Universitat Pompeu Fabra
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The early development of the inner ear:

The Role of Notch and FGF pathway on early otic neural versus non-neural patterning

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SUMMARY

Cranial sensory neurons are responsible for sensing environment inputs of the head and present a dual origin; they derive either from the neural crest cells or from the neurogenic placodes. Otic neuronal precursors are specified in the otic placode but interestingly do so only in the anterior domain of the otic placode, the *proneural* domain.

In the present study, we have explored why only this territory has the competence to undergo neurogenesis, this means the early events of otic proneural regionalization and neural commitment. The proneural and non-neural domains presented complementary gene expression patterns of transcription factors and members of the Notch pathway. Moreover, this work showed that the otic territory begins to be regionalized in the AP axis at the time of otic placode formation, much before it was reported. *Sox3* gene, involved in neural commitment, was expressed in a common proneural territory shared between the geniculate placode and the proneural otic domain, while the transcription factors *Lmx1* and *Tbx1* were restricted to the posterior pre-otic region. Fate map studies and double injections with DiI/DiO indicated that labeled cells remained confined to anterior or posterior territories with limited cell intermingling at otic cup stage. Notch pathway is one of the main signaling pathways involved in patterning and boundary formation. Notch blockade induced the expansion of non-neural genes, *Lmx1* and *Iroquois1*, into the proneural domain, and an overproduction of neuroblasts within the proneural domain. We therefore propose that Notch is required for neuroblast selection and for early otic neural/non-neural regionalization by stabilizing gene expression patterns. However, Notch signaling pathway is dispensable for the specification of a proneural territory. Since FGF signaling is one of the main pathways involved in neural induction, we analyzed the role of FGFs in otic proneural specification. Loss and gain of function experiments demonstrated the requirement of FGF signals for enhancing *Sox3* expression, inducing FGF10 and thus driving otic neural fate.

Overall, we propose that proneural character is acquired in the anterior territory by the action of localized ectodermal FGF8-FGF10 signaling that enhances Sox3 function. FGF signals through Sox3 activity would be essential for the specification of the proneural domain versus a non-neural territory, while Notch would be involved in refining this early regionalization.

INTRODUCTION

1. INTRODUCTION

1.1. The Inner Ear Of Vertebrates

1.1.1. Anatomy and histology of the inner ear

Sensory organs consist of key faculties by which information received from the environment is evaluated for producing a meaningful experience of the world and respond to changes. The ear is one of the main sensory organs of the head and is responsible for the senses of hearing, balance and detection of acceleration. It is composed by three anatomical parts: 1) the external ear that funnels sound waves to the middle ear. 2) The middle ear that transforms sound waves, through the auditory ossicles, into mechanical vibration of the endolymphatic fluid contained in the inner ear; and finally, 3) the inner ear which is responsible for the transduction of mechanical stimuli into electrical impulses and their propagation to the brain (FIG. 1A; Purves et al., 2001).

The inner ear is made up of two parts: the vestibular portion, which contains the sensory organs responsible for the senses of motion and position, and the cochlear or auditory region, which contains the sensory organ responsible for the sense of hearing (FIG. 1B; Purves et al., 2001).

Three different cell types can be considered as the functional unit of all inner ear sensory organs: the hair-cells, the otic neurons and the supporting cells (FIG. 1C; Purves et al., 2001). Hair-cells are specialized mechano-receptors that transduce the auditory and vestibular mechanical stimuli into electrical signals. Hair-cells have numerous stereocilia (hairs) projecting from the apex of the cell into endolymph and are located within a complex topological organization (reviewed in Torres and Giraldez, 1998). Movement of stereocilia cause ion channel opening/closing depending on direction, and this in turn elicits changes in the membrane potential of hair cells (Purves et al., 2001). Hair-cells are innervated by otic neurons which are bipolar primary afferent neurons that transmit the information to second order neurons in the vestibular and auditory nuclei in the brainstem. Their somas are intermingled with the glial Schwann cells forming the cochlear and vestibular ganglia (CVG) (reviewed in Rubel and Fritzsch, 2002). Supporting cells are non-sensory cells that vary greatly in morphological and functional specialization. Supporting cells maintain the correct ionic environment for the hair-cells to be functional. In addition, supporting cells release factors that maintain the trophism and survival of the hair-cells (Haddon et al., 1999). In addition, Supporting cells are also capable of serving

as progenitors to regenerate hair-cells after injury by a trans-diferentiation process (White et al., 2006; Corwin and Cotanche 1988; Ryals and Rubel, 1988; reviewed Stone and Cotanche, 2007).

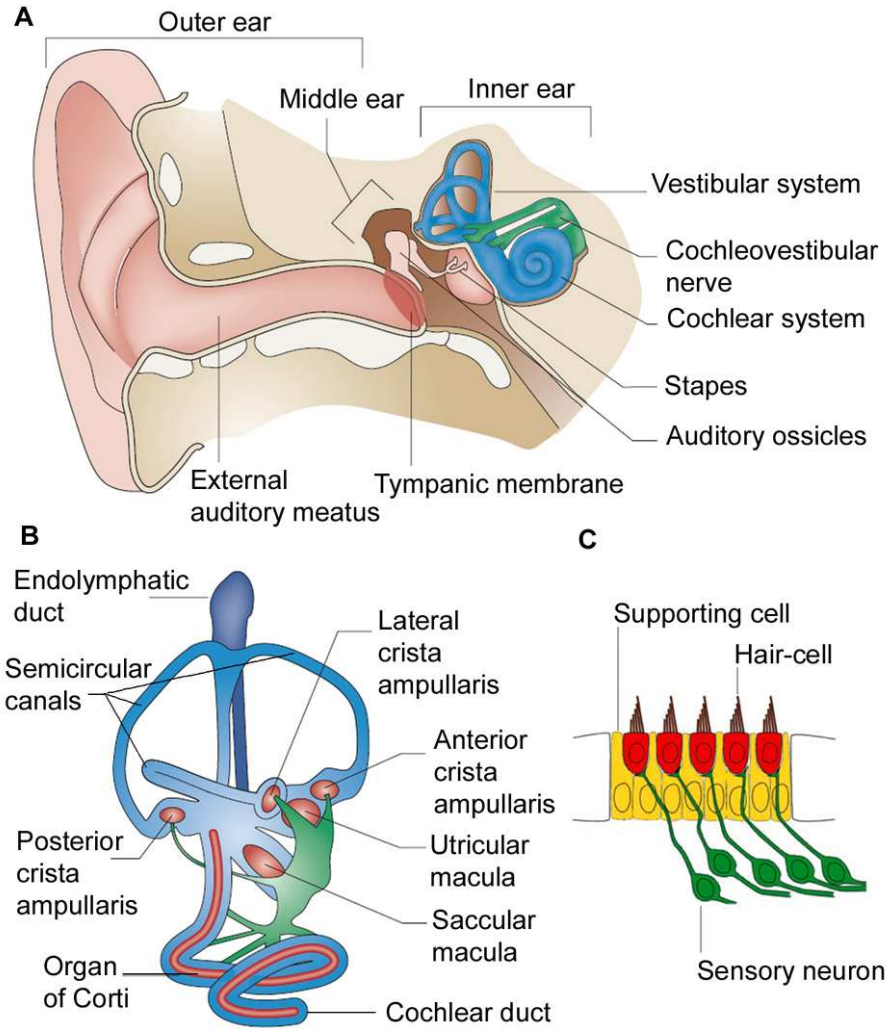

Figure1. The inner ear. (A) Schematic representation of the ear showing its different parts: the outer, the middle and the inner ear. (B) Schematic drawing showing the different parts and sensory organs of the inner ear. (C) The sensory unit is build up by three different cell types: supporting cells, hair-cells and sensory neurons. Modified from Kelley, (2006) and Adam et al., (1998).

The number of sensory organs contained in the inner ear varies between animal species, but all have at least six (FIG. 1B). The vestibular system contains three *cristae*, one per semicircular canal; and two *maculae*, one for the saccule and the other one for the utricle.

The auditory system only contains one sensory organ (organ of Corti in mammals and *basilar papilla* in birds) which is placed along the cochlear duct. In birds, amphibians, and fish, several other small maculae of uncertain function are also present (FIG 2; Fritzsch, 1999). The macula of the utricle and saccule detect linear accelerations in the horizontal and vertical axis, respectively, and the gravitational pull (Purves et al., 2001). The cristae, which are located in chambers called ampullae at the base of the semicircular canals, detect angular acceleration due to circular nature of the canals (Purves et al., 2001). In the auditory sensory epithelium, sound-wave frequency discrimination is based on the position of the hair-cells along the longitudinal cochlear axis, which is correlated with the position of the sensory neurons in the cochlear ganglion. This tonotopical order is conserved in the central auditory nuclei, where sensory neurons project, reproducing the hair-cell order in the cochlea (Purves et al., 2001; Torres and Giraldez, 1998). In addition to the sensory structures, the inner ear includes the endolymphatic duct (ED), which extends dorsally to communicate with the central nervous system (CNS).

BOX I. Sensorineural Hearing Loss

Dysfunction of the auditory sensory system is one of the most prevalent chronic disabilities of our time, affecting up to one in three elderly people. Sensorineural hearing loss occurs when there is damage to the cochlea or to the nerve pathways from the inner ear (retrocochlear) to the brain. Sensorineural hearing loss can be caused by genetic syndromes and birth injury affecting at least 1 in 500 births (Smith et al., 2005; Morton and Nance, 2006). Nevertheless, it can be also due to age- related changes caused by drugs that are toxic to the auditory system, viruses and tumors, noise exposure, head trauma and aging. Moreover, disorders of the inner ear can also disrupt the reception or processing of the vestibular sensory stimuli resulting in vertigo, nystagmus, and loss of balance (Sando et al. 2001).

1.1.2. The inner ear in different species and throughout evolution

The organization of the vestibular region of the inner ear is highly conserved among the different vertebrate classes, whereas the auditory part can vary greatly across vertebrates and it shows increasing degrees of complexity in terrestrial animals (FIG. 2). There is general agreement that ear evolution started with a gravistatic and angular acceleration sensing vestibular ear (Fritzsch, 1992; Fritzsch, 2003).

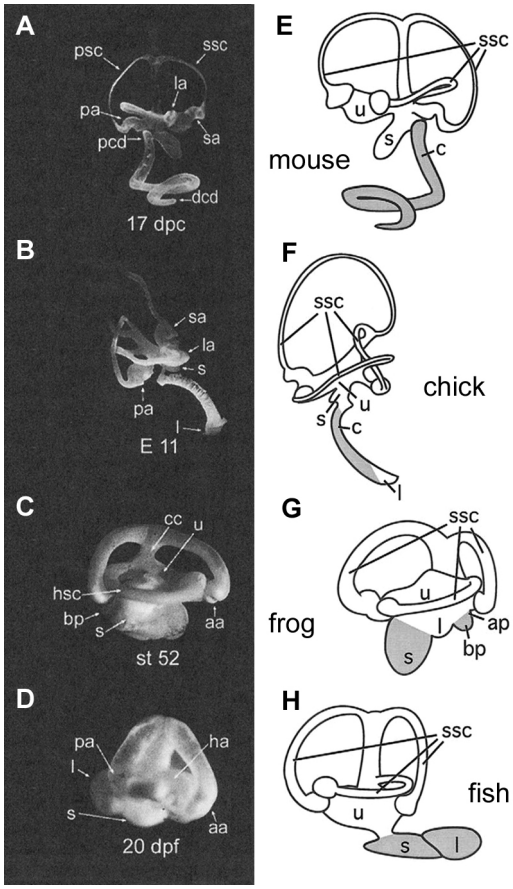

Figure 2. Inner ear in different species. (A-D) Inner ear of mouse (A), chick (B), frog (C) and zebrafish viewed by filling the inner ears with opaque paint. Lateral view, anterior to the right. (E-H) Schematic representation of the adult inner ear of mouse (E), chick (F), frog (G) and zebrafish (H). Auditory regions are shaded gray. Lateral view, anterior to the left. Abbreviations: aa, anterior ampulla; ap, amphibian papilla; bp, basilar papilla; c cochlea; cc, common crus; dcd, distal cochlear duct; dpc; days post-coitus; dpf, days post-fertilization; E, embryonic day; ha, horitzontal ampulla; hsc, horitzontal semicircular canal; l, lagena; la, lateral ampulla; pa, posterior ampulla; pcd, proximal cochlear duct; psc, posterior semicircular canals; s, saccule; sa, superior ampulla; ssc, semicircular canals; u, utricle; Adapted from Riley and Philips, (2003); Kelley et al., (2005).

Almost all animals from cnidarians to mammals have mechanosensory organs for touch and detection of vibrations and other disturbances of the environment. Within vertebrates, some mechanosensory organs evolved into auditory organs, increasing sensitivity to sound greatly through modifications of accessory structures to direct sound to the specific sensory epithelia (Friztsch et al., 2007: Molecular evolution of the vertebrate mechanosensory cell and ear). In the past decade, many genes implicated in the process of neurosensory development have been identified both in invertebrates and vertebrates (Ghysen and Richelle, 1979, Torres and Giraldez, 1998, Baker and Bronner-Fraser, 2001). Now it is clear that cellular differentiation of neurons and sensory cells requires the activity of proneural genes (Bertrand *et al.*, 2002, Kageyama *et al.*, 2005), which were first identified in *Drosophila* mutants lacking the ability to develop external sense organs and bristles (Ghysen and Richelle, 1979). Proneural genes encode for transcription factors of the *b*asic *H*elix-*L*oop-*H*elix (bHLH) class that bind to a common DNA sequence called the

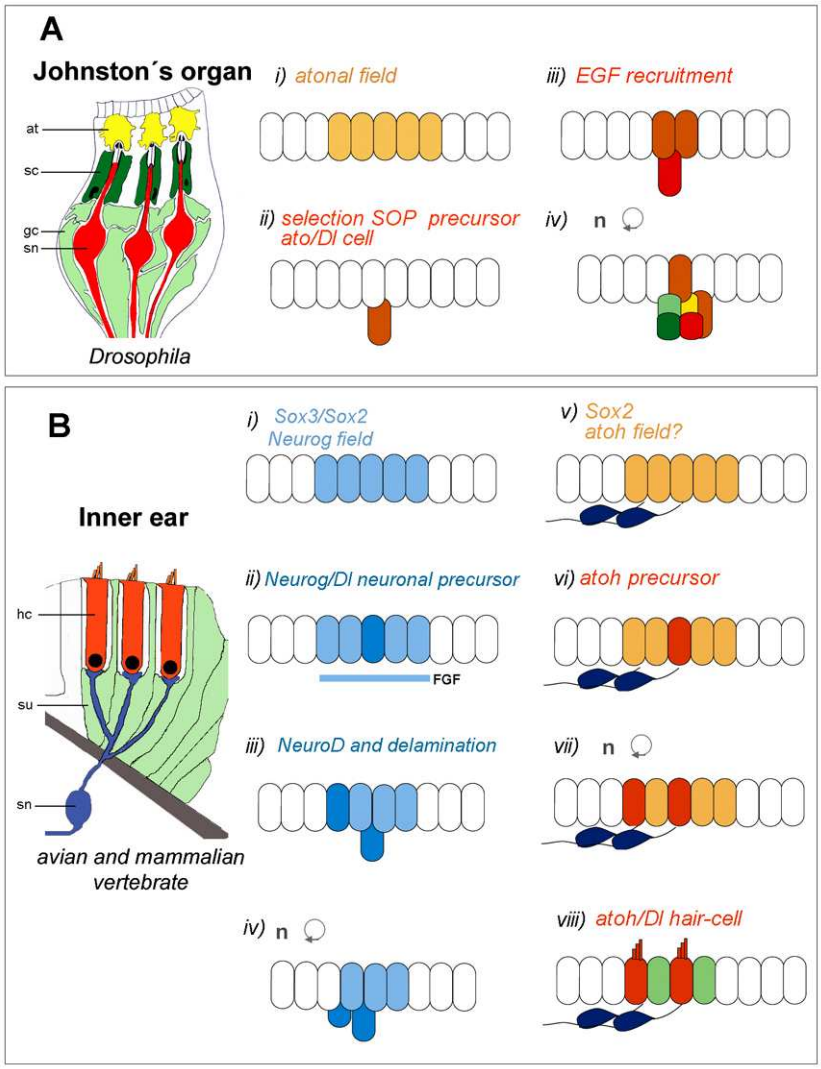

Figure 3. Comparison between the *Drosophila* Johnston's organ and the vertebrate sensory patch of the inner ear. (A) Schematic drawing of three chordotonal sensilla of *Drosophila* modified form Yager, (1999) and sequential steps of its development. i) a neurosensory field is specified by *atonal* proneural gene, ii) a SOP is singled out by the N-Dl pathway, iii) EGF recruits secondary precursors and, iv) sensory neuron and accessory cells are generated. (B) Sensory patch of the vertebrate inner ear Fettiplace and Hackney, (2006) and the main steps of its development. i) a proneural field is specified by *Neurogenin*, ii) neuronal precursors are singled out by the N-Dl pathway, iii) *NeuroD* is switch on v) neuroblasts delaminate and differentiate vi) ath starts to be expressed in a prosensory field vii) sensory precursors are singled out viii) hair-cells and supporting cells are determined by the activity of Notch pathway. Abbreviations: at, attachment cell; gl, glial cell; hc, hair-cell; sc, scolopale cell; sn, sensory neuron; SOP, sensory organ precursor; su, supporting cell. Adapted from Alsina et al., (2007).

E-box sequence, CANNTAG (Bertrand *et al.*, 2002). In *Drosophila*, two major proneural gene families control neuronal development of the Peripheral Nervous System (PNS), the *achaete-scute* (*asc*) and the *atonal* (*ato*) gene family (Simpson, 1990). *Asc* genes in *Drosophila* specify external sense organs, while *ato* gene specifies the photoreceptors and chordotonal organs, among them the specialized Johnston´s organ (Ghysen and Dambly-Chaudiere, 1989, Treisman, 2004). The latter, is located in the fly antenna and it appears to be the *Drosphila* homologous of a hearing organ as judged from its capacity to generate sound evoked potentials (FIG 3; reviewed in Caldwell and Eberl, 2002, Boekhoff-Falk, 2005;Eberl and Boekhoff-Falk). Atoh genes evolved with multicellular organisms (Seipel et al., 2004) and are highly conserved protein coding genes. Orthologues of fly and mouse, *ato* and *Atoh1*, can be mutually exchanged and show compensatory function in distant organisms (Wang et al., 2002). In vertebrates, in addition to the *asc homologues* (*ash*) and the *ato homologues* (*atoh*) ortologous genes, other related proneural families are encoded in the genome: the E proteins, Olig, NeuroD, Neurogenin (*Neurog*, also named *Ngn*) and Nscl protein families (Bertrand *et al.*, 2002). Some of them have been recruited for the peripheral sensory developmental program in craniates (Fritzsch and Beisel, 2001; Fritzsch et al., 2007).

1.2. Cranial Sensory Organs Derive From The Cranial Placodes

1.2.1. The cranial placodes

Despite the complexity of the adult inner ear, the inner ear derives from a very simple embryonic structure a cranial placode named the otic placode. Cranial placodes, also known as ectodermal placodes, are transient, discrete regions of thickened columnar epithelium that form in characteristic positions in the head ectoderm of vertebrate embryos. They contribute to the paired sense organs and cranial sensory ganglia of the head (FIG. 4A; Ariëns Kappers, 1941; Le Douarin *et al*., 1986, 1992; Vogel, 1992; Webb and Noden, 1993; Northcutt, 1996; Graham and Begbie, 2000). Cranial placodes include the hypophyseal, olfactory, trigeminal, otic and epibranchial placodes, which are neurogenic, and the lens placode, which is not. Furthermore, in amphibians and fish there is an additional series of placodes that form the lateral line system (FIG. 4; reviewed in Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser 2006; Streit 2004). Ectodermal placodes form a wide variety of cell types, including ciliated sensory receptors, sensory neurons, neuroendocrine and endocrine cells, glia, and other supporting cells (FIG. 4B; reviewed in Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser 2006; Streit 2004).

