it is also possible for the system to be non-random, biased by either external or internal factors that serve to influence the outcome of Notch signaling in a predictable direction. A relevant example to this study is the bias of Notch signaling during the development of the sensory bristles by the asymmetrically inherited cell fate determinant Numb.

The mature sensory bristle, which derives from a single SOP, consists of four cells with distinct fates: a neuron, and three accessory cells: a sheath cell, a socket cell and a bristle cell (Figure 1.8). To produce these cell types the sensory organ precursor cell (SOP), undergoes a stereotypical pattern of asymmetric divisions (Hartenstein and Posakony, 1989, Posakony, 1994). The SOP cell first divides to generate two secondary precursor cells, Ila and Ilb. The Ila cell then divides to produce two outer cells, the hair cell and the socket cell. It was thought that the Ilb cell divides shortly after the Ila division to produce two inner cells, the neuron and the sheath cell. Recently, it has been demonstrated that the mature sensory bristle actually consists of five cells (Gho et al., 1999). A fifth cell is produced by the pllb cell. This first divides unequally, to produce a small migratory glial cell and a daughter cell named plllb. The plllb cell then divides to produce the neuron and the sheath cell (Figure 1.6).

Studies using temperature sensitive alleles of Notch and Delta show that these and the other genes of the Notch pathway are required for the proper cell fate determination of the SOP progeny. A loss of Delta activity, or a loss of Notch function at the time of the first SOP division produces two Iib cells that both divide to give a neuron and sheath cell (Parks and Muskavitch, 1993; Guo et al., 1996; Wang et al., 1997). A reduction of Notch or Delta function during both the first and second divisions of the
SOP lineage leads to the production of four neurons and no accessory cells (Harteinstein and Posakony, 1990; Parks and Muskavitch, 1993).

Besides cell-cell interactions, a cell intrinsic factor Numb (Uemura et al., 1989), is also essential for the correct cell fate determination of SOP progeny (reviewed in Jan and Jan, 1998). Loss of numb causes the llb cell to be transformed into the Ila cell, whereas overexpression results in the opposite cell fate transformation (Uemura et al., 1989, Rhyu et al., 1994). Numb is also similarly involved in the subsequent divisions of the Ila and Ilb cells (after division of the SOP its expression is activated in the plIa cell). Numb is a membrane associated protein that is asymmetrically localised during mitosis of the SOP cell, and segregates into only the Ilb cell (Rhyu et al., 1994, Knoblich et al., 1995). Numb is also segregated in the Ilb (pIlb) division into the cell that differentiates as the neuron, and Numb is segregated in the Ila division into the cell that becomes the bristle cell (Rhyu et al., 1994, Frise et al., 1996, Wang et al., 1997, Gho and Schweisguth, 1998). (Figure 1.6).

The effect of Numb is opposite to that of Notch activity, suggesting that it is a negative regulator. Consistent with this idea, when assayed in cultured cells Numb inhibits the translocation of Su(H) to the nucleus upon receptor activation (Frise et al., 1996; Wakamatsu et al., 1999). Furthermore, Numb has been shown to directly bind to the cytoplasmic tail of Notch (Guo et al., 1996; Wakamatsu et al., 1999). Thus the presence of Numb in a cell can bias the Notch-mediated cell-cell interaction by inhibiting Notch activity. This may then lead to an up-regulation of Delta and the transformation of that cell into a signaling cell, activating Notch in its neighbours that do not express Numb.
Figure 1.6: Schematic representation of the cell lineage in a *Drosophila* sensory bristle (microchaete). At mitosis the sensory organ precursor cell (pl) contains a localised Numb crescent, which is segregated into one daughter cell, causing it to adopt the pllb fate. Notch activation forces the other daughter cell to adopt the plla fate. The pllb cell then undergoes an unequal division, to produce a small glial cell (which inherits Numb) and a plllb precursor cell. Next the plla cell undergoes an asymmetric division to produce a bristle/shaft cell (which inherits Numb) and a socket cell. The final division in the lineage in the plllb cell which divides asymmetrically to produce the neuron (which inherits Numb) and a sheath cell. Yellow represents cells where Notch is active and red where Notch is inhibited by the presence of Numb. It is not known if Notch is activated in the plllb cell. (Adapted from Gho et al., 1999).
1.5.3 Lateral induction

Notch activation can act as an inductive signal as well as an inhibitory one. Notch signaling at the Drosophila wing margin is perhaps the best example of inductive Notch signaling. It illustrates how Notch activation can, in some contexts, upregulate the expression of Notch ligand, in contrast to lateral inhibition. It also illustrates how the efficacy of the different Notch ligands, Delta and Serrate, can be biased through the Notch modulator, Fringe.

The Drosophila wing develops from one of the imaginal discs. During development the presumptive wing field can be divided into dorsal and ventral compartments. The wing margin forms at the interface between the dorsal and ventral compartments and acts as an organizer of wing development. Notch activity is required at the wing margin, where it creates the organizer tissue by inducing localized expression of several genes that affect wing morphogenesis, including vestigial, wingless and cut (Kim et al., 1995; Rulifson and Blair 1995; Couso et al., 1995; Kim et al., 1996; Neumann and Cohen, 1996).

Both Delta-Notch signaling and Serrate-Notch signaling are involved in establishing the wing margin. Serrate is expressed in all of the cells of the dorsal compartment, and acts as a dorsal to ventral signal (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1996; de Celis et al., 1996). Delta is expressed at high levels in ventral cells near the boundary and is principally required to signal to dorsal cells (Doherty et al., 1996 de Celis et al., 1996). In response to Serrate expressed by dorsal cells, Notch is activated and Delta is upregulated in ventral cells along the boundary. Conversely, these high levels of Delta in ventral cells signal back across the boundary and activate Notch and thereby upregulate Serrate expression in dorsal cells (de Celis and Bray, 1997; Panin et al., 1997). This results in a band of Notch-active cells.
at the dorsal/ventral boundary (de Celis et al., 1996) (Figure 1.7). This positive regulatory feedback loop in lateral induction contrasts with the negative feedback loop observed during lateral inhibition, where Notch activation downregulates the expression of Delta. Evidence in support of this positive feedback loop comes from ectopic Notch activation during early wing development, which results in ectopic ligand expression (de Celis et al., 1997).

Notch is expressed throughout the wing disc. Thus to obtain a localised region of Notch activation between the dorsal and ventral compartments, Notch ligands must be prevented from activating Notch in neighbouring cells one within the same compartment. The gene fringe has been proposed to play such a role. In the wing disc Fringe is expressed only in the dorsal cells and makes them refractory to Serrate signaling and more sensitive to Delta signaling (Fleming et al., 1997; Panin et al., 1997). Thus fringe creates an asymmetry between the two compartments and ensures that cells expressing Serrate can only signal to the ventral cells and ventral Delta preferentially activates Notch in the dorsal cells. High concentrations of Delta and Serrate in the cells adjacent to the wing margin are reported to have a cell-autonomous dominant negative effect, aiding the restriction of Notch activity to the wing margin (Micchelli et al., 1997, Klein et al., 1997). The product of another gene, nubbin, is also involved at the wing disc, and acts to sequester Notch signaling outside the wing margin. Nubbin is a POU domain protein, expressed throughout the wing disc, which has been shown to negatively regulate the activation of Notch target genes, raising the threshold of Notch activity required for their expression (Neumann and Cohen, 1998).

The function of fringe has been recently elucidated (reviewed in Blair, 2000). In a series of papers, Fringe has been shown to be a glycosyltransferase that glycosylates the Notch receptor in the Golgi, as it is being exported to the cell surface (Bruckner et al., 2000, Ju et al., 2000; Moloney et al., 2000; Munro and Freeman...
Figure 1.7: Lateral induction during wing margin formation, modulated by Fringe.

The wing margin forms between dorsal and ventral compartments of the imaginal disc. Notch induces the wing margin fate, at the boundary of Fringe expression. Serrate and Fringe are co-expressed in the dorsal domain (blue cells), while only Delta is expressed in the ventral domain (red cells). Fringe blocks the reception of Serrate and potentiates the reception of Delta. The result is that dorsal cells only signal to ventral cells, while ventral cells preferentially activate dorsal cells. This creates a band of high Notch activity (purple box), that induces these cells to become wing margin (Adapted from Greenwald, 1998).
Fringe catalyses the addition of GlcNAc (N-acetylglucosamine) to an O-fucose saccharide that is found on specific extracellular EGF repeats of Notch (Moloney et al., 2000; Bruckner et al., 2000). It has been proposed that it is this glycosylation that makes Notch less sensitive to Serrate ligand but more sensitive to Delta ligand. The mechanism by which it acts could simply depend on an enhancement of Delta-Notch binding (Bruckner et al., 2000), though cell culture binding assays argue against this (Klueg and Muskavitsch, 1999). Alternatively it has been proposed that different conformational changes affect the efficiency with which the extracellular domain of Notch is cleaved by the TACE protease (Hicks et al., 2000, Moloney et al., 2000).

1.6 Lateral inhibition operates in vertebrate neurogenesis

The different mechanisms of Notch activation that have been described in the fly also appear to operate in a similar fashion in vertebrates. Most relevant to this study is lateral inhibition, which has been demonstrated to operate during neurogenesis in the central nervous system.

In the neural tube neurons are generated from dividing precursors whose cell bodies lie close to the lumen; new-born neurons then migrate out into the mantle zone of the neuroepithelium where they differentiate. Notch1 is expressed throughout the proliferative zone: Delta1 is expressed in the outer part of that zone, in a scattered subset of cells (Myat et al., 1996). The cells expressing Delta1 are nascent neurons (Henrique et al., 1995). The expression of Notch and its ligand Delta thus suggest that Notch signaling mediates lateral inhibition during neurogenesis. By analogy with Drosophila the nascent neurons, by expressing Delta1, are thought to deliver lateral inhibition to the progenitors, preventing them from differentiating as neurons.
Although this model has not been tested during secondary neurogenesis, as described above, it has been tested during primary neurogenesis in *Xenopus* (Chitnis et al., 1995) and in the zebrafish neural plate (Haddon et al., 1998b). When Delta1 mRNA is injected into the early embryo, so that all cells strongly deliver and receive the inhibitory signal Delta1, the cells in that region are prevented from differentiating as neurons. Conversely, when all cells are forced to express a dominant negative form of Delta1, which blocks Notch signaling, they all escape inhibition and upregulate endogenous Delta1 expression, and an excess of neurons is produced. Similar results were achieved in the chick retina: here *Notch* is expressed throughout the retina and *Delta1* is expressed in scattered cells. When Delta1 is overexpressed in all cells, through a retrovirus, no neurons are produced in the infected patch. Conversely, overexpression of a dominant negative form of Delta1 results in excessive neuronal production (Henrique et al., 1997).

It has therefore been proposed that lateral inhibition mediated by Delta-Notch signaling regulates the choice between remaining as a progenitor and embarking on differentiation as a neuron in the central nervous system. This inhibition limits the proportion of cells that differentiate, thereby maintaining a balanced mixture of progenitors and neurons (Henrique et al., 1997). Furthermore these experiments on vertebrate neural plate and chick retina are consistent with the simple lateral inhibition with feedback model. The activation of Notch in a cell inhibits its expression of Delta1 as well as its tendency towards neuronal differentiation.

1.7 Does Notch mediated lateral inhibition operate in the sensory patch?

As described in the beginning of the introduction, the fine grained pattern of hair cells and supporting cells suggests that these cell types are generated through a
process of lateral inhibition with feedback, mediated by the Notch signaling pathway. Initial expression analysis by Anna Myat showed that indeed chick homologues of Notch, Delta and Serrate are expressed in the ear, suggestive of a role in lateral inhibition (Myat, 1995).

The most striking evidence that Notch signaling has a central role in the production of hair cells and supporting cells comes from the zebrafish mutant, mind bomb (mib). It displays a neurogenic phenotype that affects virtually the entire nervous system, suggesting that the mutation corresponds to a failure of Notch signaling. For example in the neural plate of mib, primary neurons are greatly overproduced, and are contiguous with each other instead of being interspersed among non-neural cells (Jiang et al., 1996). In accordance with a failure of the Notch signaling pathway, the four delta genes of zebrafish are all overexpressed (Haddon et al., 1998a). Analysis of the sensory patches in the mib ear reveals that they consist solely of hair cells, with a complete absence of supporting cells. These hair cells are produced prematurely and later die and are extruded from the epithelium (Haddon et al., 1998a). Although the gene that is mutated in the mib remains elusive, this suggests that the Notch signaling pathway is essential in inhibiting cells of the sensory patch from all differentiating as hair cells, and in delaying the production of hair cells until the proper time.

Over the past few years additional evidence has accumulated that Notch signaling is essential to the correct production of hair cells. This evidence is discussed in detail in Chapter 3.

1.8 Comparisons with Drosophila sensory bristles

As discussed, lateral inhibition mediated by the Notch signaling pathway, such as operates during the genesis of the sensory bristle of the fly, may also be operating
during the development of the sensory patches of the inner ear. In fact it has been argued that these two structures are homologous in structure, in developmental anatomy and at a molecular level. There are good data to sustain such a claim, further supporting a central role of Notch signaling in the differentiation of the sensory patch (Lewis 1991; Adam et al., 1998; Eddison et al., 2000).

First of all, a correspondence can be drawn between the cell types of these sensory organs. Thus, the sensory neuron of the bristle corresponds to the sensory (cochleovestibular) neuron of the ear, the socket cell to the supporting cell and the bristle cell to the hair cell. The neural sheath cell of the bristle, -the sister cell of the neuron-, however has no obvious counterpart in the sensory patch, as glial cells in the cochleovestibular ganglion derive from the neural crest and thus have an origin separate from that of the neurons (see below) (D’Amico-Martel and Noden, 1983). The similarity between the bristle cell and hair cell is the most impressive: both cells types have highly characteristic protrusions, containing bundles of actin filaments, and with a planar polarity corresponding to their directional selectivity as mechanosensors (Tilney et al., 1996) (Figure 1.8).

The sequences of events during the differentiation of the two types of sensory organ are also strikingly similar. Firstly, all the cell types, including the neurons, derive in both cases from the epithelium. Thus the sensory organ precursor cell (SOP) that subsequently divides to form the five cell types of the bristle, (the neuron, bristle cell, socket cell, neural sheath cell and the migratory glial cell), has its origin in a layer of epidermal cells. The SOP divides to give one precursor that migrates from the epidermis and divides to form the neuron, sheath cell and glial cell, and one that does not migrate and gives rise to the bristle and socket cell. In the same manner, during the differentiation of the sensory patch in the inner ear, the first step generates a
Figure 1.8: The sensory patches of the inner ear are reminiscent of the sensory bristles of *Drosophila*. See text for details. (From Adam et al., 1998)
neuroblast, which delaminates from the epithelium, while other epithelial precursors remain to form the hair and supporting cells (Eddison et al., 2000).

It was such parallels that originally led to the investigation of whether the development of the fly bristles and the sensory patches in the vertebrate inner ear might be controlled by homologous systems of genes. As briefly mentioned, and further elaborated on in this study, Notch and its ligands are expressed during sensory patch development in a pattern that suggests lateral inhibition is operating, in both neuronal and hair cell differentiation, as in the fly. Upstream of Notch, as in the fly, vertebrate ‘proneural’ genes are expressed in the sensory patches and are necessary for sensory differentiation. In the mammalian ear, the key proneural gene appears to be Math1, a homologue of the Drosophila proneural gene atonal. In mice, Math1 is expressed throughout the prospective sensory epithelium and is later restricted to the hair cells. Math1 knockout mice produce no hair cells (Bermingham et al., 1999), and overexpression of Math1 in cochlear explants can cause ectopic hair cells to be produced (Zheng and Gao, 2000). In Drosophila, atonal is required for the development of the chordotonal organs, sensory organs which acts as cuticular stretch receptors and are closely related to the sensory bristles (Jarman et al., 1993). The atonal gene codes for a bHLH protein similar both it structure and in its ‘proneural’ function to the bHLH proteins encoded by the achete/scute (ac/sc) genes, which are responsible for sensory bristle development. Thus, ectopic expression of the proneural genes ac/sc in areas normally without bristles leads to the differentiation of ectopic bristles, and loss of ac/sc expression leads to a loss of bristles (Skeath and Carroll, 1994).

Homologous genes are also employed downstream of Notch. The transcriptional repressors Hes1 and Hes5, vertebrate homologues of the Drosophila Enhancer of Split genes, are expressed in the sensory patch only in supporting cells (Shailam et al., 1999; Zheng et al., 2000). Furthermore, Hes1 negatively regulates Math1 expression,
and Hes1-/- homozygous mice have recently been reported to show an increase in hair cell number (Zheng et al., 2000).

Finally, additional molecular similarities lie with the transcription factor Pax-2. *Drosophila-pax2* is expressed initially in all cells of the bristle lineage but is restricted to the sheath and bristle cell, and is essential for the correct differentiation of the bristle cell (Kavaler et al., 1999). Vertebrate Pax2 is expressed in the early otic epithelium and then selectively in hair cells (Riely et al., 1998; Isabelle le Roux, personal communication). *Pax-2* is also required for correct hair cell differentiation in vertebrates. In *Pax-2* knockout mice, the cochlea is missing (Torres et al., 1996) and in the zebrafish *pax2.1* mutant hair cell differentiation is abnormal (Riely et al., 1998).

Such homologies between the insect sensory bristle and the vertebrate inner ear argue that similar developmental mechanisms control their genesis. Thus the data from *Drosophila* provide us with a working hypothesis which one can test in the inner ear. In particular, it might be expected that Notch-mediated lateral inhibition should operate in the same way in the differentiation of hair and supporting cells, as it does during the development of the sensory bristles.

### 1.9 Aim and scope of this work

The alternating pattern of hair cells and supporting cells in the sensory epithelium, the phenotype of the zebrafish *mind bomb* mutant, the expression of Notch and Delta in these cells, and the strong homologies with the insect sensory bristle, all point to a mechanism of lateral inhibition mediated by Delta-Notch signaling controlling the differentiation of these cell types. The aim of this study was to test this hypothesis. Firstly, I confirm and clarify the expression patterns of C-Notch1 and two of its ligands C-Delta1 and C-Serrate1. C-Notch1 is expressed throughout the ear epithelium
in sensory and non-sensory regions. An antibody to Serrate1 reveals that it is
directed at all of the cells of the sensory patch, and is later restricted to the supporting
cells. Delta1 is expressed in scattered cells throughout the sensory patch. Early on
some of these Delta1 expressing cells delaminate from the sensory epithelium and can
be identified as neuroblasts (Adam et al., 1998). The remainder are identified as
presumptive hair cells that migrate to the apical layer upon differentiation. These
expression data implicate Delta-Notch signaling in the singling out of hair cells from the
sensory epithelium by lateral inhibition. The role of Serrate, however, is unclear.

To functionally test the role of Delta in providing the inhibitory signal, I generated
a replication-competent retrovirus to overexpress chick Delta1 in the developing inner
ear. Parallel experiments were also conducted with a dominant negative Delta1
construct. Interestingly, overexpression of Delta1 in a patch of sensory cells did not
inhibit hair cell production, as the lateral inhibition model would predict. Likewise,
expression of dominant-negative Delta1 did not appear to cause the expected
overproduction of hair cells (although, as discussed later, appearances may have been
misleading in this case). What the experiments using dominant negative Delta1 did
show clearly was that Serrate1 expression is regulated by lateral induction, in contrast
to Delta1, whose scattered expression suggests lateral inhibition.

Failure to produce a phenotype with Delta1 led me to look into Notch signaling
more deeply. In particular, I examined the expression of the chick homologue of Numb,
a protein that is known to block Notch signaling. I found that Numb expression is
dynamic in the otic epithelium, and that it becomes concentrated in the mature hair
cells, suggesting a role of Numb in hair cell differentiation. Retroviral experiments that
overexpressed Numb did not, however, alter the number of hair cells produced.
Chapter 2

Materials and Methods

In this chapter I give the detailed protocols of the specific techniques used in the thesis.

2.1 Cryostat sections

Embryos at E6 or later were fixed in 4% formaldehyde/PBSA, usually for 1 hour, on a rocker. If Serrate1 staining was to be done on embryos earlier than E6, they were fixed for 30 minutes only. Embryos were rinsed 3 times for 10 minutes in PBS on a rocker and then embedded in a 1.5% LB agar (Gibco-BRL)/5% sucrose solution. Embedded embryos were trimmed and the blocks left overnight to equilibrate in 30% sucrose with 0.1% azide at 4°C. Cryostat sections (15μm) were cut by Jenny Corrigan on a Reichert-Jung cryomicrotome and transferred to TESPA coated slides. Sections were air dried for 2 hours at room temperature and stored desiccated with silica gel at -20°C until needed.

2.2 Antibody Staining

Sections were soaked for 30 minutes in PBS at 37°C to remove surrounding agar. Primary antibody was diluted in blocking solution and applied to the slide (75μl) which was then incubated in a humidified chamber overnight at 4°C. After three washes for 10 minutes in PTW, secondary antibody, diluted in blocking solution, was applied to
the section. These were then left in the dark at room temperature for 1 hour. The slides were then washed and mounted in Citifluor, and analysed using confocal microscopy.

2.3 Primary antibodies used and working dilutions

a) Hair cell antigen (HCA)- 1:50 (monoclonal) a kind gift of Guy Richardson (Bartolami et al., 1991).
b) Serratel - 1:100 (rabbit polyclonal) a kind gift of Isabelle le Roux, as described in Adam et al., 1998.
c) Delta1 - 1:25 (rabbit polyclonal) another kind gift of Isabelle le Roux, as described in Eddison et al., 2000. At this concentration only retroviral Delta can be detected.
d) Numb - 1:300 (affinity purified rabbit polyclonal) a kind gift of Yoshio Wakamatsu (Wakamatsu et al., 1999).
e) Islet 1/2 - 1:100 (monoclonal - 39.4D5 Developmental Studies Hybridoma Bank).
f) GFP - 1/500 (rabbit polyclonal) (gift from David Shima).

For Numb staining, an additional serial methanol fixation step was performed before the primary antibody was added:

1x 5min each for 25% methanol/PBS, 50% methanol/PBS, 75% methanol/PBS, 100% methanol, 75% methanol/PBS, 50% methanol/PBS, 25% methanol/PBS, PBS.

Reagents:
**Blocking solution**: 3% BSA, 10% Fetal Calf Serum (filter sterilise), 0.1% Triton X-100.
**PTW**: PBS, 0.1% Tween-20.
**Secondary antibodies**
Alexa Fluor 488 goat anti-rabbit - 1:500 (Molecular Probes A-11008).
Alexa Fluor 594 goat anti-mouse - 1:500 (Molecular Probes A-11005).
2.4 Syto16 staining

After the antibody staining protocol was completed, but before mounting, 75μl of a 1:1000 Syto16 (Molecular Probes S-7578) stain in H₂O was added to each slide. Then the slide was directly mounted using the Prolong antifade kit (Molecular Probes P-7481).

2.5 RNA probes

Patterns of gene transcription were determined by *in situ* hybridisation using DIG RNA antisense probes (Stratagene, RNA transcription kit). Template DNA, usually in bluescript, was linearised with an appropriate restriction enzyme (see table below) before phenol-chloroform extraction and ethanol precipitation. The DIG RNA probe was generated by transcription with the RNA polymerase indicated in the table.

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<tr>
<td>C-Notch2</td>
<td>EcoR1 or Not1</td>
<td>T7</td>
</tr>
<tr>
<td>C-Numb</td>
<td>EcoR1</td>
<td>Sp6</td>
</tr>
</tbody>
</table>

To construct a probe corresponding to the intracellular domain of C-Delta1 (C-Delta1<sup>INT</sup>) a DNA fragment that codes for just the cytoplasmic domain of Delta1, delineated by BamH1 and EcoR1 restriction sites, was subcloned into a PKS- (Stratagene) vector.
2.6 \textit{In situ} hybridisation of cryosections

\textit{In situ} hybridisations were based on a protocol described in Strahle et al. (1994), but with various modifications, so the entire protocol is described here.

1. Prepare a Perspex lid with 2 sheets of Whatmann 3MM paper wet with 1x Salts/50% formamide.

2. Defrost sections at room temperature for 15 min on Whatmann paper.

3. Dilute the probe in Hybridisation Buffer (1/200), and denature at 70°C for 10 min. Vortex to mix, then quick centrifuge.

4. Add 75μl of probe to each slide (allow for wastage when making up buffer)

   The probe may not cover all the sections completely but it does not matter because agar melts during hybridization.

5. Cover slide with a cover slip (22 x 50mm).

6. Hybridise overnight at 65°C in a sealed Perspex box with pre-wetted filter paper in 1xSalt Solution/50% formamide.

7. Prepare Washing Solution and prewarm to 65°C (1x SSC, 50% formamide (BDH), 0.1% Tween-20).

8. Transfer slides to a metal rack (accommodates 25x slides) and wash them at 65°C for 15 min in a plastic container with pre-warmed washing solution (~300 ml). Allow the coverslips to fall off.

9. Wash slides 2 x 30 min at 65°C in pre-warmed washing solution.

10. Wash slides 2 x 30 min at room temperature in 1x TBST.

\textit{Anti-DIG antibody Incubation}
1. Dry the slide off around the sections with a paper and encircle with DAKO pen (avoid
drying the sections).

2. Block with blocking reagent (2% Boehringer blocking reagent /20% heat inactivated
sheep serum in TBST) at room temperature for 1 hour.

3. Dilute anti-DIG antibody (1:2000) in blocking reagent and put 75μl on the slide and
cover with a cover slip.

4. Incubate at 4°C overnight or room temperature for 2 hour in a humidified Perspex box
(Put slides onto filter paper soaked in PBS or water).

Histochemistry

1. Wash sections at room temperature in TBST 4-5 times for 1 hour, on rocker.

2. Rinse sections 2 x 10mins in 0.1M Tris (ph8.0), on rocker.

3. Dissolve one tablet of Fast Red in 2ml of 0.1M Tris (ph8.0); filter solution through
0.45μm filter.

4. As for antibody staining, dry the slides off around the sections, put slides onto filter
paper soaked in PBS, add 75μl of Fast-Red solution and cover slip as before.

5. The stain should develop in about an hour: check under fluorescent light.

6. Wash slides in PBS 2 x 5mins, and mount in Citifluor, or alternatively add primary
antibody and proceed with the antibody staining protocol.

Reagents

Hybridisation Buffer: 50% Formamide (FLUKA); 1xSalts; 10% dextran sulphate;
Yeast RNA (1mg/ml Sigma R7125); 1x Denharts.

10x Salt: 114g NaCl (1.3X-final concentration), 14.04g Tris HCl, 1.34g Tris Base, 7.8g
NaH2PO4.2H2O, 7.1g Na2HPO4, 100ml 0.5 M EDTA, made up to 1000ml with ddH2O.

100x Denharts: 2% bovine serum albumen, 2% Ficoll, 2% polyvinylpyrrolidone.

Dextran Sulphate: Pharmacia Biotech 17-0340-01.

20xSSC: 3M NaCl, 300mM tri-sodium citrate.

Washing Solution: 50% Formamide; 1x SSC; 0.1X Tween-20.
TBST: 10 mM Tris (pH 8.0)/150 mM NaCl/0.1 % Tween-20.
Boehringer Mannheim Blocking Reagent: BM 1096176; Made up in MABT (Maleic acid buffer plus 0.1% Tween).
Alkaline phosphatase-conjugated anti-DIG antibody: Boehringer 1093274.
Fast-Red Tablets: Boehringer 1496549.

2.7 RCAS viral constructs

2.7.1 RCAS-Delta1 and Delta1<sup>dn</sup>

The RCAS(B)-DI1 and RCAS(B)-Dl<sup>dn</sup> replication-competent retroviral constructs were as described in Henrique et al. (1997). Briefly, the RCAS(B)-Delta1 construct encodes 628 amino acids, including the whole extracellular domain, the transmembrane domain and 50 amino acids of the intracellular region. The RCAS(B)-Delta1<sup>dn</sup> construct has a deletion in the intracellular domain, encoding only 13 amino acids.

In this study the viruses were used at a titre of 5×10<sup>-10</sup> - 10<sup>6</sup> IU/ml. With these two constructs a total of 184 virus-injected embryos were serially sectioned and analysed. Results are based on 36 embryos in which I saw informative patches of infection, i.e. patches that overlapped or touched sensory patches in the ear epithelium.

2.7.2 RCAS-Numb viral construct construction

The full-length chick numb gene was amplified by PCR from a pGEM plasmid containing full length Numb (pGEMNb13), such that the numb gene would be flanked by a 5'Nco1 site and a 3' EcoRI site for subsequent ligation into the Slax12 adapter plasmid.

The primers used were:
CATGCCATGGATAAATTACGGCAGAGCTTTAGG (5' with Nco1 site)
CCGGAATTCTTAAAGTTCAATCTCAAATGTGTTC (3' with EcoR1 site)

Direct ligation of the PCR product into Slax12 was not successful, so another step was introduced where the PCR product was ligated first into a T-vector (Invitrogen). The PCR product was completely sequenced (ABI sequencer) to ensure that no errors occurred during the PCR step. The numb gene was then excised from the T-Vector (by cutting with Nco1 and EcoR1) and ligated into Slax 12. This step ensures an in-frame positioning of the gene when subsequently sub-cloned into the RCAS plasmid (Morgan and Fekete, 1996). This cloning step, however, introduces a base change in the numb sequence, which if translated would result in an amino acid change from asparagine to its amide derivative aspartic acid. This could potentially alter the function of Numb. Thus the base change was converted back to the original sequence using the QuikChange Site-Directed Mutagenesis Kit (Stratagene 200518). The correct sequence was confirmed, and then the Slax12-numb plasmid was cut with Cla1 and the numb gene ligated into RCAS(B) vector (Figure 2.1).

The final titre of RCAS-Numb virus was $5 \times 10^8$ IU/ml. With this construct a total of 57 virus-injected embryos were serially sectioned and analysed. Results are based on 12 embryos in which I saw informative patches of infection.

2.8 Chick primary embryonic fibroblast preparation and revival for virus production

E10 primary chick embryonic fibroblasts (Line 0) were prepared as described in Morgan and Fekete (1996). At confluence the cells were either frozen down, or used directly for retroviral production.
Figure 2.1: Structure of RCASB/numb recombinant provirus with the sequence of chick numb inserted at the Cla1 site. Polylinker sequences (not to scale) are indicated as grey bars either side of numb. The core proteins, three enzymes (reverse transcriptase, integrase, and protease), and surface proteins are encoded by the viral structural genes gag, pol, and env respectively. LTR, Long terminal repeat. Adapted from Morgan and Fekete, 1996.
To revive primary fibroblasts from a frozen stock, culture medium was prewarmed to 37°C. The frozen cells were just thawed and added to 10ml warm medium. The cells were then spun at 900rpm for 5 minutes, and supernatant removed. The cells were resuspended in 5ml medium, plated onto a 24cm² tissue culture flask, and incubated overnight at 37°C.

**Reagents**

**E4 medium**: DMEM (Dulbecco's Modification of Eagle's Medium).

**Fetal Bovine Serum**: Helena BioSciences NS1005; heat at 56°C for 30 min to inactivate complements.

**Chick Serum**: Sigma C-5405 heat inactivate for 30 min at 56°C to inactivate complements.

**Culture Medium**: 10% FCS, 2% Chick serum/E4, filtered through 0.2-µm filter.

**Freezing Medium**: 90% FCS, 10% DMSO.

### 2.9 RCAS virus production

RCAS virus was produced by the protocol described in Morgan & Fekete (1996). An outline of the procedure, with modifications, is as follows:-

1. Briefly, when the 24cm² flasks become more than 70% confluent the cells were passed onto a 6-well tissue culture plate at a high density (40-60% confluent at the beginning) and incubated overnight. The cells were transfected, using the SuperFect Transfection Reagent (Qiagen, 301305), when the culture was 80-100% confluent.

2. When the cells had grown to confluence, the cells were passed first onto a 24 cm² flask, secondly onto a 83 cm² flask, thirdly onto a 175 cm² flask, and a Lab-Tek chamber slide (250µl of 3x dilutions), and finally onto 3x 175 cm² flasks. Process Lab-Tek chamber slides for detection of protein expression using primary antibody (either Delta1 or Numb antibody).
3. When the cells on 175 cm² flasks approach confluence, start collecting virus particles. Prepare harvesting medium (E4/2% FCS/0.2% chick serum), add 12.5 ml to each 175 cm² flask and incubate for 6-24 hours. Collect medium onto a filter unit (Nalgene 115 ml; 0.45μm membrane), filter and transfer the flowthrough medium to a 50 ml Falcon tube. Ultracentrifuge the medium at 22,000 rpm for 2.5 hours at 4°C. Pour off the supernatant into a bucket, put the tubes on ice and suspend the pellet at the bottom by pipetting (~10 times) with the rest of the medium. Cover top of the tubes with Parafilm and put the ice bucket with tubes on a rocking platform to mix well. Mix the virus as much as possible by pipetting before making aliquots. Make 20μl aliquots, freeze them on dry ice and store at -80°C. Titre each collection of virus using chick embryonic fibroblasts as described in Morgan and Fekete (1996).

2.10 Replication-defective virus

Isabelle Le Roux made both the replication-defective constructs containing Delta1 and GFP and GFP alone used in this study. Briefly, plasmids based on the LZRSpBMN-Z plasmid (Kinsella et al., 1996) were prepared by inserting cDNA of Mouse Delta1 linked to an internal ribosome entry site followed by DNA coding for green fluorescent protein (GFP). This composite coding sequence was placed under the control of a 253-bp upstream enhancer sequence from Rous sarcoma virus, within LZRSpBMN-Z. Replication defective virus was generated (by I.L.R) by transiently cotransfecting 293gp packaging cells (Qiagen) with this construct plus a plasmid coding for vesicular stomatitis virus (VSV)-G protein. The resulting virus particles contain RNA coding for Delta1 + GFP or GFP alone, with Gag, Pol, and VSV-G proteins provided by
the packaging cells. Virus released into the supernatant was concentrated by ultracentrifugation to a final titrer of $5 \times 10^8 - 10^9$ IU/ml.

2.11 Embryo culture and virus injection

Chick embryos were incubated at 38°C in a humidified oven and windowed at stage 13 (E2) (Hamburger and Hamilton, 1951). About 0.5 µl of virus solution (with 0.8 µg/µl polybrene, 3% methylcellulose and a trace of fast green dye) was injected into the lumen of the otic cup. Embryos were then placed back into the incubator and fixed at E8 (see Figure 4.1).

2.12 General strategy for analysing RCAS infections

E2 embryos were injected with virus in the both otic cups and incubated until E8. Embryos were then decapitated and the forebrain, eyes and beak were removed and fixed. The head was then embedded in an orientation that would give transverse sections, of bilateral symmetry, through both cochleas. The hindbrain was alternately sectioned: adjacent sections of chick hindbrain were put onto separate slides: section 1 onto slide A, section 2 onto slide B, section 3 onto slide A and so on, such that each slide has every other section of hindbrain. In this way adjacent sections of the same embryo could be analysed by different reagents. In general, slides were analysed either by Delta1 antibody and HCA antibody staining or by $\Delta$eltal $in situ$ hybridisation and Serrate1 antibody.

Every other slide from each potentially infected embryo was first stained with Delta1 antibody and hair cell antibody (HCA). The Delta1 antibody at the concentration used does not detect endogenous Delta1, but will detect retrovirally expressed Delta1.
or Delta1\(^{\text{th}}\). Such staining reveals, firstly, if the otic epithelium contains any patches of infection and, secondly, if a patch of infection is informative, that is, whether it was adjacent to or internal to a sensory patch, as identified by HCA antibody. For each embryo, the entire ear epithelium for both ears was checked for infection under a fluorescence microscope. The alternate slide(s) of embryos identified as having informative patches of infection as judged by the Delta1 antibody stain were then processed by \textit{in situ} hybridisation with a probe to Delta1, which would identify the same infected patch, and with the Serrate1 antibody to identify the sensory region. Delta1 and Serrate1 antibodies were both polyclonals raised in rabbit so could not be employed together. Hair cell antigen does not survive the \textit{in situ} protocol.

2.13 Electroporation

The expression vector LZRSpBMN-Z-GFP (2mg/ml in PBSA/1 mM MgCl\(_2\)) was injected into the lumen of the otic cup as described for the retroviral injections. Electrodes (TR Tech Co. Ltd, Japan) were placed between the lumen of the ear (-ve) and the midline (+ve) and an electric pulse was applied (10volts, 50milliseconds, 3 times) (Momose et al., 1999). Embryos were then incubated at 38°C for a further 24 hours.
3.1 Introduction

Hair and supporting cells are arranged in a regular pattern in which the hair cells are surrounded and isolated from each other by supporting cell processes. An obvious way in which these two cell types in this fine-grained pattern might be generated is by lateral inhibition, mediated by the Delta-Notch signaling pathway (Conlin et al., 1991, Lewis, 1991). The idea is that the nascent hair cell, by expressing Delta, inhibits its adjacent neighbours from developing as hair cells and these receivers of inhibition are then forced to differentiate as support cells.

The most striking evidence that Notch signaling has an essential role in the production of hair cells and supporting cells comes from the zebrafish mutant, mind bomb (mib), which displays a neurogenic phenotype suggestive of a loss of Delta-Notch signaling. Here, the sensory patches in the ear consist solely of hair cells, which are produced prematurely (Haddon et al., 1998a). This evidence, however, suffers from the difficulty that the nature of the mib gene product is not yet known. Other evidence has come from mice (Lanford et al., 1999) and zebrafish (Riely et al., 1999) with mutations in identified components in the Notch pathway, but the phenotypes seen in these mutants are much less extreme than in mib. These findings are discussed in detail at the end of this chapter. Evidence that Notch is important is not, however, sufficient to
prove that lateral inhibition mediated by Delta-Notch signaling is operating: Notch can also work in other ways.

To test whether lateral inhibition mediated by the Notch signaling pathway operates in the generation of hair cells and supporting cells in chick, it is first necessary to examine the gene expression patterns of Notch and its ligands during sensory patch differentiation. There are several known homologues of Notch and its ligands in chick. Here I examine the expression of all of these.

3.1.1 The timing of hair cell generation

In order to correlate the expression of Notch and its ligands with the production of hair cells one needs to know at what time hair cells are produced during inner ear development. Cell lineage studies, although inconclusive with regard to the relation between neuroblasts and hair cells and supporting cells, have demonstrated that hair cells and supporting cells can derive from sister cells (Fekete 1998, Stone 1999 & 2000). Therefore the decision to differentiate as either a hair cell or a supporting cell occurs at or shortly after a cell's terminal mitosis.

In the basilar papilla, hair cells complete their last S-phase between E5 and E8 (Katayama and Corwin, 1989) and begin to be identifiable by the earliest available marker for hair cells, hair cell antigen (HCA), from E6.25 (Bartolami et al., 1991). HCA expressing cells continue to increase in number throughout the basilar papilla up to E9/10, at which time hair cell production ceases (Bartolami et al., 1991, Goodyear and Richardson, 1997). After this time hair cells are not produced under normal circumstances, and the epithelium only exhibits proliferation at a very low rate (Oesterle and Rubel, 1993). Only after acoustic or ototoxic damage do high rates of cell proliferation occur, leading to regeneration of sensory hair cells (Cruz et al. 1987;

In the vestibular patches the hair cell antigen is first detected at E5.25. (Bartolami et al., 1991). Here, in contrast to the basilar papilla, it has been reported that there is an ongoing production of hair cells throughout life. (Jørgensen and Mathiesen 1988; Roberson et al., 1992; Kil et al., 1997; Goodyear et al., 1999).

The aim of this part of the study was to extend the work of Anna Myat and others (Myat et al., 1996; Adam et al., 1998), who had already established that c-Notch1, c-Delta1 and c-Serrate1 are expressed in developing inner ear. I examined the expression of c-Notch1 and c-Delta1 by in situ hybridisation using a more sensitive technique than used previously (Fast Red), and the expression of Serrate1 protein could now be examined with the help of a polyclonal antibody, courtesy of Isabelle Le Roux. The availability of the Serrate1 antibody also meant that simultaneous antibody and in situ staining of the same section was possible. During this analysis I examined two other genes, c-Notch2 and c-Serrate2, whose expression in the chick ear was not previously known.
3.2 Results

The expression of c-Notch1, c-Notch2, c-Delta1, c-Serrate1 and c-Serrate2 at times before, during and after hair cells are produced: from E4 (stage 24) and every day subsequently until E12 (stage 38). For each day of development a minimum of three sectioned embryos using a confocal microscope. Except for c-Serrate1, where an antibody was used to detect the expression, gene expression was detected by in situ hybridisation. I will first describe the patterns of both c-Serrate1 and c-Delta1: expression of these two alternative Notch ligands was examined simultaneously on the same section. Next I describe c-Serrate-2 expression, which could only detected in the later development of the sensory patch. Finally I show that c-Notch1 is expressed throughout the epithelium during the time of hair cell production. I was unable to detect c-Notch2 in the ear during this period of sensory patch development.

3.3 Serrate 1 expression foreshadows the development of the sensory patches in the inner ear epithelium

C-Serrate1 (Serratel) expression in restricted domains of cells in the otic epithelium throughout the period of hair cell production. These areas of Serratel expression foreshadow the appearance of the earliest known marker of hair cells, HCA and subsequently demarcate the sensory patches (Myat, 1995).

By E4, as judged by their locations, both presumptive vestibular and auditory patches could be distinguished by Serratel expression. All cells in these nascent sensory patches strongly express Serratel (Figure 3.1, A+B). By E5 the expression of Serratel in the individual patches becomes much clearer. Distinct patches of
Figure 3.1: Double-stained cryosections showing expression of Delta1 (in situ hybridisation, red) and Serrate1 (antibody, green) at E4 (A+B) and E5 (C-F). All sections are transverse through the hindbrain and parallel to the future longitudinal axis of the cochlea. Medial is to the left and dorsal is to the top of each figure. (A-F) Serrate1 is expressed in all of the cells of the future sensory patches. At E4 (A,B) (stage 24) there is only weak expression of Delta1, within the Serrate1 domain in the presumptive vestibular patches (A, prospective utricular macula (u)), while in (B) Delta1 is not expressed in the more posteriorly located presumptive basilar papilla (bp). Note the strong expression of Delta1 and the Serrate1 stripe in the adjacent neural tube (nt). By E5 (C-F) (stage 26) Delta1 is strongly expressed in scattered cells in all of the vestibular patches (C-D), including the lagena (l) (E). Expression is seen in the distal end of the basilar papilla (E), but at this time the cells in other regions of this sensory patch only express Serrate1 (F). Scale bar=100μm

s= saccular macula; c= lateral crista
expression can be clearly identified as the cristae, maculae and basilar papilla (Figure 3.1,C-F). Although these domains of expression are distinguishable, there is, however, no clear boundary between the saccular macula and the basilar papilla: between these two patches Serratel expression is continuous (Figure 3.1, D).

Serratel expression remains high during sensory patch development, up until at least E12. However, close examination of the basilar papilla at E10 and E12 reveals a change in the intensity of Serratel staining in the apical cell layer, where mature hair cells reside. (Figure 3.3, C+E). Whereas in the basally located supporting cells expression remains high and more or less uniform, in the apical layer Serratel expression appears reduced, being seen only at cell-cell boundaries, never on hair cell apical surfaces. Since each hair cell is surrounded by supporting-cell apical processes, which separate it from other hair cells, it is possible that the mature hair cells downregulate Serratel and that this staining is entirely due to supporting cells. The antibody stain leaves this uncertain: it is difficult to differentiate between staining in the plasma membranes of hair cells and staining in the apical processes of the supporting cells. *In situ* hybridisation with a *Serratel* probe has clarified this issue (Cole et al., 2000): Serratel is indeed downregulated in hair cells.

### 3.4 Delta1 expression foreshadows hair-cell differentiation within the Serratel domains

The expression of *C-Delta1* (*Delta1*) detected, from E4 to E12, was restricted to the future and actual sensory patches as marked by Serratel expression; within these patches, *Delta1* was expressed in a scattered subset of cells. The timing of onset and cessation of expression of *Delta1* in these patches differs according to the different time courses of hair cell production in individual sensory patches.
Although relatively faint, *Delta1* expression was detected at E4 within the ventral Serrat1 domain that marks the future vestibular patches (Figure 3.1, A). At this time no *Delta1* transcript expression was seen within the more posterior-medial Serrat1 domain that marks the prospective basilar papilla.

By E5, scattered cells expressing *Delta1* transcripts were detected in both the presumptive vestibular patches and the basilar papilla (Figure 3.1, C-F). This is a time when hair cells are beginning to be produced, and vestibular patches are beginning to show HCA expression. Although scattered expression of *Delta1* in the vestibular patches is widespread (Fig 3.1C), it is more restricted in the basilar papilla (Figure 3.1, E-F). Only scattered cells near the distal end of the papilla express *Delta1*. This is in accordance with the first appearance of HCA in the basilar papilla 24 hours later, in the distal region (Goodyear and Richardson, 1997). Expression of *Delta1* and HCA in the lagener macula, at the distal end of the cochlea duct/lagena, starts earlier, at the same time as in the vestibular maculae.

Between E6 and E8, *Delta1* continues to be expressed in scattered cells in all of the Serrat1 domains (Figure 3.2). It should be noted that in the basilar papilla the extent of expression of *Delta1* varied from section to section. For example, at E7 some regions of basilar papilla did not show expression of *Delta1* (Figure 3.2, B). This could reflect the position-dependent time course of terminal mitosis within this sensory patch, which follows a centre to periphery progression (Katayama and Corwin, 1989). By E8, however, *Delta1* expression was seen throughout the basilar papilla (Figure 3.2, D).

By E9 all cells in the basilar papilla have gone through their terminal mitosis (Katayama and Corwin, 1989); thus after this time no new hair cells are produced (Bartolami et al., 1991; Goodyear and Richardson 1997). Correspondingly, at E9 expression of *Delta1* in this sensory patch has almost completely disappeared. Only a weak expression in cells in the apical layer of the epithelium.
Figure 3.2: Double-stained cryosections showing expression of Delta1 (in situ hybridisation, red) and Serrate1 (antibody, green) at E7 (A+B) and E8 (C-F). All sections are transverse through the hindbrain and parallel to the longitudinal axis of the cochlea. Medial is to the left and dorsal is to the top in A and B, while medial is to the right and dorsal is to the top in C-F.

(A+B) Expression at E7 (stage 30-31), Serrate-1 continues to be expressed in both vestibular and auditory patches; Delta1 levels remain high in scattered cells of the vestibular patches. The cochleovestibular ganglion (cvg) also expresses Delta1, but does not express Serrate1. Note that Delta1 is not detected in this portion of the basilar papilla (bp) (B).

(C-F) Expression at E8 (stage 34). The pattern remains the same, except that Delta1 is now seen expressed in scattered cells throughout the basilar papilla (D). (E,F) Higher power (x20) pictures of the utricle (u) (E) and the posterior crista (c) (F) at E8. Scale bar: A-C=100μm; D-F=50μm.
Figure 3.3: Double-stained cryosections showing expression of Delta1 (in situ hybridisation, red) and Serrate1 (antibody, green) at E9 (A), E10 (B,C,D) and E12 (E,F). The basilar papilla pictures are in the left column and cristae are shown in the right. All sections are transverse through the hindbrain and parallel to the longitudinal axis of the cochlea. Medial is to the bottom and dorsal is to the left in A, C and F, while medial is to the left and dorsal is to the top in B,D and F.

At E9, when no more mitosis is detected in the basilar papilla only the newly generated hair cells, in the apical layer of the epithelium, express Delta1 (A, arrow). A day or more later and Delta1 is no longer detected in the basilar papilla (C,F). However in vestibular patches where hair cell production is continuous throughout the bird's life, Delta1 continues to be expressed in scattered cells (B,D) even up to E12 (F). Scale bar: A+C=100μm; B, D-F=50μm.
(Figure 3.3, A). This apical layer is where mature hair cells reside. *In situ* hybridisations at E10 and at E12 revealed no further expression of the *Delta1* transcript in the basilar papilla (Figure 3.3, C+E). Thus there is a strong correlation between the appearance and disappearance of *Delta1* transcripts and the differentiation of hair cells in the basilar papilla. Moreover the localisation of expression in the apical layer of the epithelium at E9 supports the view that it is the nascent hair cells that are expressing *Delta1*.

A similar correlation of *Delta1* expression and hair cell production also exists in the vestibular patches. Here hair cells are first detected by HCA at E5.25 (Bartolami et al., 1991), and their production is continuous throughout the bird’s life (Jørgensen and Mathiesen 1988; Roberson et al., 1992; Kil et al., 1997; Goodyear et al., 1999). Correspondingly, in vestibular patches *Delta1* transcripts are first detected in scattered cells between E4 and E5 and continued to see its scattered expression even up to E12 (Figure 3.3, B,D+F).

3.5 *Delta1* is expressed in delaminated neuroblasts, and remains high after neurogenesis has ceased

Neuroblasts delaminate from the otic epithelium between E2 and E3.5 (D’Amico-Martel, 1982; Adam et al., 1998). Analysis of *Delta1* at this time reveals an early wave of expression in the anterior part of the placode. 6-7 hours later neuronal markers stain basally located cells in the same anteroventral part of the otic cup. *Delta1* continues to be expressed in the scattered cells in the neurogenic region of the epithelium until E3.5. Expression of *Delta1* in the otic placode therefore foreshadows neurogenesis (Adam et al., 1998).

From E5 up to at least E9, I detected increasing numbers of *Delta1* expressing cells in the cochleo-vestibular ganglion (Figure 3.2, B+D). The developing ganglion
contains dividing neuroblasts as well as post-mitotic neurons up to E7 (D'Amico-Martel, 1982); thus it is likely that the Delta1-expressing cells are nascent or maturing neurons. This suggests a role for Delta-Notch-mediated lateral inhibition, controlling the proportion of neuroblasts and neural progenitors as in the CNS (Henrique et al., 1997). It is interesting to note that even after terminal mitosis (E7), the neuroblasts continue to express Delta1 (Figure 3.2, D). Delta-Notch signaling has been shown to be involved in axon guidance (Berezovska et al., 1999, Redmond et al., 2000), which would therefore be a possible role for this continued expression of Delta1.

3.6 Serrate2 expression is seen only in nascent hair cells

The expression of c-Serrate2 (Serrate2), the other known alternative Notch ligand in chick, was also examined during hair cell production. Despite three attempts at early stages (E7), the Serrate2 transcript could only detected during late hair cell development: at E10 and E12 Serrate2 transcripts were seen in both the utricular macula and the basilar papilla (Figure 3.4). Although the expression detected was relatively weak, the patterns in vestibular and auditory patches were similar: only cells situated in the apical layer of the epithelium express Serrate2, corresponding to the nascent hair cells. No Serrate2 transcript could detected in sensory patches preceding E10.

3.7 Notch1 is expressed widely in the otic epithelium, including all sites of expression of its ligands

Anna Myat previously completed a full analysis of Notch1 expression during ear development. Its expression is seen early, throughout the otic placode (stage11) and
Figure 3.4: Double stained cryosections showing expression of Serrate2 \textit{(in situ} hybridisation, red) and Serrate1 (green) at E10 (A-D) and E12 (E-F).  
(A) Serrate2 expression in the apical layer of the utricular macula at E10. (B) is a high power of A. (C-D) Serrate2 is expressed in cells that are apically located in the basilar papilla at E10, corresponding to the position of hair cells. (E) Continued expression, although weak, of Serrate2 in the hair cells at E12. (F) the same picture, showing Serrate2 staining only, to show more clearly that the staining is only detected in the apical layer of the epithelium. Scale bar: A=50\mu m; B=20\mu m; C-F=100\mu m
continues up to at least E10, in both sensory and non-sensory regions (Adam et al., 1998). It is, however, downregulated in the mature hair cells of the basilar papilla (A. Myat, 1995), and this has also been reported in studies of hair cell regeneration (Stone and Rubel, 1999).

Double staining by Notch-1 in situ and Serrate1 antibody at a time of hair cell production (E7-8) confirmed the findings described above (Figure 3.5). Notch1 transcripts were expressed in both sensory (marked by Serrate1) and non-sensory patches. Interestingly, at this time the intensity of expression in the maturing sensory patches is less than in the neighbouring non-sensory epithelium. A similar complementary pattern is seen in the proneural stripes of the Drosophila heminotum, from which the sensory bristles develop (Parks et al., 1997). The Notch1 receptor is therefore available at and adjacent to the sites of expression of all three Notch ligands during the period of hair cell differentiation. Corresponding to the multiplicity of Notch ligands, it is entirely possible that other Notch receptors are also expressed, and that these might be selective in their responses to the different ligands. In chick one other Notch receptor is known, Notch2. Although its expression was seen in the floor plate, no Notch2 expression was detected in the otic epithelium at any time during hair cell development.
Figure 3.5: Double-stained cryosections showing expression of Notch1 (in situ hybridisation, red) and Serratel1 (antibody, green) between E7-E8. All sections are transverse through the hindbrain and parallel to the longitudinal axis of the cochlea. Medial is to the left and dorsal is to the top of each figure. (A-C) Notch1 is widely expressed throughout the inner ear epithelium at the time of hair cell production. Note that its expression is not confined to the sensory patches as defined by Serratel1 (green). For example it is also strongly expressed in the tegmentum vasculosum (tv) (C). Scale bar: A=100µm; B-C=50µm.

(u) utricular macula; (c) lateral crista; (bp) basilar papilla
3.8 Discussion

I discuss the chick data first, before turning to the data from other vertebrates.

3.9 Expression of Notch1 and Delta1 in the chick inner ear supports a role in lateral inhibition

The highly organised nature of the sensory epithelium has led to the suggestion that the regular cellular mosaic is generated by lateral inhibition, mediated by the Delta-Notch signaling pathway. (Cotanche 1987; Corwin et al., 1991; Lewis 1991; Adam et al., 1998). According to this theory, by expressing Delta1, nascent hair cells inhibit their adjacent neighbours from becoming hair cells, with the result that they differentiate as supporting cells. A mathematical model based on the properties of the Delta-Notch signaling mechanism has shown that lateral inhibition mediated by Delta-Notch signaling can generate the fine grained pattern of hair and supporting cells that is seen (Collier et al., 1996).

The observed gene expression pattern of Delta1 in the developing chick ear is consistent with this hypothesis. Delta1 expression foreshadows hair cell generation and is expressed in scattered cells within the sensory patches against a background of widespread Notch1 expression. In the basilar papilla Delta1 appears in scattered cells between E5 and E8. This parallels the time course of terminal mitosis, which corresponds to the time when hair cell fate is decided (Katayama and Corwin, 1989). Delta1 expression is no longer detected in the basilar papilla after E10, when hair cell production is complete. Its expression does however continue, in scattered cells, in vestibular patches where hair cell production is ongoing. The appearance of Delta1 also correlates closely with the appearance of HCA, the earliest known marker for hair cell
differentiation (Bartolami et al., 1991). Thus there is a strong correlation between the onset and duration of \textit{Delta1} expression and the differentiation of hair cells in all sensory patches.

It also appears that it is the nascent hair cells that express \textit{Delta1}; at E9 \textit{Delta1} expression in the basilar papilla is only detected in the apical layer of the epithelium, where hair cells reside. This evidence is reinforced by observations of \textit{Delta1} expression during hair cell regeneration in chick (Stone and Rubel, 1999). Destruction of hair cells by an ototoxic drug triggers the surviving supporting cells (which express Notch1) to divide and generate new cells expressing \textit{Delta1}: some of these express the gene strongly and become new hair cells, while others downregulate \textit{Delta1} expression and become new supporting cells. During later stages of regeneration, \textit{Delta1} expression disappears. All these observations are consistent with the Delta-Notch mediated lateral-inhibition model.

To date, Notch1 is the only candidate receptor for \textit{Delta1} in the avian inner ear. In chick the only other identified Notch family member is Notch2, which I failed to detect in the otic epithelium. As many as four Notch family members exist in other vertebrates (Weinmaster, 1997), thus it is plausible that other chick Notch family members are also expressed in the developing inner ear.