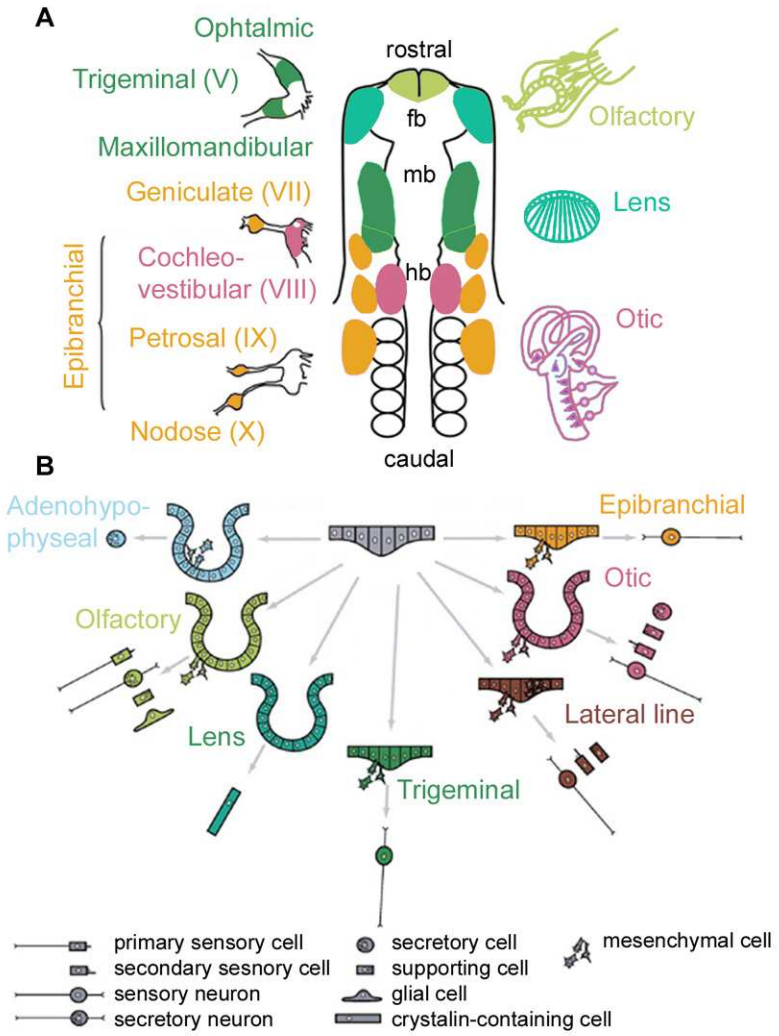

Figure 4. Cranial placodes in vertebrates. (A) Schematic representation of the sensory placodes and their derivatives at the 10 somite stage in the chick embryo (after D'Amico-Martel and Noden, 1983; Bhattacharyya*, et al.*, 2004; Streit, 2007). (B) Schematic summary of morphogenesis and cellular derivatives of various cranial placodes. Invagination occurs in adenohypophyseal, olfactory, lens, and otic placodes. Moreover, in all placodes except the lens placode, some cells migrate away from the placodal epithelium as mesenchymal cells to form sensory neurons, secretory cells, or glial cells. In lateral line placodes, another subset of cells migrates along the basement membrane and forms the lateral line primordial. Modified from Schlosser, (2005). Abbreviations: fb, forebrain; hb, hindbrain; mb, midbrain.

1.2.2. Placode development

Early in development, placodes begin to form in an area called the pre-placodal ectoderm (PPE). The PPE is located in the anterior region of the embryo, medial to the epidermis and lateral to both the neural crest and the neural plate (FIG. 5A and B; Knouff, 1935). Within the pre-placodal ectoderm, precursors for different placodes are initially interspersed, but then they separate to form the individual placodes at discrete positions along the neural tube (reviewed in Baker and Bronner-Fraser, 2001; Riley and Phillips, 2003; Streit, 2004; Schlosser, 2005; Schlosser and Ahrens, 2004; Brugmann and Moody, 2005). Fate maps of embryos of teleosts, amphibian and amniotes indeed suggest that all cranial placodes originate from this horseshoe-shaped PPE (teleosts: Kozlowski et al., 1997; Whitlock and Westerfield, 2000; Dutta et al., 2005; amphibian: Vogt, 1929; Röhlich, 1931; Carpenter, 1937; Fautrez, 1942; Jacobson, 1959; Eagleson and Harris, 1990; Eagleson et al., 1995; amniotes: Couly and Le Douarin, 1985, 1987, 1990; Streit, 2002; Bhattacharyya et al., 2004). More direct evidence for the presence of a generic placodal bias in the PPE comes from the recent identification of transcription factors like the Six1/2 and Six4/5 subfamilies (homologous to *Drosophila sine oculis* and *optix* homeobox) and the Eya (homologous to *Drosophila eyes absent)* family. *Six* and *Eya* (FIG. 5B) family of genes are initially expressed within the horseshoe-shaped domain, and later continue to be expressed in some or all cranial placodes. The importance of *Six* and *Eya* genes for normal placode development has been demonstrated by the effects of their loss-of-function in mouse, zebrafish and humans. Eya1 and Six1 have been shown to be necessary for the formation of most placode derivatives (Zou*, et al.*, 2006; Whitfield, 2005; Ozaki *et al.*, 2004; Bricaud and Collazo, 2006).

It was not until very recent that the molecular pathways that regulate cranial placode development started to be explored. Different signaling pathways converge to confer the preplacodal character to the ectoderm (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005). In chick, the head mesoderm underlying the placode territory is necessary and sufficient for placode induction. This domain expresses Fibroblast Growth Factors (FGF; see BOX III), as well as Bone Morphogenetic Protein (BMP; see section 8.2.1 for signaling pathway description) and Wnt inhibitors (Wnt, vertebrate homologous of *wingless* in *Drosophila*; see section 8.2.2 for signaling pathway description) (Litsiou et al., 2005). These factors act to protect preplacodal cells from antagonistic influences emanating from surrounding tissues. These include a high level of Wnt from mesoderm lateral and posterior to the preplacodal region and from the neural

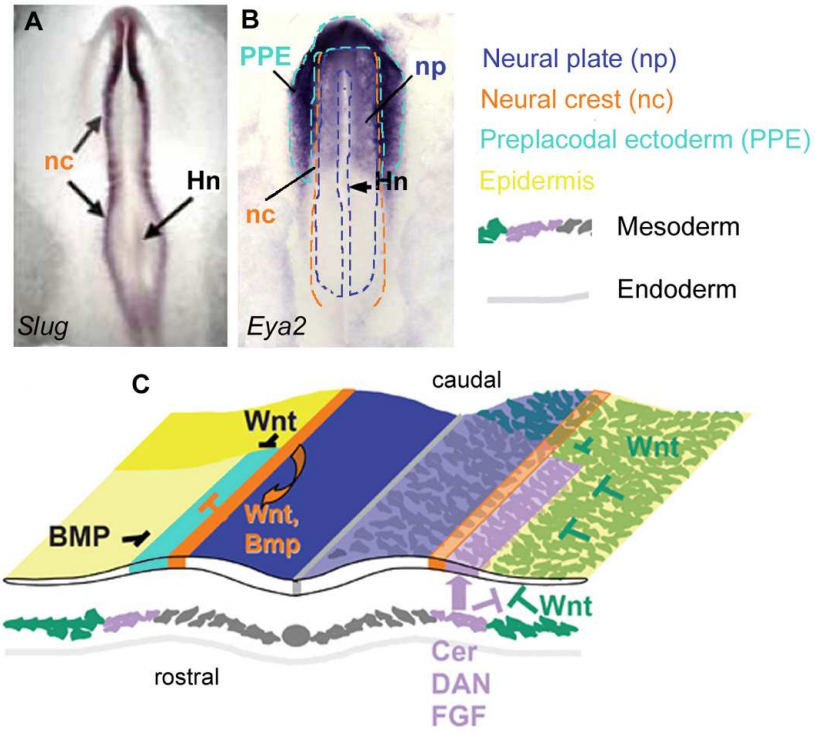

Figure 5. Model for preplacodal region induction. (A) Dorsal view of a chick embryo at the 2-4 somite stage stained for a neural crest marker, *slug* (Basch et al., 2006). (B) Dorsal view of a chick embryo at the 0 somite stage stained for a PPE marker, *Eya2* (Streit, 2004). (C) A diagram showing a cross section through a chick embryo at the 2-4 somite stage. Ectodermal signals that influence the position of the preplacodal region are schematised on the left, whereas mesoderm derived signals are shown on the right. The PPE (purple) is surrounded by inhibitory signals from the lateral (light yellow; BMP) and posterior (yellow; Wnt) ectoderm, from the neural folds (orange; Wnt, BMP) and from the lateral and posterior mesoderm (green; Wnt). FGF, Wnt antagonists and BMP antagonists (purple) from the mesoderm underlying the PPE protect the overlying ectoderm from these inhibitory signals and allow the formation of placode precursors. Modified from Litsiou*, et al.*, (2005). Abbreviations: Hn, Hensen's node.

folds flanking it medially, as well as BMP activity from the non-neural ectoderm and the neural folds (FIG. 5C). Within the preplacodal ectoderm although precursors for different placodes are intermingled, some separation of individual populations along the anterior posterior axis is apparent. Precursors for anterior placodes (adenohypophysis, olfactory, lens) are located in the rostral preplacodal ectoderm, while precursors for posterior placodes (trigeminal, epibranchial, otic, lateral line) are restricted more caudally (D'Amico- Martel and Noden, 1983; Couly and Le Douarin, 1985; Couly and Le Douarin, 1988; Kozlowski*, et al.*, 1997; Streit, 2002; Bhattacharyya*, et al.*, 2004; Litsiou*, et al.*, 2005). As development proceeds, the PPE becomes molecularly divided in successively

smaller sub-domains such that by the time placodes can be identified morphologically each one expressing a unique transcription factor code (Torres and Giraldez, 1998; Bailey and Streit, 2006; Schlosser, 2006). Signals from surrounding tissues drive the PPE to differentiate into distinct placodes with separate developmental fates (reviewed by Baker and Bronner-Fraser, 2001). For instance, FGFs play a key role in inducing olfactory, adenohypophysis, otic and epibrancial placodes (Herzog*, et al.*, 2004; Ladher*, et al.*, 2000; Vendrell*, et al.*, 2000; Leger and Brand, 2002; Maroon*, et al.*, 2002; Wright and Mansour, 2003; Nechiporuk*, et al.*, 2007; Nikaido*, et al.*, 2007; Sun*, et al.*, 2007). Wnt signalling is also involved in placode development. This is shown by the the zebrafish mutants masterblind and headless, in which Wnt signalling is overactivated. This mutants exhibit a loss of anterior placodes (lens, olfactory), but an expansion of trigeminal neurons around the anterior neural plate (Kim*, et al.*, 2000; Heisenberg*, et al.*, 2001). Thus, differential activation of the Wnt pathway along the rostro-caudal axis influences patterning of the preplacodal region.

Bailey and colleagues have postulated that the PPE is initially specified as lens tissue. These authors showed that cells from the PPE region that normally never contribute to the lens, formed lens when isolated from the embryo (Bailey et al., 2006). This suggest that the lens fate is a default state of the preplacodal ectoderm, which must therefore be repressed in the non-lens domains. In this model, FGF signals from the surrounding tissues would initiate lens suppression and simultaneously contribute to confer olfactory, adenohypophysis, epibranchial and otic character to PPE cells (Bailey et al., 2006; Ladher*, et al.*, 2000; Vendrell*, et al.*, 2000; Leger and Brand, 2002; Maroon*, et al.*, 2002; Wright and Mansour, 2003; Herzog*, et al.*, 2004; Nechiporuk*, et al.*, 2007; Nikaido*, et al.*, 2007; Sun*, et al.*, 2007).

1.3. Inner Ear Development

1.3.1. From the otic placode to the adult organ

Otic development starts with the specification of an otic field that progressively forms the otic placode. The otic placode is a transient thickening of the ectoderm adjacent to the rhombomeres 4 to 6 of the developing hindbrain that gives rise to the inner ear (FIG. 6A). The otic placode becomes visible after the events of gastrulation have laid down the body plan of the vertebrate embryo, typically once the first 5-10 pairs of somites have been generated, depending on the animal species. The placode then invaginates to form the otic cup and the otic vesicle, the, ellipsoid-shaped structure lined by a pseudo-stratified epithelium (FIG. 6A and B, see Torres and Giraldez, 1998; Groves, 2007). The otic field

progressively acquires its identity and becomes committed to the otic fate, eventually reaching an irreversible state of determination. This state can be defined as the property of the otic primordium to develop into the membranous labyrinth and to generate the cellular phenotypes of the adult organ independently of the embryonic environment, which is fully achieved at the otic vesicle stage (Waddington, 1937; Jacobson, 1963; Swanson et al., 1990; Gallagher et al., 1996). Once the otic vesicle acquires the state of determination it undergoes a period of intense proliferative growth. Cell proliferation of the otic vesicle is under the control of growth factors. Among the growth-factors involved, those belonging to the insulin family, insulin and insulin-like growth factors (IGFs), play an important role in otic development (León et al., 1995; review Isabel). Following the proliferative period, the otocyst enters a differentiation phase, during which extensive morphogenic events take place to effect the final architecture of the organ (reviewed by Bok et al., 2007).

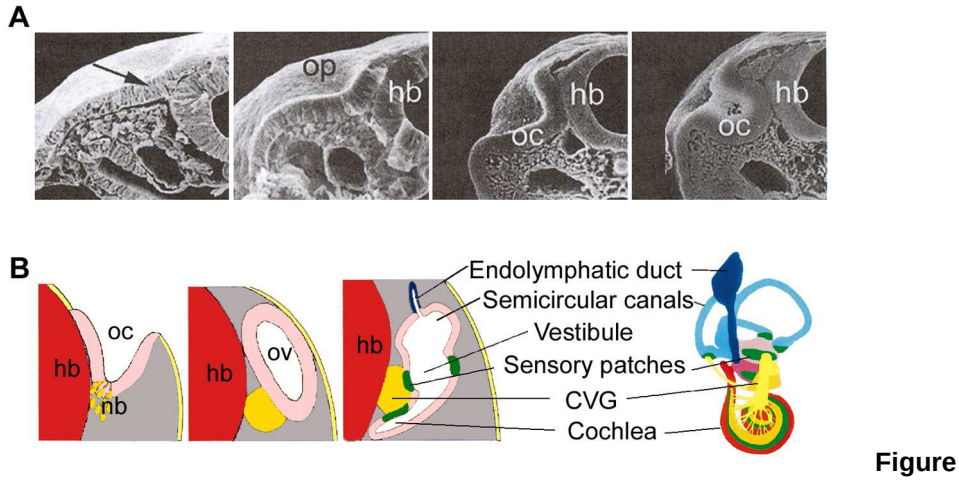

Figure 6. From the otic placode to the adult organ. (A) Electron microscopy images of chick embryos showing transversal sections from preotic placode to otic cup. Arrow points the thickening of the ectoderm adjacent to the hindbrain just prior to the otic placode invagination. Adapted from Kelley et al., (2005). (B) Drawings schematize the development of the inner ear in an idealized vertebrate and showed representation of transverse sections at the otic level from otic cup to otocyst, and the adult organ. At otic cup stage, neuroblast appears delaminating from the otic epithelium. At otocyst stage the different anatomical regions are indicated. Modified from Torres and Giraldez (1998). Abbreviations: CVG, cochleovestibular ganglion; hb, hindbrain; nb, neuroblasts; oc, otic cup; op, otic placode; ov, otic vesicle.

The cochlear and vestibular neurons are the first cell types to be specified in the inner ear of chick and mice. Neuronal progenitors can be detected in the otic field as early as otic placode/cup stage and they are only detected in the more rostral aspect of the otic

territory as revealed by the expression of *Neurog1* (Alsina et al., 2004). It is now clear that the bHLH gene *Neurog1* determines neuronal fate with downstream transcription factors such as *NeuroD* playing various roles in differentiation (see below 3.4.4). Otic neuroblasts delaminate from the ventral aspect of the otic cup forming the cochleovestibular ganglion, the CVG (VIIIth cranial nerve; FIG. 6B), which splits into the cochlear and vestibular ganglia as development proceeds. The CVG goes through a period of intense cell proliferation until neuroblasts are postmitotic and start to differentiate and innervate back inner ear hair-cells (D'Amico-Martel and Noden, 1983). The basic plan for the innervation of the ear is believed to consist of an initial gross projection of sensory fibres to the sensory epithelia, followed by the selection of synaptic contacts and the maintenance of established connections (Fekete & Campero 2007). Diffusible factors produced by the sensory epithelium are thought to mediate this process, so that cochlear and vestibular neurons are trophically dependent on their targets. (Fekete and Campero, 2007).

In parallel, the otic epithelium diversifies to generate the sensory areas of the *cristae*, *maculae* and organ of Corti (*basilar papilla* in the chick). A number of genes including *BMP4*, *FGF10, Sox2* transcription factor, the Notch ligand *Jagged1* (*Jag1*; *Serrate1* in chick) and the Notch modulator *Lunatic Fringe* glycosyltransferase (*LFNG*) are expressed in sensory domains. Functional data suggest that activation of Notch signaling pathway FGFR1 and Sox2 have a role in prosensory specification (reviewed in Kelley, 2007). Following specification of the prosensory domain, individual prosensory cells develop as either hair-cells or supporting cells. The hair-cell/supporting cell fate decision requires lateral inhibition through the Delta-Notch mechanism (See BOX IV for Notch signaling pathway description and BOX V for lateral inhibition description), which results in the characteristic cellular pattern of ear sensory epithelia. Expression of *Jag2* ligand and *Delta-like1* ligand (*Dll1*) in developing hair-cells leads to activation of Notch1 receptor (Murata et al., 2006) and expression of the down-stream targets *HES1* (*Hairy and Enhancer of Split1*) and *HES5* in neighbouring cells (Daudet and Lewis, 2005; Kelley, 2006).

1.3.2. Otic progenitors

Otic progenitors undergo several cycles of cell division and progressive specialization to generate committed cells, first neurons and later on sensory hair-cells and supporting cells. One still unsolved question is that of whether otic neurons and hair-cells originate from a common progenitor (Fekete et al., 1998; Satoh and Fekete, 2005; Fritzsch *et al.*, 2000, Ma *et al.*, 2000; Koundakjian et al., ARO 2007, Abstract 962). The loss of hair-cells reported in *Neurog1* null mutants was proposed to unveil the clonal relationship of

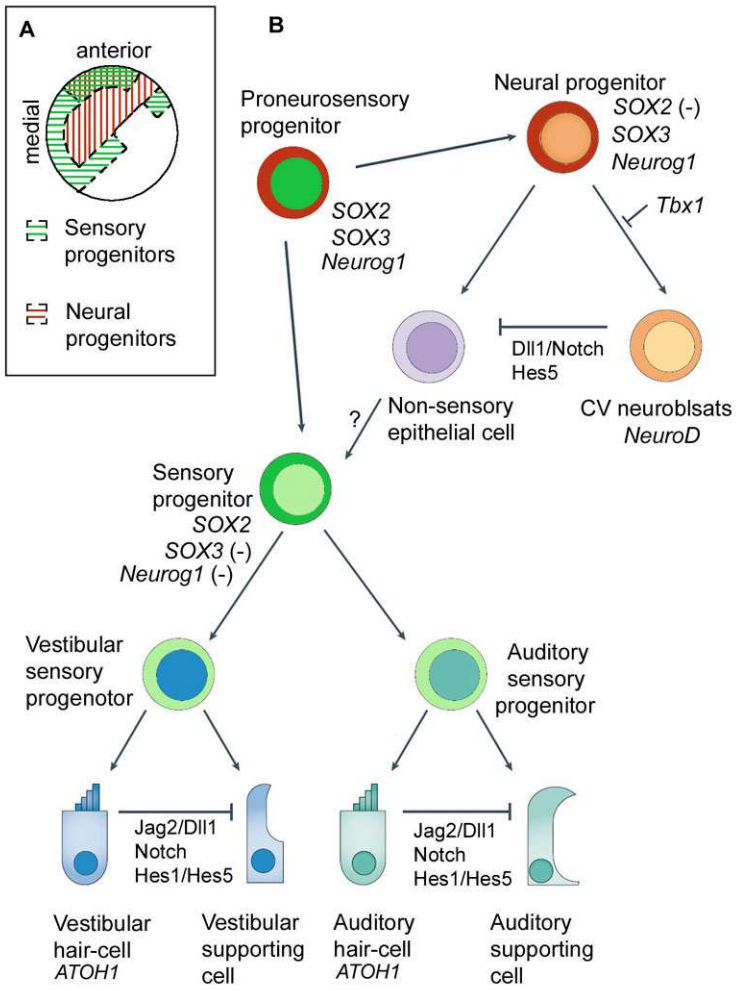

Figure 7. Otic progenitors. (A) Diagram showing: predicted fate map looking down onto the ventral floor of the otic cup/vesicle of the chicken, showing the areas for sensory (green), neural (red), proneurosensory (green and red), and non-sensory (white) progenitors. Modified from Satoh and Fekete, (2005). (B) Diagram adapted from Kelley, (2006) and Neves et al., (2007). Cell lineage studies with retoviruses revealed that there is a common sensory progenitor for vestibular and cochlear hair-cells and supporting cells; and a neural progenitor for vestibular and cochlear neurons. In addition, these experiments showed that sensory and neurogenic lineages can derive from a common cell; as well as for neurogenic and non-sensory lineages (Satoh and Fekete, 2005). Recent data has demonstrated that at least some cells thought to be in the non-sensory lineage have the potential to develop as hair cells, as a result of forced expression of Atoh1 (Landford et al., 2000; Jones et al., 2006; Zheng and Gao, 2000) or as supporting cells, as a result of proximity to an ectopic hair cell (Jones et al., 2006). It is unclear whether forced expression of Atoh1 directs non-sensory cells to enter the prosensory lineage at an undetermined point, or initiates a unique or limited hair cell/supporting cell developmental programme. Abbreviations: CV, cochleovestibular.

neuronal precursors and hair-cell precursors (Fritzsch *et al.*, 2000, Ma *et al.*, 2000). The indication is that *Neurog*1 may be possibly acting at an early stage of proneural specification and that it is required for both neuron and hair-cell specification. *Sox2*, that is restricted to sensory patches as soon as they are formed, is also expressed along with *Sox3* in the neurogenic territory, suggesting that both neurones and sensory cells derive from a common domain (Neves et al., 2007). Retroviral tracing performed in otic cups have shown that such clonal relationship may occurr in chicken (Satoh and Fekete, 2005) and in mice (Koundakjian et al., ARO 2007, Abstract 962). In the Satoh and Fekete paper, the majority of clones were restricted to a single anatomical subdivision of the sensory periphery or its associated ganglia, indicating limited clonal dispersion. Among the remaining clones, a fraction of labeled neurons were clonally related to cells of the utricular macula, but not with the other sensory organs. The sample of this work was small due to the technical difficulty of the experiments, but nevertheless it shows that it is possible that neurons and sensory cells share a common progenitor. It says little, however, on the frequency of its occurrence (Satoh and Fekete, 2005).