3.10 Expression of Serratel demarcates the future and actual sensory patches

Beginning at a very early stage, Serratel is strongly expressed in all prospective sensory patches, overlapping with the broad domain of \textit{Notch1} expression. Later, as hair-cell production begins, expression of Serratel marks the regions within which \textit{Delta1} expression is seen. Expression patterns of Serratel and \textit{Delta1} are however different: Serratel is expressed initially in all cells of the developing sensory patch while
*Delta*1 is expressed in scattered cells within this Serrat*el* domain. Because Serrat*el* is expressed in all the cells of a sensory patch the mechanism that regulates its expression is unlikely to be lateral inhibition, which would tend to create an uneven expression pattern as seen with *Delta*1. In the *Drosophila* wing margin, it has been found that Notch activation can upregulate the expression of the Notch ligand instead of inhibiting it, a mechanism termed lateral induction (de Celis and Bray, 1997). The result is that neighbouring cells stimulate each other to express the ligand strongly, creating a uniform expression of ligand in a group of cells. Thus lateral induction may control the expression of Serrat*el* in the sensory patches.

The uniform expression of Serrat*el* in the sensory patches suggests that this alternative Notch ligand serves a function different from that of Delta1. It might, for example, play a role in the regionalisation of the epithelium. It is possible that a patch of cells expressing both *Notch*1 and Serrat*el*, and with Notch1 potentially activated, takes on some special character that allows the cells to differentiate as either a hair cells or supporting cells, that is, define them as sensory patch cells. Alternatively, strong and widespread Notch activation might be important in preventing progenitor cells from differentiating into hair cells in excessive numbers or prematurely.

### 3.11 Serrate-2 is expressed in hair cells

Another alternative ligand for Notch, Serrate2, was also detected during hair cell differentiation. Unlike *Delta*1, Serrate2 expression could only be detected in mature hair cells. In mammals, as discussed below, this gene begins to be expressed somewhat earlier (Lanford et al., 1999). This discrepancy could be an artefact due to the chick Serrate2 probe, as expression did appear relatively weak, or it could reflect an actual species difference. Expression was undetectable until E10 and later at E12, when hair
cell production is already complete in the basilar papilla and has in the vestibular patches reached a plateau (Roberson et al., 1992). The expression patterns at this time were clear: only the cells at the lumenal surface of the sensory epithelium, the hair cells, expressed Serrate2. That Serrate2 is only expressed in the apical layer of cells in the vestibular patch at E10, in contrast to Delta1, which can be seen in scattered cells throughout the sensory patch, supports the view that only mature hair cells express Serrate2. Thus it appears that the nascent hair cells express Serrate2 at a time when they downregulate Delta1, and have therefore begun to differentiate. These findings also suggest that lateral inhibition controls Serrate2 expression in the same way as it does that of Delta1.

3.12 Expression patterns of Notch and its ligands are similar in other vertebrates

Expression patterns of Notch pathway components in the ear of fish, mouse and rat are strikingly similar to those seen in the chick. Firstly, hair cells express Delta1 (delta A, B, C and D in fish) and Serrate2 (called Jagged2 in mice and serrateB in fish). The expression of Delta1 appears first and is transient foreshadowing hair cell differentiation, while Serrate2 appears a little later and is more persistent (Morrison et al., 1999; Lanford et al., 1999; Shailam et al., 1999; Zine et al., 2000; Haddon et al., 1998a; Smithers et al., 2000). In mammals, as in chick, Serratel (or Jagged1 in mice) defines the domain of the sensory patch and eventually becomes restricted to the supporting cells (Lewis et al., 1998; Morrison et al., 1999; Zine et al., 2000). (The expression of the Serratel homologue, serrateA in fish has not been published.) Notch1 is expressed throughout the otic epithelium and is later downregulated in hair cells (Shailam et al., 1999; Lanford et al., 1999). In mice Notch2 (Hamada et al., 1999) and Notch3 (Lardelli et al., 1994) have also been reported to be expressed at the otic cup.
stage. It has, however, been reported that these two homologues are not expressed
during the time of hair cell differentiation (Lewis et al., 1998). However, there has to be
some doubt about this, since the same paper reported that there was no expression of
Delta1, and this is clearly incorrect (Morrison et al., 1999). No Notch expression
patterns in the ear have been published in zebrafish, though four Notch family members
are known.

This conserved expression pattern of Notch and its ligands points to an
essential role for the Notch pathway controlling the production of hair cells and
supporting cells. The expression of multiple Notch ligands in different patterns also hints
that the actual mechanism may be complex.

3.13 There is mounting evidence that Notch signaling is critical in the production of hair
cells

As mentioned in the introduction, strong functional evidence that Notch signaling
is critical in controlling the production of hair cells came first from the zebrafish mutant
mindbomb (mib). Over the past few years more genetic evidence has accumulated in
both fish and mouse. In zebrafish, the \( d\lambda A^{dr2} \) mutation produces a \( \delta\lambda A \) protein with
dominant negative activity. Sensory patches of \( d\lambda A^{dr2/dr2} \) embryos have a five-fold
increase in the number of hair cells and a severe reduction of supporting cells (Riley et
al., 1999). Thus, this supports a hypothesis that lateral inhibition mediated by \( \delta\lambda A \)
establishes the correct proportions of hair and supporting cells.

Targeted gene disruption in mice also provides good genetic evidence.
Unfortunately \( \text{Notch}1, \text{Delta}1, \) and \( \text{Jagged}1 \) knockout mice die as embryos before the
time when hair cells are produced (Swiatek et al., 1994; Hrabe de Angelis et al., 1997;
Xue et al., 1999). However, mice homozygous for a null mutation in \( \text{Jagged}2 \) survive
longer and display a significant excess of inner hair cells, and to a lesser extent outer hair cells (Lanford et al., 1999). This evidence suggests, therefore, that the loss of the inhibitory signal provided by Jagged2 allows more cells to differentiate as hair cells. It was not clear from this study whether the extra hair cells were produced at the expense of supporting cells, as would be predicted by the lateral inhibition model. Moreover the phenotype, when compared to that of mib, is relatively mild, suggesting that there is functional redundancy in the system. It is entirely possible that Delta1 is also required to inhibit the hair cell fate. Indeed in the insect sensory bristle, studies have shown that Delta is not the only Notch ligand at work: Serrate is also present and must be mutated along with Delta to give the most extreme Notch-pathway loss-of function phenotype (Zeng et al., 1998).

Thus the phenotypes of mib, dlA^{ar2/ar2}, and the Jagged2 knockout are consistent with the model where Notch-mediated lateral inhibition is required to prevent the cells in a sensory patch from all differentiating as hair cells, and provides a means to generate the fine-grained pattern. These three mutants all produce an excess of hair cells, to a greater or lesser extent. As discussed in the introduction a mouse null mutation that produces no hair cells is the Math-1 knockout (Bermingham et al., 1999), and overexpression of Math1 in cochlea explants produces ectopic hair cells (Zheng and Gao, 2000). That Math1 is a vertebrate proneural gene and these genes act in concert with the Notch signaling pathway further supports the central role of Notch signaling in hair cell differentiation. Indeed recently it has been shown that Hes1, a downstream Notch target gene, can repress Math1 expression (Zheng et al., 2000).

3.14 The standard model of Delta-Notch-mediated lateral inhibition may be simplistic
In chick the pattern of \textit{Delta1} expression in the inner ear, supports the hypothesis that \textit{Delta1} inhibits its adjacent neighbours from differentiating as hair cells. However, gene expression patterns are no proof of function. Moreover, it is clear that another Notch ligand, \textit{Serrate2}, is also expressed in the hair cells, and this has been shown to a play a functional role in the production of hair cells.

The strong expression of \textit{Serratel}, throughout the sensory patch further complicates the situation: all cells in the developing sensory patch, before, during and after hair cell production, are in contact with \textit{Serratel}-expressing neighbours and are thus liable to experience Notch activation. Recently it has been shown that \textit{Jagged1}/\textit{Serratel} does indeed play an essential role during the production of hair cells. A decrease in either \textit{Notchi} or \textit{Jagged1} expression by antisense oligonucleotide in cultures of the developing mouse sensory epithelium resulted in a dramatic increase in the number of hair cells at the expense of supporting cells (Zine et al., 2000), a phenotype more severe than that of the \textit{Jagged2} null mutant. So it appears that \textit{Serratel} does play an active inhibitory role in controlling the production of hair cells.

This result poses a further question. Do the sensory cells respond differently to the different Notch ligands? If more than one type of Notch receptor is involved in the development of the sensory patch, each type of Notch receptor might respond selectively to the different ligands and exert different downstream effects. A detailed analysis of the expression of all Notch family members at the time of hair cell development is required to entertain this possibility.

The \textit{fringe} gene family, whose products modulate the response of Notch to its different ligands, introduce another potential layer of complexity. \textit{Lunatic Fringe} is expressed in the sensory patches (Adam et al., 1998; Morsli et al., 1998; Cole et al., 2000) and recent cell culture experiments suggest that it differentially modulates \textit{Delta1} and \textit{Jagged1} signaling as \textit{Drosophila} \textit{Fringe} does in the wing margin (Hicks et al.,
On the other hand, no ear abnormalities are seen in the lunatic fringe homozygous knock-out mice (Zhang et al., 2000) (see page 115).

As discussed in the introduction, there are strong homologies between the sensory bristles of the fly and the sensory patches in the vertebrate inner ear, and lateral inhibition is thought to single out cells in both these systems. However, although the selection of the sensory organ precursor cell is often cited as an example of lateral inhibition there are several observations that do not fit with the simple lateral inhibition model. Importantly, in sensory bristle development careful examination of the expression of Notch and Delta, both at the mRNA and protein levels, shows that the regulation of transcription of Delta and commitment to cell fate is actually in contrast to the prediction of the lateral inhibition model. Selection of the sensory organ precursor cell (SOP) happens in a background of uniform Delta expression (Parks and Muskavitch, 1993; Parks et al., 1997). Also during the subsequent division of the SOP, the p1a and p1b daughter cells both continue to express Delta during this critical period (Parks et al., 1997). Similar uniform expression of Delta and Notch has been reported in the R8 photoreceptor neurons of the developing Drosophila retina (Baker and Yun, 1998) and in the embryonic Drosophila CNS. (Johansen et al., 1989; Kooh et al., 1993; reviewed in Baker, 2000). Genetic experiments show that in the embryonic central nervous system of Drosophila, transcriptional regulation of Delta is dispensable (Seugnet et al., 1997). Indeed, in the case of the sensory bristle, it has been proposed that cells within a proneural group mutually inhibit one another, and that the SOP fate is assumed by those cells that escape from this domain of inhibition, not because their neighbours cease to express Delta, but because they become somehow insensitive to Notch activation (Parks and Muskavitch, 1993).

Finally, observations of the initial pattern of hair cells and supporting cells in the chick basilar papilla indicate that the final and precise alternating pattern of cell types
cannot be solely due to lateral inhibition. The mosaic is initially irregular with hair cells widely spaced and occasional transient hair-cell-hair-cell contacts, and achieves its final precision through cell rearrangements (Goodyear & Richardson, 1997).

So although the expression pattern of Delta1 encourages one to believe in the relatively simple mechanism for hair cell production, the expression of Serrate1 and the evidence discussed above suggest that the actual mechanism for hair cell production might be more complicated. To disentangle the functions of this array of Notch ligands, and test whether Delta-Notch signaling provides the inhibitory signal to supporting cells, it is necessary to functionally test the role of Delta1 and examine what happens when Delta1 activity is manipulated artificially. In chick this is made possible by the use of retroviruses as vectors for gene misexpression. This was the aim of the next part of this study.
Chapter 4

Functional analysis of Delta1 using retroviruses

4.1 Introduction

Having identified Delta1 as a candidate-signaling molecule that may be involved in patterning the inner-ear epithelium, the next step is to test its function directly. At present, manipulation of gene expression using standard genetics is not feasible in chick due to the long generation time and lack of an efficient technique for making transgenic birds. However, retroviral vectors provide a valuable alternative means to alter gene expression (Morgan and Fekete, 1996). A retrovirus genetically engineered to contain a specific genetic construct has the advantage that it can be injected into the embryo at a chosen time and place, circumventing problems of early embryonic lethality that may arise if the gene in question is essential to the survival of the early embryo.

The main experiments in this chapter make use of a replication competent retrovirus (RCAS) – a retrovirus, which, upon infection, can generate new infectious particles that will spread to adjacent cells. This technique of retrovirus-mediated gene transfer is nicely applicable in the ear, since it is possible to inject the virus directly into the otic cup before or after it closes, heavily infecting the otic epithelium (Fekete and Cepko, 1993). With the RCAS virus it has been estimated that expression of the transgene is detectable by immunohistochemistry within 18 hours of injection into the neural tube (Homburger and Fekete, 1996). In my experiments, RCAS virus containing either Delta1 or a truncated form of Delta1 (Delta1<sup>ΔN</sup>) was introduced into the otic cup at embryonic day two (stage12). This is 72 hours before the appearance of the first hair
cells (Bartolami et al., 1991), thus allowing plenty of time for the infection to spread and for the transgene to be expressed in patches of cells in the otic epithelium (Figure 4.1). Previously, these two RCAS constructs were used to demonstrate that lateral inhibition mediated by the Delta-Notch signaling pathway controls the number of neurons and neural progenitors in the chick retina (Henrique et al., 1997). It should be noted that to accommodate the Delta1 sequence into the RCAS vector, which is limited to the size of insert it will accept, a slight truncation in Delta1 was necessary. This, however, had no discernible effect on the inhibitory function of Delta1 in the chick retina (Henrique et al., 1997).

To test the lateral inhibition hypothesis, I infected the developing otic epithelium with the RCAS virus overexpressing either Delta1 or Delta1\(^\text{dn}\). The results were not as predicted. Delta1 overexpression did not inhibit hair cell production and the dominant negative form of Delta1 did not produce excess hair cells, at least as judged from the numbers of hair cells seen at E8. RCAS-Delta1\(^\text{dn}\) did, however, shed some light on how Serratel expression is regulated, which I shall discuss first. The results I obtained in the otic epithelium using replication competent retrovirus were also confirmed using a replication defective retrovirus constructed by Isabelle Le Roux (Le Roux et al., in press, Eddison et al., 2000), as discussed later.

Finally, I also used a green fluorescent protein (GFP) -expressing replication defective retrovirus as a control for non-specific viral effects. It proved to be a good marker of cells, and thus has great potential for cell lineage analysis. The expression of GFP revealed the detailed morphology of the developing hair cell. Such observations show some interesting morphological changes that occur during hair cell maturation.
Figure 4.1: Schematic diagram of the technique of retroviral infection in the developing inner ear. Virus is injected at E2 (stage 12) when the prospective inner ear is an otic cup. Infected dividing cells begin to express the transgene after 10 hours, protein expression can be detected at 18 hours. More infectious particles are then made which spread to adjacent cells. Hair cells are first detected at E5.25 (Bartolami et al., 1991). Chicks are killed at E8, when the majority of hair cells have been produced, and the ears are then sectioned (15μm) for analysis.
4.2 Results

I shall first discuss the results that concern the effects of overexpression of the RCAS-Delta1\(^{dn}\) on Serratel expression and *Delta1* transcription before addressing the central question of hair cell production. I will then go on to discuss the effects of overexpression of RCAS-Delta1 on hair cell production.

4.3 Infections with RCAS-Delta1\(^{dn}\)

For the series of experiments with RCAS-Delta1\(^{dn}\), which include the Serratel results and the subsequent results on hair cell production, I injected a total of 185 E2 embryos with RCAS-Delta1\(^{dn}\) into both otic cups and incubated them until E8. Of these, only 75 survived (40%). The embryos that died could be roughly classified into two groups: (1) those that died soon after injection and (2) those that died around E5. Notch signaling is involved in many developmental processes and it is possible that the embryos that died around embryonic day 5 did so because the virus infected the vascular system and prevented its correct development. Indeed, many embryos that died around E5 showed extensive haemorrhaging.

Initial analysis of every other slide (see Materials and Methods) to check for infection by staining with Delta1 revealed that of the 75 embryos that survived, 38 embryos (50%) had infected patches within the otic epithelium either in sensory or nonsensory regions, as determined by HCA staining. Of these infected embryos, 15 had a good-sized patch or patches of informative infection which lay within or directly adjacent to a sensory patch. A total of 33 patches of infection were used in the analysis of the results.
4.3.1 Serratel is regulated by lateral induction

All the cells of a sensory patch initially express Serratel; only later after they have differentiated do the hair cells downregulate Serratel (Cole et al., 2000). This uniform expression of Serratel in contiguous cells suggests that the mechanism that controls the levels of its expression is lateral induction – that is, a positive feedback of Notch activity on ligand expression (de Celis and Bray, 1997), in contrast to lateral inhibition. If Serratel expression is positively regulated by Notch activation, it follows that in cells expressing Delta^{dn}, which blocks Notch activation in a cell-autonomous fashion (Henrique et al., 1997), one would expect to see a downregulation of Serratel. Indeed this appeared to be the case.

From a total of 33 RCAS-Delta^{dn} infected patches, 16 (48%) lay entirely within or were "marginal" to a sensory patch, as judged by Delta1 and HCA antibody. ("Marginal" means that an infected patch partially overlapped a sensory patch). The alternate sections of these infected embryos were then subsequently processed by Delta1 in situ hybridisation and Serratel antibody. Unfortunately 5 of the sets of sections that were processed in this way either did not physically survive the in situ treatment and were lost from the slides, or were not useful because the Serratel staining did not work well, even in sensory epithelia far from the site of infection. A total of 10 Delta^{dn} infected patches occurring within a sensory patch survived the treatment and showed good Serratel staining. Of these, 8 showed a complete absence or dramatic reduction of Serratel expression at the site of infection (Figure 4.2, Table 4A).

This result was strengthened by a parallel set of experiments conducted by Isabelle Le Roux. She infected the otic epithelium with RCAS virus expressing an alternative Notch pathway inhibitor, a dominant negative form of Suppressor of Hairless. Sections were analysed by an anti-viral antibody to detect infection (p27, Life
Science) and Serratel1 antibody. Here too she found that in 8 out of 10 infected patches in a Serratel domain, a down regulation of Serratel was seen, in agreement with my findings (Eddison et al., 2000).

I analysed embryos infected with RCAS-Delta1, in the same way as those infected with RCAS-Delta1dn. If Notch activation regulates Serratel1 expression positively, as suggested by the results with RCAS-Delta1dn, and ubiquitous Serratel is already serving to activate Notch1 maximally in all susceptible cells, the addition of virally-expressed Delta1 would not be expected to have any effect on Serratel expression. On the other hand, if the results with Delta1dn reflect merely a non-specific effect of viral infection, one would expect to see a reduction of Serratel1 staining within the Delta1 infections similar to that seen with Delta1dn. In fact, in the embryos infected with RCAS-Delta1, in the large majority of cases, (19 out of 24 patches) no down regulation of Serratel1 was seen in the RCAS-Delta1 infected patches (Figure 4.3, Table 4C). Thus the reduction of Serratel1 staining in the RCAS-Delta1dn specimens is not likely to be a non-specific effect of viral infection. One can therefore conclude that, in the otic epithelium, Serratel1 is regulated by lateral induction. That is, Notch activity upregulates the expression of Serratel1 (Eddison et al., 2000).

4.3.2 Effects on Delta1 transcription in Delta1dn expressing cells could not be easily determined

I also examined the level of Delta1 transcription in a Delta1dn infected patch. According to the lateral inhibition model, expression of Delta1 is inhibited by Notch activity. Thus when Notch activity is suppressed by Delta1dn, one might expect to see an up-regulation of Delta1 in infected cells. The pattern of expression here would be in contiguous cells, not scattered like the normal expression. To examine this point, I
Figure 4.2: Blocking Notch signaling causes a downregulation of Serrate1. (A-H) Four cases of infection with RCAS-Delta1⁰ⁿ virus, blocking signaling via Notch. Consecutive sections at E8 through the utricle (A+B and C+D), crista (E+F), and lagena (G+H). In the left-hand column the expression of Delta1⁰ⁿ is shown by in situ hybridisation with a Delta1 probe (red), coupled with detection of Serrate1 expression by antibody (green). In the right hand column expression of Delta1⁰ⁿ is detected by Delta1 antibody (green) and the distribution of hair cells is detected by HCA (detecting the hair bundles, red). Serrate1 expression is lost or clearly reduced at sites of infection. Presence of hair cells proves that these sites lie within the sensory patch, where Serrate1 is normally expressed. Scale bar=50μm.
Figure 4.3: Infection with RCAS-Delta1 in sensory patches does not affect expression of Serratel. Sensory patches infected with RCAS-Delta1 were analysed for Serratel expression. (A-C, utricle infections) The expression of RCAS-Delta1 is shown by in situ hybridisation with Delta1 probe (red). Serratel expression is shown by antibody stain in green. Serratel expression is not affected by Delta1 overexpression. Where the two stains are superimposed a yellow colour is produced. Scale bar=50µm.
made an RNA probe that corresponds to the last 153 amino acids of the intercellular domain of Delta1, a region that is absent in the truncated form of Delta1\(^{dn}\). The probe should, therefore, only detect the endogenous full length Delta1. Three Delta1\(^{dn}\) infected patches within sensory regions, from three separate embryos, were identified by staining with Delta1 and HCA antibodies. However, in situ hybridisation on the alternate slides to these patches of infection produced staining that was very weak with a high background. A final attempt on one other Delta1\(^{dn}\) infected patch within a sensory region was performed under more stringent in situ hybridisation conditions (0.7xSSC in the hybridisation mix as opposed to 1.3xSSC – see Materials and Methods) and still no firm conclusions could be drawn. A plausible reason is that this probe was short (460 nucleotides) and that secondary structure in this region hindered hybridisation to Delta1.

4.3.3 Effects of Delta1\(^{dn}\) expression on hair cell production are difficult to decipher

The original purpose of the retroviral experiments was to see what effect the retroviral constructs had on the production of hair cells. The expectation was that the Notch receptor would be inactive in a patch of cells infected by RCAS-Delta1\(^{dn}\). These infected cells would thus be rendered insensitive to Delta1-mediated inhibition by adjacent nascent hair cells, and, as a consequence, differentiate as hair cells. The predicted result of infection, therefore, is an overproduction of hair cells at the expense of supporting cells, similar to the phenotype seen in the zebrafish mutants mindbomb or deltaA\(^{dx2/dx2}\) (Haddon et al., 1998a; Riely et al., 1999) or the Notch1 antisense experiments in rat (Zine et al., 2000).

Interestingly, of the informative patches roughly half (17/33) directly abutted sensory patches, as marked by expression of Serratel1 and hair cell antigen (Figure
This cannot be explained as mere chance: when I analysed RCAS-Delta1 infected patches in a similar way, I found that only 9% of informative infected patches (3 out of 32) abutted sensory patches, as against 91% that overlapped with or were internal to sensory patches (Table 4C).

At least two interpretations of the "abutting" phenomenon might be proposed. First, infected cells that were originally destined to be sensory became diverted to a non-sensory fate as a result of viral infection: upon expressing Delta1^dn they were unable to differentiate as sensory patch cells since maintenance of sensory patch character requires activated Notch. The cells therefore became non-sensory in character, leading to a juxtaposition between uninfected sensory and infected non-sensory cells. However, by this line of argument one would expect to find not only patches of non-sensory infected cells abutting a sensory patch, but also non-sensory infected patches within the sensory patch. This phenotype was not seen in any of the 33 informative Delta1^dn cases. A more satisfactory explanation of the "abutting" phenomenon is that the initial infected patch of cells included both sensory and non-sensory cells and that all the infected sensory cells blocked from Notch activation differentiated prematurely as hair cells. By analogy with the mind bomb mutant, these hair cells without accompanying supporting cells would have soon been excluded from the epithelium and then would have died (Haddon et al., 1998a). This would leave, at the time of analysis (E8), the residual non-sensory part of the infected patch abutting the residual non-infected part of the sensory patch. An earlier analysis, for example at E4, might be expected to reveal small patches of supernumerary hair cells. This prediction remains to be tested.

What about the other half of informative patches of RCAS-Delta1^dn (16/33) that were seen inside or overlapping sensory patches? Hair cells (detected with HCA antibody) were counted per μm length of sectioned epithelium in the infected regions,
Figure 4.4: Infections with RCAS-Delta1<sup>dn</sup> often produced an "abutting" phenotype with infected non-sensory cells on one side of a boundary confronting non-infected sensory cells on the other side. Infection of the virus and sensory character appeared to be mutually exclusive. Two examples are shown (A+B) Sections through the utricle at E8. Delta1<sup>dn</sup> expression is revealed by an antibody against Delta1 (green) and the sensory patch is detected by the presence of hair cells (red). (B') the next adjacent section to (B) but processed by in situ hybridisation for Delta1 (red) and Serrate1 antibody (green).

Scale bar=50μm.
and compared with the adjacent uninfected sensory tissue (Figure 4.5). The perplexing result was that, in contrast to predictions, hair cell counts on a total of 11 Delta1Dn infected patches showed no significant increase in number (see Table 4B and Figure 4.5: 0.22±0.07 hair cells per μm infected regions, as compared with 0.23±0.05 in uninfected sensory tissue). There are several plausible interpretations of why we didn’t see the expected phenotype. These include the possibility (1) that several members of the Notch receptor family may be expressed in the ear, and that Delta1Dn, while blocking the one that regulates Serratel, may fail to block the one that regulates hair cell differentiation; (2) that there are different thresholds of response for Serratel transcription and cell fate control; and (3) that the infection occurred too late to influence the cell fate decision. These possibilities are discussed in detail later in this chapter.

4.3.4 RCAS-Delta1Dn infected cells were often seen in the cochleovestibular ganglion

I found that in many embryos that had good infection with RCAS-Delta1Dn, large numbers of infected cells often ended up as neurons in the cochleovestibular ganglion (Figure 4.6). This observation fits with the lateral inhibition hypothesis: cells in which Notch activation has been blocked during the early, neurogenic, phase of ear development should all differentiate as neurons or neuroblasts and delaminate from the otic epithelium if they lie in the neurogenic (antero-ventral) region.

4.4 Ectopic expression of Delta1 does not inhibit hair cell production

According to the simple lateral inhibition hypothesis, Delta1 provides the inhibitory signal that prevents cells from adopting the hair cell fate. To test this, I
Figure 4.5: Infection with RCAS-Delta1<sup>dn</sup> did not affect hair cell population density. Sections at E8 through the utricle (A,D), lateral crista (B) and basilar papilla (C). Delta1<sup>dn</sup> expression was detected by Delta1 antibody (green), hair cells by HCA (red). Despite expression of Delta1<sup>dn</sup> in all of the cells of the infected patch, an excess of hair cells was not produced. (E) High power (x60) of (D); infection occurs across the boundary of sensory and non-sensory epithelium. Within the sensory patch, cells within what appears to be the supporting cell layer, are strongly expressing Delta1<sup>dn</sup> (white arrows). Scale bar: A-D=50µm; E=20µm.
Figure 4.6: Delta1<sup>dn</sup> expressing cells often ended up as neurons in the cochleovestibular ganglion. Sections through the basilar papilla and underlying ganglion at E6. Two examples are shown. In the left column (A+C) the section is stained by in situ hybridisation for Delta1<sup>dn</sup> (red) and Iselt1/2 antibody (green) marking the nuclei of neurons in the cochleovestibular ganglion. In the right hand column (B+D) the adjacent section is stained by Delta1 antibody, revealing the dendrites of infected neurons with Delta1<sup>dn</sup> in their membranes. Scale bar=50μm.
overexpressed Delta1 in the developing sensory patch. The expectation was that, overexpressing Delta1 in the sensory patch should inhibit the production of hair cells.

In total, I injected 252 E2 embryos with RCAS-Delta1 into both otic cups. Of these, 109 (43%) survived until E8. Reasons for the high death rate are probably similar to those discussed for RCAS-Delta1<sup>rin</sup>. Every other slide from each embryo was then initially analysed for infection in the otic epithelium by staining with Delta1 and HCA antibodies. Of the 109 embryos that survived, 30 embryos had patches of infection within the otic epithelium in either sensory or non-sensory regions. Of these 30 infected embryos, 21 embryos had a good size patch or patches of informative infection, that lay within or directly adjacent to a sensory patch. From these 21 infected embryos, a total of 32 patches of infection were used in the analysis of the results. The vast majority of the infected patches were situated in the utricle.