Based on the data summarized above, Bernd Fritzsch hypothesized that there are at least three proneural populations in the otocyst: one expressing *Neurog1*, which gives rise only to neurons, a second population that is positive to *Neurog1* and *Sox2* and gives rise to neurons and sensory cells and, finally, a third population that expresses only *Sox2* and gives rise to sensory cells (FIG. 7B; Fritzsch et al., 2006). Current work in several laboratories is testing this model for which there is yet no direct proof. The specification of different progenitor types may be the result of intrinsic mechanisms related to the rounds of cell divisions, it may be caused by environmental signals, or both. On the other hand, positional cues may specify an initially multipotent progenitor and give rise to more restricted and committed progenitor populations (reviewed in Abelló and Alsina, 2007).

1.3.3. Inner ear induction

The development of the inner ear from a simple epithelium involves a series of changes in the cranial ectoderm in order to reach the irreversible state of otic determination. Otic specification and determination is the result of consecutive inductive signals emanating from neighboring tissues and within the ear itself.

The competence of a tissue is defined by its ability to acquire a specific fate in response to appropriate inducing signals. In chick, the ectoderm lying along the neural plate of the head and anterior trunk is competent to express ear specific markers and to form an otic vesicle when kept in contact with otic inducing signals, i.e. transplanted to the future otic

placode site (Groves and Bronner-Fraser, 2000). Competence in embryonic ectoderm is present from the end of gastrulation and decreases until the 10-12 somite stage, when the ectoderm becomes refractory to otic inducing signals. Similar results have been reported in amphibian embryos (Gallagher et al., 1996). Otic competnece correlates with the expression of PPE genes (Martin and Groves, 2006). Many of the genes are transcription factors of the Six, Eya, Dlx, Dach, and Foxi families, and it is possible that some of these genes act individually or in concert as "competence factors" in the chick (reviewed in Streit, 2004; Groves, 2005). This has been also suggested for *Foxi* and *Dlx3b* and *Dlx4b* in zebrafish (Hans et al., 2004; Hans et al., 2007). Genes that are expressed at preplacodal stages, but restricted to the otic field are *Pax2* in mouse, chick and zebrafish; *Pax8* in mouse and zebrafish; *Lmx1b* in chick and zebrafish; *BMP7*, and *Sox3* in chick and *Dlx3*, *Six4.1* and *Eya1* in zebrafish (Abello et al., 2007; Ekker et al., 1992; Giraldez, 1998; Groves and Bronner-Fraser, 2000; Kobayasi, 2000; Krauss et al., 1991; O'Hara et al., 2005; Pfeffer et al. 1998; Sahly et al., 1999; Wright and Mansour, 2003). In chick, specification assays for the expression of the otic markers *Pax2* and *BMP7* have been carried out by Groves and Bronner-Fraser (2000). In these experiments, pieces of presumptive otic ectoderm were collected at different developmental ages and placed in culture in the absence of inducing signals such as growth factors or serum. Presumptive otic ectoderm in such "neutral condition" expresses *Pax2* at the 5-6 somite stage (ss) and *BMP7* at 7-8 ss. Thus, in both cases specification appeared to occur at the approximately same time as they are expressed in vivo, 5 ss and 7ss respectively. It is important to note that other as yet identified genes in chick may be expressed prior to *Pax2*.

BOX II. Development Biology Definitions

Differentiation implies changes in cellular biochemistry and function of cells. Those changes are preceded by a set of instructions that specify the commitment of the cell to a certain fate. Cells in the embryo progressively loose their initial extended potential and transit through successive states in which they restrict their fate by the selective expression of a set of genes and the suppression of others. When the state of commitment is spontaneously expressed in a neutral environment, but still reversible or modifiable, it is called specification, and the cell is said to be specified for a certain fate. When the process of commitment is irreversibly fixed, then it is called determination (Slack, 2007). The process of commitment can be induced by external signals or can be

autonomous. Jacobson in his 1966's review in Science (Jacobson, 1966) defined induction as: *"an interaction between one tissue (the inductor) and another responding tissue as a result of which the responding tissue takes a course of differentiation it would not have followed had the interaction not occurred".* On the contrary, it is said to be autonomous when the fate of a cell is fixed with independence of the interactions with neighbouring cells, i.e. based only on the information inherited after each cell division (Slack, 2007). A tissue is said to be specified when it has already received inducing signals and can express markers for a specific fate in the absence of any additional signals.

There is compelling evidence for the contribution of the hindbrain, mesoderm and endoderm to the development of the otic placode, although their specific roles appear to vary from one species to another (Kil et al., 2005; Waskiewicz et al., 2002; Kwak et al., 2002; Giraldez, 1998; Zhang et al., 1998; Gritsman et al., 1999; Phillips et al., 2001; Leger and Brand, 2002; Kil et al., 2005; Ladher et al., 2005).

Members of the FGF gene family are among the prime candidates to control inner ear induction since they show a spatiotemporal expression pattern consistent with playing a role during this process (see BOX III for FGF signalling description and FIG. 8). FGF signals have been shown to be necessary for otic placode induction and otic vesicle formation in fish, chick and mouse. In fish, FGF8 and FGF3 have been proposed to be the main FGF candidates for ear induction (Brand, 2002; Phillips et al., 2001; Leger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003). In mouse, FGF3 and FGF10 are considered as potential inducers, with *FGF3* being expressed in rhombomere 5 and 6 of the hindbrain and *FGF10* in the mesoderm underlying the presumptive placode (FIG. 8D-F; Mahmood et al., 1995; McKay et al., 1996; Wright and Mansour, 2003; Ohuchi et al., 2000; Mansour et al., 1993; Alvarez et al., 2003). In the chick, FGF3 and FGF19 are expressed in the mesoderm and hindbrain and play a major role in inner ear induction (FIG. 7A-C; Ladher et al., 2000; Martin and Groves, 2006; Represa et al., 1991; Vendrell et al., 2000). But FGF8 is the first known inductive signal involved in placode induction in chick and mouse. It is expressed in cranial endoderm, and is necessary for the mesodermal expression of other FGFs, *FGF19* in chick and *FGF10* in mouse (FIG. 7A-F; Ladher et al., 2005; Zelarayan et al., 2007). It is worth noting, however, that some aspects of otic placode induction are independent of FGF signaling. For example, in zebrafish induction of *Foxi1*, *Dlx4* and *Sox9b* are unaffected in mutants lacking both

FGF3 and *FGF8* (Solomon et al., 2004). Indeed, *Foxi1* has been proposed to act as a competence factor that allows the ectoderm to respond to FGF, but not itself being regulated by FGFs (Hans et al., 2004; Hans et al., 2007). Also in chick, two otic markers, *Dlx3* and *BMP7*, are unaffected by the FGF receptor inhibitor SU5402 (Martin and Groves, 2006).

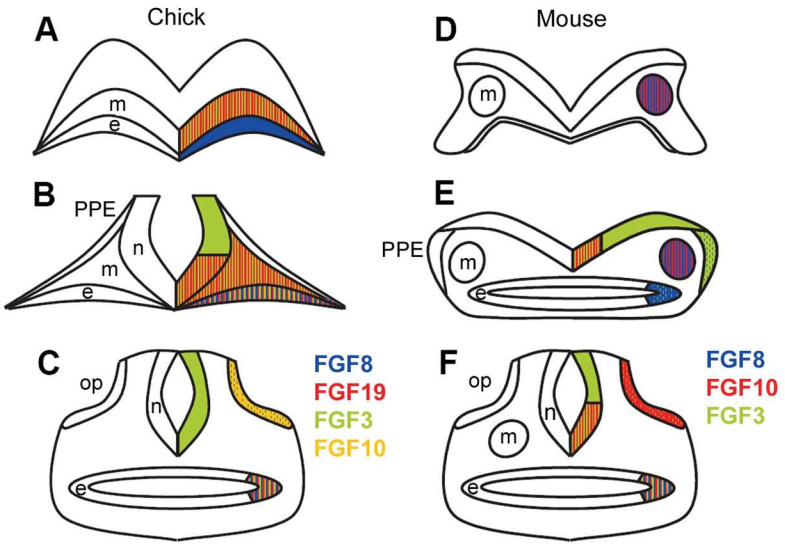

Figure 8. Otic induction in chick and mouse. Schematic sections of embryos taken at the level where inner ear induction takes place in chicken and mouse. (A, D) Expression of FGFs in the endoderm and/or mesoderm is observed during the first phase of induction. (B, E) A second phase of induction is defined by the onset of FGF expression in neural tissue together with the initiation of otic placode formation in the surface ectoderm. (C, F) Finally, a third phase is characterized by the completion of placode formation and the initiation of placode invagination, a period when expression of some FGFs expressed during placode induction is still maintained whilst other FGFs initiate their expression in the placode itself or in the endoderm. Modified from Schimmang, (2007). Abbreviations: e, endoderm; m, mesoderm; n, neural tube; op, otic placode; PPE, preplacodal ectoderm.

Recent experiments suggest that the acquisition of preplacodal identity is necessary for subsequent induction of the otic placode by FGFs. Naïve epiblast is able to up-regulate preplacodal genes (such as *Eya* and *Dlx* gene family members) between 4 and 8 hours after being grafted into the preplacodal region of chick embryos. When such grafts are removed prior to their expression of preplacodal genes, they are unresponsive to FGF signals, but they respond readily when challenged with FGF after they have expressed preplacodal genes (Martin and Groves, 2006). These experiments indicate that the competence to respond to FGF signalling correlates with the expression of preplacodal

genes in otic induction. Many of the genes known to be expressed in the preplacodal domain are transcription factors of the *Dlx*, *Six*, *Eya*, *Dach* and *Foxi* families (Streit, 2004, Groves, 2005) and it is possible that some of these genes act individually or in concert as "competence factors", as has been suggested for Foxi and Dlx3b and Dlx4b in zebrafish (Hans *et al.,* 2004, Hans *et al.,* 2007). It is not clear whether these competence factors act downstream of FGF signaling, or whether they themselves enable FGF signaling to occur by, for example, up-regulating components of the FGF pathway.

Studies in chick and mouse show that the Wnt pathway also participates in the induction of the otic placode. Ladher and colleagues reported the first evidence of the involvement of Wnt signaling in otic placode induction. They cultured presumptive chick otic ectoderm with FGF19 or Wnt8c-soaked beads, and observed that the induction of otic markers like *Pax2* by *FGF19* was potentieted by Wnt8c. They hypothesized that Wnt8c is induced in the hindbrain by mesodermally-secrteted FGF19, and that it acts synergistically with FGF19 in inducing otic genes (Ladher *et al.,* 2000). In the mouse, Ohyama et al. (2006) have shown that Wnt signals are active in the *Pax2*-positive ectodermal cells that are adjacent to the neural plate, but they are inactive in the lateral ones. Conditional inactivation of beta-catenin in *Pax2*-positive cells causes an expansion of epidermal markers at the expense of the otic placode. Conversely, the conditional activation of beta-catenin in these cells causes an expansion of the otic placode at the expense of epidermis (Ohyama et al., 2006). Although Pax2 is commonly regarded as one of the earliest markers of the otic placode, studies in chick and mouse reveal that *Pax2*-expressing cells can adopt otic placode as well as epidermal and epibranchial placode fates, suggesting that *Pax2*-expressing cells are not yet committed to an otic fate (Streit, 2002; Ohyama and Groves, 2004b, Baker and Bronner-Fraser, 2000; Krauss et al., 1991; Nechiporuk et al., 2007). In fact, Pax2 can be better considered a 'pre-otic field' marker. The current view is that FGF signaling is required for the induction of the *Pax2*-positive pre-otic field, while additional signals such as Wnts are required to pattern the pre-otic field into the otic placode and non-otic territory (Ohyama, 2006). Several studies suggest a potential crosstalk between Wnt and FGF pathways. For instance, the increased phosphorylation of GSK3β by FGF signalling (possibly via Akt) enhances the stabilization of β-catenin (Hashimoto *et al.,* 2002, Holnthoner *et al.,* 2002, Israsena *et al.,* 2004, Dailey *et al.,* 2005). But it is also possible that FGF and Wnt signaling act independently during otic placode induction. For example, recent studies show that activation of Wnt signaling represses the epidermis-specific transcription factor *Foxi2*, whereas loss of Wnt signaling

causes an expansion of *Foxi2* expression (Ohyama *et al.,* 2006). Wnt signaling could therefore simply be acting as a permissive factor that defines the size of the otic placode by repressing *Foxi2*, thus giving FGF free rein to induce otic genes in a *Foxi2*-negative domain (Ohyama *et al.,* 2006).

In summary, three major steps punctuate the induction of the otic placode. The first step is the formation of a region of competence for all craniofacial sensory placodes, the PPE. The second step, driven by FGF signaling, is the induction of a "pre-otic field" within this PPE, which is subsequently refined into the otic placode and the surrounding non-otic epidermis in a third inductive step, that require the Wnt pathway and perhapsr other unknown factors.

BOX III. Fibroblast Growth Factors Signalling (FGF)

Receptors. FGF signalling is transduced through a family of four transmembrane receptor tyrosine kinases (FGFR1–4) in all vertebrates. The extracellular domain contains the three immunoglobulin loops (IgI, IgII and IgIII), and the acid box (AB), which is located between IgI and IgII. There is a transmembrane domain (TM), and intracellular sequences include a split tyrosine kinase enzyme domain (TKI and TKII). Alternative splicing can generate a range of FGFR1–4 isoforms, some of which are secreted proteins. Most significantly, alternative splicing within the region encoding the C-terminal part of the third extracellular Ig loop in FGFR1–3 generates IIIb or IIIc isoforms, dramatically affecting ligand–receptor binding specificity. The AB is removed in certain splice variants. Heparan sulphate proteoglycans (HSPGs) are obligate co-factors in the activation of FGFRs by FGFs, and mutations in various components of the heparan sulphate biosynthesis pathway affect FGF activity during development (Ornitz and Itoh, 2001). The binding site for FGF ligands comprises the C-terminal part of IgII and the N-terminal portion of IgIII, whereas the binding site for CAM ligands includes the acid box.

Ligands. Sequencing of the human and murine genomes has revealed a total of 22 *FGF* secreted molecules in each species (Ornitz and Itoh, 2001). Phylogenetic analyses have arranged FGFs into seven subfamilies. Some are subject to alternative splicing, and this can alter their receptor affinity (Olsen et al., 2006). A number have been shown to be modified post-translationally, particularly by glycosylation (Baird and Klagsbrun, 1991). It should also be noted that several other ligands can activate FGFRs, including a subset of cell adhesion molecules (CAMs) which includes N-CAM, N-Cadherin and L1 (Sanchez-

Heras et al., 2006). However, the significance of the CAM/FGFR partnership to vertebrate neural development is currently unclear (reviewed in Mason, 2007)

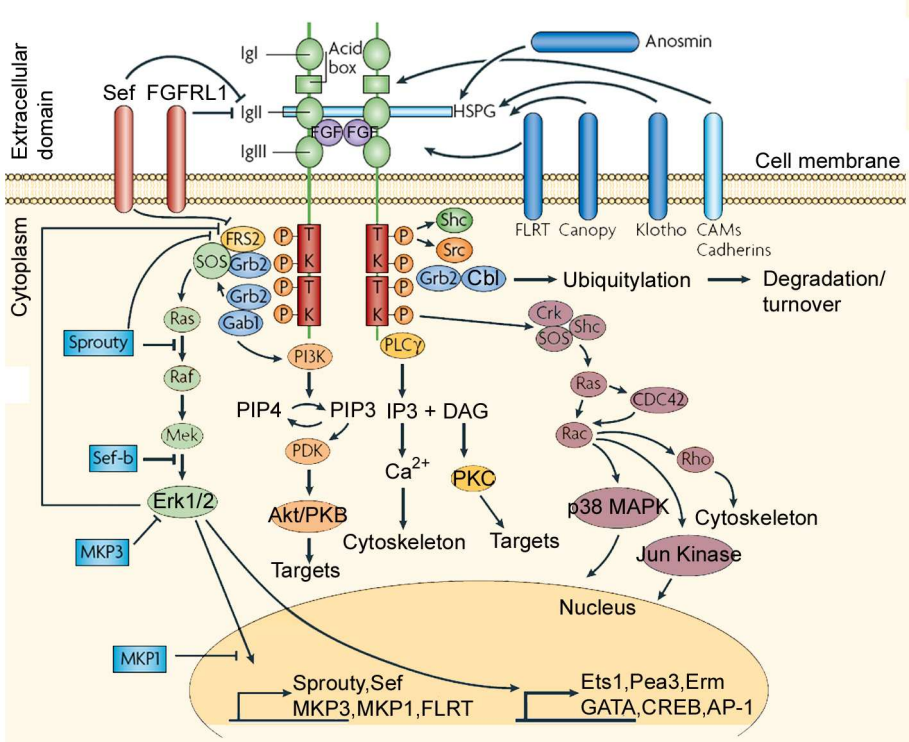

Figure BOX III. Scheme of the diversification of the FGF signaling pathway. Adapted from Mason, (2007).

Intracellular signalling pathways and targets. Several intracellular signalling pathways are activated downstream of FGFRs, a process that is mediated through a range of adaptor proteins that dock with the activated receptor to facilitate the recruitment and activation of downstream enzymes (Eswarakumar et al., 2005; Tsang & Dawid , 2004).

Erk1/2 MAP kinases. Docking of FGF receptor substrate (FRS) adaptors and/or Grb2 with the receptor leads to the activation of the Ras pathway, and ultimately to activation of mitogen activated protein (MAP) kinases. Activation of Erk1/2 MAP kinases seems to be common to responses mediated by all FGFRs.

Akt/PKB. Activation of phosphatidylinositol 3 (PI3) kinase in the presence of Gab1/Grb2 results in stimulation of the Akt/protein kinase B (PKB) pathway, which may mediate the anti-apoptotic effects of FGFs in the developing nervous system.

PKC/Ca^{2+}. Phospholipase C is also activated by FGFs, and it hydrolyses phosphatidylinositol to produce diacylglycerol (DAG) and inositol tris phosphate (InsP3),

with DAG activating protein kinase C and InsP3 stimulating calcium release. p38 MAPK; Jun Kinase. Other responses to FGF signalling include recruitment of Shc and Crk adaptors, which have the potential to activate a number of downstream pathways, and activation of p38 and Jun kinases, which may be activated in a cell type specific manner (Dailey et al., 2005).

Cbl. Gbr2 can bind the ubiquitin ligase Cbl, which can direct degradation of the receptor complex promoting FGFR turnover.

Among the genes whose transcription is regulated by FGFR activity are several that function in the feedback regulation of intracellular signalling (Dailey et al., 2005; Tsang & Dawid , 2004). Most serve as negative regulators, particularly of the Ras/Erk pathway, which they modulate at several levels: these include Sprouty proteins, Sef and MAP kinase phosphatase 3 (MKP3). In addition, active Erk itself can antagonize FRS activity. By contrast, stimulation of the expression of transmembrane fibronectin leucine rich transmembrane (FLRT) proteins results in positive regulation of FGF signalling (Tsang & Dawid, 2004). In addition to promoting the transcription of feedback regulators of signalling, the expression of a number of families of transcription factors is stimulated primarily via Erk activation. These include members of the *Ets* family (notably *Ets1*, *Pea3* and *Erm*), *GATA* genes, *Creb* and *AP-1* (Dailey et al., 2005). In addition, active Erk can phosphorylate and activate Irx proteins (Dailey et al., 2005).

1.3.4. Otic neural development

1.3.4.1. An overview of neural induction

The primordium of the nervous system consists of a uniform epithelium, the neural plate that subsequently develops into the neural tube (reviewed in Wilson and Edlund, 2001; Stern, 2005). The Peripheral Nervous System (PNS) arises from the lateral neural plate, a border region between the neural plate and the lateral ectoderm, which gives rise to the neural crest and ectodermal placodes (reviewed in Crane and Trainor, 2006).

Neural fate specification starts before gastrulation and it is indicated by the onset of expression of genes characteristic of neuroepithelium, which include members of the SoxB family of transcription factors (Pevny et al., 1998; Rex et al., 1997; Wood and Episkopou, 1999). During neural induction, the “organiser”, or the equivalent signaling centre of the vertebrate embryo, instructs neighbouring ectodermal cells to become nervous system rather than epidermis (Streit et al., 2000; Stern, 2005; Stern, 2006). During the nineties the prevailing model for neural induction suggested that ectodermal

cells differentiate into neural fate by default, but they do not do so because they are normally inhibited by BMPs (see section 8.2.1 for BMP signalling description). The emission of BMP antagonists by the organizer would allow cells in its vicinity to execute their default neural programme (Wilson and Hemmati-Brivanlou, 1997; Schier and Talbot, 1998).