Typical results of infection with RCAS-Delta1 are shown in Figure 4.7. In total 32 informative patches were examined (see Table 4C) 29 of these RCAS-Delta1-infected patches lay within sensory patches or overlapping them, and 3 lay directly abutting sensory patches. Of the 29 patches that lay within or overlapping the sensory patch, the vast majority (90%) displayed no obvious reduction in hair cell number. To check this impression more quantitatively, I counted individual hair cells in 18 representative infected patches and compared them to adjacent or nearby uninfected patches. Values of hair-cell counts of infected and adjacent non-infected epithelium were 0.23±0.08 and 0.24±0.08 hair cells per μm (see Table 4D). It appears then that the cells are not influenced by expression of Delta1 by their neighbours, and develop normally as hair cells despite being surrounded by an excess of Delta1.
Figure 4.7: Overexpression of Delta1 by RCAS virus in a sensory patch does not inhibit hair cell production. (A-F) Sections through the utricle (A-D), crista (E) and basilar papilla (F) infected with RCAS-Delta1. The infection is detected by a Delta1 antibody (green) and the hair cells are detected by HCA (red). Hair cells are produced normally despite being exposed to Delta1. Scale bar=50μm.
4.4.1 Ectopic expression of Delta1 by a replication defective virus also did not inhibit hair cell production

Results from the RCAS-Delta1 infections were checked by use of a replication-defective retrovirus to drive ectopic expression of full length Delta1. Replication-defective retroviral vectors contain deletions of the viral genes required for replication and hence can accommodate larger inserts of DNA, but are unable to generate new infectious particles. The retroviral construct used was based on the LZRSpBMN-Z plasmid (Kinsella et al., 1996) with the Delta1 gene linked to an internal ribosome entry site followed by the gene encoding green fluorescent protein (GFP). Thus both Delta1 and GFP will be expressed in infected cells. Isabelle Le Roux both made the virus and performed injections into the otic cup. I then analysed the sections by immunostaining with a GFP antibody (which gave stronger fluorescence than GFP alone) and HCA. A total of 8 embryos were analysed of which 2 had multiple patches of informative infections. Although this virus generates smaller patches of infection than the replication-competent virus, it has the advantage that one can assume that all infected cells have become infected at the same early time, soon after the virus is injected into the embryo. In total, hair cell counts were taken from 8 patches where infected cells lay in contact with each other. Again, hair cell counts from infected patches and neighbouring non-infected patches showed no significant difference: values of hair-cell counts of infected and adjacent non-infected epithelium were 0.24±0.05 and 0.25±0.05 hair cells per μm (Figure 4.8, Table 4E). Thus it can be concluded that Delta1 does not inhibit prospective hair cells from differentiating as hair cells. Therefore the simple lateral-inhibition model based on the regulation of Delta1 is not the correct explanation of how hair and supporting cells come to be generated in the observed proportions.
Figure 4.8: Infection with a replication-defective virus expressing Delta1 and GFP in a sensory patch does not inhibit hair cell production. (A-E) Sections through crista (A), basilar papilla (B), and utricle (C-E, x60). Infection is detected by a GFP antibody (green), hair cells by HCA (red). Hair cells appear to be produced normally even within clusters of contiguous cells all expressing Delta1. Scale bar: A+B=50μm; C-E=20μm.
4.5 GFP reveals that hair cells change shape during their development

As a control for non-specific effects of retroviral infection I injected a replication-defective retrovirus with only a GFP transgene into the otic cups of 10 embryos, all of which survived until E8. Sectioned embryos were then analysed by GFP and HCA antibody staining. Half of these displayed a good infection. Analysis showed that sensory patch development was normal and infection with this replication defective-GFP virus did not bias differentiation: infected cells became either supporting cells or hair cells. What these experiments did reveal, in great detail, was the morphology of the developing hair cells, lit up by GFP (Figure 4.9).

During hair cell development, the nuclei of hair cell progenitors migrate from the basal layer of the epithelium toward the lumen as they progress through M-phase of the cell cycle (Katayama and Corwin 1993; Tsue et al., 1994); at the same time, the cells round up, withdrawing the processes that connect them to the basal lamina. In the mature sensory patch, cell bodies of hair cells are confined to the lumenal portion of the epithelium; they do not contact the basal lamina, unlike supporting cells, which span the entire depth of the epithelium. Surprisingly, the recently formed GFP-expressing hair cells could be clearly seen to extend a thin process down to the basal lamina even though they were already expressing hair cell antigen apically. Recently, Stone and Rubel (2000) have reported similar hair-cell shapes in the regenerating ear epithelium. These workers also report that there is a period in the differentiation of a hair cell between its birth and its adoption of its final lumenal position when the hair cell is elongated, with its nucleus in the support cell layer and processes extending to both apical and basal surfaces. Thus in this period, the hair cell has a morphology similar to that of a supporting cell, yet it expresses hair cell antigen.
Figure 4.9: Expression of GFP by replication defective viral infection reveals detailed morphology of hair cells during their development. A set of cells is arranged in a series to suggest the likely course of events in the maturation of an individual hair cell. (A-E) GFP antibody (green) and HCA (red) at E8. A variety of cell shapes were detected probably reflecting the stage of development of the hair cell. (A,B) Hair cells, identified by expression of HCA, contact both the luminal and abluminal surface of the epithelium, with the nucleus in the supporting cell layer. (C) The luminal portion of the hair cell is swollen and presumably the nucleus migrates to this region (white arrow). (D) The nucleus is now situated at the luminal surface and the cell no longer contacts the basal lamina. (E) The basal 'tail' of the hair cell then retracts. Scale bar=10\(\mu\)m.
My observations, together with those of Stone and Rubel, show that loss of attachment to the basal lamina is a consequence and not a cause of the commitment to differentiate as a hair cell: the new-born committed hair cell, after emerging from mitosis close to the lumen, sends a process back down to the basal lamina just as a progenitor or supporting cell does, and only withdraws that process after hair-cell differentiation is well under way.
Table 4A: Results of infection with RCAS-Delta1<sup>dn</sup>.

<table>
<thead>
<tr>
<th>Embryo</th>
<th>No. of informative DI&lt;sup&gt;1&lt;/sup&gt;&lt;sup&gt;dn&lt;/sup&gt; infected patches</th>
<th>No. of infected patches abutting sensory patch</th>
<th>No. of infected patches internal to the sensory patch</th>
<th>No. of infected patches with a marginal overlap with the sensory patch</th>
<th>Expression level of Serrate1 in internal or marginal patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dn7</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Dn10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>No data x1</td>
</tr>
<tr>
<td>Dn12</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>No data x2</td>
</tr>
<tr>
<td>Dn19</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>Dn25</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>Dn27</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Dn28</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>Reduced x4</td>
</tr>
<tr>
<td>Dn29</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Dn31</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>Dn34</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>Normal x1</td>
</tr>
<tr>
<td>Dn40</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>No data x1</td>
</tr>
<tr>
<td>Dn43</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>Normal x1</td>
</tr>
<tr>
<td>Dn44</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>Dn45</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>No data x1</td>
</tr>
<tr>
<td>Dn61</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>No data x1</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>17</td>
<td>9</td>
<td>7</td>
<td>No data x6 Reduced x2 Normal x2 Reduced x8</td>
</tr>
</tbody>
</table>

Abutting means that the infected cells were non-sensory but were juxtaposed to an uninfected sensory patch (Serrate1 expressing cells), with no intermingling. Internal means that the infected cells were located within the sensory patch, as judged by HCA and Serrate1 expression. Marginal means the infected patch partially overlapped a sensory patch. Where the infected cells were judged to be of sensory character, because they expressed HCA, Serrate1 was down regulated.

Infected patches that were within a sensory patch and expressed both HCA and Serrate1 at normal levels were classified as normal. Reduced means that Serrate1 expression was reduced or absent.
Table 4B: Counts of hair cells in RCAS-Delta1<sup>dn</sup> infected and non-infected regions of sensory patches

<table>
<thead>
<tr>
<th>Embryo</th>
<th>No. of hair cells per 10 μm in Infected patch</th>
<th>No. of hair cells per 10 μm in Uninfected patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dn10</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Dn25</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Dn28</td>
<td>1.4, 1.8, 2.1</td>
<td>1.8, 2.1, 2.1</td>
</tr>
<tr>
<td>Dn31</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Dn34</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dn40</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Dn43</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>Dn45</td>
<td>3.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Dn61</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Total</td>
<td>24.6</td>
<td>25.6</td>
</tr>
<tr>
<td>Average</td>
<td>24.6 / 11</td>
<td>25.6 / 11</td>
</tr>
<tr>
<td>Per 10 μm</td>
<td>2.24</td>
<td>2.33</td>
</tr>
</tbody>
</table>

Thus 0.22±0.07 hair cells were counted per μm length of sectioned epithelium in the infected regions, as compared with 0.23±0.05 per μm in the adjacent uninfected sensory tissue (mean ± SD, n=11 in both cases; counts from representative sections showing patches ≥ 20 μm wide).
Table 4C: Results of infection by RCAS-Delta1

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Number of informative Delta1 infected patches</th>
<th>Infected patches showing normal hair cell numbers</th>
<th>Infected patches showing reduced hair cell numbers</th>
<th>No. of infected patches abutting sensory patch</th>
<th>Expression level of Serratel</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>No data x1</td>
</tr>
<tr>
<td>D24</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>No data x1</td>
</tr>
<tr>
<td>D33</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>Normal x3, Reduced x1</td>
</tr>
<tr>
<td>D50</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D55</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D58</td>
<td>6</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>Normal x4, Reduced x1</td>
</tr>
<tr>
<td>D60</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>D61</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D68</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>D74</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D75</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D78</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D80</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>No data x1</td>
</tr>
<tr>
<td>D82</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D83</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>Normal x2</td>
</tr>
<tr>
<td>D87</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Normal x1, Reduced x1</td>
</tr>
<tr>
<td>D88</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>D95</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D102</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Normal x1</td>
</tr>
<tr>
<td>D105</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>No data x1</td>
</tr>
<tr>
<td>D109</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>No data x1</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>26</td>
<td>3</td>
<td>3</td>
<td>No data x5, Reduced x5, Normal x19</td>
</tr>
</tbody>
</table>

Abutting means that the infected non-sensory cells were juxtaposed to an uninfected sensory patch (Serratel expressing cells), and did not intermingle.
Table 4D: Counts of hair cells in RCAS-Delta1 infected and non-infected patches

<table>
<thead>
<tr>
<th>Embryo</th>
<th>No. of hair cells per 10 µm of Infected patch</th>
<th>No. of hair cells per 10 µm in Non-infected patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18</td>
<td>3, 2.1</td>
<td>3.2, 2.3</td>
</tr>
<tr>
<td>D33</td>
<td>2.3, 3.4, 1.3, 1.4</td>
<td>2.3, 3.2, 1.3, 1.1</td>
</tr>
<tr>
<td>D50</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>D55</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>D68</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>D74</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>D75</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>D78</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td>D83</td>
<td>2.5, 4.3</td>
<td>2.7, 4.3</td>
</tr>
<tr>
<td>D87</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>D88</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>D95</td>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>D105</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Total</td>
<td>40.8</td>
<td>43.4</td>
</tr>
<tr>
<td>Average per 10µm</td>
<td>40.8/18</td>
<td>43.4/18</td>
</tr>
<tr>
<td></td>
<td>2.26</td>
<td>2.41</td>
</tr>
</tbody>
</table>

Thus 0.23±0.08 hair cells were counted per µm length of sectioned epithelium in the infected regions, as compared with 0.24±0.08 per µm in the adjacent uninfected sensory tissue (mean ± SD, n=18 in both cases; counts from representative sections showing patches ≥ 30 µm wide).
Table 4E: Counts of hair cells in replication-defective-Delta1 infected and non-infected patches

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Hair cell number per 10µm infected patch</th>
<th>Hair cell number per 10µm uninfected patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>E</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>19.5</td>
<td>20.2</td>
</tr>
<tr>
<td>Average per 10µm</td>
<td>19.5 / 8</td>
<td>20.2 / 8</td>
</tr>
</tbody>
</table>

Thus, 0.24±0.05 hair cells were counted per µm length of sectioned epithelium in the infected regions, as compared with 0.25±0.05 per µm in the adjacent uninfected sensory tissue (mean ± SD, n=8 in both cases; counts from representative sections showing patches ≥ 10µm wide)
4.6 Discussion

From these results and the discussion in the previous chapter, it is clear that regulation of hair cell production is not as simple as the lateral inhibition model suggests. Misexpression of Delta1 and Delta1^{dn} constructs with the RCAS virus did not produce the predicted effects on hair cell number. From other evidence, nevertheless, it is clear that Notch signaling is of central importance in controlling the production of hair cells. Thus Notch signaling operates in this context, but in a more complicated way than was originally proposed.

4.7 Delta1^{dn} blocks Notch activation but supernumerary hair cells were not detected

Expression of Delta1^{dn} in sensory epithelial cells caused Serratel to be downregulated in these cells. This result, while providing strong evidence that Serratel transcription is positively regulated by Notch activation, also acts as a positive control showing that, in this context, Delta1^{dn} is able to block Notch signaling. However, in contrast with the chick retina, where Delta1^{dn} expression causes over-production of neurons at the expense of progenitors (Henrique et al., 1997), I failed to see any overproduction of hair cells. Notch signaling in the retina is a simpler system: the only Notch ligand expressed is Delta1, while Serratel1 and Serratel2 have not been detected (I. Le Roux, personal communication). Thus one explanation for the lack of an effect in the ear might be that Delta1^{dn} fails to block activation of Notch by Serratel1 or Serratel2, while still blocking the activity required to maintain Serratel1 expression. Before opting for this interpretation, however, one needs to consider more closely the results of overexpressing Delta1^{dn} in the ear, since these are not straightforward.
Half the informative patches of infection lay directly abutting a sensory patch – a much higher proportion than could be explained by chance. As mentioned, this could be interpreted as the consequence of patches of infection that initially spanned both prospective sensory and non-sensory regions, causing the infected sensory cells to differentiate prematurely into hair cells, which, without surrounding supporting cells, are excluded from the epithelium and die (Haddon et al., 1998a & 1999). At E8, when infected ears were analysed, no traces of these hair cells would remain. Only through earlier analysis would one be able to see this phenotype. Also in support of Delta1^dn being able to transform the cell fate, many infected cells ended up as part of the cochleovestibular ganglion.

However, in many cases an infected patch was seen within a sensory patch and despite expression of Delta1^dn, the normal amount of hair cells was seen. This is surprising given the strong evidence that Delta1^dn blocks Notch signaling (Henrique et al., 1997) and that Notch signaling has a central role in the production of hair cells, as discussed in the previous chapter (Riley et al., 1999; Zine et al., 2000). How Delta1^dn actually works to block Notch signaling remains a mystery; it is only known that it acts cell autonomously, somehow preventing activation of Notch within the Delta1^dn expressing cell (Henrique et al., 1997). Explanations as to how Delta1^dn could be expressed in the sensory patch without affecting hair cell fate, while yet influencing expression of Serratel, are several. Firstly, hair-cell commitment and Serratel expression could have differing thresholds of response to Notch activity. In both lateral inhibition and lateral induction, Notch has two functions, to direct cell fate and to control ligand transcription and it is possible that these two different responses require different threshold levels of Notch activation. Delta1^dn could have a 'leaky' effect, allowing Notch to be activated to the extent required to prevent cells from adopting the hair cell fate, but not sufficiently to drive normal levels of transcription of Serratel.
Secondly, as discussed in the previous chapter, it is possible that more than one Notch receptor may be involved in development of the sensory patch. Delta1\textsuperscript{dn} may only be able to block the activity of one Notch family member – the one responsible for stimulation of Serrate1 transcription - while not affecting another Notch family member that governs cell fate choices. For example, one Notch receptor could be involved in the mechanism of lateral induction while another could be involved in the mechanism of lateral inhibition.

Another possibility is that the cells seen expressing Delta1\textsuperscript{dn} within a sensory patch may have become infected late, so that Delta1\textsuperscript{dn} did not begin to be expressed until after the cells were already committed to become hair or supporting cells. Thus the block of Notch activation would only effect ligand transcription and not cell fate. Virus-infected nerve fibres innervating a sensory patch of epithelium are a possible source of late infection: in infected embryos it was common to see infected neurons that innervate a sensory patch. However, for the retrovirus to be integrated into the genome and to be expressed, the host cell must undergo a division cycle and hair cell and supporting cell commitment occurs at the terminal mitosis. Therefore this interpretation requires that cells within sensory patches frequently became infected during a narrow time window just before their terminal mitosis.

A fourth possibility is that appearances were deceptive, and that even though the population density of the hair cells in the infected patches seemed normal, the Delta1\textsuperscript{dn} infection had actually altered the pattern of cell differentiation. This possibility is discussed in the next chapter in connection with Numb overexpression experiments, where similar phenomena were seen.
4.8 *Delta1 expression cannot account for the choice between hair cell and supporting cell fate.*

The simple model predicts that overexpression of Delta1 should inhibit hair cell production. I was unable to see such an effect. Cells that become hair cells therefore do so regardless of whether their neighbours express Delta1. If the choice of fate depends ultimately on levels of Notch activation, as implied by the data from Notch pathway mutants, then factor(s) other than Delta1 must be operating to allow some cells to escape inhibition and become hair cells while their neighbours do not. These factors could only be present in hair cells and are necessary for the efficacy of the Notch ligands that these cells produce, or these factors could be antagonists of Notch signal reception, expressed only by the nascent hair cells, and thereby protecting them from inhibition.

4.9 *Inhibition signaling might depend on an activation of Delta1 in the Delta1-expressing cell*

A positive effector could, for example, modify or process Delta1 protein to enable it to activate Notch. If nascent hair cells express such a factor, so that it is only in these cells that truly functional Delta1 is produced, ectopic Delta1 in the adjacent supporting cells, which do not express such a factor, would be unable to inhibit selection of hair cell fate. If this modulatory factor were itself negatively regulated by Notch activity the pattern-generating mechanism of the original simple model would still hold true, but with the modulatory factor playing the key role instead of Delta1. Little is known about such factors that might modify Notch ligands. The protease Kuzbanian is known to be necessary for production of active Delta in cell culture (Qi et al., 1999), but
there is no evidence that it is differentially regulated in adjacent cells. Another interesting candidate is the neurogenic gene neuralized. In Drosophila, neuralized is expressed selectively in neuroblasts and other cells that escape lateral inhibition, yet its loss-of-function phenotype is an overproduction of these cells, suggesting that it is required to enable them to deliver lateral inhibition to their neighbours (Price et al., 1993; Boulianne et al., 1991). Indeed, recently in Drosophila, Neuralized (Neu) has been shown to be a membrane localised protein (Yeh et al., 2000). Paradoxically, however, Yeh et al. (2000) report that Neu acts cell autonomously and is required in the cells that receive inhibition. The true mode of action of Neu is therefore still unclear.

At the beginning of my PhD research I made serious efforts to clone the chick homologue of neuralized by degenerate PCR and later used the human neuralized homologue (Nakamura et al., 1998) to screen a chick embryonic library. These efforts were not successful (for reasons that remain unclear), so I abandoned work in neuralized in the chick. It would be interesting to return to this gene and examine its expression in the ear.

4.10 Escape from inhibition might depend on a loss of sensitivity to Notch signaling in the prospective hair cell

It could also be that the nascent hair cells are insensitive to the expression of ectopic Delta1 from adjacent cells. By analogy with Drosophila there are three ways that a cell could become unresponsive to Notch ligands. First, the cell could down regulate the expression of the Notch1 receptor. In fact, the hair cell does this (Myat, 1995; Stone and Rubel, 1999), but apparently only after the decision to become a hair cell has been made. Thus this level of control may operate too late to control the cell fate decision. Second, a cell could make itself insensitive to Delta signals from its
neighbours by expressing high levels of Delta itself (Jacobsen et al., 1998). Since nascent hair cells normally express Delta1, this might be sufficient to make them refractory to the ectopic Delta1 from adjacent cells in the misexpression experiments. However, one could argue then that the supporting cells expressing ectopic Delta1 should also be insensitive to Delta1 expressed by their neighbours and so should differentiate as hair cells, which is not what happens. Thirdly, and most likely, the nascent hair cell may express a protein that acts as a Notch antagonist, making the cell insensitive to Delta1 expressed by neighbouring cells. Possible candidate proteins that could serve this function are Hairless (Bang and Posakony, 1992) or Numb (Uemura et al., 1989). The Hairless protein interacts physically with Suppressor of Hairless and antagonises the transduction of the Notch signal (Brou et al., 1994). The Numb protein physically interacts with the intracellular domain of Notch and blocks its activation (Frise et al., 1996; Wakamatsu et al., 1999). Moreover, in Drosophila, Numb and Hairless physically interact (Wang et al., 1997).

Using the sensory bristle analogy discussed in the Introduction, one could imagine that hair cells of the otic epithelium are akin to the sensory organ precursors (SOP), which despite being surrounded by Delta1 in the proneural cluster (Parks and Muskavitch., 1993; Parks et al., 1997) manage to become determined. Indeed, these authors argue that it is the cell that becomes resistant to Notch activation that adopts the SOP fate. Likewise the hair cell of the inner ear, despite being surrounded by ectopic Delta1, or for that matter the alternative Notch ligand Serrate1, still manages to adopt the hair cell fate. In the Drosophila sensory bristle Hairless promotes SOP commitment by antagonising the activity of the Notch pathway, protecting the future SOP from inhibitory signals from its neighbours in the proneural cluster (Bang et al., 1995). Thus by analogy, the nascent hair cell might make itself unresponsive to any Notch ligands expressed in its neighbour by expressing a homologue of Hairless. There
is, however, one difficulty with this proposal: vertebrate genomes do not appear to contain any Hairless homologues, as far as one can tell from BLAST searches of the sequences in GenBank and HTGS database.

Alternatively, the analogy with the bristle could be drawn at a later stage of bristle development, where the four types of cell that constitute the bristle are generated from the SOP through asymmetric divisions. As well as Hairless, which plays a role in bristle cell formation, Numb is also inherited by the bristle cell (Wang et al., 1997). Thus in vertebrates, Numb might also be present as a potential Notch antagonist inherited by the hair cell. The role of Numb in the ear is examined in detail in the next chapter.

A third candidate for the role of modulation of Delta-Notch signaling, in addition to Hairless and Numb, is Lunatic fringe (L-Fringe). In the larval Drosophila wing disc and in cell culture, Fringe has been shown to glycosylate Notch in such a way as to antagonise the function of Serrate and potentiate that of Delta as an activator of Notch signaling (Fleming et al., 1987; Panin et al., 1997; Hicks et al., 2000). The modulatory effect of Fringe, however, appears to be context dependent: in the developing fly bristle, for example, Fringe has been shown to interfere with both Delta and Serrate mediated signaling (Jacobsen et al., 1998). The expression of L-fringe in the sensory patch (Adam et al., 1998; Cole et al., 2000) suggests that it could be modulating the cells’ response to Delta1 and Serrate1 in a similar way in the ear. However, several lines of argument suggest that the role of L-fringe is not essential to hair cell production. First and foremost, there is no abnormality of hair cell production in the L-fringe null mutant mouse, suggesting any effect L-fringe has on Notch signaling is mild (Zhang et al., 2000). Zhang et al., (2000) were able to see an affect of L-fringe only in a Jagged2 -/- mutant background, where loss of L-fringe gave a rescue of the Jagged2 -/- mutant phenotype (i.e. prevented over-production of hair cells). Thus L-fringe appears to weakly inhibit Jagged2-independent Notch signaling but has only a minor influence in
normal development. Finally, chick ear development is entirely normal when \textit{L-fringe} is overexpressed by RCAS virus (I. Le Roux personal communication). In summary it seems unlikely that \textit{L-fringe} plays an essential role in the correct pattern of production of hair cells, though genetic redundancy is a possibility and one cannot exclude a role for other fringe family members (Johnston et al., 1997).

4.11 Signaling by \textit{Serratel} may serve to prevent premature hair cell production

From the results discussed it is clear that the correct production of hair cells and supporting cells cannot be simply determined by the levels of expression of \textit{Delta1} as proposed by the simple lateral inhibition model with feedback. Overexpression of \textit{Delta1} does not inhibit hair cell production. Moreover, \textit{Delta1} is not the only Notch ligand present: in the normal developing sensory patch all cells, including the nascent hair cells, are also exposed to \textit{Serratel}. An unresolved question is whether \textit{Serratel} and \textit{Delta1} activate Notch in the same fashion in this context in the vertebrate, as they appear to be capable of doing in neuroblast specification and bristle development in \textit{Drosophila} (Gu et al., 1995; Zeng et al., 1998; Jacobsen et al., 1998).

But what might be the function of \textit{Serratel}? An attractive possibility is that \textit{Serratel}, expressed throughout the prospective sensory patch from an early stage, might serve to prevent the premature production of hair cells by maintaining a high background level of Notch activation. This might be important in enforcing the delay between neuroblast delamination and hair cell differentiation. This idea receives strong support from the \textit{Jagged1} antisense experiments in rat (Zine et al., 2000).

According to this hypothesis, all sensory cells are exposed to \textit{Serratel} and consequently experience Notch activation during their development; overexpression of \textit{Delta1} does not significantly increase Notch activation beyond the level already evoked
by Serrate1. Assuming that Serrate1 and Delta1 can activate the same Notch receptor, as in the fly, all cells in the developing sensory patch would have a high level of Notch activation. It seems plausible to assume, then, that a cell that differentiates as a hair cell does so because it has become resistant to Notch activation, by analogy with the cells of the fly sensory bristle. In the next chapter I explore the possibility that it is the presence of Numb that allows a cell to differentiate into a hair cell.
Chapter 5

Expression of Numb and Numb overexpression

5.1 Introduction

A plausible explanation as to why exposure to ectopic Delta1 did not prevent sensory cells from differentiating as hair cells is that the prospective hair cells contain a protein that blocks Notch signaling. Indeed, all sensory cells are normally exposed to Serrate1, making it likely that only those that are immune to its Notch-activating action will be able to become hair cells.

With these facts in mind, we have proposed a new working model of hair cell production (Eddison et al., 2000). Supporting cells express Serrate1; hair cells express Delta1 and Serrate2. Serrate1 is positively regulated by Notch activation (lateral induction); Delta1 and Serrate2 are negatively regulated by Notch activation (lateral inhibition). Supporting cells, in contact with one another and hair cells, have a high level of Notch activation and thus down regulate Delta1 and Serrate2 and upregulate Serrate1. Hair cells contain a Notch antagonist, which allows these cells to upregulate Delta1 and Serrate2 and downregulate Serrate1. The absence of Notch activity then permits the cell to differentiate as a hair cell (Figure 5.1).

A good candidate Notch antagonist is the protein Numb. As discussed in the introduction, numb was first characterised in Drosophila as a membrane associated protein that is asymmetrically localised at mitosis and is essential for correct cell fate specification of the sensory bristle lineage (Uemura et al., 1989; Rhyu et al., 1994; Knoblich et al., 1995). The asymmetric localisation of Numb at mitosis is coupled to the
Figure 5.1: A new model for Notch signaling during hair cell generation (see text for details).
orientation of the cleavage plane (Knoblich et al., 1995). Upon division of the SOP, Numb is specifically segregated into the IIb cell. Overexpression of Numb forces both cells from the SOP division to adopt the IIb fate (Rhyu et al., 1994), while mutations in numb cause both cells to adopt the Ila fate (Uemura et al., 1989). Numb also acts in later cell divisions of the sensory bristle lineage. Importantly in this context, it is segregated in the Ila division into the cell that becomes the bristle cell (Rhyu et al., 1994; Frise et al., 1996; Guo et al., 1996; Gho and Schweisguth, 1998; Wang et al., 1997). Numb is thought to determine cell fate by interacting with the cytoplasmic domain of Notch and blocking its activation (Guo et al., 1996; Frise et al., 1996). Thus vertebrate Numb protein could confer hair cell fate in the ear: it is a good candidate for this role not only because it blocks Notch, but because it controls an analogous cell-fate decision in the Drosophila sensory bristle, where it is required for bristle cell differentiation.

Recently, a chicken homologue of numb (c-Numb) has been cloned (Wakamatsu et al., 1999). In mitotic neuroepithelial cells it is asymmetrically localised to the basal edge of the cell. Moreover, it has been shown to directly bind the cytoplasmic domain of Notch1, and inhibit Notch activation (Wakamatsu et al., 1999). In this chapter I explore the possibility that c-Numb is biasing the outcome of Notch signaling during the development of the sensory patch of the inner ear. First, I examine its expression pattern during otic development and find that this is consistent with the hypothesis that the nascent hair cell inherits c-Numb and that this blocks Notch signaling and allows hair cell differentiation. Using the same retroviral technique of gene misexpression as in the previous chapter, I managed to overexpress Numb during sensory patch development. However, overexpression of Numb, like overexpression of Delta1, did not appear to disturb the normal production of hair cells.
5.2 Results

5.3 Expression of Numb during otic development

To investigate the possibility that Numb may make the hair cell insensitive to Notch signaling, I analysed the expression of Numb during sensory patch development, from E2 (stage 13) to E12 (stage 38). As the Numb protein has a dynamic distribution pattern, an antibody was used to visualise the protein distribution in the otic epithelium (a kind gift of Yoshio Wakamatsu). The antibody was a polyclonal raised in rabbit against a 20-amino acid peptide in the carboxy terminus of c-Numb, and recognised a bacterially expressed, carboxy-terminal fragment of c-Numb by Western blot analysis (Wakamatsu et al., 1999). Sectioned embryos from each stage were stained with this chick Numb antibody and a nuclear marker, Syto16 (Molecular Probes), and analysed using a fluorescence microscope. For each stage a minimum of 3 embryos were analysed.

5.3.1 Early expression of Numb between E2 and E4

Immunostaining of the developing otic epithelium at daily intervals between E2 and E4 reveals that Numb is expressed throughout the otic epithelium, in both presumptive sensory and non-sensory regions (Figure 5.2+5.3). The staining pattern is similar to, but not as intense as, the Numb staining in the neuroepithelial cells of the CNS (Figure 5.2A). With regard to the staining in the CNS, most of the Numb is basally localised even in cells that are in interphase of the cell cycle, in contrast to the previous report that c-Numb is basally localised only at mitosis (Wakamatsu et al., 1999).
Figure 5.2: Numb expression at E2 and E3

Double-stained cryosections showing expression of c-Numb (antibody, green) and a nuclear marker (Syto16, red) at E2 (stage 13) (A-C) and E3 (stage 18), (D+E). All sections in this and the following figures in this chapter are transverse through the hindbrain and parallel to the future longitudinal axis of the cochlea.

(A-E) Numb is expressed in all of the cells of non-sensory and future sensory patches. (A) Low power picture showing the hindbrain and adjacent otic vesicles. Numb is expressed throughout the otic epithelium and neuroepithelium. Most of the staining is localised to the basal edge of the cells, though there is some staining of lateral membranes (B,C) E2: higher power (x20) photos of otic epithelium showing basal localisation of Numb in a mitotic cell (B), and in an asymmetric division were Numb is preferentially segregated into one of the two daughter cells (C). (D) E3: the central mitotic figure shows a symmetric division where Numb will be divided equally between two daughter cells. (E) a putative asymmetric division were Numb will be segregated into one daughter cell due to the orientation of the metaphase plate. Scale bar: A=50μm; B-E=20μm
Figure 5.3: (A+B) Double-stained cryosections showing expression of c-Numb (antibody, green) and a nuclear marker (Syto16, red) at E4 (stage 23).

(A) Numb is expressed in all of the cells of the future basilar papilla (bp), and interestingly, just as strongly in the non-sensory region (presumptive tegmentum vasculosum, tv). Basal Numb crescents can be seen in mitotic cells at the apical surface. (B) The same future basilar papilla, showing basal Numb crescents in mitotic cells.

Scale bar: A=50μm; B=20μm.
In the otic epithelium, Numb is expressed in the majority of cells and is strongly localised to the basal surface, even in cells that are in interphase. At mitosis, when cells migrate to the apical surface, Numb is still strongly localised to the basal edge of the cell. This persistent basal localisation of Numb is not only independent of the cell cycle but is also independent of the orientation of the cleavage plane of the cell, which were quite variable. Orientations of the cleavage plane were assumed to be the same as the orientation of the metaphase plate, which was visualised with the nuclear marker, Syto 16. Cleavage planes were either vertical to the plane of the epithelium, producing two cells that lay side by side in the epithelium, or were tilted (though rarely horizontal) such that a division produced one cell more apically and one cell more basally. Because Numb is always basal, its equal or unequal segregation into daughter cells depends on the angle of the cleavage plane. Thus in a vertical plane of division both daughters would inherit Numb (symmetrical), while in a tilted plane of division only the basal cell inherits Numb (asymmetrical) (Figure 5.2 B-D). Using these criteria, the number of asymmetric divisions was counted, between E2 and E4 in the prospective sensory patches (urticular macula or basilar papilla) and correlated with the basal Numb localisation.

Interestingly 18 out of 27 (66%) cell divisions were asymmetric, as judged by the segregation of Numb into only one of the two prospective daughter cells. It is not clear whether the asymmetric divisions at these early stages give rise to daughter cells with different fates. One possibility is that they correspond to the production of neuroblasts, which are indeed destined to migrate basally and delaminate from the otic epithelium (D'Amico-Martel, 1982).
5.3.2 *Numb is expressed in delaminated neuroblasts*

Neuroblasts delaminate from the anterior part of the otic placode epithelium between E2 and E3.5 and can be identified by staining with Islet1 antibody (D'Amico-Martel, 1982; Adam et al., 1998). Two sectioned E3 embryos were double stained with Islet 1/2 and Numb. This revealed that the delaminated neuroblasts also contain Numb (Figure 5.4). This suggests that Numb here is involved in neuronal differentiation or commitment. Interestingly, the expression of Numb in these neuroblasts also has an asymmetric localisation within the cell even after delamination (Figure 5.4). This agrees with the observations of asymmetric Numb localisation in cells in developing dorsal root ganglia (Wakamatsu et al., 2000); these may be cells that are destined to divide asymmetrically to produce a neuronal and a non-neuronal daughter.

5.3.3 *Numb expression at E6*

At E6 a change in the Numb expression pattern in the mitotic cells was noted. Numb crescents could no longer be detected in mitotic cells in the basilar papilla, and were less prominent than before in the vestibular patches (Figure 5.5). In total 12 mitotic cells in the basilar papilla were counted from three separate embryos, and none had significant Numb staining, or any hint of a Numb crescent. The absence of staining was not merely an artefact of the staining procedure, staining could still be seen in the basal region of the sensory epithelium. In the utriclular macula, though numbers are again small, 3/9 mitotic cells also showed an absence of Numb staining, again from three separate embryos. The remaining 6 mitotic cells had clear basal Numb crescents.
Figure 5.4: (A+B) Double-stained cryosections showing expression of c-Numb (antibody, green) and a nuclear neuronal marker (red, Islet1/2) at E3 (stage 18).
(A) Numb is expressed in the delaminated neuroblasts of the otic epithelium, marked by Islet1/2. (B) High power (x60) reveals that c-Numb is localised to one pole of the neuroblast (white arrows).
Scale bar: A=50μm; B=20μm.
Figure 5.5 (A-D) Double-stained cryosections showing expression of c-Numb (antibody, green) and a nuclear marker (red, Syto16) at E6 (stage 29). (A,B) Numb expression in the developing utricle (u). (A) Numb is still expressed throughout the epithelium and has a basal localisation. (B) High power of A (x60); in the majority of mitotic cells, basal Numb crescents can still be seen. (C,D) Numb expression in the developing basilar papilla (bp). (C) Numb is basally localised in the epithelium, and is also seen in the cochleovestibular ganglion (cvg). (D) High power of C (x60); in apically located mitotic cells no c-Numb crescents are detected. Scale bar: A,C= 50μm; B,D=20μm.
5.3.4 Numb expression at E7

By E7 many hair cells have differentiated in the vestibular patches, while only a few hair cells can be detected in the basilar papilla. At this time the expression pattern of Numb is remarkably different (Figure 5.6). In vestibular patches Numb expression is concentrated in the hair cells, and in these cells it no longer has a basal localisation (Figure 5.6 A-C). Instead it is expressed diffusely throughout the cytoplasm. In the basal supporting cells, there is no strong Numb expression, though some weak staining is apparent at the basal edge of the epithelium. This expression pattern therefore suggests that the hair cells and not the supporting cells either inherit Numb or switch on its expression at an increased level.

In the basilar papilla, E7 is a time of hair cell generation (Katayama and Corwin, 1989; Bartolami et al., 1991). Numb is still expressed in all the cells, but at higher levels in the hair-cell layer than in the supporting cell layer. Its localisation, though, is no longer predominantly basal (Figure 5.6 D). Staining now appears to be present in both apical and basal aspects of each cell, suggesting that Numb has lost its basal anchorage. This loss of basal localisation in all of the cells was only seen in the basilar papilla and may be peculiar to it.

5.3.5 Numb expression at E10 and E12

By E10 the basilar papilla has its full complement of hair cells (Katayama and Corwin 1989). The expression of Numb in the basilar papilla at this time is similar to the staining seen in the vestibular patches at E7. Numb is concentrated in hair cells with little staining in supporting cells. Furthermore, Numb appears diffuse throughout each
Figure 5.6: (A-D) Double-stained cryosections showing expression of c-Numb (antibody, green) and HCA, detecting mature hair cells (red) at E7 (stage 31). (A-C) Vestibular patches showing Numb expression mainly in the hair cells of the saccule (s) (A), utricle (u) (B), and crista (c) (C). Within the hair cell Numb is no longer localised, and is diffuse throughout the cytoplasm. In the basal supporting cells, Numb is absent or greatly reduced. Any Numb in these cells is basally located. Numb is also seen in some non-sensory epithelium. (D) In the basilar papilla (bp), Numb expression is still in the majority of the cells, and is distributed throughout each cell that expresses it. In some basal cells (support cells) the expression is reduced (white arrow). Note also expression of Numb in the blood vessel (bv). Scale bar=50μm
Figure 5.7: (A-D) Double-stained cryosections showing expression of Numb (antibody, green) and HCA, detecting mature hair cells (antibody, red) at E10 (stage 36). (A+B) Expression of Numb in the hair cells of the utricle (u). (B) High power photo showing clear expression of Numb only in the apical, hair cell layer. Within these cells Numb expression is not localised. (C+D) Expression of Numb in the basilar papilla (bp). As in the vestibular patches, strong Numb expression is found only in the mature hair cells and within these cells it is diffuse throughout the cytoplasm. Scale bar: A,C=50μm; B,D=20μm.
Figure 5.8: (A-C) Double-stained cryosections showing expression of c-Numb (antibody, green) and HCA, detecting mature hair cells (antibody, red) at E12 (stage 38). In both vestibular (A) and auditory patches (B+C) Numb remains diffusely expressed in the mature hair cells. Scale bar: A=50µm; B,C=20µm. c = crista; bp = basilar papilla
hair cell (Figure 5.7). The diffuse Numb expression pattern in the hair cells persists in both vestibular and auditory patches till at least E12 (Figure 5.8).

5.4 Overexpression of Numb does not produce supernumerary hair cells

To test whether overexpression of Numb would bias the number of hair cells produced during sensory patch differentiation, the same approach was used as described in the previous chapter. I engineered an RCAS virus (see Materials and Methods) that contained the full-length chick *numb* gene. Virus was then generated (see Materials and Methods) and used to infect early otic epithelium at E2 (stage 13). Embryos were killed 6 days later and alternately sectioned for subsequent analysis. Embryos were initially checked for infection by staining every other slide with c-Numb and HCA antibodies. Alternate slides from embryos with informative infections subsequently underwent *in situ* hybridisation with a *numb* probe followed by a Serratel antibody stain. A total of 57 embryos were injected, of which 23 survived until E8 (40%). Of these 23 potentially infected embryos, 12 had a total of 24 informative patches of infection that lay within or adjacent to a sensory region.

According to the obvious hypothesis suggested by *Drosophila* analogy, Numb should make cells insensitive to Notch activation and permit them to differentiate into hair cells. Thus one might expect to see an overproduction of hair cells in a patch of sensory cells infected with RCAS-Numb. Alternatively, one might expect a phenotype similar to that seen following infection with RCAS-Delta1<sup>dn</sup>, since Numb and Delta1<sup>dn</sup> are both supposed to block Notch activity.

Of the 24 informative patches, 14 of them (58%) lay within a sensory patch; 10 (42%) lay abutting a sensory patch. The great majority of infections within sensory patches were found in the utricle, though a few were seen in the basilar papilla (Figure 135).
Figure 5.9: (A-I) Overexpression of c-Numb by RCAS viral infection in a sensory patch does not affect hair cell number. Sections at E8 through the utricle (u) (A-C and G-I), lateral crista (c) (D), and basilar papilla (bp) (E+F). C-Numb overexpression was detected by c-Numb antibody (green), hair cells by HCA (red). Despite overexpression of Numb in a patch of sensory cells, the number of hair cells produced per unit length of epithelium was not affected. (C) is a high power (x60) of (B) and (F) is a high power (x60) of (E). Overexpressed Numb is not asymmetrically localised within the cell. Levels of Numb overexpression in a patch do vary, but all infected cells have an excess of Numb far more than in a normal cell. Scale bar: A,B,D,E=50 μm; C,F,G-I=20 μm.
Individual hair cells (detected by HCA) were counted in all 14 patches of infection within sensory patches and compared to adjacent or nearby uninfected sensory epithelium. Values of hair cell counts for infected and non-infected epithelium were not significantly different: 0.18±0.05 and 0.19±0.05 hair cells per μm, respectively (see Table 5B). Analysis of the infected patches with the Numb antibody revealed very strong expression of Numb in the infected cells, and this virally-generated Numb was not asymmetrically localised or concentrated at the basal ends of the infected cells. Thus one can assume that the localisation machinery of Numb did not function for the virally-generated Numb, and in a cell division Numb is likely to be inherited equally by both daughters. It should be noted that the level of expression of Numb differed, quite considerably, between cells within an infected patch. This is in contrast to the expression of both the Delta1 constructs which was more uniform throughout an infected patch. Why this might be is unclear, but if one normalises the pictures of infection so as to see the endogenous Numb in hair cells (using Adobe Photoshop) one can see that the infected cells, even those with relatively low-levels of Numb in comparison with their infected neighbours, all had a huge excess of Numb, in comparison with normal levels (Figure 5.9 (J)).

Given the apparent lack of effect on hair-cell production, the results with Numb were similar to those seen with Delta1<sup>dn</sup> for infected patches within sensory epithelia. As mentioned above, 58% of infected patches lay within a developing sensory patch. The remaining informative patches of infection –10 out of 24 (42%) were found abutting a sensory patch, as with RCAS-Delta1<sup>dn</sup> infections. This abutting relationship was seen both in specimens stained with HCA and Numb antibodies and in those stained by Serrate1 antibody and numb in situ hybridisation (Figure 5.10). As with Delta1<sup>dn</sup> this phenotype is not likely to be merely the result of chance: infection with
Figure 5.9: (J) Comparison between a photo of a patch of sensory cells infected with RCAS-Numb, and the same photo adjusted by Adobe Photoshop so one can see endogenous Numb expression in uninfected hair cells (white arrows). Using this adjustment one can see that all RCAS-Numb infected cells have a very high level of Numb as compared to the uninfected hair cells.
RCAS-Delta1 only gave 9% of patches with such a phenotype. As explained with Delta1^{dn}, the abutting phenotype could have been created by the death of infected cells that have prematurely differentiated as hair cells and subsequently died.

Some infected patches did show a down regulation of Serrate1 expression (Figure 5.11). This phenotype, however, was not as common as it was with Delta1^{dn} overexpression. Out of 10 Numb-infected patches only 4 showed a loss or reduction in the expression of Serrate1, while 6 showed a co-expression of Serrate1 and numb. This suggests that Numb is a less potent inhibitor of Notch activity than is Delta1^{dn}. 

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Figure 5.10: Infections with RCAS-Numb often produced an abutting phenotype. Two examples are shown, in consecutive sections through the lateral crista (c) (A,B) and the utricle (u) (C,D) at E8. Numb is either detected by *in situ* hybridisation (red) in the left column or by antibody (green) in the right column. The sensory patch is detected by Serratel antibody (green) in the left column or by HCA (red) in the right column. In these cases infection by the virus and sensory character appear to be mutually exclusive. Scale bar = 50μm.
Figure 5.11: Numb overexpression shows a downregulation of Serrate 1 (in some cases—see text). Two examples are shown. Consecutive sections through the utricle (u) at E8. In the left column numb overexpression is detected by in situ hybridisation (red), and Serratel by antibody (green). Note that where numb is overexpressed Serratel is down regulated. The right column shows the adjacent section stained with c-Numb antibody (green) and HCA.

Scale bar =50μm.
Table 5A: Results by infection with RCAS-Numb.

<table>
<thead>
<tr>
<th>Embryo</th>
<th>No. of Informative c-Numb infected patches</th>
<th>No. of infected patches abutting sensory patch</th>
<th>No. of infected patches internal to sensory patch</th>
<th>Expression level of Serratel in internal patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>N7</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>No data x3</td>
</tr>
<tr>
<td>N8</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N11</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>Reduced x2</td>
</tr>
<tr>
<td>N13</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>Normal x2</td>
</tr>
<tr>
<td>N14</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N18</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>Normal x2</td>
</tr>
<tr>
<td>N20</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>Normal x2</td>
</tr>
<tr>
<td>N21</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>No data x2</td>
</tr>
<tr>
<td>N22</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>Reduced x1</td>
</tr>
<tr>
<td>N23</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>No data x1</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>10</td>
<td>14</td>
<td>No data x6, Reduced x4, Normal x6</td>
</tr>
</tbody>
</table>

Table 5B: Counts of hair cells in RCAS-Numb infected and non-infected patches

<table>
<thead>
<tr>
<th>Embryo</th>
<th>No. of hair cells per 10 μm in Infected patch</th>
<th>No. of hair cells per 10 μm in Uninfected patch</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>N7</td>
<td>1.8, 3.4, 1.7</td>
<td>2.2, 3.4, 1.9</td>
</tr>
<tr>
<td>N11</td>
<td>1.2, 1.6</td>
<td>1.3, 1.6</td>
</tr>
<tr>
<td>N18</td>
<td>1.8, 2.1</td>
<td>1.4, 2.4</td>
</tr>
<tr>
<td>N20</td>
<td>2.0, 1.6</td>
<td>1.7, 1.8</td>
</tr>
<tr>
<td>N21</td>
<td>1.4, 1.3</td>
<td>1.7, 1.8</td>
</tr>
<tr>
<td>N22</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>N23</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>24.8</td>
<td>26.4</td>
</tr>
<tr>
<td>Average per 10 μm</td>
<td>24.8/14</td>
<td>26.4/14</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Thus 0.18±0.05 hair cells were counted per μm length of sectioned epithelium in the infected regions, as compared with 0.19±0.05 per μm in the adjacent uninfected sensory tissue (mean ± SD, n=14 in both cases; counts from representative sections showing patches ≥ 20 μm wide).
5.5 Discussion

5.6 Numb expression in the developing sensory patches is dynamic and suggests a role in the production of hair cells

Chick Numb is expressed from very early stages during otic development. At E2 it is strongly expressed throughout the otic cup epithelium, in both presumptive sensory and non-sensory regions. The expression pattern is very distinctive: Numb is strongly localised to the basal end of each cell. This basal localisation is independent of the cell cycle: it is seen in cells that are in either interphase or mitosis. Thus, whether two daughter cells inherit equal or different amounts of Numb is dependent on the plane of cell division, and between E2 and E6 Numb can been seen to be segregated sometimes into both daughter cells and sometimes into just one.

In the vestibular patches, many hair cells can be detected at E7. Here Numb is absent or much weaker in the supporting cells, while in hair cells Numb expression is much stronger. Moreover, in the hair cells it is now no longer basally localised, but appears to be diffuse throughout the cytoplasm. In the basilar papilla at E7, where only a few hair cells can be detected, there also appears to be a loss of basal localisation of Numb. Moreover, in some cells at basal positions within the epithelium - presumably nascent supporting cells - Numb staining appears significantly weaker than in the apically located cells, which are presumably nascent hair cells. By E10, when hair cell production in the basilar papilla is complete, Numb is only expressed in the hair cells and is seen diffusely throughout their cytoplasm. Thus at the time of hair cell differentiation it appears that supporting cells lose expression of Numb, while nascent hair cells retain Numb but lose its basal localisation.
However, at E6 a puzzling observation was made that does not fit with expectations based on the \textit{Drosophila} analogy. This was most marked in the basilar papilla. Here, no Numb was detected in the apically located mitotic cells during the period when hair cells complete their last S-phase, between E5 and E8 (Katayama and Corwin, 1989). A mitosis seen at E6 in the basilar papilla probably corresponds to a cell’s final division before the daughter cells differentiate. The absence of Numb in such cells could suggest that Numb is not required for hair-cell fate determination, at least in the basilar papilla. However, later Numb expression exclusively in hair cells would suggest that Numb does promote hair cell development. It seems that Numb is downregulated in all cells at terminal mitosis, and later upregulated in the hair cell. This disparity between Numb expression during terminal mitosis and in a mature hair cell is at present difficult to interpret. It could be that the mitotic cells seen were undergoing symmetrical divisions, not requiring Numb, and that the cell fate decision is taken after the final cell division has occurred. Supporting such a view, evidence from chick regeneration studies has shown that terminal mitoses in the basilar papilla sometimes produce two hair cells, sometimes two supporting cells, and sometimes a hair cell plus a supporting cell (Stone and Rubel, 1999). Moreover these occur in roughly equal proportions. This suggests that a process of asymmetric cell division does not exclusively generate hair cells in the basilar papilla.

Indeed if each cell in the developing sensory patch went through a final asymmetric division, yielding one daughter that inherited Numb and became a hair cell and one daughter that did not, the result would be a 1:1 ratio of hair cells to supporting cells. This is quite different from the observed ratio of hair cells and supporting cells: in the basilar papilla, the measured ratio of hair cells to supporting cells ranges from 1:1.7 to 1:3.9 (Goodyear and Richardson, 1997).
To summarise, in the otic epithelium Numb is expressed basally in all cells before hair cell differentiation, but during hair cell differentiation Numb expression is lost in cells that become support cells and either remains or is upregulated in cells that differentiate as hair cells. In the hair cells Numb is no longer localised to the basal surface, and is instead found throughout the cytoplasm.

5.7 The loss of basal localisation may have a significant functional consequence

During the proliferative phase of otic development Numb is present in all cells. Numb is thought to directly bind cytoplasmic Notch and block its activation (Guo et al., 1996; Wakamatsu et al., 1999). However all the cells of a sensory patch express Serrate1, and in the previous chapter I have shown that Serrate1 expression is positively regulated by Notch. This apparent paradox can be simply explained by the basal localisation of Numb. This localisation renders most of Numb inactive, as it is sequestered away from the site of Serrate-Notch signaling, which occurs over the entire cell surface, judging from the distribution of the Serrate1 protein. Only when Numb is released from its basal anchorage does it become functionally significant. Upon release it can now associate with the cytoplasmic domain of Notch throughout the cell membrane, and effectively block Notch signaling.

5.8 The asymmetric distribution of Numb is governed by different rules in chick, fly and mouse

The asymmetric localisation of Numb in the cells of the otic epithelium of the chick is reminiscent of the asymmetric localisation of Numb in Drosophila. There are, however, some important differences.
In the chick ear, Numb is basal, and the same is true in the developing CNS of
the fly (Rhyu et al., 1994; Spana et al., 1995). In the asymmetric divisions that give rise
to the fly bristle, however, the asymmetry is at right angles to the apico-basal axis of the
epithelium and is controlled by the planar-polarity gene frizzled (Gho and Schweisguth,
1998). In the chick basilar papilla, as discussed above, it seems that there is no
detectable Numb protein in the cells going through the final mitosis that gives rise to the
hair cells. But there are major differences from the Drosophila phenomena even before
this, in the period when Numb is clearly asymmetrically localised in the chick ear
epithelium. Firstly, the basal localisation in the chick does not depend on the stage in
the cell cycle; in Drosophila, by contrast, in both the CNS and the PNS, Numb is only to
be found asymmetrically localised at mitosis, while during interphase it is symmetrically
distributed over the cell membrane (Uemura et al., 1989; Rhyu et al., 1994). Secondly,
the position of Numb is independent of the plane of cleavage in chick but in the
Drosophila SOP lineage the position of the Numb crescent is coupled to the orientation
of the cleavage plane (Knoblich et al., 1995). The asymmetric localisation of mouse
Numb (m-Numb) is also independent of cleavage plane. However, unlike c-Numb, m-
Numb is asymmetrically localised to the apical membrane of dividing ventricular
progenitors (Zhong et al., 1996). This discrepancy between mouse and chick has not
yet been fully resolved, or completely understood. However, in any case, my
observations make it clear that Numb localisation is governed by different rules in
different species.

5.9 C-Numb antibody might cross react with a putative chick Numb-like

In mouse another protein similar to m-Numb, called Numb-like, is known to be
present and may be confusing the issue. In mouse, Numb-like is only expressed in
differentiating neurons outside the mitotically active zone, and is not localised to the membrane (Zhong et al., 1997). The sequence similarity between Numb and Numb-like makes cross reactivity with the chick Numb antibody a distinct possibility. Although no Numb-like is known in chick, Zhong et al., (2000) argue that the anti-c-Numb antibody is unlikely to be c-Numb specific. The peptide used to make the c-Numb antibody is highly conserved in mouse Numb-like, and this may account for the disparity between basal localisation of c-Numb in chick and apical localisation in mouse. However, this explanation is far from satisfactory, as Numb-like has no reported basal localisation.

Cross reactivity does raise another possibility. As mentioned, Numb-like is only expressed in postmitotic differentiating neurons and is not membrane-associated. In the chick ear, the c-Numb expression in the mature sensory patch is in the postmitotic hair cells, and it is diffuse throughout the cytoplasm. Thus it is possible that the antigen seen with the c-Numb antibody in the mature hair cells is in fact a chick Numb-like and not c-Numb. Mouse Numb-like, however, can mimic the action of Drosophila-Numb when misexpressed in flies with a numb loss-of-function mutation, (Zhong et al., 1997) suggesting that Numb and Numb-like are functionally interchangeable in their effects on cell fate, at least to some extent.

5.10 Numb overexpression within a sensory patch does not appear to affect hair cell production

Overexpression of full length Numb in developing sensory patches did not make a significant difference to the number of hair cells per unit length of epithelium in the infected patches. As Numb inhibits Notch, one would have expected to see an increase in hair cell number. The results seen with Numb overexpression are reminiscent of those seen with Delta^{1\text{st}}, not only because excess hair cells were not seen in infected
patches internal to the sensory patches, but also because a large proportion of infected patches lay directly abutting a sensory patch.

The most straightforward way to explain why excess hair cells were not seen is to suppose that Numb cannot completely inhibit Notch activation, and that as suggested in the previous chapter, only a total absence of Notch activity allows the differentiation of a hair cell. At least two lines of evidence support this. Firstly, in over half of the Numb infected patches that lay within a sensory patch Serratel was still expressed, which, as previously argued, implies that Notch was active. Secondly, it has been reported that in vitro the presence of Numb is not sufficient to completely inhibit nuclear translocation of activated Notch1 (Wakamatsu et al., 1999). It could also be that although the retroviral Numb protein is being expressed, it somehow is not functioning as the native protein. It has been established that chick Numb can promote neurogenesis in chick neuroepithelial cells (Wakamatsu et al., 1999), thus it would be good to test the virus in the CNS as a positive control.