There is growing evidence, however, that a more complex model is required to explain neural induction. In the chick embryo, naïve epiblast cells do not respond to BMP antagonists unless previously exposed to the Hensen's node signals such as FGF (Streit et al., 1998). FGF signals repress BMP expression which leads to induction of neural tissue, and when FGFs are inhibited, BMP expression is maintained and neural fate is blocked (Wilson et al., 2000). Moreover, in Xenopus, BMP antagonists do not induce neural tissue in the presence of dominant-negative FGF receptors (Launay et al., 1996; Sasai et al., 1996). Two key reports had elucidated how FGF and BMP signaling pathways converge intracellularly during early embryogenesis. The first, shows the direct MAPK-dependent phosphorylation of Smad1 that results in the inhibition of BMP signaling (Pera *et al.*, 2003). The second, reports the finding that *Sip1*, the Smad-interacting protein 1, is regulated by *Churchill*, a gene induced by FGF (Sheng *et al.*, 2003). However critical in neural induction, FGFs and BMP antagonists may only be able to induce neural tissue in cooperation with other signals, since neither FGF nor 5h node exposure followed by BMP inhibition are sufficient to generate induction of *Sox2* or other neural markers (Streit et al., 1998; Streit and Stern, 1999). The current view on this process is that FGF signals together with Wnt and BMP antagonists are required to potentiate neural fate over epidermal fate (Stern, 2005). Nuclear localization of the Wnt transduction protein β-catenin marks cells exposed to active Wnt signals. In chick gastrula embryos, β-catenin is detected in nuclei of lateral epiblast tissue that acquires epidermal fate, but is excluded from the medial epiblast cells that generate neural tissue (Roeser et al., 1999). This observation suggested that differential distribution of Wnt signalling may regulate neural differentiation. Medial epiblast explants grown in isolation express neural markers such as *Sox2* and *Sox3*, while lateral epiblast explants express epidermal markers, *Msxl/2* and *GATA2*. Medial epiblast exposed to Wnt3A and Wnt8 generated *Msx1/2* and *GATA2*-positive cells, but negative to *Sox2* or *Sox3*. Interestingly, medial epiblast explants exposed jointly to FGF2 and Wnt3A generated *Msx1/2*-positive cells but no *Sox2* or *Sox3*-positive cells.

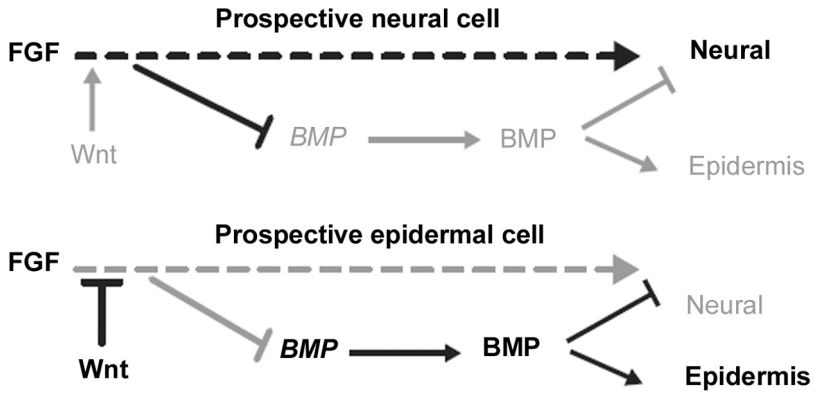

Figure 9. A model for neural induction. This model, based on studies in chick, is proposed to reconcile findings on the roles of BMP, FGF and Wnt signalling in neural induction. At the blastula stage, medial epiblast cells (prospective neural cell) express FGFs but not Wnts. FGF signalling activates two transduction pathways in epiblast cells: repression of BMP expression (solid line) and the promotion of neural fate by an independent pathway (broken line from FGF). Lateral epiblast cells (prospective epidermal cell) express both FGFs and Wnts. High Wnt levels block the response of epiblast cells to FGFs, BMPs are expressed, and BMP signals promote epidermal fate and repress neural fate. When Wnt signalling is attenuated, Wnts block the ability of FGFs to repress BMP expression, but the independent pathway (broken line) promoting neural fate is preserved. Under these conditions, BMP antagonists are able to induce neural fate. Modified from Stern, (2005).

This series of experiments served as a basis for a model, which states that a lack of exposure of the epiblast to Wnt signals permits FGFs to induce a neural fate (FIG. 9A). Conversely, continual Wnt signalling blocks the response of epiblast cells to FGF signals, permitting the expression and signalling of BMP to direct an epidermal fate (FIG.9 B); Wilson et al, 2001).

Among the genes that are first expressed after neural induction are the *SoxB1* genes (*Sox1*, *Sox2* and *Sox3*). Sox proteins belong to a family of High Mobility Group (HMG) Box transcription factors that are related to SRY, the mammalian testis determining factor. The HMG domain is a DNA binding motif of approximately 80 amino acids that bind into the minor groove (Bianchi and Agresti, 2005). The C-terminal region of the Sox protein carries a cryptic transactivating domain that uncovers only after specific interaction with partner factors (FIG 10). By convention, HMG domains of Sox proteins are at least 50% identical to the HMG domain of SRY. There are twenty-four members of *Sox* family grouped into seven subgroups (A-G) on the basis of sequence similarity, both

in the DNA-binding domain and in other, group-specific conserved motifs. As mentioned above, Sox proteins bind to specific DNA sequences via their HMG box domain; however, their target specificity depends on cooperative binding with other transcription factors. Presented below are examples of the partnerships that Sox proteins make. For instance, Sox2-Pax6 parternship acts to induce δ-crystallin expression during lens differentiation (Kamachi et al., 1995; Kamachi et al., 1999), Sox2-Oct3/4 induce the expression of FGF4 and UTF-1 (undifferentiated embryonic cell transcription factor 1), both required for establishing the ES cell state (Yuan et al., 1995; Nishimoto et al., 1999), Sox11-Brn1/2 partnership is involved in olygodendrocyte specification, and Sox10-Oct6 for Schwann cell specification (Kuhlbrodt et al., 1998; reviewed in Kamachi et al., 2000). Furthermore, depending on cellular context, Sox proteins can function as activators or repressors, adding more versatility to their potential functions (Kamachi et al., 1999; Wilson and Koopman, 2002).

Throughout evolution the expression of the *Sox1-3* genes, directly correlates with uncommitted ectodermal cells that develop into neuroectoderm in response to inductive signals (Wegner and Stolt, 2005; Pevny and Placzek, 2005). Direct evidence for the involvement of *SoxB1* genes in neural commitment comes from *in vitro* stem cell studies, where it was shown that the *Sox1* expression induces neural fate in competent ectodermal cells (Pevny *et al.*, 1998). SoxB1 factors are modified by neural inducing signals (Streit *et al.*, 2000), and strong evidence for the link between neural inducing signals and *Sox* activation is derived from the identification of FGF and Wnt response binding domains in the same enhancer region of the *Sox2* locus (Takemoto *et al.*, 2006). Furthermore, *Sox1-3* genes are expressed by most progenitor cells within the developing CNS and are down-regulated as they exit the cell cycle and start to express proneural genes (Uwanogho et akl., 1995; Rex et al., 1997; Pevny et al., 1998; Kamachi et al., 1998 lens).

SOX1-3 maintain neural progenitor cells in an undifferentiated state by blocking the capacity of proneural bHLH proteins to induce down-stream events of neural differentiation. Furthermore, the capacity of proneural proteins to direct the generation of neurons from precursor cells seems to be based on their ability to repress *Sox1−3* expression (Bylund et al., 2003). Moreover, in the nascent PNS, *SoxB1* genes also mark a subset of cranial placodes (Groves and Bronner-Fraser, 2000; Abu-Elmagd et al., 2001; Iishi et al., 2001; Schlosser and Ahrens, 2004). The *Drosophila* SoxNeuro, a putative ortholog of the vertebrate SOX1-3 proteins, is one of the earliest transcription factors to

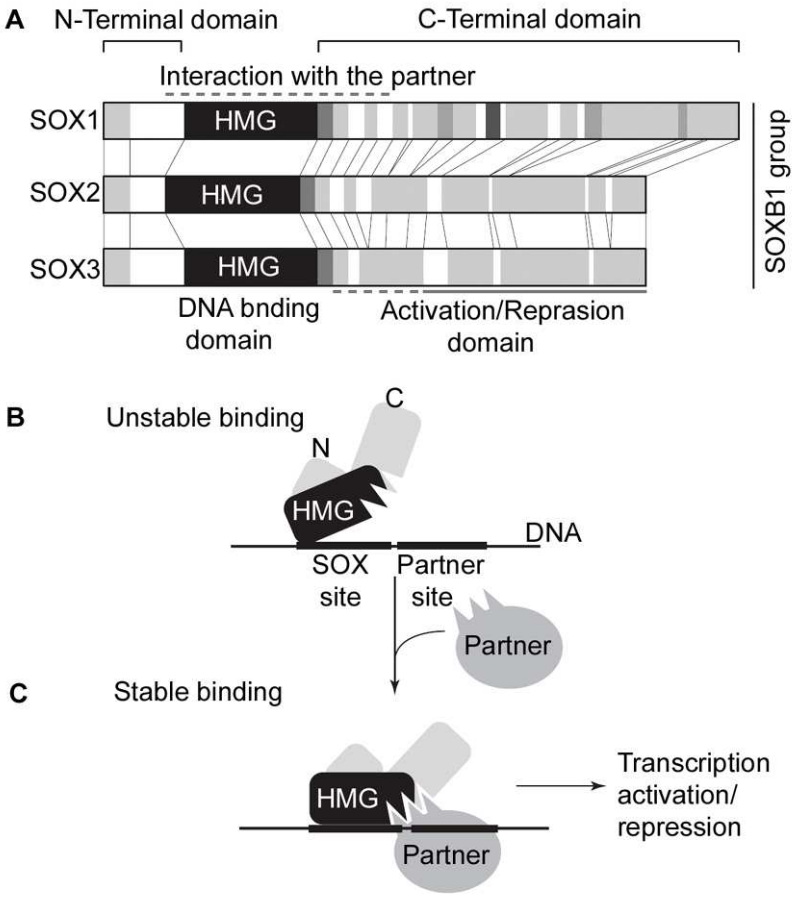

Figure 10. SOX B1 proteins. (A) Representative SOX proteins of Groups B1 are shown schematically. A typical SOX protein has three functional domains: an HMG domain to interact with DNA; an activation domain or a repression domain usually close to the C-terminus; and a region which can include a part of the HMG domain for interaction with the partner factor. In the model, when a SOX protein binds to a SOX-binding site through its HMG domain alone (B), the binding is probably unstable. When the partner factor interacts with the SOX protein and binds to the DNA site next to the SOX site (C), then the SOX protein will be stabilized on the DNA and exhibit its activity of transcriptional activation or repression. Modified from Kamachi et al., (2000). Abbreviations: N, N-terminal; C, C-terminal.

be expressed pan-neuroectodermally (Cremazy *et al.*, 2000), and it acts upstream and in parallel with the *achaete-scute* genes. Interestingly in *Drosophila*, SoxNeuro is only involved in CNS but not in PNS development, being suggested that recruitment of SOX proteins into placode development is a novelty of craniates to expand the ectodermal anlage rapidly (Fritzsch *et al.*, 2006b).

1.3.4.2. An overview of neurogenic placodes development

On top of neural induction, FGF signals and *SoxB1* genes have also been involved in neurogenic placode development. In olfactory development, signalling by FGF8 from the Anterior Neural Ridge (ANR) appears to suppress lens fate and dictate the choice towards olfactory fate from cells in the PPE (Bailey et al., 2006). During epibranchial placode development, FGF3 and FGF8 from the neural tube and the underlying endoderm are required for epibranchial-placode induction (Sun et al. 2007; Nechiporuk et al., 2007, Nikaido et al., 2007). *Sox2* and *Sox3* are expressed in the otic and epibranchial placodes in chick (Abu-Elmagd et al., 2001; Ishii et al., 2001, REF SOX2 otic) and zebrafish (Okuda et al., 2006), but only *Sox3* has been reported to be expressed in epibranchial placodes in frogs (Mizuseki et al., 1998) and medaka (Koster et al., 2006). In zebrafish, S*ox3* expression is completely abolished in the otic-epibranchial placode in *acerebellar* (*ace*) homozygous mutants, in which the *FGF8* gene is disrupted, as it is in embryos treated with the FGF receptor inhibitor SU5402 (Reifers et al., 1998; Sun et al., 2007; Nikaido et al., 2007). Implanted FGF8-soaked beads near the presumptive anterior rhomboencephalic region restored placodal *Sox3* expression *ace* mutants (Nikaido et al., 2007). *Sox3* expression was totally abolished after double injection of *FGF3* and *FGF8* morpholinos (Sun et al., 2007). These findings suggest a requirement of FGF8 and FGF3 for *Sox3* expression in the otic-epibrancial placode. Furthermore, once epibranchial placodes are formed, FGF3 and BMP signals from the endodermal pouches contribute to induce epibranchial neurogenesis (Begbie et al., 1999; Holzschuh et al., 2005; Neichiporuk et al., 2005; Trokovic et al., 2005). FGF signaling has been also implicated in adenohypophyseal (De Moerlooze et al., 2000; Ohuchi et al., 2000; Herzog et al., 2004) and lens placode induction (Faber et al., 2001; Hayashie et al., 2004). Besides, *Sox2* genes also control the final stages of lens differentiation, directly activating delta-crystallin expression in the embryonic lens through Sox2-Pax6 partnership (Kamachi et al., 1998).Finally, FGF signals and SoxB1 play also a role in otic placode development. FGFs are involved in otic placode induction in chick, zebrafish and mouse (see section 3.3). In medaka, ectopic *Sox*3 is sufficient to induce supernumerary otic vesicles (Koster et al., 2000). In mouse, *Sox2*-deficient mice like light coat and circling (*Lcc*), and yellow submarine (*Ysb*), show hearing and balance impairment. *Lcc/Lcc* mutant mice fail to establish a prosensory domain and neither hair cells nor supporting cells differentiate. While Ysb/Ysb mice show abnormal development with disorganized and fewer hair cells (Kiernan et al., 2005). This work correlates with the fact that mutations of Sox2 in humans cause sensory-neural hearing loss (Hagstrom et al., 2005).

1.3.4.3. An overview of neurogenesis: the determination of the neural fate

Building a nervous system involves the production of a vast array of neuronal and glial cell types that must be produced in the correct numbers and at appropriate positions. In *Drosophila*, neural development requires the transformation of ectodermal cells into progenitor cells, which undergo a limited number of divisions through fixed cell lineages before differentiating into neurons and glia. In vertebrate embryos, neuroepithelial cells have the self-renewing properties of stem cells. They produce intermediate progenitors that are restricted to a neuronal or glial fate, and proliferate to some extent before differentiating (Bertrand et al., 2002). By the late 1970s, a complex of genes, named proneural genes, that are involved in the early steps of neural development in *Drosophila were* identified (García-Bellido, 1979). Molecular analysis led to the isolation of the four genes of this complex, namely *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*lsc*) and *asense* (*ase*) , all containing a bHLH domain (Villares and Cabrera, 1987). A further *Drosophila* proneural gene, *atonal,* was isolated later in a PCR-based screen to identify bHLH sequences. Also the vertebrate bHLH genes homologous to *Drosophila* proneural genes AS-C and *atonal* have been characterized and their function explored: AS-C homologues (*Mash*, *Cash*, *Xash)* and *atonal* homologues (*Math*, *neurogenin, NeuroD and Olig*). Proneural genes are key regulators of vertebrate neurogenesis as they coordinate all features inherent to the process of neuronal differentiation. First, they coordinate the transition from a proliferating neural progenitor to a post-mitotic neuron, generally by activating the expression of Cyclin-dependent kinase (CdK) inhibitors, which promotes cell cycle exit (Farah et al., 2000; Ohuma et al., 2001; Bertrand et al., 2002; Nguyen et al., 2006). Subsequently, proneural proteins also coordinate the acquisition of both generic and specific neuronal characters, as they trigger the expression of cascades of other transcription factors that regulate pan-neuronal and subtype specific characters (reviewed in Bertrand et al., 2002). Neurogenins have a similar proneural function to that of their *Drosophila* counterparts, whereas other proneural bHLH, such as NeuroD, are involved in specifying neuronal fates or in neuronal differentiation and survival, but have not conserved proneural role (Bertrand et al., 2002; Cau et al., 2002). Thus, vertebrate neurogenesis is driven by proneural genes in individual neural progenitors that promote full neuronal differentiation as a result of the induction of a cascade of downstream bHLH genes. During cranial sensory neuron development, *Neurog1* or *Neurog2* is required for the expression of *Math3* and *NeuroD* (Fode et al., 1998), while *Mash1* acts upstream of *Neurog1* and *NeuroD* in the olfactory sensory epithelium (Cau et al., 2002). Finally, they inhibit their own expression in adjacent cells, thereby preventing these cells from

differentiating. This is achieved through activation of the Notch signalling pathway, in a process termed lateral inhibition (See BOX V). Expression of Delta in the fated neurones?? activates the Notch signalling cascade in neighbouring cells, resulting in the expression a set of bHLH repressors encoded by *Hairy* and *Enhancer of split* (*Espl*) which antagonizes the proneural bHLH factors and inhibits neuronal differentiation. Two mouse genes homologous to the *Drosophila Hairy* and *Espl* genes, are *Hes1* and *Hes5*, and they play important roles in neurogenesis. *Hes1* is expressed by precursor cells in the nervous system and, in the absence of *Hes1*, the differentiation of the precursors is accelerated causing neural tube defects (Ishibashi et al., 1994, 1995; Tomita et al., 1996; Ohtsuka et al., 1999; reviewed in Kageyama and Nakanishi, 1997). In addition, in both *Notch1* and *RBP-J* transcription repressor null mutant mice (see BOX IV), *Mash1* expression is upregulated prematurely in the regions where *Hes5* expression disappears, thus suggesting that Hes5 normally downregulates *Mash1* expression under the control of the Notch pathway (de la Pompa, et al., 1997).

Summarizing, both in invertebrates and vertebrates, proneural genes are initially expressed in groups of equivalent neurectodermal cells (Ma et al., 1996; Henrique et al., 1997). Through lateral inhibition, this initial pattern is refined and proneural gene expression is restricted to single cells that enter a neural-differentiation pathway, while the surrounding are inhibited to do so (Lewis, 1998; Artavanis-Tsakonas et al., 1999). The balance between these two antagonistic groups of bHLH factors, proneural and Hairy and Espl, is critical for the timing of differentiation and for generation of the correct number of neurons (Bertrand et al., 2002; Kageyama and Nakanishi, 1997).

BOX IV. Notch Signalling

Receptors. Mammals have four Notch receptors (Notch 1-4), while avian have only two (Notch1-2). Notch is a large type-I transmembrane receptor that accumulates at the plasma membrane as a heterodimer, composed of the Notch Extracellular Domain (NECD) and a membrane bound intracellular domain (NTM). These two polypeptides are formed in the trans-golgi as the result of proteolytic activity by a Furin protease that constituitively cleaves Notch molecules at the S1 site. The Notch receptor heterodimer is then formed trough a non-covalent Ca2+ dependent bound (Mumm and Kopan, 2000; Shweisguth, 2004). The Drosophila Notch receptor is not an heterodimer as in vertebrates, but composed of just one single polipeptide (Artavanis-Tsakonas, 1999). Notch receptor contains a large extracellular domain with 36 tandem epidermal growth

factor (EGF)–like repeats and three cysteine-rich Notch/LIN-12 repeats (Wharton et al., 1985; Yochemet al., 1988). Six tandem CDC10/ankyrin repeats (Breeden and Nasmyth, 1987), and one or two nuclear localization signals, a glutamine-rich domain (opa) and a PEST domain rich in proline, gutamate, serine and threonine (Stifani et al., 1992) are found within the intracellular domain (reviewed in Artavanis-Tsakonas *et al.*, 1999).

Ligands. Notch receptors bind to type I transmembrane proteins known collectively as DSL proteins (Delta and Serrate for *Drosophila* and Lag2 for *Caenorhabditis elegans*). Mammals have five DSL ligands (Jagged 1-2 homologous of Serrate and Delta-like 1-3 homologous to Delta). While avian have Serrate1-2 and Delta-like 1 and 4 (Abello and Alsina, 2007). In the extracellular domain they contain a DSL region and several EGF repeats, while the intracellular region is much smaller than in the Notch receptor and is poorly conserved among DSL family members (Alton et al., 1989; Fleming et al. 1990; Xu et al., 1990; Fleming, 1998).

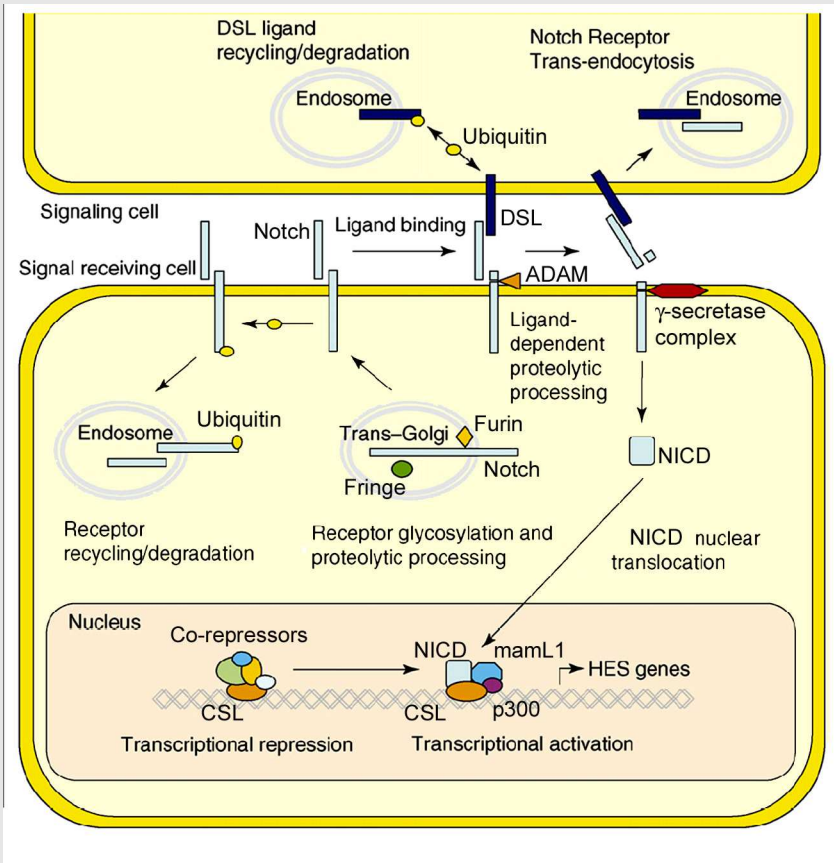

Figure BOX IV. Schematic drawing showing the Notch signaling pathway. Adapted from Hurlbut et al., (2007).