Another possibility is that Numb function may require the presence of other proteins that are restricted to the nascent hair cell along with Numb. There are several proteins that could bind directly to c-Numb, which has several domains that could mediate protein-protein interactions. The amino terminal phosphotyrosine binding (PTB) domain, is known to bind two proteins: the putative serine/threonine kinase NAK (Numb associated kinase) and the PDZ domain-containing protein LNX (Dho et al., 1998). NAK has been shown to antagonise the function of Numb in vivo (Chien et al., 1998). Moreover, Numb also possesses a proline-rich carboxy-terminal region containing several putative Src-homology 3 domain-binding sites (Verdi et al., 1996), and an Eps15 homology (EH) domain-binding motif. (Such Eps proteins are thought to regulate endocytosis (Salcini et al., 1997). Alternatively, there could be some factor that is segregated to the hair cell in parallel with Numb and is independently required for hair
cell differentiation. For example, in *Drosophila* CNS, Prospero colocalises with the Numb crescent during cell division (Knoblich et al., 1995) and acts as a critical transcriptional regulator of the transition from mitotically active cells to terminal differentiated neurons (Li and Vaessin, 2000). Indeed, preliminary evidence suggests that *cProx1*, a chicken homologue for *Drosophila* Prospero, may be involved in hair cell development and regeneration (Stone and Rubel, 2000a).

5.11 *Numb may nevertheless influence cell fate in the chick ear*

It should be emphasised that a remarkably high proportion of the Numb-infected patches—42%—contained no hair cells but directly abutted uninfected sensory patches. Just as with the Delta1<sup>dn</sup> infections, this is a much higher proportion than would be expected by chance, and it strongly suggests that Numb did exert some effect on the pattern of cell differentiation. It is possible that in such cases infection occurred early, and the cells were either prevented from adopting a sensory character or differentiated prematurely as hair cells and disappeared from the epithelium, as proposed for Delta1<sup>dn</sup>.

How can this be reconciled with the apparent lack of an effect on hair-cell numbers in Numb-infected patches internal to a sensory patch, discussed in the preceding section? The answer may lie in a redundancy of mechanisms, such that the effects of Numb (or of Delta1<sup>dn</sup>) are sometimes weak in relation to other factors and sometimes strong, depending on the time and site of infection. There are, however, other possibilities.

All the explanations put forward in the preceding section for the apparent lack of an effect of Numb overexpression presuppose that this phenomenon should be taken at face value: that overexpression of Numb—or of Delta1<sup>dn</sup>—within a sensory patch really
causes no change in the proportion of cells that become hair cells or supporting cells. The evidence for this comes from counts of the hair bundles per unit length of epithelium in sections of infected as compared with uninfected regions. Since, at the stages analysed, the apices of supporting cells normally occupy a substantial part of the apical surface of the sensory epithelium, one would expect that an absence of supporting cells and their replacement by hair cell bundles should give a substantial increase in the number of hair bundles seen per unit length of epithelium in a section through an infected patch. There are at least two ways in which this expectation might be mistaken.

First, it is conceivable that where supporting cells are absent, the hair cells may adopt a different shape, each one occupying a larger area of epithelium than normal in such a way as to compensate for the absence of supporting cells. This would give an apparently normal number of hair bundles per unit area of epithelium. Observations of patches of hair cells without supporting cells in the zebrafish mind bomb mutant (Haddon et al., 1998a) suggest that this possibility is not quite as far fetched as it might seem. In the absence of a good marker for supporting cells at the relevant stages of development, it would be instructive to count the numbers cell nuclei per unit length of epithelium in infected as compared with uninfected regions of sensory patches. If the infected patches indeed consist entirely of abnormally large hair cells, with no supporting cells, the number of cell nuclei per unit length of epithelium should be reduced. Unfortunately, this possibility was not foreseen when the original sections were prepared, and no nuclear stain was applied. Thus it is difficult to count the cell nuclei in the existing preparations. This is a task that remains for the future.

A second possibility is that overexpression of Numb or Delta1\textsuperscript{th} may indeed have caused all the cells in an infected patch to develop as hair cells, but that subsequent cell movements may have caused these hair cells to become dispersed
among the surrounding uninfected supporting cells, restoring an apparently normal mixture of the two cell types. In this case, one should find on close analysis that the supporting cells within an infected region of a sensory patch are uninfected while the hair cells are infected. Because the two cell types are so intimately mingled at the stages analysed, this is not easy to resolve with the markers that I used, and images such as those in Figure 4.5 or (more debatably, perhaps) Figure 5.9 do not encourage the idea. The matter could be settled by allowing infected embryos to develop to a later stage, such as E10, when the hair cells have normally retracted their basal processes and become confined to the apical layer of the sensory epithelium.

5.12 Four isoforms of mammalian numb exist

The recent identification of different isoforms of mammalian numb raises the interesting possibility that the different isoforms serve different functions during development. In human and mouse 4 different Numb isoforms, generated by alternative splicing have been isolated (Verdi et al., 1999; Dho et al., 1999). The isoforms vary in two domains of the Numb protein, the PTB domain and a proline rich (PRR) domain. The PTB domain can contain an 11 amino acid insert, while the PRR domain can contain a 48/49 amino acid insertion. These different isoforms have been shown to be differentially expressed during development (Verdi et al., 1999; Dho et al., 1999). Moreover the amino acid insertion in the PRR affects the behaviour of the cell in vitro and in vivo. The short PRR isoform promotes neuronal differentiation and the long PRR isoform, while not affecting differentiation, promotes cell proliferation (Verdi et al., 1999). Analysis of the protein sequence of chick numb revealed that it does not contain the insert in the PRR region. By analogy with the human numb isoforms then, overexpression of chick Numb should have an effect on cell differentiation. Indeed, the
Numb I overexpressed has been shown to promote neurogenesis in the chick neuroepithelial cells (Wakamatsu et al., 1999) and in neural crest cells (Wakamatsu et al., 2000). However, the PTB domain in chick Numb also lacks the short amino acid insert and this has been shown, in vitro, to localise the protein to the plasma membrane (Dho et al., 1999). Thus, the overexpressed Numb may not be localised correctly to the plasma membrane and is unable to successfully block Notch activity. Isolation of all potential chick numb isoforms and antibodies specific to these different isoforms would greatly enhance our understanding of the role of numb in otic development. The c-Numb antibody used in this study does not distinguish between the potential different isoforms of c-numb.

5.13 Conclusion

In summary, overexpression of Numb alone does not produce the expected phenotype of an excess of hair cells at the expense of supporting cells. This may be because Numb is unable to completely block Notch activity: for example, the Numb that was expressed lacks the sequence shown in mouse to localise Numb to the membrane and thus enable it to act on Notch (Dho et al., 1999). Numb may not be able to act alone: it may be necessary but not sufficient to cause a cell to differentiate into a hair cell, because other factors are required in conjunction with it for hair cell differentiation. There is also the possibility that it is not required at all, as suggested by the absence of Numb in cells of the basilar papilla undergoing terminal mitosis. Loss-of-function experiments would need to be conducted to completely rule out a central role of Numb in hair cell production. This could be achieved by mis-expressing a dominant negative c-Numb that only contained the PTB domain (Verdi et al., 1996).
On the other hand, there is evidence to suggest that, in some cases at least, Numb overexpression does alter cell fates in the otic epithelium. This, and the close similarity between results of Numb overexpression and Delta1\textsuperscript{dn} overexpression, suggests that appearances might not be quite as they seem. More careful analysis of infected regions within sensory patches, using a new series of specimens stained in different ways, might reveal that there are, after all, effects on the developmental choice between a hair-cell fate and supporting-cell fate.
6.1 Notch signaling is central to hair cell production, but the manner in which it operates is complex

Although the evidence from a wide variety of vertebrates points to a central role for Notch signaling in the differentiation of hair cells, the way in which it operates remains elusive. As demonstrated in this study, it is clearly not as the original lateral inhibition model supposed. The complexity corresponds to the multiplicity of Notch ligands expressed in the ear.

At least three Notch ligands are expressed during the development of the chick inner ear: Serratel is initially expressed in all cells of the developing sensory patch, and persists in supporting cells after hair cell differentiation. Delta1 is expressed in scattered cells in two phases, firstly in the delaminating neuroblasts and later in the presumptive hair cells, and it is downregulated soon after hair cell differentiation. Only Serrate2 appears to be expressed in differentiated hair cells and persists in them until at least E12. Notch1 is expressed throughout the otic epithelium. A similar pattern of expression of Notch and its ligands is seen in other vertebrates, such as mice, rats and fish (Morrison et al., 1999; Lanford et al., 1999; Zine et al., 2000; Haddon et al., 1998a).

The expression pattern of Serratel suggests a mechanism of lateral induction, whereby Notch activation up-regulates Serratel expression. Indeed, inhibition of Notch signaling, by expression of a dominant negative form of Delta1, down-regulates the
expression of Serratel, supporting the hypothesis that Serratel expression is normally stimulated by Notch activation.

It is possible that different Notch receptors respond selectively to the different Notch ligands, providing a simple way in which the different Notch ligands could exert different effects. However, all three Notch ligands are likely to activate Notch1 - the only known receptor to be expressed in chick otic epithelium (Shimizu et al., 2000). This suggests that all sensory patch cells initially contain activated Notch during their development, a hypothesis supported by the fact that several days before hair cell differentiation, all cells persistently express Serratel, which requires Notch activation. This early Notch activation may define the domain of the sensory patch. In such circumstances, it is difficult to see how a cell expressing Delta1 could influence a neighbour by activating Notch, since Notch is already active in the neighbour because of the continual presence of Serratel on other adjacent cells. Indeed, Delta1 expression in the nascent hair cell does not inhibit adjacent cells from differentiating as hair cells: artificial overexpression of Delta1 in a patch of sensory cells does not prevent or even reduce the production of hair cells. Thus the simple model of lateral inhibition with feedback, mediated by Delta-Notch signaling, does not explain how hair cell production is controlled.

Serratel seems to have a more clearly definable role in sensory patch development. In rats Jagged1 (Serratel) antisense oligonucleotides produce an excess of hair cells (Zine et al., 2000). It is tempting to propose on this basis, in the light of the gene expression data, that all the cells of a sensory patch are Notch active before the time of hair cell differentiation and that this inhibits commitment to a hair-cell fate, preventing premature differentiation. A cell must escape the state of Notch activation in order to differentiate as a hair cell. The question then is - how does a nascent hair cell escape Notch activation?
An answer is suggested by the observation that the Notch antagonist Numb is expressed in mature hair cells. However, overexpression of Numb in a sensory patch does not appear to affect the number of hair cells produced. As argued in previous chapters, it may be that only a total absence of the Notch signal will allow a cell to adopt the hair cell fate, and Numb alone may not be sufficient to completely block Notch activation. A similar hypothesis might also explain the absence of a hair cell phenotype with RCAS-Delta1<sup>Δn</sup>.

I have presented evidence, on the other hand, that the RCAS-Delta1<sup>Δn</sup> and RCAS-Numb constructs used in this study do, in some cases, have effects on hair-cell commitment. I have also argued that the phenotype in other cases may have been subtle and may have escaped detection by the method of analysis used. Indeed, in the Jagged2 and Hes1 knockout mice (Lanford et al., 1999; Zheng et al., 2000) the phenotype is relatively mild, and could quite possibly be missed by taking transverse sections through the cochlea, instead of an en face view. Interestingly, the hair cell phenotype of both these mutants was restricted to inner hair cells of the auditory epithelium, perhaps reflecting a further refinement and specialisation of the mechanism according to the type of hair cell being produced.

6.2 Other factors may also be involved in hair cell production

Other pathways interact with Notch, and may also play a role in hair cell generation and patterning. The epidermal growth factor (EGF) pathway is one example. Like Notch, the Drosophila EGF receptor is involved in a large number of developmental decisions (reviewed in Schweitzer and Shilo, 1997), and EGF growth factor signaling acts in conjunction with the Notch pathway to control cell fate in the developing Drosophila chordotonal sense organs (zur Lage and Jarman, 1999).
In vertebrates, an excess-hair-cell phenotype is produced by retinoic acid. In the mouse there are several reports of retinoic acid and its receptors being expressed in the developing organ of Corti. (Kelley et al., 1993; Romand et al., 1998; Raz and Kelley, 1999), and addition of exogenous retinoic acid to organ cultures of mouse auditory epithelium produces an excess of hair cells and supporting cells (Kelley et al., 1993). Conversely, addition of a retinoic acid receptor antagonist significantly reduced the number of hair cells (Raz and Kelly, 1999). However, the precise role of retinoic acid is unclear. In the inhibition studies the expression of myosin VI, an early marker for hair cell differentiation, is not affected by the disruption of retinoic acid signaling, suggesting that initial commitment of hair cells is not dependent on retinoic acid (Raz and Kelley, 1999). 

It could be that the final pattern of hair cells and supporting cells is not a reflection of the initial pattern of cell fate specification. Indeed some hair cell rearrangement does occur (Goodyear and Richardson, 1997), and in regenerating sensory epithelium it has been demonstrated that symmetric divisions can occur such that sister cells both become hair cells, implying that subsequent cell movements take place to separate them (Stone and Rubel, 2000a). Another factor that could affect the final pattern is programmed cell death, which has been reported during the development of sensory patches (Lang et al., 2000). Finally, the phenomenon of transdifferentiation has been reported in chick, and is also a possibility in generating the fine-grained pattern. Here supporting cells differentiate into hair cells without passing through mitosis (Alder and Raphael, 1996; Roberson et al., 1996; Baird et al., 2000).
6.3 Why do hair cells express Delta1?

A cell expressing Delta1 in the ear does not inhibit its neighbours from adopting the hair-cell fate. Why then is Delta1 expressed in the nascent hair cells? It may be that its expression serves no function and is just a by-product of a blockade of Notch activation. However, there are other possibilities. Delta1 could, for example, act in collaboration with Serrate2/Jagged2 to deliver lateral inhibition, but be ineffective on its own. It is also conceivable that it, and Notch signaling more generally, may have a role in the planar polarity of hair cells. In the mouse Jagged2 knockout, the hair cell bundles are not correctly aligned (Lanford et al., 1999), and in the Notch1 and Jagged1 antisense experiments too, the hair cells display a wide variation of planar polarity (Zine et al., 2000). On the other hand, there is evidence that Notch does not play a role in planar polarity from the zebrafish mutant mind bomb, where the hair cell bundles do not show gross departures from the normal orientation (Haddon et al., 1999).

6.4 Possible future experiments

Retrovirus mediated gene overexpression provides a good means to test gene function in the inner ear. However, production of the virus can be time consuming, and only high titres of virus ensure a reasonable infection in the otic epithelium. With some constructs, such as RCAS-Delta1, high titres of virus are difficult to obtain, because the gene in question is toxic to the primary fibroblasts used to grow up the virus.

Electroporation is a new method that can potentially circumvent these problems. Here the DNA construct is directly introduced into the cells of the inner ear by injecting the DNA into the otic cup and applying a transepithelial voltage with small electrodes (Momose et al., 1999). Using an expression vector with a GFP reporter gene, I
managed to see good transient transfection of the otic epithelium (Figure 6.1). Moreover, initial electroporation experiments using the DNA of the RCAS construct also show that good infection in the otic epithelium, including the basilar papilla, is possible (Alex Davies, personal communication). Thus electroporation may provide a more efficient means to test the function of genes in the inner ear.

An easily achievable experiment, which may yield important results, is to test the effect on hair cell production of overexpressing a dominant-negative form of Suppressor of Hairless (Su(H)) (Eddison et al., 2000). Good titres of RCAS-Su(H)\textsuperscript{dn} virus are difficult to obtain by cell culture (Isabelle Le Roux, personal communication) and electroporation of the corresponding DNA provides an alternative means to achieve infection. If this construct completely blocks Notch activation in a sensory patch, then one would expect to see an over-production of hair cells. Such an experiment alongside the RCAS-Delta data would provide a further test of the 'escape from Notch signaling' hypothesis against the lateral inhibition with feedback model.

Another retroviral experiment that could also be easily conducted is lineage analysis using the replication-defective GFP virus. Present cell lineage of the neuroblasts that delaminate from the otic epithelium is still unclear. Using this virus, it should be possible to elucidate the lineage and determine how neuroblasts are related (if at all) to hair cells and support cells. The phenotype of the zebrafish mutant \textit{mind bomb} (mib) suggests the neuroblast lineage may be separate from the hair-cell/supporting cell lineage. Here one sees a 50% increase in neurons, as well as supernumerary hair cells at the expense of supporting cells (Haddon et al., 1998a). If the neurons and hair cells all derive from a common ancestor resembling a fly sense organ precursor, then one might expect that in mib there should be a massive overproduction of neurons and no production of hair cells, as in the severe Notch loss-of-function mutants in the \textit{Drosophila} sensory bristle (Hartenstein and Posakony, 1990).
Figure 6.1: Electroporation of the DNA of the replication-defective GFP-expressing virus in the inner ear. LZRSpBMN-Z-GFP plasmid was electroporated into the inner ear at E2, and analysed a day later using immunofluorescence. Good expression of GFP is seen in most of the otic epithelium.
To fully decipher the contribution of Numb, several further experiments are necessary, as discussed in the previous chapter. A more thorough analysis of the endogenous Numb-staining pattern would be valuable, especially around the time of terminal mitosis in the basilar papilla. Also, a positive control for the effects of RCAS-Numb retrovirus is essential if one is to be truly confident that the retroviral Numb is functioning like wildtype Numb. This could be achieved by infecting the neural tube and observing the effect on neuroepithelial cells. Alternatively, the chick retina might provide a simpler system to manipulate. To date, no Numb expression has been reported in the retina, and it would be interesting to see if it is present and has effects here.

In order to decipher the exact role of each Notch ligand, over-expression experiments are not enough: gene knockouts are also needed. Mouse null mutants for Delta, Serrate and Numb all die before hair cells differentiate (Hrabe de Angelis et al., 1997; Xue et al., 1999; Zhong et al., 2000), but this problem may be overcome by the use of organ culture. Here the otocyst could be transplanted from the homozygous mouse embryo at a time before its death, either into an organ-culture dish or into a host animal such as a chick or a nude mouse. Since the factors necessary for the differentiation of the sensory patch are intrinsic to the otic epithelium (Swanson et al., 1990), a transplanted ear rudiment should differentiate relatively normally, allowing one to assess the contribution of each gene to the control of hair cell fate.

6.5 Clinical implications

Finally, it is hoped that this work will add to the growing body of information that may, one day, help to relieve deafness. In the British population, some 16% of adults suffer some kind of hearing impairment and in a substantial proportion of cases this is due to a loss of hair cells or to other defects of the inner ear (Steel and Brown, 1994).
Any advances in our understanding of the developmental biology of the inner ear will improve the chances of finding ways to repair that loss.

The genes that control ear development and patterning are well conserved across the vertebrates, and hence analysis of gene function in the chick can be related to human development. Mouse mutants are good models for human deafness (reviewed in Fekete, 1999). Chick and zebrafish provide good complementary systems in which one can carry out manipulative experiments for which the mouse is unsuitable. Chick, moreover, has a special interest because it is able to regenerate its auditory hair cells after damage caused by drugs or noise, unlike humans (reviewed in Stone and Rubel 2000a). Importantly, the mechanisms controlling hair cell production during regeneration appear similar to those operating during hair cell differentiation in the embryo.

Regardless of whether the study of Notch signaling in the ear leads to advances in the treatment of deafness, it has clinical importance as a model system or paradigm for the analysis of Notch signaling in general. This signaling pathway affects cell fate decisions in almost every tissue where it has been examined, and what we discover in the ear can hardly fail to have implications elsewhere in the body.
References


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The sensory patches in the ear of a vertebrate can be compared with the mechanosensory bristles of a fly. This comparison has led to the discovery that lateral inhibition mediated by the Notch cell–cell signaling pathway, first characterized in Drosophila and crucial for bristle development, also has a key role in controlling the pattern of sensory hair cells and supporting cells in the ear. We review the arguments for considering the sensory patches of the vertebrate ear and bristles of the insect to be homologous structures, evolved from a common ancestral mechanosensory organ, and we examine more closely the role of Notch signaling in each system. Using viral vectors to misexpress components of the Notch pathway in the chick ear, we show that a simple lateral-inhibition model based on feedback regulation of the Notch ligand Delta is inadequate for the ear just as it is for the fly bristle. The Notch ligand Serrate1, expressed in supporting cells in the ear, is regulated by lateral induction, not lateral inhibition; commitment to become a hair cell is not simply controlled by levels of expression of the Notch ligands Delta1, Serrate1, and Serrate2 in the neighbors of the nascent hair cell; and at least one factor, Numb, capable of blocking reception of lateral inhibition is concentrated in hair cells. These findings reinforce the parallels between the vertebrate ear and the fly bristle and show how study of the insect system can help us understand the vertebrate.

Almost all animals, from cnidarians to mammals, have mechanosensory organs for touch and detection of vibrations and other disturbances of the air or water in which they live. This sensory capability, it seems, is as important and as universal as sensitivity to light, suggesting that the apparatus of mechanosensation, like that of photoreception, may have a very ancient evolutionary origin. Mechanosensory organs such as the ear may be elaborate and highly specialized according to the animal’s way of life, but at their core they must always have a set of mechanosensory transducer cells to perform the fundamental task. At the level of these cells and their immediate companions one may hope to find conserved features reflecting evolution from a common prototype: homologous cell types, homologous developmental processes, and homologous molecular mechanisms.

In the vertebrate ear, the core structures are the sensory patches, consisting of hair cells (the transducers), supporting cells (which form the epithelial framework in which hair cells are held), and the adjacent cochleovestibular sensory neurons (which synapse with the hair cells). How are these cell types, especially the hair cells and supporting cells, generated in the correct pattern and proportions? Parallels with Drosophila provide a route toward an answer: the sensory patches in the vertebrate ear have a counterpart in the sensory bristles of the fly, suggesting that homologous mechanisms may operate. This approach has revealed that lateral inhibition mediated by the Notch signaling pathway, a key mechanism for controlling cell diversification in fly sense organs, has a similarly crucial role in the vertebrate inner ear.

In the first part of this paper we review the published evidence on Notch signaling in the ear and see how it fits with a simple model that has been proposed to explain the patterning of the ear’s sensory patches. This model does not, however, correspond accurately to the way in which Notch signaling governs development of sensory bristles in the fly. The vertebrate ear and the insect bristle not so closely homologous after all, or is the simple model proposed for the ear misleading? We review the arguments for homology, and in the second half of the paper we present experimental evidence to test the role of Notch signaling in the ear. We show that Notch signaling in the ear is more complex than was originally suspected: at least three Notch ligands are at work; they are regulated in contrary and complementary ways; and, contrary to previous suggestions, the pattern of cell determination cannot simply be explained in terms of lateral inhibition rules governing Notch-ligand expression. These corrections to previous ideas, far from undermining the arguments for homology with Drosophila sensilla, make the parallels seem even closer than before.

The Hair Cells, Supporting Cells, and Neurons of a Sensory Patch Have a Common Origin in the Otic Placode. The inner ear derives from the otic placode, a thickening of the epidermis adjacent to the hindbrain in the early embryo. Are the sensory patches of the vertebrate ear and the fly bristle not so closely homologous as was previously supposed? We review the arguments for homology, and in the second half of the paper we present experimental evidence to test the role of Notch signaling in the ear. We show that Notch signaling in the ear is more complex than was originally suspected: at least three Notch ligands are at work; they are regulated in contrary and complementary ways; and, contrary to previous suggestions, the pattern of cell determination cannot simply be explained in terms of lateral inhibition rules governing Notch-ligand expression. These corrections to previous ideas, far from undermining the arguments for homology with Drosophila sensilla, make the parallels seem even closer than before.

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The Alternating Mosaic of Hair Cells and Supporting Cells Suggests a Simple Lateral Inhibition Model for Genesis of Spatial Pattern. The ratio of hair cells to supporting cells in a mature sensory patch is variable from one region to another. In the auditory epithelium (the basilar papilla) of the 12-day chicken embryo, for example, it ranges from 1:1.7 at the distal end to 1:3.9 at the inferior-proximal end (18). Despite this variation, the distribution of hair cells among supporting cells seems almost everywhere to obey the same simple rule: with few exceptions, every cell that lies in contact with a hair cell is a supporting cell, and any cell that escapes all contact with hair cells is itself a hair cell. This immediately suggests a pattern-generating mechanism based on lateral inhibition (Fig. 1) (3, 19). According to this hypothesis, the cells in the undifferentiated sensory patch have a choice of two fates (hair cell or supporting cell), and the choice is governed by interactions between nearest neighbors. The default or primary fate, in the absence of interaction, is to become a hair cell, but in the interacting system each nascent hair cell delivers an inhibitory signal to the cells in contact with it, deterring these from becoming hair cells, too, and at the same time preventing them from producing inhibitory signals that would act back on the nascent hair cell. Neighbors thus compete to be hair cells, and the losers in the competition, the cells surrounding hair cells, become supporting cells.

Lateral inhibition of this sort is well documented in Drosophila and Caenorhabditis elegans, and studies in these species have revealed the molecular mechanism that transmits the inhibitory signal (5, 20). The receptor on the cell receiving inhibition is the transmembrane protein Notch, and the ligand on the adjacent cell that delivers inhibition is the transmembrane protein Delta. Loss-of-function mutations in the Delta–Notch signaling pathway abolish lateral inhibition and allow an excessive proportion of the population to adopt the primary fate.

On this basis, a simple formal model for pattern generation can be proposed (21–23): an increase of Delta in one cell causes increased activation of Notch in its neighbor, and Notch activation in the neighbor down-regulates Delta expression in that same cell, as well as inhibiting commitment to the primary fate (Fig. 1). The feedback control of Delta expression has the effect of amplifying contrasts between adjacent cells: a sheet of initially similar cells, with only small random differences between them, will spontaneously develop into a mosaic of alternate cell types of just the type seen in the sensory patches of the ear (6).

Lateral Inhibition in the Sensory Patches of the Ear Is Mediated by the Notch Signaling Pathway. In the vertebrate central nervous system, or at least in the neural plate (24, 25) and retina (26), the production of neurons from neuroepithelial progenitor cells seems to be controlled in just the way that the simple lateral-inhibition-with-feedback model proposes. All of the progenitors express the Notch homolog Notch1, while nascent neurons appear within this population as scattered cells expressing the Delta homolog Delta1 (D1) at higher levels than their neighbors. When D1 is artificially overexpressed, so that all cells strongly deliver and receive the inhibitory signal, the cells all are prevented from differentiating as neurons. Conversely, when all of the cells are forced to express a dominant-negative form of D1, blocking Delta–Notch signaling, they all escape inhibition and differentiate as neurons prematurely. Moreover, expression of the endogenous D1 gene is regulated in accordance with the simple feedback model. All of the requirements for pattern generation by lateral inhibition with feedback, and all of the predictions of the model, seem to be satisfied.

To see whether the same is true in the ear, the first step is to examine the normal gene expression patterns in the developing sensory patches (4, 27, 28). Notch1 is expressed, as in the central nervous system, throughout the population of progenitor cells (and in fact even more widely in the otic epithelium). D1 is expressed in a scattered subset of the population, and these D1-expressing cells can be identified as the nascent hair cells (4, 27). These observations are backed up by similar circumstantial evidence for Notch signaling during hair-cell regeneration in birds (63).

Direct functional evidence comes from zebrafish and mice. Thus in zebrafish with a mutation in the deltaA gene—one of four zebrafish Delta homologs, all normally expressed in nascent hair cells—hair cells are produced in increased numbers, as though inhibition has been reduced (29). Likewise, there is some overproduction of hair cells in mice with a knockout mutation in a gene coding for another Notch ligand, Serrate2 (also known as Jagged2, but henceforward in this paper Serrate2 to match chicken and Drosophila terminology) (28). Serrate2 (Ser2) is a homolog of the Drosophila Serrate gene, closely related to Delta and likewise coding for a protein capable of activating Notch; in the sensory patches of the ear it is expressed with D1 in the nascent hair cells (although more persistently). The phenotypes of the deltaA and Ser2 mutations are relatively mild but consistent with the model. The zebrafish mind bomb mutant provides more spectacular evidence. This mutant takes its name from its neurogenic phenotype: in the central nervous system, it grossly overproduces neurons at the expense of neural progenitor cells, in a manner that is diagnostic of a failure of lateral inhibition. The excess of neurons goes with an up-regulation of Delta expression: the cells are deaf to the inhibitory signal that Delta normally delivers. In the ear, the result of this failure of Notch signaling is dramatic; each of the prospective sensory patches differentiates in its entirety into a uniform mass of hair cells, with no supporting cells (7). The hair cells are not only produced in great excess, as much as 30-fold, but also prematurely. In the absence of supporting cells to hold them in place, the hair cells all are then extruded from the epithelium and die and disappear within a day or two after their first appearance (30).

The mind bomb phenotype tells us that Notch signaling is required (i) to prevent the cells in a developing sensory patch from all differentiating alike as hair cells, and (ii) to delay production of hair cells until the proper time. It shows that lateral inhibition mediated by the Notch pathway is essential, but it falls short of proving that lateral inhibition with feedback is the
mechanism that generates the normal orderly mixture of hair cells and supporting cells. Indeed, there are several reasons to be cautious in adopting this simple model mechanism, seductive as it may seem. Goodyear and Richardson (18) have shown, for example, that the orderliness in the basilar papilla of the chick depends to a large extent on cell rearrangements occurring after cell differentiation. While Dll and Ser2 are concentrated in nascent hair cells, there is yet another Notch ligand, Ser1, that is strongly expressed in supporting cells (4, 27, 31). Most significantly, in the sensory bristles of Drosophila, Delta expression is not regulated in the way the model postulates, even though lateral inhibition via Notch is as essential as in the ear (8, 9). Is the Drosophila bristle a misleading paradigm, or is the simple theoretical model wrong? To answer these questions, we first review the parallels between insect bristles and the sensory patches of the vertebrate ear.

The Sensory Patches of the Vertebrate Ear Resemble the Sensilla of a Fly in Function and Developmental History. Each insect bristle is a miniature sense organ, or sensillum, consisting of a set of four cells: a neuron, a neural sheath cell, a bristle socket cell, and a bristle shaft cell. These cells normally all derive, along with a migratory glial cell (32), from a single sensory mother cell, or SMC. Lateral inhibition mediated by Notch operates repeatedly in development of the bristle, first to single out the SMC within a cluster of competent cells in the epidermis, and then at each subsequent cell division to drive the diversification of the progeny of the SMC to form the differentiated cells of the bristle (8, 10, 33, 34).

The insect bristle resembles the sensory patch in the vertebrate ear in several respects. First of all, the mechanosensory function is essentially the same, with the same kinetics of response and adaptation (35).

Second, the cell types correspond, in part at least: neuron with neuron, bristle shaft cell with hair cell, bristle socket cell with ear supporting cell. Bundles of actin filaments form the shaft of the bristle shaft cell, just as bundles of actin filaments form the stereocilia of the hair cell (36). Both these cell types have a well-defined planar polarity, essential for directional sensitivity.

Third, the developmental anatomy is similar. Just as the component cells of the bristle have a common origin in the epidermis, so do the neurons, hair cells, and supporting cells of the ear have a common origin in the otic placode ectoderm. Just as the first division of the SMC gives rise to a neuronal precursor, which delaminates from the epidermis, and an epithelial precursor, which stays behind to generate the socket and shaft cells, so also the first step in differentiation of the sensory patch is production of neuroblasts, which delaminate from the otic epithelium, and sensory epithelial precursors, which stay behind to form hair cells and supporting cells. In both systems, the final differentiated cell types are the products of a series of dichotomous cell-fate choices, and this series of choices is similar in the fly sensillum and the vertebrate ear.

Lastly, and most crucially, the molecular mechanisms underlying these choices are similar.

Corresponding Events in Development of Insect Sensillum and Vertebrate Inner Ear Are Controlled by Homologous Systems of Genes. In fly sensilla, basic helix-loop-helix (bHLH) transcription factors of the Achaete/Scute family and the related Atonal family have a key role, both in initiating the program of sensory development (the "proneural" function) and as differentiation factors for the final cell types (22). Examples of the latter include the products of the aense and cousin of atonal (cato) genes, which drive differentiation both in bristles and chordotonal organs (a closely related type of sensillum) (37). In the ear, although bHLH genes serving the early proneural function have not been identified, it has been shown that the atonal homolog Math1 is expressed selectively in hair cells and drives hair-cell differentiation: hair cells fail to develop in a Math1 knockout mouse (38), and nonsensory cells in the cochlea differentiate into hair cells when transfected with Math1 (39). Upstream from the proneural genes in the fly, controlling their domains of expression, lie transcription factors of the Iroquois family; these, too, have vertebrate homologs that are expressed at early stages in the future sensory epithelium of the ear (40).

Components of the Notch signaling pathway lie downstream from the proneural genes and play a central and recurrent role in the subsequent development of the fly sensillum. In each of the series of cell-fate decisions in the sensillum lineage, from the singling out of the SMC onward, lateral inhibition mediated by Notch signaling is required (8, 33, 34).

The Notch signaling pathway has a similarly central and recurrent role in the development of the sensory patches of the vertebrate ear. Notch1 is expressed throughout the otic placode at the very beginning of ear development and thereafter throughout the developing sensory epithelium, persisting into adult life in the supporting cells (4, 16). Dll is expressed at each of the sites where cell-fate choices are being made (4). During the segregation of neural and epithelial sublineages of the ear, Dll RNA is seen in scattered cells in the neurogenic region of otic epithelium, apparently the nascent neuroblasts. Subsequently it is expressed in the ganglion formed by the neuroblasts, and, as we have seen, in the sensory-patch epithelium as hair cells are being generated and becoming different from supporting cells. The block of Notch signaling in the mind bomb mutant not only causes overproduction of hair cells, but also neurons (7).

Finally, the molecular data reveal additional similarities between the differentiated cell types. In particular, the Pax gene D-pax2 is expressed specifically in the bristle shaft cell and required for its correct differentiation (41); the vertebrate homolog Pax2 is expressed in the early otic epithelium and then selectively in hair cells and also is required for their correct differentiation. In the mouse Pax2 knockout, no cochlea forms (42), and in a zebrafish pax2.1 mutant, hair-cell differentiation is abnormal (29).

The parallels between insect bristles and ear sensory patches that we have summarized above add up to a persuasive argument that these mechanosensory organs are indeed homologous—that they resemble one another because they have evolved from a common ancestral prototype. There are, of course, also important differences, as one might expect after 800 million years of divergent evolution. The fly bristle is ensheathed in a semirigid cuticle, making the mechanics of stimulus delivery quite different from that in the ear. In the ear, the hair cells are the transducers and synapse with the neurons; in the bristle, there is no synapse and the dendrite of the neuron acts as the transducer. In the development of the ear, there is no step corresponding to the singling out of the SMCs from the epidermis; instead, the future sensory patch behaves like a uniform mass of contiguous SMCs. The numbers of cell divisions elapsing between one cell-fate choice and the next are different in the two systems, and the cell lineage patterns are not identical. Almost all of these developmental differences, however, correspond to plausible evolutionary variations and have parallels in differences between the various, but unmistakably related, types of sensilla in the fly (see ref. 4 for details).

The Pattern of Cell Types in the Insect Bristle Is Not Controlled Through Regulation of Delta Expression. Notch signaling in the developing bristle shows three important departures from the simple lateral-inhibition model sketched in Fig. 1. First, recent studies have shown that Delta is not the only Notch ligand at work. Serrate is also present and must be mutated along with Delta to give the most extreme Notch-pathway loss-of-function phenotype (43). Second, Delta is not regulated in the way the simple model
postulates, either at the mRNA or at the protein level. Delta expression remains high in the winning and losing cells during many of the lateral-inhibition interactions that decide cell fate (8, 9, 34), implying that some other factor(s) must create a difference by modulating the efficacy of Delta or the ability to respond to it. Third, factors acting in this way to bias Notch signaling have been identified. In particular, the intracellular protein Numb is distributed asymmetrically between the daughters and granddaughters of the SMC and makes the cells that contain it deaf to lateral inhibition (10, 44).

We now turn to our experimental observations on Notch signaling in the sensory patches of the chick ear.

Materials and Methods

Viral Constructs. The RCAS-Dll and RCAS-Dll<sup>dn</sup> replication-competent retroviral constructs were as described (26). RCAS-X-Su(H)<sup>h</sup> was a gift from J.-C. Izpisua-Belmonte (Salk Institute, La Jolla, CA) and contains a form of the Xenopus Suppressor-of-Hairless (Su(H)) cDNA with its DNA binding mutated, as described (45). The viruses were used at a titer of 5 × 10<sup>7</sup>-10<sup>8</sup> cfu/ml.

The pseudotype virus will be described in detail elsewhere (I.L.R., unpublished work). Briefly, plasmids based on the LZRspBMN-Z plasmid (46) were prepared by inserting cDNA for the product of interest (Dll or Dll<sup>dn</sup>) linked to an internal ribosome entry site followed by green fluorescent protein (GFP); this composite coding sequence was placed under the control of a 253-bp upstream enhancer sequence from Roux sarcoma virus, within LZRspBMN-Z. Pseudotype virus was then generated by transiently cotransfecting 293gp packaging cells (Qiagen, Chatsworth, CA) with this construct plus a plasmid coding for vesicular stomatitis virus (VSV)-G protein. The resulting pseudotype virus particles contain RNA coding for Dll + GFP or Dll<sup>dn</sup> + GFP, with Gag, Pol, and VSV-G proteins provided by the packaging cells. Viruses released into the supernatant was concentrated by ultracentrifugation to a final titer of 5 × 10<sup>7</sup>-10<sup>8</sup> cfu/ml.

Embyos and Chicken Injection. Chick embryos were windowed at stage 13 [embryonic day 2 (E2)], and ~0.5 µl of virus solution (with 0.8 µg/µl polybrene, 3% methyl cellulose, and a trace of fast green dye) was injected into the lumen of the otic cup. Embryos were fixed between E6 and E9.

In Situ Hybridization and Immunohistochemistry. In situ hybridization was performed on 15-µm cryosections of fixed embryos as described (26), by using FastRed (Boehringer) for detection by fluorescence. For immunostaining, the cryosections were incubated overnight at 4°C in blocking solution (PBS with 3% BSA, 10% FCS, 0.1% Triton) containing the primary antibody. For Numb, sections were taken through an additional methanol fixation before the primary antibody was added. Chick anti-Numb antibody (47) was a gift from Y. Wakamatsu, Tohoku University, Japan. Ser1 was detected with a polyclonal antiserum as described (4). Dll and Dll<sup>dn</sup> were detected with a rabbit polyclonal antiserum directed against amino acids 325-462 of chicken Dll (26). In the specimens shown here, this antiserum was used at a concentration sufficient to detect the high levels of exogenous Dll or Dll<sup>dn</sup>, but not endogenous Dll. Hair cells were detected by using the hair-cell antigen (HCA) antibody (48). Co lateral inhibition (10) with a rabbit polyclonal antiserum (gift from D. Shima, Imperial Cancer Research Fund, London). Secondary antibodies were labeled with Alexa488 or Alexa594 (Molecular Probes). Images were collected by confocal microscopy. A total of ~180 virus-injected embryos were serially sectioned and analyzed. Results are based on 47 embryos in which we saw informative patches of infection, i.e., patches that overlapped or touched sensory patches in the ear epithelium.
later. Patches of infection within sensory regions of the otic epithelium were analyzed for Ser1 expression (Fig. 2). Ser1 expression was lost or clearly reduced in 8 of 10 patches of infection with RCAS-Dll^"", and in 8 of 10 patches of infection with RCAS-X-Su(H)^"". A control for nonspecific effects of viral infection was provided by a parallel series of experiments (see below) using another virus, RCAS-Dll1, containing the full-length form of Dll. In the majority of these cases (20 of 25 patches) no down-regulation of Ser1 was seen. Taken together, these data indicate that Ser1 is indeed positively regulated by Notch activity.

**Effects of Dll^"" Expression on Hair Cell Differentiation Are Difficult to Decipher.** It might be expected that hair cells should be seen in excess in regions where Notch signaling was blocked with RCAS-Dll^"" or RCAS-X-Su(H)^"". We have examined hair cell production in our experiments with RCAS-Dll^"", in which embryos were infected at 2 days of incubation and fixed 4 or 6 days later. Altogether, we found 33 potentially informative infected patches. Strikingly, just over half (17/33) of the informative patches of infection directly abutted sensory patches, without any overlap (Fig. 3A and B): infection with the virus and sensory character appeared to be mutually exclusive. By comparison, in parallel experiments using the RCAS viral vector to misexpress full-length Dll (see below and Fig. 4), only 10% of informative infected patches abutted sensory patches in this way, as against 90% that overlapped with or were internal to sensory patches. At least two interpretations are possible. It could be that blockade of Notch signaling by RCAS-Dll^"" converted prospective patch cells to a nonsensory character. Alternatively, the loss of Notch activity may have caused premature hair-cell differentiation as in *mind bomb*, and as in *mind bomb* this may have lead to early death and disappearance of any infected cells that lay in a prospective sensory patch (7, 30). Where an early infection partially overlapped a prospective sensory patch, the loss of cells in the region of overlap would bring infected nonsensory cells into juxtaposition with uninfected sensory cells.

Patches of infection with RCAS-Dll^"" were seen inside sensory patches, or overlapping them, in 16 of 33 informative cases. Contrary to expectation, none of these patches showed an excessive density of hair cells (see Fig. 2B): 0.22 ± 0.07 hair cells were counted per μm length of sectioned epithelium in the infected regions, as compared with 0.23 ± 0.05 in the adjacent uninfected sensory tissue (mean ± SD, n = 11 in both cases; counts from representative sections showing patches ≥20 μm wide). Again, several interpretations are possible. Blocking Notch activity with RCAS-Dll^"", although it affects Ser1 expression, may fail to affect cell differentiation (different Notch family members with different sensitivities to Dll^"" and different downstream actions could be involved, for example, or Ser1 expression and cell differentiation might have different thresholds of response to Notch activity). Alternatively, the cells may have been already irreversibly committed as hair cells or supporting cells before they became infected. Lastly, the cells may...
not have been irreversibly committed, but may only recently have become infected by the replication-competent virus as it spread through the tissue and may not have had time yet to change their phenotype. More experiments will be needed to decide between these interpretations.

A further observation is more straightforward in conformity with the expected effects of blocking Notch activity. Infected cells expressing Dll\textsuperscript{th} often ended up as neurons in the cochleovestibular ganglion (Fig. 3 C and D). Indeed, in 6 of a set of 6 embryos fixed at E6, infected cells were found only in the ganglion. This finding suggests that the infected cells were biased toward a neuronal fate during the early phase of ear development when neuroblasts delaminate from the otic epithelium. Virus-infected nerve fibers innervating a sensory patch of epithelium are a possible source of late infection for the epithelial cells.

**Ectopic Expression of Dll Does Not Inhibit Hair-Cell Production.** There are two ways in which a cell might escape lateral inhibition so as to become a hair cell: its neighbors might not deliver inhibitory signals, or it might be deaf to signals that it receives. In the sensory patches of the ear, all cells are in contact with neighbors expressing Ser1, suggesting that the nascent hair cells are deaf to at least this Notch ligand. They are not, however, normally exposed to Dll, because Dll is not expressed by the supporting cells. To test whether forced expression of Dll throughout the cell population would block cell differentiation, as it does in the retina, we once again used viral vectors, this time containing the full-length Dll CDNA. We found 29 RCAS-Dll-infected patches that lay within sensory patches or overlapping them (Fig. 4A–D), and three that lay directly abutting sensory patches. We counted the numbers of hair cells per unit length of sectioned epithelium in infected as compared with adjacent uninfected sensory epithelium. No significant difference was seen. The values were respectively 0.23 ± 0.08 and 0.24 ± 0.08 hair cells per μm (mean ± SD, n = 18 in both cases; counts from representative sections showing patches ≥30 μm wide).

The observations using RCAS-Dll to misexpress Dll were confirmed by using a pseudotype replication-defective virus for the same purpose. This virus gives smaller patches of infected cells, but has the advantage that they can be assumed to have all become infected at the same early time. Again, hair cells could be seen to develop normally even where all of the cells in the neighborhood expressed Dll (Fig. 4E).

The cells that become hair cells therefore do so regardless of whether their neighbors express Dll. The simple lateral-inhibition model based on regulation of Dll expression cannot be the correct explanation of why some cells escape inhibition to become hair cells and others do not. Some other factor must operate, either interacting with Notch ligands in the neighbors of the nascent hair cell and blocking their ability to deliver an inhibitory signal, or interacting with the Notch pathway in the nascent hair cell and blocking its ability to respond.

**Numb Protein May Make Nascent Hair Cells Deaf to Notch Signaling.** Almost nothing is known about molecules that might interact with Notch ligands in cis to prevent them delivering a signal to an adjacent cell (see Discussion). Analogies with Drosophila suggest, however, at least three factors that might act in nascent hair cells to make them insensitive to signals received. First, the hair cells might down-regulate their expression of Notch1 itself—and, indeed, they are known to do so, although this may occur too late to control the cell-fate decision (16). Second, Delta protein in large quantities can make cells that contain it unresponsive to signals from neighbors (51), and hair cells contain Dll. Lastly, there are proteins such as Numb that interact with Notch to block its activity (51). We have used immunohistochemistry to look for expression of a chick Numb homolog in the inner ear. As shown in Fig. 5, the chick Numb protein is localized to the basolateral membranes of the epithelial cells at stages before hair-cell differentiation. Once hair cells have differentiated, it is seen at high concentration in hair cells. These observations are preliminary. They clearly suggest, however, that Numb protein acts in nascent hair cells to make them immune to Notch signaling.

**Discussion**

The implications of our experiments are summarized diagrammatically in Fig. 6, showing the patterns of expression of the various Notch ligands in a newly differentiated sensory patch and their regulatory interactions. Supporting cells express Ser1; hair cells express Dll and Ser2. Ser1 expression is regulated positively by Notch activity; Dll and Ser2 are regulated negatively (7). Supporting cells contact one another, so that mutual lateral inductive signals, as well as signals from hair cells, keep them all in a state of high Notch activation, which maintains high expression of Ser1 and low expression of Dll and Ser2. Hair cells contain Numb and

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**Fig. 5.** C-Numb expression during sensory patch development. (A) Section of otic epithelium at E3, stained with Numb antibody (green) and counterstained with the nuclear dye Syto16 (red). (B) Section of basilar papilla at E12, stained with Numb antibody (green) and HCA (red). The hair cells preferentially contain Numb, and it is no longer basally localized. (C) Section showing patches >30 μm wide).

**Fig. 6.** Summary diagram of Notch-mediated interactions in a newly differentiated sensory patch in the chick ear. See Discussion for commentary.
down-regulate their Notch1 expression; thus despite their exposure to Ser1 from all sides, their level of Notch activation is low, their expression of Ser1 is low, and their expression of Dll and Ser2 is high. The cells that differentiate as hair cells do so because their level of Notch activation is low; those that differentiate as supporting cells do so because their level of Notch activation is high.

This system of regulatory interactions is robust and consistent with all of the observations. Our account is, however, based on some tacit assumptions and raises several questions that need to be discussed. The first concerns Ser1.

**Signaling by Ser1 May Serve to Prevent Premature Hair-Cell Production.** An attractive possibility is that Ser1, expressed throughout the prospective sensory patch from a very early stage, serves to prevent premature or ectopic hair-cell production by maintaining a high background level of Notch activation. This suggestion is supported by the recent finding that hair cells are overproduced in mouse cochlea explants when Ser1 (Jag1) production is inhibited with antisense oligonucleotides (52). Ser1 thus may be important in enforcing the long delay that occurs between neuroblast delamination and hair-cell differentiation in birds and mammals and in preventing the peripheral regions of growing sensory patches from differentiating prematurely in fish.

The supporting cells in sensory patches also express Lunatic fringe (Lfng) (4, 31), a homolog of the Fringe protein that has been shown in *Drosophila* to interact with Notch in cis and make Notch refractory by activation by Serrate (53, 54). This might be taken to suggest that Ser1 function normally is blocked by Lfng, in which case removal of Lfng should drastically alter the pattern of Notch activation and cell differentiation. However, ear development appears entirely normal in mice with a knockout mutation of the Lfng gene (55), and we see no abnormalities in ears overexpressing Lfng (4, 31), a homolog of the Fringe protein that has been located companion proteins (61, 63) may confer immunity to the Drosophila sense organs (62).

**More Than One Notch Homolog May Be Involved in Ear Development.** A questionable assumption is that the various Notch ligands all antagonize Notch signaling in the development of the ear. The data leave no doubt, however, that the Notch pathway has a central role in controlling cell fate in this system. Our findings reveal a more complex picture than initially suspected, but reinforce the parallels between the sensory patches of the vertebrate ear and the sensilla of a fly, adding weight to the argument for homology. Thus in both systems, both the Delta and the Serrate subfamilies of Notch ligands are involved; cell fate choice is not simply dictated by the level of Notch ligand production in neighboring cells; and the cells that escape Notch-mediated inhibition contain high levels of Numb, a factor that can block Notch activity by binding to Notch.

Homologies between insects and vertebrates are commonplace at the molecular level. There are innumerable examples of homologous proteins serving the same molecular functions. It is also true that Notch signaling is important in many different tissues, both in vertebrates and invertebrates. But the homologies we have pointed out for the mechanosensory structures go deeper, including function, multicellular anatomy, development, and molecular controls. Indeed, there are few, if any, other multicellular structures where correspondences between insect and vertebrate seem so clear, detailed, and extensive. Of course, there are many differences: the *Drosophila* data can only provide us with hypotheses as to the workings of the vertebrate system, not dogmatic answers. By testing these hypotheses, as we have attempted to do in this paper, we gain a better appreciation of the evolutionary relationship, and a better understanding of the evolutionary relationship opens the way to new hypotheses and new insights into the development of the inner ear.

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Cell fate choices and the expression of Notch, Delta and Serrate homologues
in the chick inner ear: parallels with Drosophila sense-organ development

Julie Adam*, Anna Myat*, Isabelle Le Roux, Mark Eddison, Domingos Henrique, David Ish-Horowicz and Julian Lewis

Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK
*These two authors contributed equally to the work
†Present address: Department of Biochemistry, Imperial College, London SW7 2AZ, UK
‡Present address: Institute Histologia e Embriologia, Faculdade Medicina Lisboa, Av. Prof Egas Moniz, 1699 Lisboa Codex, Portugal
*Author for correspondence (e-mail: j.lewis@icrf.icnet.uk)

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SUMMARY

The sensory patches in the vertebrate inner ear are similar in function to the mechanosensory bristles of a fly, and consist of a similar set of cell types. If they are truly homologous structures, they should also develop by similar mechanisms. We examine the genesis of the neurons, hair cells and supporting cells that form the sensory patches in the inner ear of the chick. These all arise from the otic epithelium, and are produced normally even in otic epithelium cultured in isolation, confirming that their production is governed by mechanisms intrinsic to the epithelium. First, the neuronal sublineage becomes separate from the epithelial: between E2 and E3.5, neuroblasts delaminate from the otocyst. The neuroblasts then give rise to a mixture of neurons and neuroblasts, while the sensory epithelial cells diversify to form a mixture of hair cells and supporting cells. The epithelial patches where this occurs are marked from an early stage by uniform and maintained expression of the Notch ligand Serrate. The Notch ligand Delta is also expressed, but transiently and in scattered cells: it is seen both early, during neuroblast segregation, where it appears to be in the nascent neuroblasts, and again later, in the ganglion and in differentiating sensory patches, where it appears to be in the nascent hair cells, disappearing as they mature. Delta-Notch-mediated lateral inhibition may thus act at each developmental branchpoint to drive neighbouring cells along different developmental pathways. Our findings indicate that the sensory patches of the vertebrate inner ear and the sensory bristles of a fly are generated by minor variations of the same basic developmental program, in which cell diversification driven by Delta-Notch and/or Serrate-Notch signalling plays a central part.

Key words: Notch1, Delta1, Serrate1, Chick, Ear, Bristle

INTRODUCTION

Peripheral sense organs can be grouped into two fundamentally different classes according to the developmental relationship between the peripheral sensory cells and the sensory neurons that innervate them. Organs of one class, typified by the mechanosensory structures of the skin and the spindle organs of muscles, are innervated by neurons generated at a remote central site in the embryo, such as the neural crest. Organs of the other class, by contrast, derive both their sensory neurons and their peripheral transduction apparatus from one and the same peripheral source. The inner ear is an organ of the latter type: its sensory neurons, as well as its sensory hair cells and supporting cells, derive from the otic placode (D'Amico-Martel and Noden, 1983; Hemond and Morest, 1991; Haddon and Lewis, 1996). In this basic feature the mechanosensory patches of the inner ear – the basilar papilla, sensing sound vibrations; the maculae, sensing linear acceleration and gravity; and the cristae, sensing rotation – resemble the mechanosensory bristles and other sensilla of insects, whose neurons and ancillary cells likewise have a common origin from peripheral ectoderm (Hartenstein and Posakony, 1989). This provides the basis on which to draw a developmental as well as a functional parallel between the system of neurons, hair cells and supporting cells that form a sensory patch in the ear, and the system of neuron, shaft cell, socket cell and sheath cell forming a sensory bristle on a fly (Fig. 1A,B; Lewis, 1991).

For both structures, the central developmental question is how these cell types are generated and organised in the correct spatial pattern (Fig. 1C) (Fekete, 1996; Goodyear and Richardson, 1997). If all the cells arise from a common source, how are they caused to become different? One mechanism for such cell diversification is lateral inhibition, whereby a cell that is becoming committed to a particular pathway of differentiation inhibits its immediate neighbours from doing likewise (Corwin et al., 1991; Corwin et al., 1991; Lewis, 1991). In many developmental systems, including insect bristles, this type of interaction is mediated by Delta-Notch signalling, with the Notch protein acting as a transmembrane receptor in the cell receiving inhibition, and the Delta protein acting as a...
transmembrane ligand in the cell delivering inhibition (Hartenstein and Posakony, 1990; Artavanis-Tsakonas et al., 1995; Lewis, 1996). We have previously reported the cloning of homologs of Notch, of Delta and of the Delta-related gene Serrate in the chick, and have shown that Delta-Notch-mediated lateral inhibition controls production of neurons in the vertebrate central nervous system as it does in the fly (Chitnis et al., 1995; Myat et al., 1996; Henrique et al., 1997; Haddon et al., 1998). Here we examine how the neurons, hair cells and supporting cells of the chick inner ear are generated, with special reference to the role of these genes - C-Notch1 (N1), C-Delta1 (D1) and C-Serrate1 (Ser1) - in their production. Our findings allow us to spell out a detailed system of developmental parallels between the sensory patches of the ear and the sensory bristles of insects.

MATERIALS AND METHODS

Embryos and staging

Fertile hens’ eggs (Light Sussex × Rhode Island Red) were incubated in a humidified atmosphere at 38°C. The Hamburger-Hamilton tables were used for staging and to relate the stage number to the age in notional hours of incubation (Hamburger-Hamilton hours, or HH-hours).

Descriptions of normal development are based on at least three embryos for each stage, for each antibody or in situ hybridisation probe.

Antibody labelling

Embryos were prepared for cryostat sectioning (10-15 μm) and immunostaining essentially as described by Strathle et al. (1994). Antibodies were: TuJ1 (Lee et al., 1990; Memberg and Hall, 1995); Islet1/2 antibody (originally designated Islet1; Tsuchida et al., 1994); BEN (Chedotal et al., 1996); 3A10 (Furley et al., 1990); and hair-cell antibody (HCA) (Bartolami et al., 1991). Ser1 polyclonal antiserum was raised against a 310 bp fragment of the Ser1 intracellular domain, as described by Varnum-Finney et al. (1998). FITC-labelled rabbit anti-mouse IgG (Dako) and Texas-Red-conjugated goat anti-mouse IgG (Jackson) were used as secondary antibodies.

Sections were counterstained with the nuclear dye 7 amino-actinomycin D (7AAD; Molecular Probes; 50 μg/ml in PBS) and viewed either on a confocal microscope (BioRad MRC600) or on a conventional fluorescence microscope. In all cases controls were prepared, omitting either the primary or the secondary antibody. Immunostaining for Ser1 or Islet1/2 was combined with in situ hybridisation for the expression of other genes as described by Henrique et al. (1997).