Intracellular signalling pathways and targets. Notch receptor is activated by binding to DSL membrane protein ligands. Therefore, the system operates as short-range signals between cells (de Celis and Bray, 2000; Hicks et al., 2000; Moloney et al., 2000; Panin et al., 1997). Upon ligand-receptor interaction, the Notch receptor undergoes successive proteolytic cleavages that lead to the release of the Notch intracellular domain (NICD). Cleavage at the S2 site is triggered by ligand binding to NECD and is carried out by the ADAM/TACE/Kuzbanian family of metaloproteases. This S2 cleavage generates an activated membrane-bound form of Notch, NEXT (Notch Extracellular Truncation). Subsequently, NEXT is further processed at two more cleavages sites – S3 and S4, releasing the NICD into the cytoplasm and a small peptide (Nb) to the extracellular space (the fate and possible signalling activity of the Nb peptide is unknown). These S3 and S4 cleavages sites are located within the transmembrane domain and are catalyzed by the γ-secretase activity of the Presenilin-Nicastrin-Aph1-Pen2 protein complex (reviewed in Mumm and Kopan, 2000; Schweisguth, 2004).

The NICD fragment is the active form of the receptor, acting in the nucleus as a transcription co-activator. NICD translocates to the nucleus (through its nuclear localization signals) and binds to the CSL transcription factor (mammalian C-promoter binding factor 1 CBF-1 or RBP-jkapa, Drosophila Supressor of Hairless and C. elegans Lag-1) and to the Master mind (MAM and C. elegans Lag-3) co-activator, forming a ternary complex. In the absence of NICD, the CSL transcription factor promotes the assembly of a repressor complex at the cis-regulatory regions of the CSL/NICD target genes (named Su(H) or S binding boxes), which are therefore transcriptionally inactive (Bailey and Posakony, 1995; Nellesen, 1999; Cave et al., 2005; Lamar and Kintner, 2005; Ong et al., 2006). When NICD translocates to the nucleus and binds to CSL, it is able to recruit HAT (Histone Acetylase) and displace the co-repressor complexes, relieving repression. But it is only when MAM binds to NICD/CSL, forming the ternary complex, that transcription is activated (reviewed in Mumm and Kopan, 2000). Therefore, in the absence of Notch activity, the Notch target genes are repressed by CSL. When Notch signalling is initiated, NICD makes the switch from CSL-mediated repression to NICD/CSL/MAM activation, triggering transcription of the Notch target genes (Bray, 1998; Castro et al., 2005).

There are many binding sites for the CSL transcription factor throughout the genome (Rebeiz et al., 2002), and it is not clear which actually represent Notch targets. The best-characterized Notch targets are the bHLH transcription repressors of the Enhancer of split (Espl) genes in *Drosophila* and the hes (Hairy and Espl) and hrt (hes-related type) family

genes in vertebrates (Jarriault et al., 1995; Schroeter et al., 1998; Struhl and Adachi, 1998). In addition to this core CSL-dependent Notch pathway, in which the key signalling molecule is NICD and the ultimate output is transcription, there is also evidence for a CSL-independent Notch signalling (reviewed in Martinez Arias et al., 2002). This CSL-independent Notch signalling seems to rely on a Deltex dependent activity and, in some cases, it relies on different ligands that do not belong to the DSL family, like Contactin and DNER (Eiraku et al., 2005). Interactions between Notch an either of its ligands can be differentially modulated by the glycosyltransferase Lunatic Fringe, located in the Golgi apparatus. Lunatic Fringe glycosylates EGF repeats of Notch protein before its maturation and localisation to the cell membrane (REF). Notch functions might differ by the modulation on the amount of the receptor or the ligand on the cell surface, by feedback loops that potentiate or shut off the signal, or by tissue specific co-factors (Schweisguth, 2004).

1.3.4.4. Otic neurogenesis

The generation of otic neurons is a sequential process, which includes first the specification of otic precursors in the otic epithelium. Transplantation experiments in the chick have shown that neither the potential to generate neurons nor the identity of the neurons generated by the otic placode is determined before the placodal stage (Vogel and Davies, 1993). Specification of the neuroblast lineage must therefore occur simultaneously with, or immediately after, the irreversible determination of the otic placode, and it marks the starting point of the cell-fate specification period. Initially the neural domain is the anterior-medial aspect of the otic placode to end up, after invagination, in an anterior-medial and ventral position of the otic vesicle (FIG. 11A-C). Thereafter, epithelial neuroblasts delaminate along the posterior margin of the proneural domain forming the CVG (FIG. 11D; Alsina et al., 2004). Finally, there is a proliferative expansion of ganglionar neuroblasts followed by the differentiation of the neurons that innervate back the vestibular and choclear sensory organs (Hemond and Morest 1991b; Adam et al. 1998; Noden and van de Water, 1986). The sequence in the onset of gene expression during otic neurogenesis is FGF10>Neurog1/Delta1/Hes5>NeuroD/M. *FGF10* expression defines an early regional domain that anticipates proneural and neurogenic gene expression in the otic placode (Alsina et al., 2004). In parallel to CNS neurogenesis,

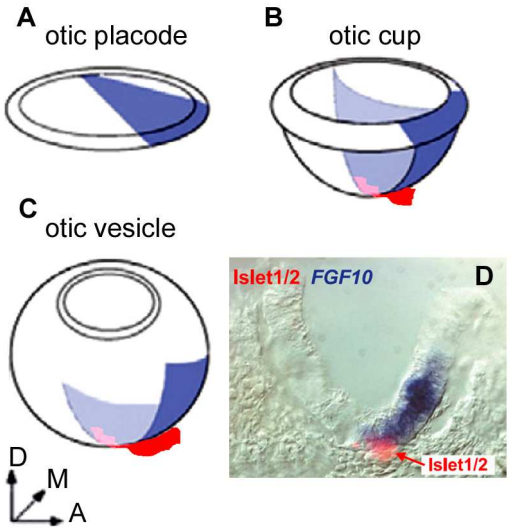

Figure 11. Otic proneural domain from otic placode to otic vesicle stage. (A-C) Schematic drawing illustrating the dynamics of the proneural/non-neural domains at otic placode (A), otic cup (B) and otic vesicle (C) stage. Blue indicates the proneural territory, while in white is depicted the non-neural region, and red the delaminating neuroblasts. (D) ISH of an otic cup showing *FGF10* (blue) expression as a proneural marker, and immunostaining specific for islet1/2 (red) as a neuroblast marker. Neuroblast delamination only takes places along the ventral neural/non-neural interface. Modified from Alsina et al., (2003). Abbreviations: A, anterior; D, dorsal; M, medial.

subsequent expression of proneural genes is involved in the selection of the neural progenitors that become competent to acquire specific cell fates and commit to differentiation (Cole et al., 200; Cau et al., 2002; Alsina *et al.*, 2003, Fritzsch *et al.*, 2006; Sanchez-Calderón et al., 2007; Kelley, 2007; Bertrand *et al.*, 2002). Two bHLH transcription factors, *Neurog1*, and *NeuroD*, play critical roles in inner ear neurogenesis (Fritzsch, 2003): Mice lacking *Neurog1* lack all sensory neurons in the inner ear (Ma et al., 1998; 2000). Mice lacking *NeuroD* exhibit a near-complete loss of cochlear ganglia and a significant loss of vestibular ganglia (Kim et al., 2001). The neuronal loss in the CVG is due to a perturbed delamination of the neuroblasts from the otic vesicle epithelium and significant apoptosis among those neurons that do delaminate to form the CVG (Kim et al., 2001; Liu et al., 2000). The surviving vestibular ganglia displayed disorganized fiber projection onto the vestibular sensory epithelia, suggesting that *NeuroD* may be important for differentiation of these neurons or their pathfinding properties (Kim et al., 2001). *NeuroD* is also required for the survival and differentiation of the inner ear sensory neurons during later stages of development (Kim et al., 2001; Liu et al., 2000). In summary, bHLH gene *Neurog1* function is the determination of neuronal fate, with downstream transcription factors such as *NeuroD* playing various roles in differentiation and perhaps identity (Ma et al., 1998; 2000; Kim et al., 2001; Liu et al., 2000).

The overexpression of *FGF10* in otic explants increases the number of cells expressing *NeuroD*, and on the contrary, FGF receptor inhibition by SU5402 results in a reduction of

NeuroD, *Delta1*, *Neurog1*, and *Hes5* expressing cells, suggesting that FGF signaling is required at initial stages of neuronal determination (Alsina et al., 2004). In parallel, FGF8 and FGF2 soaked beads placed anterior to the otic placode resulted in an increased CVG positive for NSCL2, a ganglionar marker, without affecting the proliferation rate, suggesting a role on FGFs in promoting neural fate (Adamaska et al., 2001). The zebrafish *ace* mutant embryos (FGF8 mutants) show small CVGs and a reduced number of otic *Neurog1*-positive cells (Leger and Brand, 2002 OR Maroon et al., 2002). These results suggest that FGFs are required to regulate otic neurogenesis.

In chick and mouse, *Notch1* is expressed ubiquitously in the entire otic epithelium, from placode to late otocyst stage (Groves and Bronner-Fraser 2000). *LFNG,* a regulator of Notch signalling, is expressed throughout the proneural domain, whereas *Dl1* is detected in a salt and pepper pattern (Adam et al., 1998; Cole et al., 2000; Alsina et al., 2004). *Hes5* is expressed in cells adjacent to *Dl1*-positive cells (Abello et al., 2007). As expected from the lateral inhibition model, disruption of Notch signalling leads to the production of excess neuronal precursors in the inner ear concomitantly to the suppression of *Hes5* activation (Haddon et al., 1998; Abello et al., 2007; Daudet et al., 2007). Altogether, as shown in the results section, Notch signalling is required in otic neurogenesis to regulate the number of neural cells committed to neuronal differentiation.

BOX V. Notch Pathway: The Control Of Cell Fate Choices

The Notch pathway serves for communication between cells that are next-door neighbours: the receptor, Notch, and ligands, Delta and Serrate, are transmembrane proteins, making signaling to relie on direct cell to cell contact. Binding of ligand triggers proteolytic cleavage of Notch, releasing an intracellular fragment, NICD, that enters the nucleus and regulates transcription of specific target genes (for reviews, see Baron, 2003; Lai, 2004). The most obvious role of Notch pathway is its ability to influence its neighbouring cells by a mechanism named lateral inhibition, whereby a cell that is committed to a particular and default fate inhibits its immediate neighbors from doing likewise. Activation of Notch in a given cell diminishes the ability of that cell to produce functional ligands that can activate Notch in the neighbours (Heitzler and Simpson, 1991). A cell that signals more strongly thereby causes its neighbours to signal more weakly and the effect is to amplify differences between adjacent cells. If this negative feedback loop is sufficiently steep, the predicted outcome is a mosaic of cells in sharply different states (Collier et al., 1996). Thereby, cells that express functional Notch ligands alternate with

cells that do not; the cells that express ligands escape Notch activation because their neighbours do not express ligands, and vice-versa (Brooker et al, 2006). If either Notch or Delta is downregulated, supernumerary cells with the default fate will differentiate (Haddon et al., 1998; Eddison et al., 2000). Lateral inhibition operates in vertebrate central nervous system neurogenesis (Henrique et al., 1995; De la Pompa et al., 1997), and in *Drosophila* neurogenesis (Campos-Ortega and Jan, 1991). The same mechanism generates the alternating arrangement of hair-cells and supporting cells in inner ear sensory organ, and the mechanosensory unit of the insect bristles (Adam et al., 1998; Eddison et al., 2000; Daudet and Lewis, 2004).

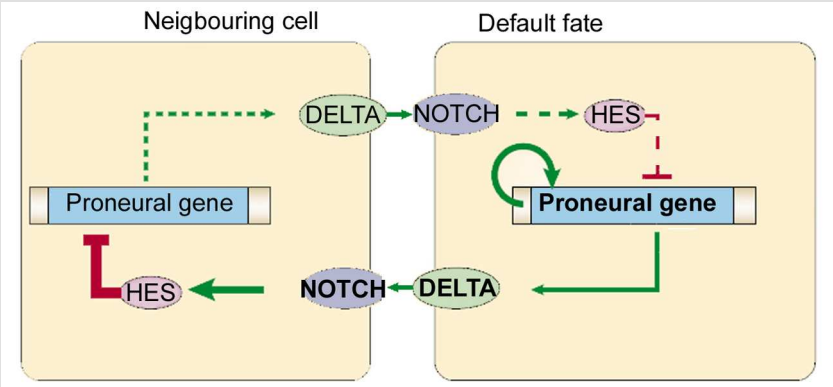

Figure BOX V. Lateral inhibition. Schematic drawing illustrating the mechanism of lateral inhibition. Notch-DSL binding activates Hes target genes to the receiving cell, as a result it can not acquire the same fate as the signalling cell. In this model a negative feedback loop promotes a mosaic distribution of two different cell types.

1.3.5. Otic patterning

1.3.5.1. General mechanisms for generating diversity

During development, morphogenesis and cell fate specification have to be coupled in order to give rise to different cell types and functional structures in the correct space and time coordinates. One strategy to generate cell and structure diversity is to segregate the initial and homogeneous territory into distinct compartments. Each compartment is composed by a group of cells that develop independently from adjacent territories; they are specified by selector genes and, as a result, the cells within a compartment express the same combination of genes. Furthermore, compartments can exhibit lineage restrictions and can generate specialized cells along the boundary between them that are instructive in shaping future development. Early demonstration of the existence of compartments and their relevance for development was put forward by García-Bellido et

al (1979) for the development of the imaginal disc of the *Drosophila*. In vertebrates, similar principles operate in the segmentation of the CNS (Lumsden and Krumlauf, 1996; Fraser et al., 1990; Lumsden, 1999), the formation of somites (Sato et al., 2002), and the generation of the dorsal and ventral compartments in the developing limbs (Chen and Johnson, 1999). There are similarities between the situation in flies and vertebrates, but they may not be identical (Vincent, 1998).

Recent studies have begun to shed light on the molecular mechanisms that underlie boundary formation in vertebrates. Members of the cadherin superfamily of cell adhesion molecules are expressed differentially in subdivisions of the brain, and they are candidate mediators of affinity differences between neuroepithelial compartments (Redies and Takeichi, 1996; Redies et al., 2000). Likewise, diverse cell adhesion molecules are expressed differentially in the developing inner ear. In chick, BEN, a cell adhesion molecule of the Ig superfamily is expressed within the proneural domain at otic cup-vesicle stage (Goodyear et al., 2001; BEN: Pourquie´ et al., 1990, 1992; SC-1: Tanaka et al., 1991; DM-GRASP: Burns et al., 1991). At stages HH26 -29 BEN demarcates all the sensory patches of the vestibular and the cochlear systems (Goodyear et al., 2001). In mouse, α3 and α6 integrins, a class of α/β heterodimers that mediate cell adhesion to proteins in the extracellular matrix, are expressed differentially in sensory/non-sensory epithelium of the inner ear. From E12.5 until birth, α6 integrin is detected in the sensory regions of the vestibular and cochlear structures, while α3 integrin expression is reciprocal to α6 integrin (Davies and Holley, 2002; Davies, 2007).

Signalling through ephrin and Eph receptors (erythropoetin-producing hepatoma) is known to regulate contact-mediated repulsion in both the nervous and the vascular systems (Palmer and Klein, 2003; Poliakov et al., 2004). Boundaries might act as mechanical barriers between populations of cells by generating specialized boundary cells or by increasing the deposition of extracellular matrix, either of which could act like a fence (reviewed in Kiecker and Lumsden, 2005).

1.3.5.2. Inner ear patterning and regionalization

In jawed vertebrates, the adult inner ear is highly regionalised along its three axes. In addition to the DV subdivision into vestibular and auditory regions, an asymmetry along the mediolateral axis is also obvious with, for instance, the endolymphatic sac and duct located in the medial part, close to the brain, and a pronounced anteroposterior (AP) asymmetry. These subdivisions are established during the developing inner ear and accompanied by assymmetric gene expression patterns. For instance, *Tbx1* and *Lmx1*

are expressed in the posterior and dosrsal domains of the otocyst, while FGF10, *LFNG* and *NeuroD* are restricted to the anterior and ventral domains (FIG. 12). In amniotes, otic regionalisation most likely begins after (about?) otic placode formation and involves early cell fate decisions -neural against non-neural, and neuronal against sensory fates. Sensory and non-sensory fated cells most likely interact with each other to coordinate the morphogenetic process (reviewed in Alsina et al., 2007). Tissues surrounding the inner ear, such as the

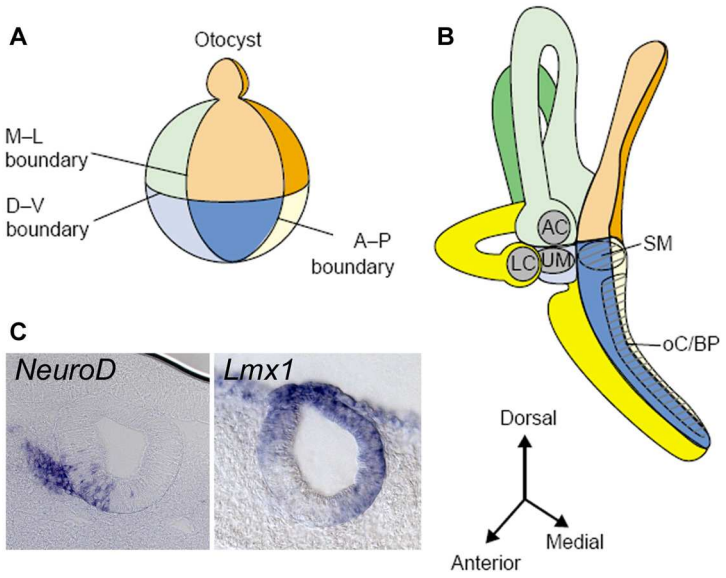

Figure 12. The developing inner ear is highly regionalised along its three axes. Compartment-boundary model of ear morphogenesis. (A) Model of the compartmentalized otocyst viewed from an anteromedial perspective, shown bisected by three boundaries (A–P, M–L and D–V) into eight developmental compartments (posterodorsolateral and posteroventrolateral not visible). The budding endolymphatic duct arises near the dorsal pole. (B) Predicted fate map for the early labyrinth, showing where cells from each compartment of the early otocyst are likely to reside after morphogenesis. The ear is viewed from an anteromedial perspective. The possible location of sensory patches relative to compartment boundaries is indicated. The fate map is especially hypothetical in the cochlear duct, as very few data are available to define the location of compartments and boundaries in this part of the ear. It is assumed that the posterior part of the cochlear duct arises from the posteroventrolateral and posteroventromedial compartments. (C) Sagittal sections of otic vesicles showing *NeuroD* expression profile in the proneural (anteroventral) domain and in the CVG, and *Lmx1* expression pattern in the posterolateral aspect of the otocyst. Adapted from Fekete and Wu, (2002). Abbreviations: A, anterior; AC, anterior crista; BP, basilar papilla; D, dorsal; L, lateral; LC, lateral crista; M, medial; oC, organ of Corti; P, posterior; SM, saccular macula; UM, utricular macula; V, ventral.

hindbrain, mesoderm and endoderm are potential sources of signals required for inner ear patterning (Fekete 1999; Giraldez 1998).

During development, gene expression profiles are dynamic, therefore, there are not *bona fide* markers for labeling the origin of specific cell types, which require of careful fate map analysis. . Fate maps give information not only about the end fate of a given domain in the embryo, but also on how it behaves during development. Fate maps of the developing inner ear have been generated for three species of vertebrates, yet, a complete picture is still missing.

The first attempt to fate map the otocyst was done by Li et al. (1978), who performed fate map studies in *Mus musculus* embryos by culturing pieces of different parts of the otocyst that were isolated by microdissection. Their study is better described as a specification map (Slack, 1991). Dorsal, ventral, anterior, posterior, medial, or lateral halves of days 10, 11, 12, and 13 otocysts were cultured separately *in vitro* and after 10 days, the explants were analyzed for differentiation of sensory structures based on morphology. Otocysts used in this study were much more advanced in development than those used in the studies discussed below. Sensory structures did not differentiate from E10 mouse otocysts when cultured in isolation, suggesting that external factors are necessary for the specification of inner ear sensory organs before E10. In contrast, from E11 onwards fragments of otocysts were able to differentiate into all the sensory organs and membranous labyrinth chambers expected. Data from E11 and E12 mouse otocysts demonstrate that the dorsal half of the otocyst is specified to form the semicircular canals and their associated cristae, while the ventral half is the sole source of cochlear structures. Anterior halves gave rise to two semicircular canals (anterior and part of the lateral) and their associated cristae, while posterior halves gave rise to the remaining canals (posterior and lateral) and to the posterior cristae. The medial and the lateral walls of the otocyst give rise to all the sensory structures, suggesting that by E11-E12 the anteroposterior and dorsoventral axes are fixed (FIG. 13C; Li et al., 1978).

Kil and Collazo (2001) generated a fate map of the *Xenopus laevis* inner ear at placode and otocyst stages to determine the origins of inner ear sensory organs. Vital dye was injected into one of four quadrants and the embryos analyzed at stages when sensory organs are developed. Results indicate that every region of the placode and otocyst gave rise to sensory organs, but that the range of sensory organs formed was not uniform. For example, the posterior quadrant tended to develop into non-sensory organs, anterior and ventral domains developed two anterior sensory organs, and dorsal injections resulted in labeled cells in both anterior and posterior sensory structures. Time-lapse

videomicroscopy experiments provides direct proof that cells from different regions of the inner ear do intermingle during early inner ear development, mixing occurring when injections where performed at early placode stages. Taken together, these results suggest that a single sensory organ may arrise from cells located in different parts of the placode (FIG. 13A; Kil and Collazo, 2001).

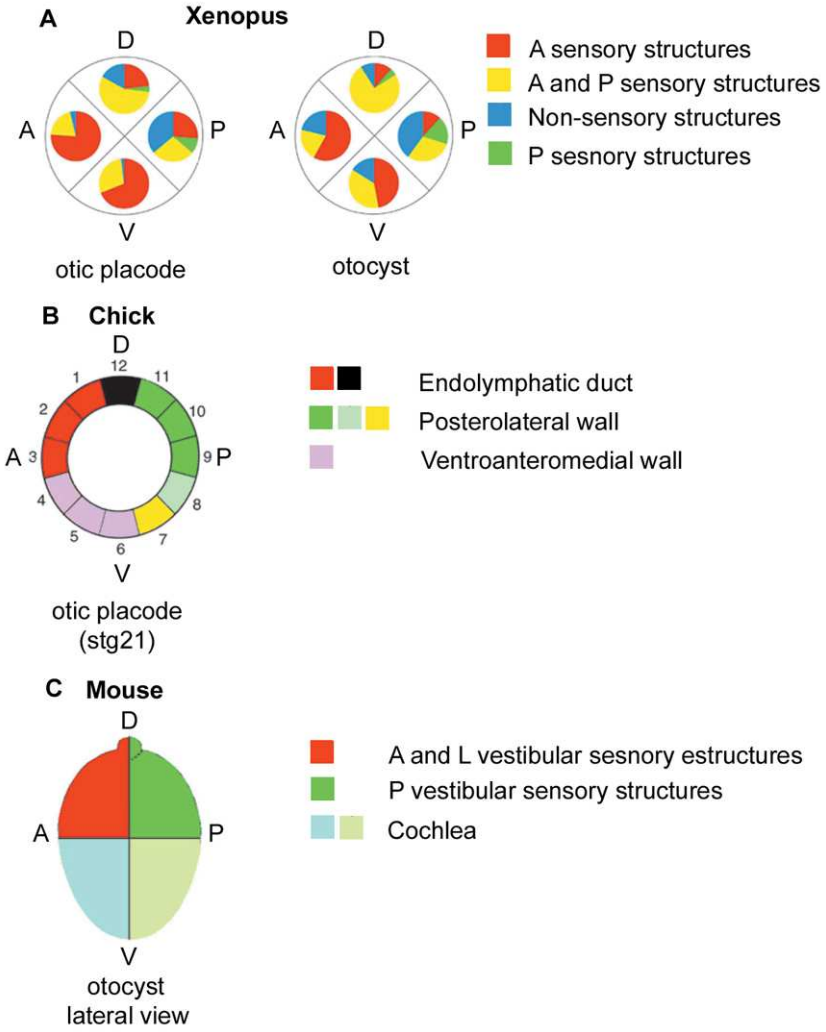

Figure 13. Inner ear fate maps. (A) Pie graphs summarizing the results of the frog fate map at otic placode and otocyst stages (Kil and Collazo, 2001). (B) Fate map of chick otic rim at placode stage (Brigande et al., 2000a). (C) Specification map of mouse otocyst (Li et al., 1978). Adapted from Kill and Collazo, (2002). Abbreviations: A, anterior; D, dorsal; L, lateral; stg, stage; P, posterior; V, ventral.

Brigande and colleagues (2000) mapped the *Gallus gallus* inner ear by injecting fluorescent dyes in the otic cup rim (HH13.5 and HH16.5) and analyzed the progeny at

otocyst stage (HH17 and HH21). The results revealed that the medial rim of the otic cup (the otic region in contact with the neural tube) maps to the nascent endolymfatic duct (ED). The anterior-lateral rim of the otic cup maps to anteroventral bend of the otic vesicle, and the posterior rim to the lateral wall of the otocyst. By simultaneous labelling with two different dyes, these authors identified a putative boundary of lineage restriction along the anterior-posterior axis of the ED. With this data they hypothesize that boundary between compartments were crucial for specifying endolymphatic duct outgrowth, and that it may also be involved in the location of the sensory organs and delamination of neuroblasts (FIG 13B; Brigande et al., 2000).
Summarizing, fate map studies indicate that there are no clear lineage restriction boundaries until late placode stages, and cells seem to mix extensively at these early stages. At late placode stages, when the otic cup has formed, there are potentially two boundaries for prospective dorsal structures of the otocyst. The first boundary bisects the endolymphatic duct into anterior and posteriordomains, while the second separates the lateral base of the endolymphatic duct.

In addition to fate map studies and gene expression data, there is evidence from both transplantation studies and gene knockout experiments to support the hypothesis that adult inner ear structure depends upon both otocyst gene expression domains and gene expression within the surrounding tissues. The hindbrain and the otic placode keep an invariant spatial relation in all species. The importance of the hindbrain for ear development has been demonstrated by the analysis of hindbrain ablation, as well as by the analysis of several mutants for genes that are expressed in the hindbrain, but not in the otic primordium (Schneider-M & Pujades 2007).
Hindbrain ablation after otic induction causes severe defects in vestibular structures, including the lack of semicircular canals and the fusion of the utricle and saccule, with little alteration of the basilar papilla (Bok et al., 2005; Hutson et al., 1999). However, ablation of the ventral part of the hindbrain together with the notochord results in a lack of the basilar papilla formation and normal vestibular morphogenesis (Bok et al., 2005). These experiments strongly suggest that signals from the dorsal hindbrain are involved in vestibular structures development, while signals from the ventral midline (ventral hindbrain and notochord) are involved in cochlear development (FIG. 14).
Hindbrain segmentation genes, such as *MafB*, *vHnf1* and *Hoxa1*, which are expressed in rhombomeres 4 to 6, have strong effects on otocyst development. While there are discrepancies among the interpretation of the results obtained in different animal species,

available data point to an essential role of hindbrain signals, and particularly FGF, Wnt and Hedgehog (Hh), in otic regionalisation (FIG. 14; reviewed in Alsina et al., 2007; Bok, et al., 2007; Shneider-Maunoury and Pujades, 2007;). Several *FGF* genes are expressed in the hindbrain, with species-specific patterns (Wilkinson et al 1988; Crossley and Martin, 1995; Ladher et al. 2005). *Hh* genes are expressed in the floor plate and underlying notochord (Jessel ad Dodd, 1990; Placzek, 1995). In mouse and chick, *Wnt* genes, in particular *Wnt1* and *Wnt3a* are expressed in the roof plate in all vertebrates (Parr et al., 1993), while *Wnt8a and Wnt8c* are expressed in r4 (Boullite et al., 1996; Hume and Dodd, 1993; Kil et al., 2005; Niederreither et al., 2000).

The function of FGFs in otic development has been extensively studied and its role in otic induction was reviewed above (3.3). I shall discuss here its role in otic patterning. Both in amniotes and in fish, the loss of function of *FGF3, FGF8* and *FGF10* leads to smaller and malformed otic vesicles, demonstrating a role for this signalling pathway in otic formation (Alvarez et al., 2003; Wright and Mansour, 2003; Zelarayan et al., 2007; Schimmang, 2007). Most *FGF10* mutants completely lack semicircular canals (Pauley et al., 2003; Ohuchi et al., 2005). *FGF3*-null mutants undergo normal otic vesicle formation, but then go on to develop highly variable and incompletely penetrant inner ear dysmorphologies in endolymphatic duct (ED) and semicircular canals, and poor coiling of the cochlea, phenotypes that are very similar to those of *Hoxa1*, *Mafb* and *Gbx2* mutants (Pasqualetti et al., 2001; Lin et al., 2005; Choo et al., 2006). The initial molecular patterning of *FGF3* mutant otocysts was normal, but by E10-10.5 these ears lacked or had reduced domains of dorsal otic genes, suggesting a loss of the cells fated to form the ED. Ventrally expressed genes important for cochlear development were not affected, but markers of the developing vestibular sensory domains, particularly those expressed in the posterior otic vesicle, were downregulated or absent (Hatch et al., 2007; Mansour et al., 1993). Furthermore, the role of FGF signalling in inner ear development has been examined using both *FGFR1* hypomorphs and conditional deletion of *FGFR1* using *FoxG1-Cre* (Pirvola et al., 2002). In each case, a dose dependent decrease in the size of the organ of Corti, and the expression of *Atoh1* (a proneural gene involved in hair-cell specification), was observed. In contrast, the vestibular system was normal. These results suggest a role for FGFR1 in cochlear development. On the contrary, mice lacking FGFR2b form otic vesicles subsequently develop dysmorphologies initiating at ED outgrowth and include failure of semicircular canal formation (Pirvola et al., 2000). In zebrafish, the function of FGF in inner ear patterning is attributed mainly to FGF signals coming from the hindbrain, while in amniotes, other surrounding tissues such as the mesenchyme and endoderm are

also sources of FGFs (Ladher et al., 2005). Ace-/- mutant embryos showed a dramatic reduction in the number of hair-cells (Leger and Brand, 2002) and, more recently, Millimaki et al (2007) demonstrated that FGF3 and FGF8 are required for *Atoh1* expression in the zebrafish otocyst, suggesting a role for the FGF pathway in hair-cell commitment.

The role of canonical Wnt signalling from the dorsal neural tube has been studied in mouse (Riccomagno et al., 2005; Ohyama et al., 2006). Nuclear localisation of beta-catenin indicates that Wnt signaling is active in dorsal regions of the otic vesicle (Riccomagno et al., 2005). By knocking out beta-catenin in *Pax2*-positives cells, hindbrain ablation or Wnt1-/-;Wnt3a-/- double mutant embryo analysis, they showed that Wnt signalling functions to positively regulate the expression of some otic dorsal genes such as *Dlx5/6* and *Gbx2*, and negatively regulate some otic ventral genes such as *Neurog1*, *NeuroD* and *LFNG* which conforms the neurogenic domain (Riccomagno et al., 2005; Ohyama et al., 2006). Moreover, they showed in Wnt1-/-;Wnt3a-/- double mutant embryos Wnt signalling requirement for vestibular morphogenesis (Riccomagno et al., 2005). Interestingly, hindbrain expression of *Wnt3a* was ventrally expanded in *FGF3* mutants, suggesting that FGF3 also serves to focus inductive Wnt signals on the dorsal otic vesicle, highlighting a new example of cross-talk between the two signaling systems (FIG. 15).

Shh is required for the ventral patterning of the inner ear in both chicken and mice (Liu *et al.*, 2002, Riccomagno *et al.*, 2002, Bok *et al.*, 2005). In *Shh* -/- mouse embryos result in the complete absence of ventral inner ear structures (Riccomagno *et al.*, 2002). In chicken, injecting hybridoma cells, which secrete antibodies blocking Shh bioactivity, into the ventral midline at the otic cup stage also resulted in inner ears devoid of ventral structures (Bok *et al.*, 2005). Shh signaling is mediated by the Gli family of transcription factors (Ingham and McMahon, 2001). Analyses of several mouse lines carrying various genetic combinations of mutant alleles associated with the Shh/Gli signalling pathway suggest that a proper balance of Gli3 repressor and Gli2/Gli3 activators along the DV axis is critical for mediating graded levels of Shh signaling in the inner ear (Bok *et al.*, 2007).

1.3.5.3. Otic neural versus non-neural patterning

We have described in the previous section the importance of the hindbrain for inner ear morphogenesis by the analysis of hindbrain ablation, and several mutants for genes that are expressed in the hindbrain, but not in the otic primordium. Therefore, it is possible that the hindbrain confers also AP axial identity to the inner ear. To determine if the

unique arrangement of the rhombomere segments in the hindbrain plays a role in conferring AP axial identity to the inner ear, Bok et al., (2005) reversed the AP polarity of the hindbrain close to the otic tissue between r4 and r7 chicken embryos. However, AP orientation of the inner ear was normal based on the expression patterns of proneural domain markers *LFNG* and *NeuroD*, indicating that changing the AP axis of the rhombomeres adjacent to the inner ears does not affect the AP axial orientation of the inner ear. Contrastingly, rotation of the DV axis of the neural tube changed the location of the expression domain of *LFNG* and *NeuroD* from anteroventral to anterodorsal, indicating that signals from the ventral hindbrain are important for placing the proneural domain in the ventral aspect of the otocyst and signals from the dorsal hindbrain may inhibit the dorsal expansion of the neurogenic domain markers (FIG. 14; Bok et al., 2005). Accordingly, Shh -/- mutant embryos showed a reduction of the expression domain of *Neurog1* and *NeuroD.* In addition, *FGF3* mutant embryos and *FGF3*;mox2Cre directed deletion of *FGF8* mutant embryos showed a reduced or no CVG (Mansour et al., 1993; Zelarayan et al., 2007). Besides, neurogenic markers in the ventral otocyst such as *LFNG*, *NeuroD1* and *Neurog1* were absent in conditionally activated β-catenin mutant embryos (Ohyama et al., 2006). Moreover, the expression of *Neurog1* was expanded ectopically along the lateral wall of the otocyst in explants lacking the dorsal hindbrain (Ricomagno et al., 2005). However, treatment of ablated embryos with LiCl, which activates the canonical Wnt pathway, did not fully restore the expression of *Neurog1* (Ricomagno et al., 2005), suggesting that Wnts are not the only dorsal hindbrain signals involved in this process. Another family of secreted molecules that may serve as signaling molecules for the inner ear patterning are BMPs, which are expressed in the roof plate of the hindbrain and dorsal ectoderm (Lee and Jessell, 1999). Bmp family members have previously been implicated in vestibular morphogenesis (Chang et al. 1999; Gerlach et al. 2000). The perturbation of Bmp4 function in the presumptive cristae of chick embryos using the Bmp2/4 antagonist Noggin resulted in a loss of semicircular canal formation. Since BMP and Shh inhibit each other in the neural tube and inhibitors of BMPs, expressed in the notochord, also modulate Shh functions in the ventral neural tube (Liem *et al.*, 1995, Liem *et al.*, 2000, Patten and Placzek, 2002), it is possible that similar opposing interactions between Shh and BMPs occur in the inner ear. Thus, a similar multi-signaling hypothesis for the hindbrain DV patterning could be achieved for establishing neural versus non-neural patterning in the ear (Maklad and Fritzsch, 2003).

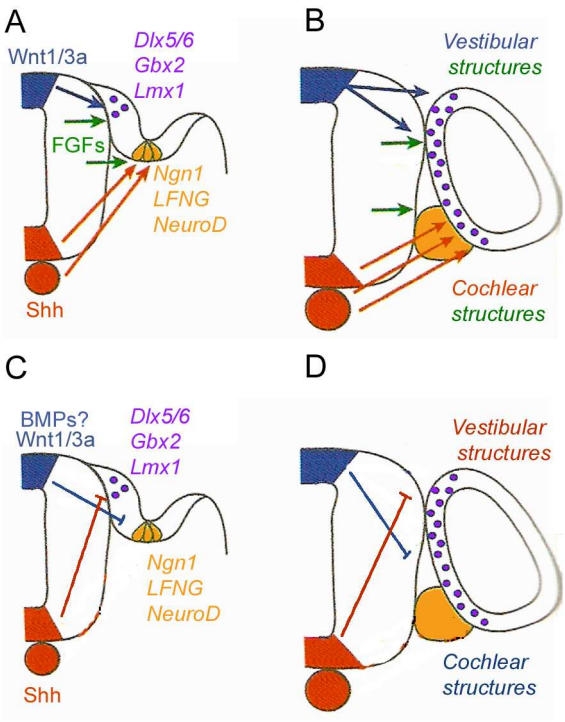

Figure 14. inner ear patterning. (A, C) At early stages of otic development only the dorsomedial part of the otic cup makes intimate contact with the dorsal half of the neural tube. (B, D) As invagination proceeds, medial otic cells are now located in more ventral positions and receive signals from the ventral aspect of the hindbrain. (A, B) Midline inducing signals are involved in inner ear patterning. Dorsal signals are involved in inducing dorsal otic markers as *Dlx5/6*, *Gbx2* and *Lmx1* (Wnts), and in later vestibular structures formation (Wnts and FGFs), while ventral signals (Shh) are required for the expression of ventral and neurogenic markers such as *LFNG*, *Neurog1* and *NeuroD*, and in later development of cochlear structures. (C, D) Midline inhibiting signals are involved in inner ear patterning. Additionally, Dorsal signals (Wnts and BMPs) are required for restricting proneural markers such as *LFNG*, *Neurog1* and *NeuroD* in the ventral aspect of the otic cup-vesicle, while ventral signals (Shh) are involved in restricting expression *Dlx5/6*, *Gbx2* and *Lmx1* in the dorsal region of the otic cup-vesicle. Modified from Schneider-Maunoury and Pujades, (2007).

Furthermore, ablation studies also analysed the expression profile of *Lmx1b* (hereafter *Lmx1*; a transcription factor of the LIM family, homologous to the *Drosophila apterous* gene) which is normally detected complementary to the neurogenic domain. Interestingly, the experiments showed that *Lmx1* is induced and maintained by dorsal neuroectoderm signals, whereas it is downregulated by the ventral neural tube (FIG. 14; Giraldez, 1998). All in all, this data suggests that patterning signals from the hindbrain are regionalized along the DV axis and that they can act as positive and negative regulators. A balance of FGFs and Shh from the ventral midline positively regulates neurogenic identity in the

ventral aspect of the otic cup-vesicle, whereas Wnt and BMPs from the dorsal hindbrain restricts the proneural domain into the ventral region of the otic vesicle (FIG. 14).

As described above, changes in extrinsic signalling result in the disruption to AP patterning in the otic vesicle. I will describe here the role of Tbx1 since it could be a key downstream target of extrinsic signals having a role on neural versus non-neural patterning (Vitelli *et al.*, 2003, Raft *et al.*, 2004, Arnold *et al.*, 2006). Tbx1 is a Brachyury related member of the DNA-binding T-box gene family of transcription factors that plays a role in the formation of prosensory domains. *Tbx1* is initially expressed in a posteroventral region of the otocyst that correlates with the location of the first expression of *BMP4* (Raft et al., 2004). However, expression of *Tbx1* is significantly reduced by E12.5 and it is not clear if *Tbx1* is ever expressed in the elongating cochlear duct. Individuals suffering from DiGeorge syndrome, velocranial facial syndrome and conotruncal anomaly face syndrome experience a number of abnormalities, including auditory deficits, as a result of a chromosomal deletion on 22q11.2 (reviewed by Baldini, 2003). Several studies have demonstrated that the basis for these defects is the deletion of the *Tbx1* gene (Yagi et al., 2003). Analysis of the effects of either deletion of *Tbx1* or overexpression of *Tbx1* in the mouse otocyst between E9 and E13.5 indicates a role in the regulation of neurogenesis (Raft et al., 2004). Specifically, overexpression of *Tbx1* in the otocyst decreases the number of *Neurog1*-positive neuronal precursors and reduces the size of the CVG ganglion. Conversely, in *Tbx1* mutants, *Neurog1*-postive precursor numbers and ganglion size increase. Contrastingly, expression of *BMP4* in *Tbx1* mutant otocysts is reduced and sensory epithelia fail to form (Raft et al., 2004). In addition, ectopic expression of *Tbx1* throughout the otocyst results in larger and ectopic sensory epithelia (Funke et al., 2001). These results clearly demonstrate a role for Tbx1 in the specification of inner ear neural and sensory epithelia. However, considering that neuronal precursors arise from the anterior half of the otocyst, and that *Tbx1* is apparently not expressed in those precursors, it is not clear whether Tbx1 plays a direct role in prosensory specification or acts indirectly through regulation of AP patterning within the otocyst (Raft et al., 2004). In fact, several markers of anterior-posterior identity are altered in *Tbx1* mutants (Raft et al., 2004), suggesting a role in otocyst axial patterning. Axial patterning markers are also altered in mice from a bacterial artificial chromosome (BAC) transgenic line *316.23,* in which *Tbx1* is broadly expressed throughout the otocyst and the size of sensory regions is increased. These results suggest that changes in the size of the sensory epithelia could be a result of axial re-specification (Raft et al., 2004). Novel genetic approaches, which include *Tbx1* ablation from the time of otic cup closure and fate mapping, indicate that

Tbx1 identifies a cell population that forms most of the otocyst, excluding the neurogenic and the endolymphatic duct territories. *Tbx1* is required for localization, expansion and fate of this cell population, but it is not required to establish its AP identity. This Tbx1-dependent cell population does not normally contribute substantially to the cochleo-vestibular ganglion, but deletion of *Tbx1 at* otic cup closure results in the expansion of the Delta-like1-Notch1 activation domain, and in change of fate of at least some of the *Tbx1*-traced cells towards a neurogenic fate (Xu et al., 2007). Overall, we can say that the main functions of Tbx1 in the inner ear are to control the contribution, size and fate of a large population of otic epithelial cells, and cochlear morphogenesis.