For whole-mount immunostaining of the basilar papilla, chick embryos were partially dissected in PBS, fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight, rinsed in PBS and further dissected to expose the basilar papilla. The tissue was immunostained with HCA as described by Bartolami et al. (1991) and then counterstained for actin using 2.5 mg/ml FITC-phalloidin (Sigma) in PBS at room temperature in the dark for 2 hours overnight; specimens were rinsed and mounted in PBS and viewed with a BioRad MRC600 confocal microscope.

Culture technique

Otic placodes and otocysts were dissected from embryos at stages 8 to 25. The otic placode does not become visibly distinct until Stage 10 (36 HH-hours); before this, we identified the prospective placode by position relative to hindbrain segments and somites. Each dissected fragment was transferred to 0.25% trypsin (Flow Laboratories) in PBS with 0.02% EDTA at 4°C (5-10 minutes for otic epithelium from 2-day embryos; 30-40 minutes for epithelium from 5-day embryos) and stripped of mesenchyme using tungsten needles. The epithelium was then flattened out on a feeder layer of mitomycin-C-treated fibroblasts (Freshney, 1993), or directly on laminin-coated glass. Cultures were incubated at 37°C, in a humidified 5% CO2 atmosphere, in medium 199 (modified with Hanks salts) with 20% foetal calf serum, 2 mM glutamine, 1.1 g/l NaHCO3, 2% chick embryo extract; no antibiotics were added. After 1-5 days, the cultures were fixed in 4% formaldehyde in PBS and stained with 3 A10 or with HCA and Ser1 antisera; 7AAD was used as a counterstain.

In situ hybridisation

In situ hybridisation was performed with DIG-labelled RNA probes and an alkaline-phosphatase-coupled anti-DIG antibody, which was then detected with NBT/BCIP or with Fast Red to give a fluorescent product, as described by Henrique et al. (1997). For whole-mount embryos, we followed the protocol of Henrique et al. (1995) and for cryostat sections, that of Strathle et al. (1994) with minor modifications. Details of the D1, Ser1 and N1 probes are given in Henrique et al. (1995) and Myat et al. (1996); the Lamprich fringe probe was as described by Lauter et al. (1997).

RESULTS

Fig. 2 gives an outline of inner ear development in the chick. The otic placode is first visible at 1.5 days of incubation (E1.5) as a thickening in the head ectoderm next to the hindbrain at the level of rhombomeres 5 and 6. The placode invaginates to form a cup, which closes and pinches off from the head ectoderm to become a pear-shaped otocyst. Over the following four or five days complex shape changes take place, converting the otocyst

![image of inner ear development in the chick](image-url)
into a membranous labyrinth with semicircular canals and utricle dorsally and saccule and banana-shaped cochlea ventrally. Neurogenesis occurs at the beginning of this period (E2–E3.5), hair-cell differentiation towards its end (from E5 onwards).

**Expression of N1 and Dll in the otic placode foreshadows neurogenesis**

By early stage 11 (40 HHhours), expression of Notch1 marks out an ectodermal patch that includes the whole otic placode; within the patch, expression is uniform (Fig. 3A). By the end of stage 11 (about 3 hours later), a few scattered cells expressing Dll begin to be seen in the anterior part of the placode. The number of cells expressing Dll increases rapidly, but these remain confined to the anterior half of the placode (becoming anteroventral in the cup) and continue to be only a scattered subset of the cell population in that region (Fig. 3B).

To follow neurogenesis and to see how it relates to Dll expression, we have used four antibodies: Islet1/2, TuJ1 and BEN, which detect neuronal antigens whose expression is reported to begin early in neuronal differentiation, before neurofilaments are seen in the cell, and 3A10, which binds to a neurofilament-associated epitope (see Materials and Methods).

In the otic epithelium, we first see immunostaining with Islet1/2, TuJ1 and BEN at stage 12/13 (48-49 HHhours), 6-7 hours after Dll expression begins. By stage 14/15 (52 HHhours), staining with Islet1/2, TuJ1 and BEN is concentrated in the anteroventral part of the otic cup. Islet1/2, as a nuclear marker, gives the most precise indication of the behaviour of individual cells: within the anteroventral domain, the cells expressing Islet1/2 antigen form a scattered subset of the epithelial population (Fig. 3C). The cells expressing Islet1/2 and TuJ1 are concentrated basally in the epithelium, and some can be seen straddling the basal lamina, as though in the act of delaminating (Fig. 3D,E,F).

Doubly stained sections show that the Islet1/2 domain coincides with the Dll expression domain, but that the individual cells in this region never express both markers simultaneously (Fig. 3H,J). By analogy with the embryonic central nervous system (Chitnis et al., 1995; Henrique et al., 1995; Myat et al., 1996), this suggests that the Dll-expressing cells in the ear epithelium are neuronal precursors, expressing Dll transiently before switching on expression of markers of neuronal differentiation.

At stage 14-15 (52 HHhours), we begin to see a few cells expressing Islet1/2, TuJ1 and BEN antigens that have escaped to form the first rudiment of the cochleovestibular ganglion, pressed close against the anteroventral otic epithelium. On the basis of these observations, we can identify the anteroventral patch of expression of Dll, Islet1/2, TuJ1 and BEN in the otic epithelium as the site of neurogenesis. A similar pattern of labelling persists in this neurogenic patch for about 36 hours, up to stage 21-22 (84-90 HHhours), and throughout this period there is a continuing exodus of cells expressing Islet1/2, TuJ1 and BEN from the otic epithelium into the developing ganglion.

**The delaminating cells are neuroblasts rather than postmitotic neurons**

3A10 staining is not seen in the ear until at least stage 17 (58 HHhours), 6 hours after delamination has begun, and it is confined to the ganglion. The 3A10-positive cells are identifiable as young bipolar neurons, with axons and dendrites (Fig. 3J). Over the subsequent days, the number of these neurons in the ganglion steadily increases.

From previous [3H]thymidine studies of cell division in the ganglion (D’Amico-Martel, 1982), it is clear that the ganglion precursor cells delaminating from the otic epithelium are not postmitotic nascent neurons, but neuroblasts capable of dividing before they differentiate (see also Memberg, 1995). Our observations confirm this: Islet-positive cells can be seen in mitosis both in the neurogenic patch in the otic epithelium (Fig. 3D) and in the developing cochleovestibular ganglion (Fig. 3E), and this matches findings with BrdU labelling (data not shown).

**BEN expression persists in the target epithelium as well as in the developing neurons**

The Islet1/2 and TuJ1 antigens continue to be expressed in the ganglion, but in the otic epithelium cells expressing them have disappeared by stage 21-22 (84-90 HHhours), corresponding to the end of delamination of neuronal precursors from the otocyst (Fig. 3K).

BEN, however, behaves differently. In the ganglion, its expression is similar to that of Islet1/2 and TuJ1, but in the otic epithelium it persists after Islet1/2 and TuJ1 have disappeared, and marks the region that is invaded by processes from the ganglion cells, i.e. the presumptive sensory area (Fig. 3L). Thus BEN labels both the neurons and their peripheral target epithelium. Homophilic interactions mediated by BEN (a cell-surface adhesion molecule) may help the dendrites of the cochleovestibular neurons, as they grow back into the otic epithelium, to recognise the sensory patch in which they must make synapses (Chedotal et al., 1996).
Ser1 expression foreshadows the development of sensory patches in the inner ear epithelium

The epithelial sensory patches are defined by the presence of hair cells, which do not begin to differentiate overtly until E5, more than a day after neuroblast delamination has ended. Unexpectedly, expression of the Notch ligand Ser1 foreshadows this process, marking out what appear to be the prospective sensory patches long before hair cells differentiate. The patches of Ser1 expression in the otic epithelium serve as landmarks that can be followed through from as early as stage 21 (E3.5) to at least stage 38 (E12).

Expression of Ser1 in the ear rudiment begins at or before stage 11, as reported previously (Myat et al., 1996, Figs 2F-1), but the pattern is at first a rapidly changing one, with expression originally strongest in the peripheral parts of the otic placode/cup, resolving over a period of a day or two into a domain in the ventral part of the otic vesicle. By stage 21 (E3.5), the cochlea has begun to develop as a ventral downgrowth of the otic vesicle while the dorsal wall of the vesicle is thinning and beginning the shape changes that will create the semicircular canals (Bissonnette and Fekete, 1996). Ser1 expression at this stage appears as a single disc - is also expressed in the developing inner ear (Laufer et al., 1997). We have compared its expression with that of Ser1 at E5 and find that the two patterns overlap exactly (Fig. 4F).

Ser1 expression persists at least up to E12, both in the vestibular sensory patches, where hair-cell production continues indefinitely, and in the basilar papilla (the sensory patch of the cochlea), where hair-cell production ceases by E9/10 (Fig. 5). In both types of sensory patch, Ser1 appears to be expressed in all the cells, with no obvious change in expression when the hair cells differentiate. We cannot, however, exclude the possibility that Ser1 may be down-regulated in the hair cells themselves: these are closely apposed to and interdigitated with the supporting cells, which certainly do express the gene, and it is difficult to be sure whether apical staining is in the hair cells or in the apical processes of the supporting cells.
**N1 is expressed widely in the otic epithelium, including all sites of expression of its ligands**

As noted above, N1 is already expressed throughout the otic placode at stage 11 (E1.5), and it continues to be expressed up to at least E12, in both sensory and non-sensory regions. Throughout this period, expression is strong in the whole of the ventral, thicker region of otic epithelium—the region that includes the sensory patches (Fig. 4C-E). Expression does, however, become somewhat less intense in the maturing sensory patches than in neighbouring regions (Fig. 4E). N1 expression disappears only in the thin-walled dorsal regions that form the semicircular canals and neighbouring non-sensory parts of the utricle and saccule (Fig. 4C). Thus the receptor N1 is available at and adjacent to all sites of expression of the ligands Ser1 and, as we now discuss, Dll also.

**Dll1 expression foreshadows hair-cell differentiation within the epithelial Ser1 domains**

From E3.5 (stage 21) to at least E12, expression of Dll1 in the otic epithelium is restricted to the prospective or actual sensory patches, as marked by Ser1 expression; and within these patches, Dll1 is restricted to a scattered subset of the cells. The timing of expression differs between patches, according to their different time courses of hair-cell production.

In the region of Ser1 expression that corresponds to the future basilar papilla, expression of Dll1 is absent or very weak at E3.5-E4 (Fig. 5A), but by E5 scattered cells expressing Dll1 can be clearly seen (Fig. 5B). From E6 to E8, these continue to be visible, though with a varying distribution along the length of the basilar papilla (Fig. 5C). By E9, cells expressing Dll1 have almost disappeared, with weak expression remaining only in the apical layer of the epithelium (Fig. 5D); this is the layer occupied by the bodies of the hair cells, which lose their connections to the basal lamina and move to the apical face of the epithelium as they differentiate (see Fig. 1A). By E12, expression of Dll1 in the basilar papilla has disappeared completely (Fig. 5E).

Previous studies have shown that hair cells of the basilar papilla complete their last S-phase at a range of times between E5 and E8 (Katayama and Corwin, 1989) and begin to be identifiable by immunofluorescent staining for hair-cell antigen.
HCA (at E6.25 (stage 29) (Bartolami et al., 1991). HCA-expressing cells are plentiful all along the length of the basilar papilla by E7.5, and the numbers continue to increase up to E9/10, after which hair-cell production ceases (Bartolami et al., 1991; Goodyear and Richardson, 1997). Comparison with the present observations indicates, therefore, that the cells expressing DL1 within the Ser1 patch are nascent hair cells, and that these, like nascent neurons, express DL1 transiently, switching expression of the gene on as they become committed to their fate, about the time of their final mitosis, and switching it off again as they differentiate overtly, approximately one day later.

In the vestibular patches — the cristae and maculae — a similar relationship between DL1 expression and hair-cell differentiation is seen. Hair cells begin to be identifiable at or soon after E5 (stage 26) by immunofluorescent staining for HCA (Bartolami et al., 1991) and hair-cell production then continues throughout life (Kil et al., 1997). Correspondingly, scattered cells expressing DL1 are already visible in the prospective vestibular patches at stage 21 (E3.5), and they continue to be seen in these patches at all subsequent stages (Figs 4C-E, 5F-H).

In the cochleovestibular ganglion, meanwhile, we also see cells expressing DL1 (though in this site there is no expression of Ser1). At stage 21 (E3.5), the DL1-expressing cells in the ganglion are few and sparsely scattered; from E5 up to at least E9, large numbers are visible (Figs 4E, 5C). Since the developing ganglion contains dividing neuroblasts up to E7 (D'Amico-Martel, 1982), it is possible that the DL1-expressing cells are nascent or maturing neurons.

Fig. 5. Double-stained specimens showing expression of DL1 (in situ hybridisation, shown as red fluorescence using Fast Red detection), in relation to Ser1 (green immunofluorescence). (A) Prospective basilar papilla (bp) at E4 expresses Ser1 but not yet DL1. (B) At E5: DL1-expressing cells begin to be seen in basilar papilla (l, lagena macula). (C) At E8: DL1-expressing cells plentiful in basilar papilla; cochleovestibular ganglion (cvg) also expresses DL1, but does not express Ser1. (D) At E9 in basilar papilla; last vestiges of DL1 expression are seen in hair-cell layer (arrow). (E) At E12 in basilar papilla, DL1 expression is no longer seen, but Ser1 persists. (F) Crista at E8. (G) Crista at E12. (H) Macula at E12.

Fig. 6. Otic epithelium in culture. (A,B) Explants taken from stage 13 and cultured for 13 hours, then stained with 3A10 antibody (green) to show differentiating neurons and counterstained with 7AAD for nuclei. (C) Explant taken from stage 12 and cultured for 48 hours, then stained with 3A10. (D) Explant taken from stage 13 and cultured for 4 days, then stained with Ser1 antibody to show sensory patches. (E) Detail of explant similar to that in D, stained for Ser1 (green) and HCA (red): the Ser1 patches correspond to patches of hair cells. (A,B,D and E are confocal images). (F) Graph of neuron production in cultured otic epithelium explants. The results are plotted as a function of age (HHhours) of the embryo from which the explant was taken and the final age following culture (HHhours) when they were fixed. Distance above the diagonal dotted line represents time spent in culture. The approximate numbers of neurons in each explant as visualised with 3A10 antibody were scored, as indicated in the key. 3A10-positive cells are first seen in the cochleovestibular ganglion at Stage 17 (58 HHhours) and this is indicated in the graph. Each point represents one culture dish, containing several (1-12) explants. Cultures were scored '0' if some or all explants produced no neurons and none produced more than 5.
Expiants in 48 separate cultures were scored according to the number of neurons produced and the results plotted as a function of age of the embryo from which the explant was taken and of the time in culture (Fig. 6F). The graph shows that explants of otic epithelium isolated from stage 9/10 up to stage 21/22 are capable of generating neurons, without need of any signal from adjacent head tissues, and do so according to the normal in ovo timetable.

To see whether hair-cell production occurred normally in isolated otic epithelium, we studied explants from embryos at E2 (stage 13), 36 hours before the first hair cells can be identified (Bartolami et al., 1991). The cultures were maintained for up to 5 days, equivalent to E7, a stage when large numbers of vestibular and auditory hair cells have developed in vivo. After this period in vitro large numbers of hair cells were readily identifiable by their HCA expression. As in vivo, the hair cells were grouped in several discrete patches, which stained by immunofluorescence for Ser1 (Fig. 6D,E).

**DISCUSSION**

Before examining the detailed parallels with the development of insect sensory bristles, we must first consider how our present findings compare with and add to previous observations on ear development and Delta-Notch function in vertebrates.

**Cochleovestibular neurons originate from neuroblasts in the otic epithelium**

We have confirmed previous studies reporting that the ear’s neurons are derived by delamination from the otic epithelium (D’Amico-Martel and Noden, 1983; Carney and Couve, 1989; Hemond and Morest, 1991; Haddon and Lewis, 1996). Quail/chick grafting has shown that while almost all the cochleovestibular neurons derive from the placode, the associated glial cells derive from the neural crest (D’Amico-Martel and Noden, 1983). Thus the cells that we see delaminating from the otic epithelium are specifically neuronal precursors. Moreover, as we have seen, they are capable of dividing before they differentiate: they are neuroblasts rather than postmitotic neurons. Cochleovestibular neurons are born from the neuroblasts in the ganglion over a prolonged period, up to E7 in chick (D’Amico-Martel, 1982) and in fish throughout life (Presson and Popper, 1990). The embryonic ganglion thus consists of a mixture of neurons and neuroblasts, with a common origin from neuroblasts in the otic epithelium.

**The control machinery for neurogenesis is intrinsic to the otic epithelium**

Our culture experiments show that neurons are generated from the otic epithelium even when it is isolated from its usual surroundings: the otic epithelium is programmed for neurogenesis from as early as stage 9 (31 HHhours), before the otic placode is even identifiable morphologically. No signal from the hindbrain or other neighbouring tissues is needed to trigger neurogenesis at the appropriate time, at least from stage 9 onwards; on this point, we contradict the speculative suggestion that neurogenesis is induced by a signal from the hindbrain (Repsa et al., 1991). The mechanisms that regulate genesis of neuroblasts in the otic epithelium seem to be intrinsic to the epithelium itself.
Expression of Dll1 in the early otic epithelium foreshadows expression of neuronal differentiation markers in the same way as in the embryonic CNS

What are the regulatory mechanisms for neurogenesis in the ear? We have found that Dll1 is expressed in scattered cells in the neurogenic patch in the otic epithelium, in a pattern suggesting that Dll1 is expressed transiently in the neuroblasts at or soon after their commitment, before they begin to express other neuronal markers. The relationship of Dll1 expression to expression of the neuronal markers TuJ1, Islet1/2, BEN and other neuronal markers. The relationship of Dll1 expression to expression of the neuronal markers TuJ1, Islet1/2, BEN and 3A10 is very similar to that seen for nascent motoneurons in the spinal cord (Erickson et al., 1992; Tsuchida et al., 1994; Chitnis et al., 1995; Henriques et al., 1995; Memberg and Hall, 1995; Haddon et al., 1998b), where commitment to a neuronal fate is regulated by Delta-Notch signalling, delivering lateral inhibition from the nascent neural cells to their neighbours.

Our data provide circumstantial evidence that the same is true in the ear. There is, however, one striking difference: for the motoneuron, expression of Dll1, Islet1/2, BEN and TuJ1 is associated with the cessation of cell division; for the otic neuroblast it is not (see Memberg and Hall, 1995, for other examples). We shall see that this has a parallel in Drosophila bristle development.

Hair cell determination is correlated with a second round of expression of Dll1

The differentiation of hair cells and supporting cells is anticipated by a second round of Dll1 expression, occurring against a background of continuing widespread N1 expression, and we have shown that the Dll1-expressing cells are most probably nascent hair cells. This conclusion is supported by preliminary observations in transgenic mice carrying a Dll1:lacZ reporter gene, whose product is detected in differentiating hair cells (Alastair Morrison, personal communication).

Hair cells and supporting cells arise side by side, forming, in the basilar papilla at least, a regular alternating pattern (see Fig. 1C). The precision of this pattern is refined by cell movements through which the newly differentiated hair cells and supporting cells become rearranged (Goodyear and Richardson, 1997); but the pattern is already evident when the differentiated cells first appear. This suggests that the initial pattern of cell commitment is regulated by lateral inhibition, whereby each nascent hair cell inhibits its neighbours from becoming hair cells, with the result that they become supporting cells instead. A simple mathematical model based on the known properties of the Delta-Notch signalling mechanism has shown that lateral inhibition mediated by Delta-Notch signalling can indeed generate an alternating pattern of just the type observed (Collier et al., 1996).

The timing of Dll1 expression is as expected if this mechanism is operating in the ear. The decision to differentiate as either a hair cell or a supporting cell is presumably taken at or after a cell's terminal mitosis, since there is evidence from several systems that a hair cell can have a supporting cell as its sister (Stone and Cotanche, 1994; Weisleder et al., 1995; Jones and Corwin, 1996); and we have seen that Dll1 expression, present in scattered cells in the developing basilar papilla from E5 to E8/9, parallels the time-course of terminal mitoses of hair cells, which complete their final S-phase between E5 and E8 (Katayama and Corwin, 1989). A similar correlation between Dll1 expression and hair-cell production is seen in the vestibular patches.

The accompanying paper (Haddon et al., 1998a) provides evidence from a zebrafish mutant, mind bomb, that Delta-Notch-mediated lateral inhibition does indeed control the hair-cell/supporting-cell decision in the ear.

Loss of Delta-Notch signalling is unlikely to be the trigger for cell proliferation during hair cell regeneration

In the basilar papilla of a bird, there is normally no post-embryonic cell division or hair-cell production. If hair cells are destroyed, however, even in the adult, the mitotically quiescent supporting cells re-enter the division cycle and new hair cells are generated (Cotanche et al., 1994). An obvious suggestion is that the process is controlled by a signal from hair cells that normally acts continuously, so long as hair cells are present, to inhibit supporting cells both from dividing and from differentiating into hair cells. If so, the inhibitory signal must be something other than Dll1, since we have shown that expression of this gene is transient, ceasing as the hair cells become fully differentiated. Delta-Notch signalling may, however, play a part in controlling cell fate once the regenerative response has been set in motion (Jennifer Stone, personal communication), just as it appears to be involved, according to our observations, in the continuing generation of hair cells from stem cells in the vestibular sensory patches.

Ser1 expression is an early and persistent marker of sensory patches

We have shown that Ser1 is a very early marker for the prospective sensory patches, reflecting, perhaps, a 'prosensory' cell state (Kelley et al., 1993) analogous to the proneural state preceding neuronal commitment in neurogenic epithelium. BMP4 expression has been reported to be a similarly early marker of all sensory patches (Wu and Oh, 1996), although in our hands, as also for the mouse embryo (Morsli et al., 1998), the BMP4 domain does not exactly coincide with the Ser1 domain or with the location of all the sensory patches (I. Le Roux, unpublished observations).

Tracing the domains of Ser1 expression backwards through development gives an indication of how the pattern of sensory patches is set up. At E3.5 the saccular macula and the utricular macula appear to be one continuous ventral patch. The neurogenic region of the otocyst corresponds to the anterior part of this patch, i.e. the future utricular portion. The prospective anterior and lateral cristae appear initially continuous with the utricular macula, and the posterior crista, lagenar macula and basilar papilla appear initially continuous with the macula of the saccule (see also Fekete, 1996). This matches observations in fish embryos (Becerra and Anadón, 1993; Haddon and Lewis, 1996), where the corresponding maculae and cristae similarly appear to derive from one continuous primordial patch – a macula comminvis, whose form and location are closely similar to those of the Ser1 expression patch in the E3.5 chick ear. Thus, despite their different final forms, the chick ear and the fish ear may be fundamentally alike in the way they organise their global pattern of sensory patches.

Our tissue culture experiments, as well as previous grafting experiments (Swanson et al., 1990) show, moreover, that the discrete sensory patches develop through mechanisms intrinsic to the otic epithelium, in agreement with results for neurogenesis at earlier stages. This contrasts with tissues such as the feather tracts
in the chick skin, which also depend on Delta-Notch signalling, but in which interactions between epidermises and mesenchyme play a crucial role (Crowe et al., 1998; Viallet et al., 1998).

The function of Ser1 in the otic epithelium remains to be determined. The Dil expression associated with hair-cell production is precisely restricted to the Ser1 domains, suggesting that the two genes are functionally coupled in some way, and in these domains Ser1 is coexpressed with Lunatic fringe (in agreement with observations of Lunatic fringe expression in the mouse ear; Morsli et al., 1998). This situation is reminiscent of the Drosophila wing disc, where Serrate, Fringe, Delta and Notch interact to define the special band of organiser tissue at the wing margin (Irvine and Vogt, 1997), in whose neighbourhood, incidentally, arrays of close-packed sensory bristles develop (Hartenstein and Posakony, 1989). By analogy with the wing disc it is possible, for example, that confrontation between cells that express Ser1 and Lunatic fringe and cells that do not plays a part in organising development of the ear's sensory patches. Recent work has shown, however, that Serrate also acts in conjunction with Delta in the development of individual sensory bristles elsewhere on the insect's body (Zeng et al., 1998); so it may be wrong to look for specific parallels with the insect wing margin.

**Sensory bristles in Drosophila and sensory patches in the ear are generated by essentially the same developmental program**

Each insect sensory bristle is a functional unit formed from the progeny of a sensory mother cell (SMC): this cell is singled out from a proneural cluster in the insect epidermis, and (in the case of a standard mechanosensory bristle) divides twice to generate four different cells. At each division of the SMC and its progeny, Delta and Serrate, acting together in a quasi-redundant fashion as ligands for Notch, mediate lateral inhibition to force the sister cells to adopt different fates (Hartenstein and Posakony, 1990; Ghyessen et al., 1993; Parks and Muskavitch, 1993; Jan and Jan, 1995; Zeng et al., 1998). In the first division, one daughter (the daughter delivering lateral inhibition) becomes committed as a neuroblast while the other becomes committed as a sensory epithelial precursor. In the second division, the daughters of the neuroblast become, respectively, a neuron (delivering lateral inhibition) and a neural sheath cell, while the daughters of the sensory epithelial precursor become, respectively, a bristle sheath cell (delivering lateral inhibition) and a bristle socket cell. Some SMCs follow variants of this program: in chemosensory bristles, for example, the neuroblast divides several times to generate 3-5 neurons (Notebohm et al., 1994); in non-innervated bristles of the posterior wing margin, conversely, neurons are missing (Hartenstein and Posakony, 1989).

The sensory neuron of the bristle corresponds to the sensory neuron of the ear; the shaft cell, presumably, to the hair cell of the ear (see Tilney et al., 1995, for the structural similarities in cytoskeletal organisation); and the socket cell to the supporting cell. The neural sheath cell has no such obvious counterpart: the glial cells in the cochleovestibular ganglion derive from the neural crest, not the otic epithelium (D’Amico-Martel and Noden, 1983). One might, however, compare the neural sheath cell of the bristle to a second-generation neuroblast in the cochleovestibular ganglion – both of them are daughters of first-generation neuroblasts but have not differentiated into neurons.

Assuming these correspondences between the cell types, the correspondences in the developmental program can be inferred directly (Fig. 7). At the outset, however, there is a contrast. Whereas each bristle is typically isolated from the next by intervening epidermis, each sensory patch in the ear consists of a mass of contiguous hair cells and supporting cells, with no non-sensory cells between them. Thus it seems that the counterpart of the SMC is not a single isolated cell, but a cluster of contiguous sensory precursor cells (SPCs) that coexist instead of competing by lateral inhibition. (In Drosophila, SMCs likewise develop in contiguity, exceptionally, at the wing margin; Hartenstein and Posakony, 1989.) If we draw the ear/bristle parallel in this way, we can relate all the subsequent steps of sensory patch development to those of bristle development, with Delta/Serrate-Notch signalling acting repeatedly in a similar way in the two systems. The singling-out of neuroblasts in the ear corresponds to the determination of one of the two daughters of an SMC as a neuroblast. The production of neurons from neuroblasts in the cochleovestibular ganglion corresponds to the production of a neuron or of several neurons from the bristle neuroblast. And the genesis of hair cells and supporting cells from otic sensory epithelial precursors corresponds to the genesis of bristle shaft cells and supporting cells from sensory epithelial precursors in the insect epidermis. The two systems differ, it is true, in the numbers of cell divisions that occur at each step; but this is variable even between types of bristles in the fly.

In addition to the above systematic parallels in the developmental program, there are other facts that suggest a conserved process. For example, Pax2, along with its close relatives Pax5 and Pax8, is strongly expressed in the early ear rudiment (Pfeffer et al., 1998) and is required for development of the cochlea (Torres et al., 1996); and its homolog in Drosophila, Pax258, is strongly expressed in the precursor cells of the sensory bristles (Czerny et al., 1997).

It seems clear that the mechanosensory organs of flies and vertebrates are fundamentally similar, not only in function and architecture, but also in the developmental programs that generate their precisely patterned arrays of cell types. The precise correspondence that we have identified should help in the search for other molecules that have a conserved role in the two systems.

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No references were found in the provided text.

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