Box VI. Notch Pathway: The Control Of Territory Regionalization

Notch is another signalling factor that mediates communication between populations of cells. Completely different behaviour is predicted if Notch activation regulates ligand expression in an opposite way than in lateral inhibition. In this case, which is called lateral induction, Notch activation in a given cell increases the ability of that cell to produce functional Notch ligands, (Eddison et al., 2000; Lewis, 1998). A cell that expresses Notch ligands strongly will make its neighbours to do the same (FIG. BOX VI). The effect of the lateral induction is the cooperation between neighbours, instead of competition. This positive feedback loop has the effect of amplifying one territory with a common identity that differs from the adjacent territory as occurs during the specification of the sensory patches of the inner ear (See 3.5.5; Eddison et al., 2000);

can be responsible for generating morphological boundaries, as for example the excision of somites from the presomitic mesoderm (PSM) in vertebrate somitogenesis (Jiang et al., 2000); and can aslo restrict Notch pathway activity along the boundary cells between adjacent territories as occurs, for example, at the *Drosophila* wing margin, in the mouse and zebrafish interrhonbomeric boundaries and in the mouse Zona limitans intrathalamica (ZLI; Bray, 1998; de Celis and Bray, 1997; Amoyel et al., 2005; Cheng et al., 2004; Baek et al., 2006; Riley et al., 2004). Although the mechanisms of compartmentalization through the action of Notch signaling in flies are well understood, the situation in vertebrates is not fully unveiled and might differ. In *Drosophila*, Notch is expressed in the entire wing disc, while *LFNG* and *Serrate* are expressed in the dorsal region of the wing disc. On the contrary, *Delta* is expressed in the ventral part. LFNG promotes Notch binding to Delta rather than to Serrate, as a result, Serrate is only able to activate Notch in neighbouring cells that do not express *LFNG,* which are the cells in the ventral side of

the dorsoventral (DV) interface. In parallel, Delta only signals to neighbouring cells that are expressing *LFNG*, which are the ones in the dorsal side of the DV border. As a consequence, Notch is activated in the cells placed at the DV boundary of the imaginal disc and induces *wingless* (Wg) expression (Bray, 1998). Wg from the DV boundary organizes subsequent outgrowth and patterning of the wing, and promotes expression of *Delta* and *Serrate* in flanking cells, which in turn provide feedback to maintain Wg at the margin (Couso et al., 1994, 1995; Kim et al., 1996; Rulifson and Blair, 1995; de Celis et al., 1996; Rulifson et al., 1996; de Celis and Bray, 1997; Micchelli et al., 1997).

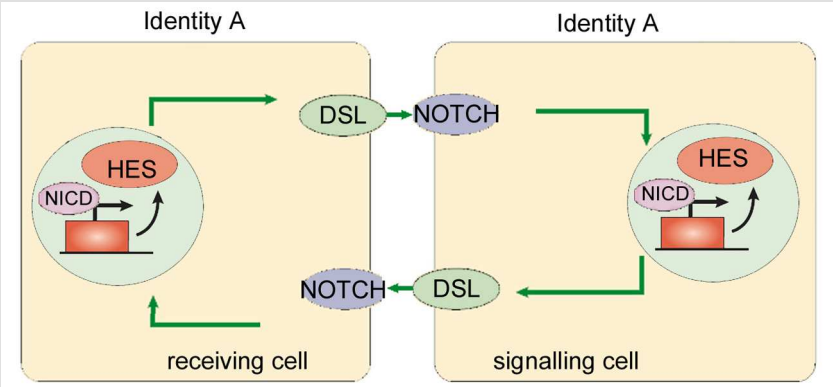

Figure BOX VI. Lateral induction. Schematic drawing illustrating the mechanism of lateral induction. Notch-DSL binding activates the expression of DSL in the receiving cell, as a result, both cells (signaling and receiving cell) acquire the same identity. In this model a positive feedback loop induces neighbourings cell to have the same identity.

On top of that, *Hairy* and *Enhancer of Split* homologs (*Hes/Her*) can also act as prepattern factors in the zebrafish and mouse midbrain-hindbrain boundary, mouse olfactory placode and the inter-proneural stripes of the zebrafish neuroectoderm by repressing proneural genes expression (Cau et al., 2000; Geling et al., 2003; Bae et al., 2005, Baek et al., 2006; Ninkovic et al., 2005; Hirata et al., 2001). However, in this mechanism Hes and Her do not act as downstream effectors of Notch signalling but rather, as prepattern factors. *Hes* and *Her* are regulated by positional cues and regulate the spatial pattern of proneural gene expression, in a manner reminiscent of neurogenesis in the *Drosophila* peripheral nervous system (Cau et al., 2000; Bae et al., 2005; Geling et al., 2004; Davis and Turner, 2001; Fisher and Caudy, 1998).

1.3.5.4. Notch pathway in inner ear sensory patterning

The inner ear regionalisation is the result of consecutive inductive signals emanating from neighbouring tissues. The prevailing view is that Notch/Jag1 signaling initially specifies sensory versus non-sensory epithelium within the ear, placing the limits by a lateral inductive mechanism (FIG. 15A; Adam *et al.*, 1998, Lewis, 1998, Eddison *et al.*, 2000, Landford and Kelley, 2005). Subsequently, Notch/Jag2-Dll1 pathway inhibit hair-cell differentiation and establish a mosaic cellular pattern by lateral inhibition (FIG. 15B; Daudet and Lewis, 2005).

Three Notch signalling pathway elements that are expressed in patterns that are largely consistent with a role in otic prosensory specification are *Notch1*, *Jag1* and *LFNG.* Although they are initially expressed in more diffuse patterns in the otic cup, each ultimately resolves to the developing prosensory regions (Lindsell et al., 1996; Haddonet al., 1998; Wu et al., 1996, Morsli et al., 1998; Adam et al., 1998; Cole et al., 2000). *Jag1* expression is first localized to the prosensory domain, while later on is expressed exclusively in supporting cells. However, an important exception is the expression of *Jag1* in the mammalian cochlea which abuts with, rather than overlaps, the prosensory domain until well past the developmental stages at which prosensory specification takes place (Kiernan et al., 2006; Chen et al., 2002). This observation suggests that the mechanisms underlying the specification of the organ of Corti may differ from the one driving the vestibular sensory regions. Recent experiments have illuminated a role for Notch signalling, in the specification of prosensory domains. In particular, analysis of inner ears from mice in which *Jag1* has either been specifically deleted at the early otocyst stage using a *FoxG1*-dependent *Cre* expressing line, or made hypomorphic, reveals that most of the vestibular organs, with the exception of the saccular maculae, are essentially absent, and within the cochlea, a reduced number of mis-patterned hair cells are restricted to the apical region of the duct (Kiernan et al., 2001; Tsai et al., 2001; Kiernan et al., 2006). Similarly, deletion of *RBP-Jk*, a transcriptional repressor that is required for Notch function (Mizutani et al., 2001), leads to a complete absence of all vestibular epithelia and to a nearly complete loss of all cochlear hair cells (Yamamoto and Kelley, unpublished). Finally, inhibition of γ-secretase activity, a component of the Notch signalling pathway, inhibits prosensory formation in the chick otocyst (Daudet et al., 2007). Conversely, over-expression of an activated form of chicken Notch1, cNotch1-intracellular domain (NICD), in non-sensory regions of the chick otocyst leads to the formation of ectopic sensory patches (Daudet and Lewis, 2005). All these results are

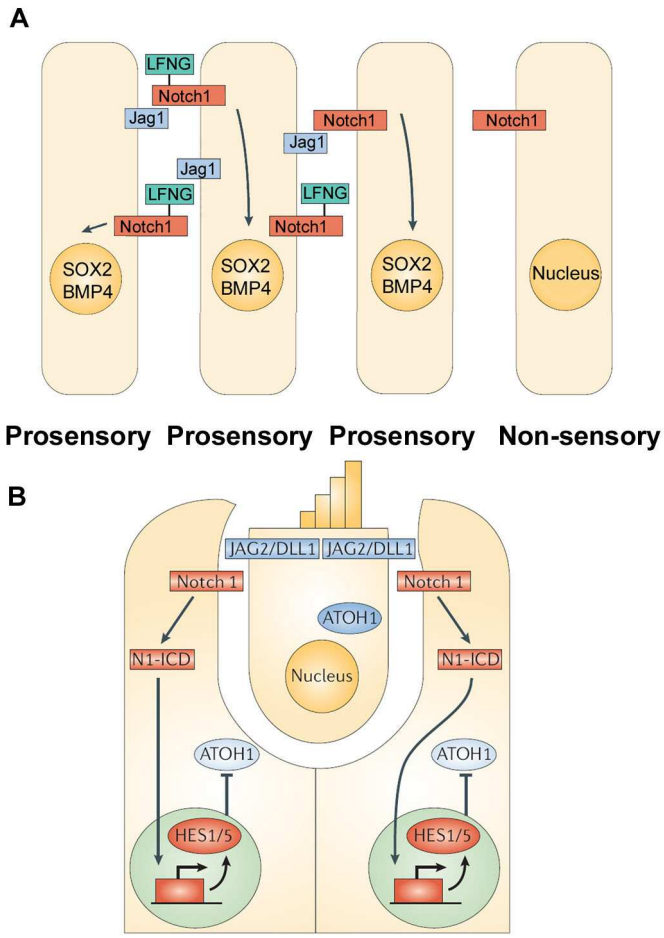

Figure 15. Two contrasting roles for Notch pathway during inner ear sensory development. (A) Proposed interactions that specify the prosensory patches. Serrate1-Notch signalling maintains and extends prosensory patches. At early stages of inner ear development, Serrate1 activates Notch1, which induces *Sox2* and *BMP4.* As a result, a normal sensory patch differentiates. (B) Once a prosensory patch has been specified, interactions between individual cells in the patch determine which cells will develop as hair cells or supporting cells. Developing hair cells express Jag2 and Dll1. Both ligands bind to Notch 1 in adjacent cells, leading to the generation of Notch intracellular domain (NICD). This upregulates the expression of two inhibitory basic helix–loop–helix proteins, HES1 and HES5, which block the effects of proneural genes such as Atoh1, leading to inhibition of the hair-cell fate. Inhibited cells subsequently develop as supporting cells. Adapted from Kelley, (2006); and Daudet et al., (2007).

consistent with a role for Jag1-dependent Notch activation in the specification of prosensory domains throughout the ear including the cochlear duct. However, additional factors might be required for sensory specification in the cochlear system, since all these mutants showed a reduction instead of a complete loss of the hair-cell positive domain. In

contrast, inner ear deletion of *Notch1*, again using the *FoxG1-Cre* line as a driver, results in an over-production of hair-cells in both the vestibular and cochlear epithelia (Kiernan et al., 2005a). The mechanism for this effect is most likely related to the role of Notch signalling in the determination of individual cell fates within prosensory domains by lateral inhibition as discussed above.

Consistent with a role of Notch in cell fate specification in the inner ear sensory epithelium, nuclear localization of NICD, and *Hes5*, *LFNG* and *Jag1* expression are detected in supporting cells, while *Jag2* and *Dll1* expressions are restricted to hair-cells (Adam *et al.*, 1998; Haddon *et al.*, 1998; Eddison *et al.*, 2000; see revision in Landford and Kelley, 2005). Null mutations for *Jag2*, *Hes5* and/or *Dll1* in mouse embryos and *mind bomb* (E3 ubiquitin involved in ubiquitinylation and endocytosis of Delta required for Notch function) in zebrafish embryos results in overproduction of hair-cells at the expense of supporting cells, indicating that Notch signaling regulates sensory cell fate specification by a mechanism of lateral inhibition (Haddon *et al.*, 1998; Lanford *et al.,* 1999; Zine *et al.*, 2000; Kiernan *et al.*, 2001). Thus, Jag2/Dll1 ligands lead to the activation of Notch pathway and *Hes5* in adjacent cells that blocks hair-cell determination.

Altogether, it has been proposed that Notch activity is required for sensory development to first, make cells competent to form a prosensory patch conferring them a prosensory character and, subsequently, inhibit hair-cell differentiation and establish a mosaic cellular pattern (Daudet and Lewis, 2005).

AIMS AND SCOPE OF THIS THESIS

2. AIMS AND SCOPE OF THIS THESIS

As outlined in the introduction, a lot of work has been addressed in order to elucidate the first event of inner ear development, which is otic induction. Besides, several laboratories have addressed the question on how the otic vesicle is patterned in the three axes. Fekete and co-workers have suggested that otocyst regionalization into distinct gene expression domains can be important for otic patterning (Brigande et al., 2000). Moreover, it has been shown the role of Notch pathway in specifying the inner ear sensory patches and in regulating cell fate decision between hair-cell and supporting cell (Adam *et al.*, 1998; Lewis, 1998; Eddison *et al.*, 2000; Landford and Kelley, 2005; Kiernan et al., 2001; Tsai et al., 2001; Kiernan et al., 2006; Mizutani et al., 2001; Haddon *et al.*, 1998, Lanford *et al.*, 1999, Zine *et al.*, 2000, Kiernan *et al.*, 2005; Daudet and lewis, 2005; Daudet et al., 2007). More to the point, it has been described the requirement of FGF signalling not only for otic induction but also for proper morphogenesis of the endolymphatic duct and semicircular canals, and CVG formation within zebrafish, chick and mouse (mouse: Alvarez et al., 2003; Wright and Mansour, 2003; Zelarayan et al., 2007; Schimmang, 2007; Pauley et al., 2003; Ohuchi et al., 2005; Hatch et al., 2007; Mansour et al., 1993; Pirvola et al., 2002; Pirvola et al., 2000; zebrafish: Leger and Brand, 2002; Millimaki et al., 2007). However, little work has been done to uncover the patterning events that drives the first otic cell lineage to develop, the neural lineage. The Major aims of this work are:

A) To analyze the early steps of otic regionalization: Description of the candidate patterning genes involved in inner ear regionalization.

B) Fate map of early otic placode and cup.

C) To examine the role of Notch signaling in early otic regionalization.

D) To elucidate the mechanisms underlying the decision of the otic territory to adopt a neural fate with special emphasis to FGF signal and SOX3 transcription factor.

This work dissects the first steps of inner ear regionalization, showing that the otic territory is regionalized in the AP axis much before it was reported. Our work presents data indicating that the ear primordium is already prepatterned at the time of the development of the otic placode. At otic cup stage, the anterior proneural region is

characterized by the expression of *Sox3*, *FGF10* and *LFNG*, whereas *Iroquois1* (*Irx1*) and *Lmx1* transcription factors are restricted to the complementary posterior non-neural domain. Moreover, we show that *Sox3* is expressed in the pre-otic region foreshadowing the proneural domain before the otic placode is morphologically visible. By DiI/DiO injections we revealed that these two domains exhibit limited cell intermingling. The work presented here also shows that Notch is required for restricting the expression of *Lmx1* and *Irx1* to the non-neural domain and for neuroblast selection in the proneural domain but not for the establishment of the proneural domain. These results led us to investigate other signals implicated proneural induction. Our results suggest that a cascade of local ectodermal FGF8-FGF10 signaling was required to enhance *Sox3* which in turn was essential for the specification of the proneural domain versus a non-neural otic territory. Furthermore, *Sox3* was required for the down-regulation of non-neural genes such as *Lmx1* but not *Tbx1*. All in all, we can say that the first inner ear regionalization event is the specification of the proneural territory versus a non-neural domain which requires FGF signals and the functional integrity of Notch pathway for its stability.

In this research project we have used the chick as a developmental model for studying inner ear development. The chick inner ear resembles other higher vertebrates, and the chick model system is accessible during the stages when inner ear developmental decisions are occurring, thus allowing experimental intervention *in vivo*. As a result, a large variety of methodologies can be used to analyze the genetic regulation of many different developmental processes such as *in ovo* cell tracing, *in ovo* electroporation, organotypic explants and embryo culturing. Moreover, in collaboration with other laboratories we have take advantage of the mouse and zebrafish as developmental models (Thomas Schimmang, de la Pompa and Paul Scotting laboratories).

Figure 16 shows dorsal views of chick embryos from stage 8HH to 18H which are the stages of chick development where the events that we are focus on are taking place.

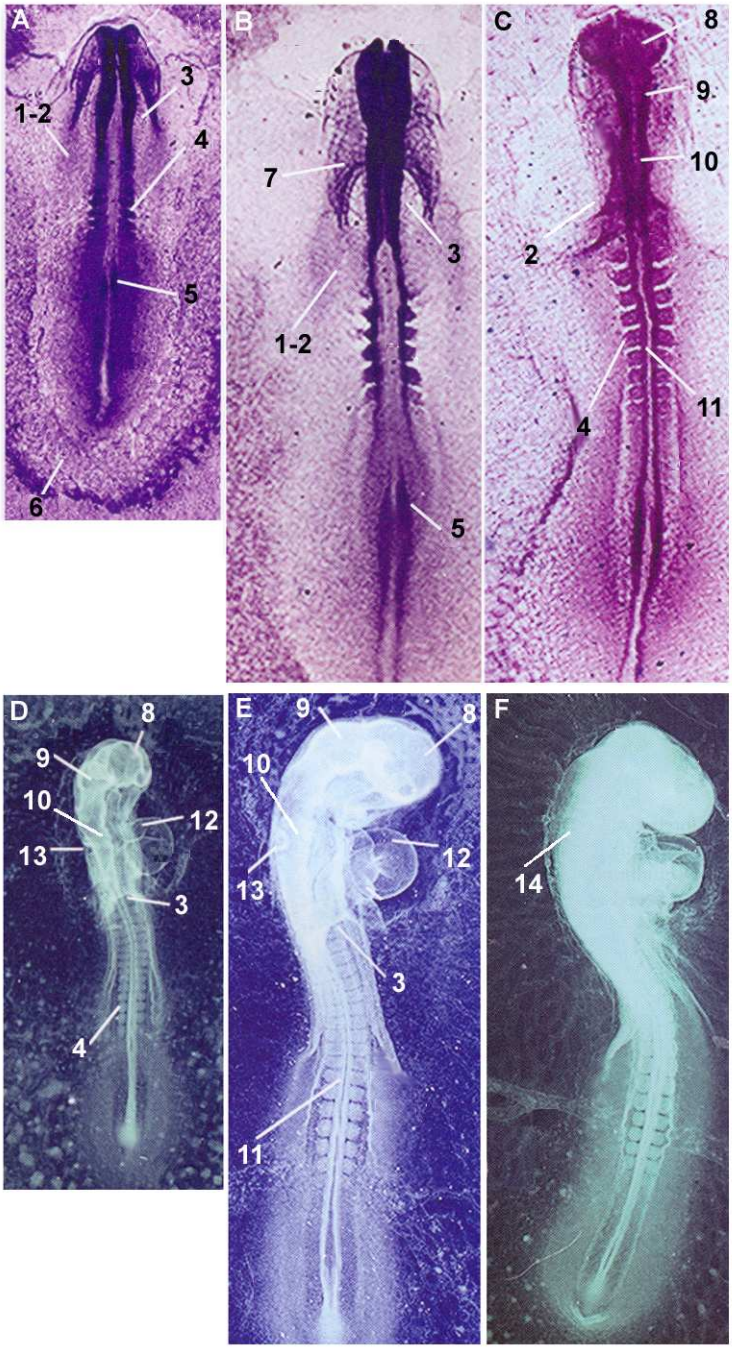

Figure 16. Chick development from otic placode to early otic vesicle. Dorsal view of embryos stage (A) HH8, (B) HH9, (B) HH10, (C) HH13, (D) HH14 and (E) HH15. 1, heart- forming area in the mesoderm; 2, pre-otic ectoderm; 3, anterior intestinal porta; 4, somite; 5, Hensen's node; 6, area vasculosa; 7 foregut; 8, forebrain; 9 midbrain; 10, hindbrain; 11, neural tube; 12, heart; 13 otic cup; 14, otic vesicle. Modified from Bellairs and Osmond.

RESULTS

3. RESULTS

3.1. Regionalization During Early Steps Of The Inner Ear Development

3.1.1. The otic cup is regionalized into a proneural and a non-neural domain

Otic neurogenesis in the chick starts at the otic placode/cup transition (HH11, 13ss) as revealed by the expression of *Neurog1* and *Delta1* (Henrique et al., 1995; Adam et al., 1998; Alsina et al., 2004). Expression of these genes is detected only in a subdomain of the otic cup, suggesting that specification of the proneural region has already taken place by otic cup stage. Previous work showed that *FGF10* and *Lunatic Fringe* (*LFNG*) are confined to the proneural domain, and *Lmx1b* and the HNK1 epitope to the non-neural (Giraldez, 1998; Cole et al., 2000; Alsina et al., 2004). The experiments that follow analyzed the expression of other genes with a similar regional expression pattern.

As previously mentioned, the *Sox3* gene (Sry like HMG-box) belongs to a large family of transcription factors, expressed at early stages of neural plate induction and placode development (Rex et al., 1997). *Sox3* expression precedes the expression of neurogenic genes and is downregulated by neuronal differentiation genes (Abu-Elmagd et al., 2001; Bylund et al., 2003). In the otic cup of HH13 stage embryos, *Sox3* was predominantly expressed in the anterior proneural domain, in a similar manner to *FGF10* and *LFNG* (FIG. 17A, B and C). Two other genes involved in neural patterning in *Drosophila* and vertebrates were studied during otic proneural regionalization. The *Iroquois* (*IRO* in *Drosophila* and *Irx* in vertebrates) genes are a family of homeodomain proteins within the TALE class (Burglin, 1997) that are involved in neural prepatterning (Gomez-Skarmeta and Modolell, 2002; Gomez-Skarmeta et al., 2003). In the otic cup, expression of *Irx1* was restricted to the posterior domain, in a complementary manner to *Sox3* and the proneural region (compare FIG. 17D with 17A-C). A second gene analyzed was *Lmx1b* (hereafter *Lmx1*), which is a transcription factor of the LIM family, homologous to the *Drosophila apterous* gene. *Apterous*/*Lmx1* are involved in the specification of the dorsal limb character in *Drosophila* and vertebrates (Blair et al., 1994; Riddle et al., 1995; Blair, 1995; Vogel et al., 1995b), and in roof plate and isthmus development in mouse (Adams et al., 2000; Chizhikov and Millen, 2004). In the otic cup, *Lmx1* was detected in the posterior domain and in the otic ridge all around the border between otic and non-otic ectoderm (FIG. 17E), except at the anterior-lateral position (red arrowhead in FIG. 17E). Several genes of the Notch pathway were expressed in the non-neural territory, complementary to the expression of the Notch modulator, *LFNG* (see FIG. 24 and 25). As an example,

Hairy1 (*Hes1* in mammals), a member of the *Hairy* and *Enhancer of Split* family of genes, was expressed in the posterior domain of the otic cup (FIG. 17F).

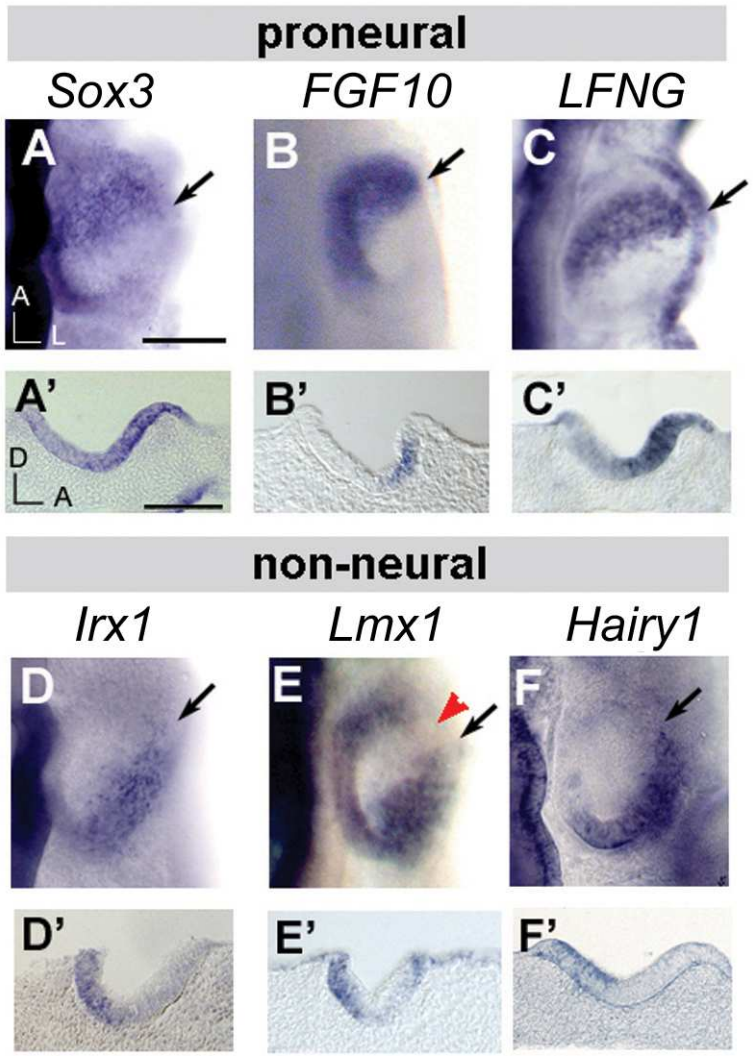

Figure 17. Regionalization of the otic cup into a proneural and a non-neural domain. (A-F) Dorsal views of HH13 otic cups showing expression of *Sox3* (A), *FGF10* (B), *LFNG* (C) in the proneural domain and complementary expression of *Irx1* (D), *Lmx1* (E) and *Hairy1* (F) in the non-neural domain. Note that *Lmx1* was also expressed in the medial and anterior otic ridge but excluded from the anterior lateral ridge (red arrowhead). Arrows indicate antero-posterior expression boundary. (A'-F') Sagittal sections of HH12-13 otic cups showing higher expression of *Sox3* (A'), *FGF10* (B'), *LFNG* (C') in the anterior proneural domain and restricted expression of *Irx1* (D'), *Lmx1* (E') and *Hairy1* (F') in the posterior non-neural territory. Abbreviations: A, anterior; D, dorsal; L, lateral. Scale bars in A and A' =100 μm.

Para-sagittal sections in Figure 17A'-F' show complementary expression patterns and illustrate that all genes were confined to the otic epithelium and not expressed in the surrounding mesenchyma. The proneural/non-neural boundary viewed dorsally at otic cup stage is 45 degrees tilted with respect to the anterior-posterior (AP) embryonic axis (Figure 17A-F). The proneural domain is, strictly speaking, the anterior-medial aspect of the otic placode, and the non-neural domain is posterior-lateral. However, for convenience they will be referred to as anterior (proneural) and posterior (non-neural) domains. As shown in Figure 18A (modified from Alsina et al., 2004), the proneural domain (blue), is initially a flat triangle. As the otic placode invaginates and the lateral wall grows, the proneural domain ends up in the antero-ventral aspect of the otic vesicle. *Lmx1* expression is excluded from the proneural domain and, in the medial wall, is always dorsal to the proneural region.

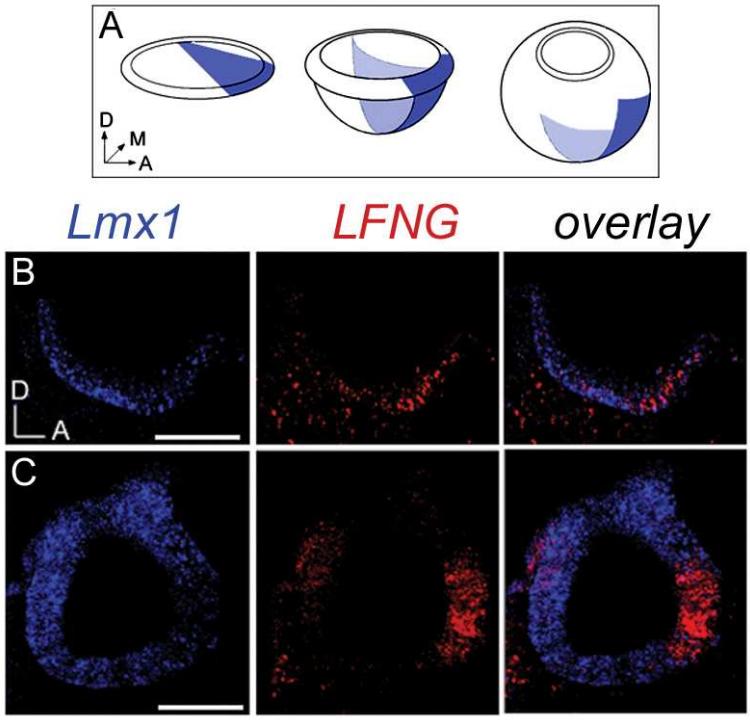

Figure 18. (A) Schematic drawing illustrating the dynamics of the proneural/non-neural domains from otic placode stage to otic vesicle viewed laterally. Blue indicates the proneural territory, while in white is depicted the non-neural region. (B-C) Double fluorescent ISH of an otic cup and otic vesicle showing complementarity of expression between *LFNG* (red) and *Lmx1* (blue). Abbreviations: A, anterior; L, lateral; D, dorsal; M, medial. Scale bars in B and C =100 μm.

The complementary gene expression patterns are better illustrated by the double fluorescent in situ hybridization for *LFNG* and *Lmx1* from otic cup (FIG. 18B) to otic

vesicle (FIG. 18C). *LFNG* probe was detected with a tyramide-Cy3 fluorochrome (red) and *Lmx1* with a tyramide-Cy5 fluorochrome (blue). Taken together, the results show that at early cup stages, patterning genes and members of the Notch signaling pathway are differentially expressed between the neural and non-neural regions.

3.1.2. Otic regionalization occurs at the onset of otic placode formation

Having described that the otic cup is subdivided into a proneural domain and a non-neural domain, each of them expressing a specific combination of transcription factors and signalling molecules, the following step was to explore the developmental time of otic regionalization. It has been reported that the onset of expression of *Sox3* in the head ectoderm is at 6 somite stage (ss), after the appearance of the pre-otic field marker *Pax2* (Groves and Bronner-Fraser, 2000). We therefore went at pre-otic placode stages to carefully examine whether *Sox3* was only detected in a subdomain of the *Pax2*-positive territory, such in a way that *Sox3* might be delineating a neural competent field in the pre-otic territory. In parallel, we wanted to know whether *Lmx1* and *Tbx1* were also expressed in the presumptive otic territory and whether *Sox3* and *Lmx1*/*Tbx1* were initially overlapping domains that become regionalised after placode formation. The first observation was that *Sox3*, at 6 ss, appeared in a broad region within the *Pax2*-positive domain (FIG. 19Aa-a'' and Ab). On the contrary, low levels of *Lmx1* and *Tbx1* were also present but in few cells. In the case of *Lmx1*, expressing cells were mainly detected adjacent to the neural tube, while *Tbx1*-positive cells were confined in the caudal region of the *Pax2* domain (FIG. 19Ac and d). The relative broad expression of *Sox3* progressively got refined and enhanced to the more rostral part of the preotic territory from 7-8 to 9-10 ss. The highest levels of *Sox3* draw a lateral to medial band encompassing the geniculate placode (FIG. 19Ab' and b''; lateral red arrowhead) and part of the otic field (FIG. 19Ab''; white arrowhead), suggesting that this band could be foreshadowing the neural competent territory of both placodes. At this time, strong *Tbx1* expression was confined to the posterior region of the presumptive otic ectoderm at the level of r5-r6 (FIG. 19Ac'-c''). In contrast, higher expression of *Lmx1* in the posterior otic territory was only evident at 9-10 ss (FIG. 19Ad'-d'').

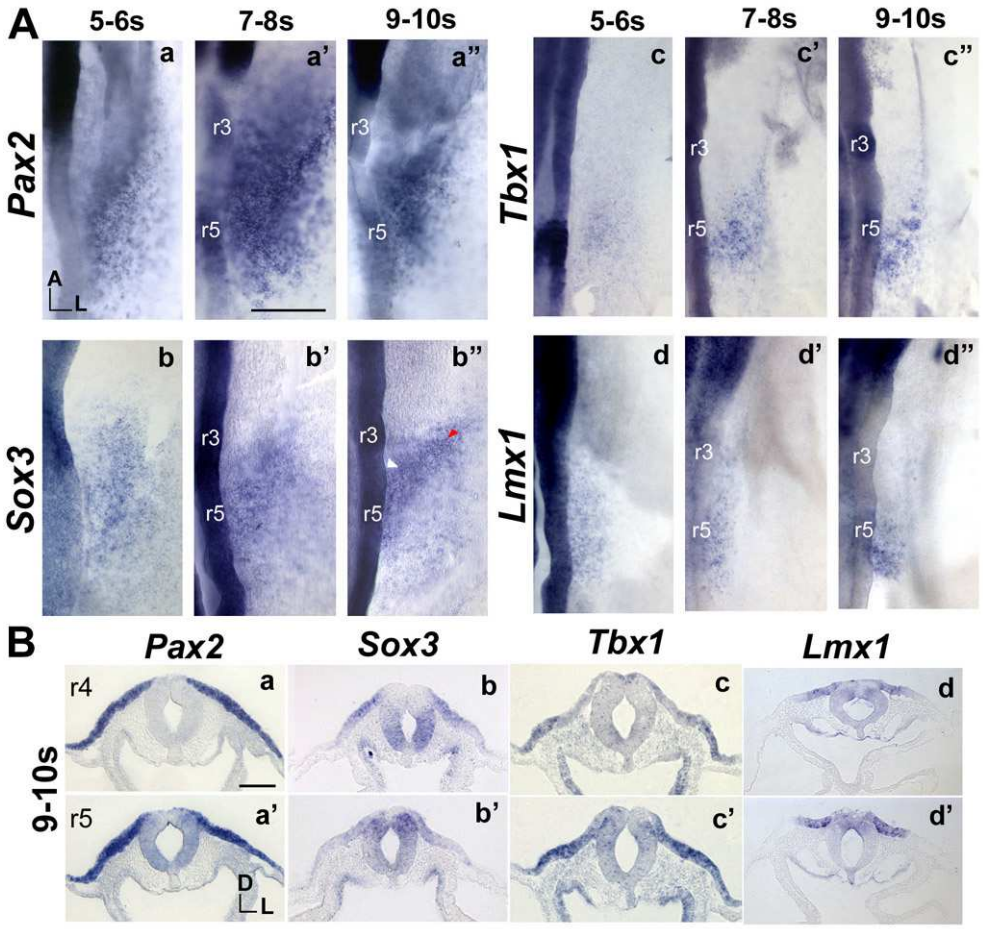

Figure 19. Otic regionalization occurs at the onset of otic placode formation. (A) Dorsal views of otic region from 5 to 10ss showing expression of *Pax2* (a-a''), *Sox3* (b-b''), *Tbx1* (c-c'') and *Lmx1* (d-d''). Note that *Sox3* was expressed in a band encompassing the otic territory and the geniculate placode (white and red arrowheads). (B) Transversal sections of 9-10 ss showing otic expression of *Pax2* (a-a'), *Sox3* (b-b'), *Tbx1* (c-c') and *Lmx1* (d-d'). *Pax2* is expressed in the otic placode from anterior to posterior. *Sox3* is highly expressed on the rostral sections of the otic placode, while *Tbx1* and *Lmx1* are highly expressed on caudal sections. Abbreviations: A, anterior; L, lateral; D, dorsal; s, somite; rhombomere. Scale bars in a' =200 μm, in a =100 μm.

Several observations can be extracted from these results. First, that most of the transcription factors regionalized in the otic cup in the neural and non-neural domains are already expressed before the otic placode is morphologically visible. Second, that *Tbx1* expression appears already only in the posterior otic field, while *Sox3* and *Lmx1* appear in a broader domain to progressively restrict their patterns. Third that *Sox3* high expression band encompasses a subdomain of the otic territory and the geniculate

placode, suggesting that this territory foreshadows a field with proneural character. Finally, that *Lmx1* regionalization is the latest event of this preotic regionalization.

3.2. Fate Mapping The Early Inner Ear

3.2.1. Common origin for geniculate placode and otic neurogenic.

Sox3 labels the neurogenic epibranchial and otic placodes (Rex et al., 1997; Abu-Elmagd et al., 2001; Ishii et al., 2001). The three epibranchial placodes develop close to the otic placode. The one anterior to the otic placode (geniculate) will generate sensory neurons from the VII ganglion, the two posterior to the otic territory (petrosal and nodosal) will generate the IX and X sensory ganglia, respectively. Ishii and coworkers had previously shown by fate mapping different populations of cells from the ectodermal *Sox3*-positive domain at 10 ss, that this area contained prospective otic and geniculate placodes (Ishii et al., 2001). However, we have shown that *Sox3*-positive cells comprised only the anterior region of the otic placode foreshadowing the proneural domain. In order to elucidate whether a common proneural domain was shared between the proneural competent otic domain and the geniculate placode, we performed DiI labeling in ovo in embryos of 8-11 ss and followed the DiI progeny until stage HH16. Only one population of cells within the *Sox3* domain was DiI-labeled and mapped by reference to the rhomobomere 4 and rhombomere 5 positions. After 22-24 hours of incubation, the position of DiI fluorescent cells was analyzed in relation to *NeuroD* or *NeuroM* expression patterns that were used as neuronal markers. Figure 20A shows a representative experiment (n=4) in which DiI cells were found in both, otic and geniculate, neuronal populations, but excluded from the non-neural region of the otic vesicle. The extension of the *Sox3* positive domain in a representative HH10 embryo is shown in FIG. 20Aa, and the initial DiI labeling of a different specimen in FIG. 20Ab. DiI-labeled cells were detected in the otic epithelium, the otic ganglion and the lateral ectoderm. Close analysis at different confocal microscope planes revealed DiI cells in the CVG labeling in a more medial focus (FIG. 20Ac and d), and in the neurogenic region of the geniculate ganglion in a lateral plane (FIG. 20Ae and f). Note that ventral neuroblasts adjacent to the branchial arches were not labeled (asterisk in FIG. 20Ae and f). Those cells were probably derived from the most anterior *Sox3* positive domain that was not injected with DiI (asterisk in FIG. 20Aa). Figure 20Ba-c illustrates the separation of the otic-geniculate proneural domain from HH10 towards HH14.

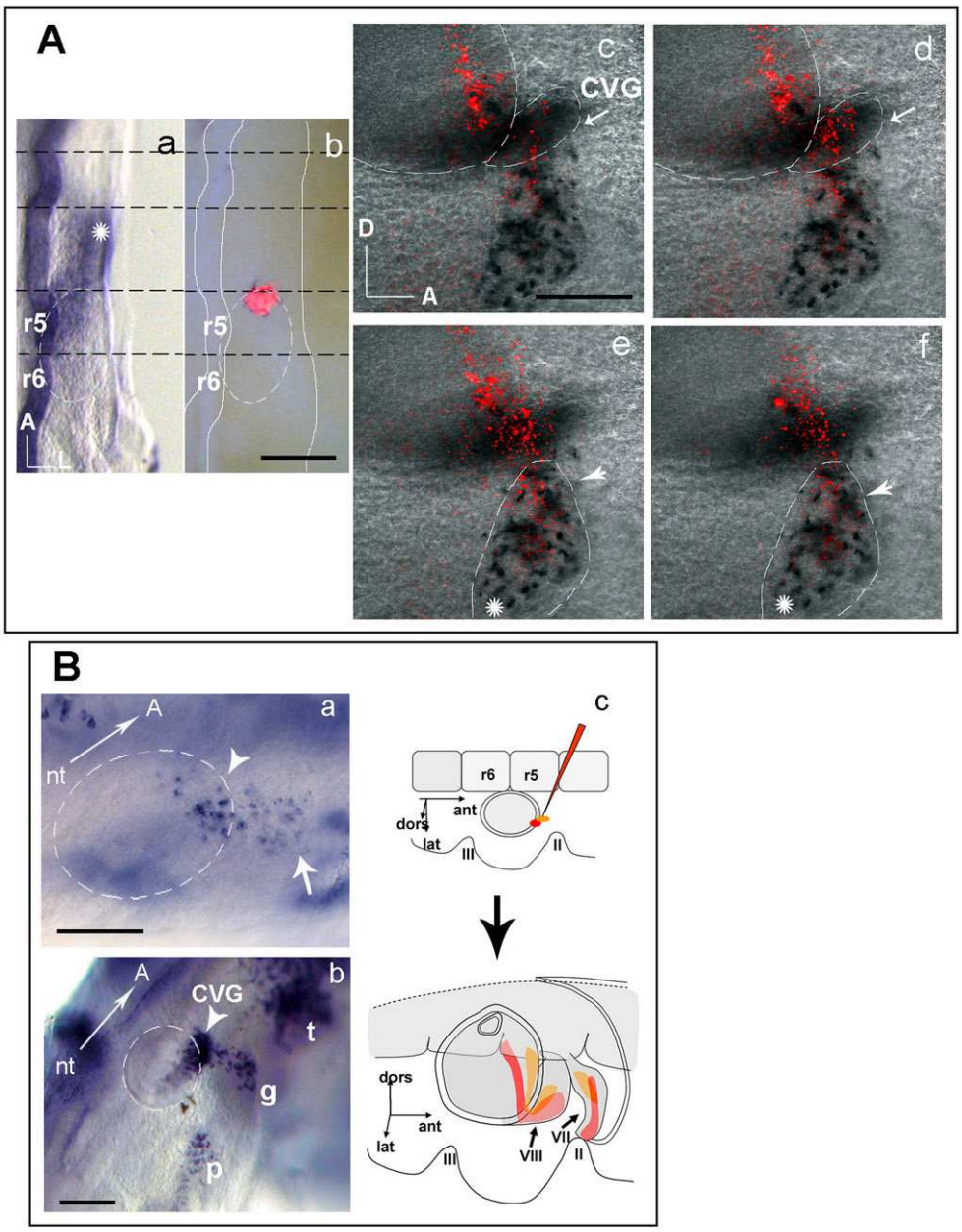

Figure 20. Shared otic and geniculate proneural domain. (A) *Sox3* and *NeuroD* expression stained by in situ hybridization. (a) Dorsal view of an otic placode at stage HH10. *Sox3* expression territory comprising the anterior otic region and the geniculate placode (asterisk). (b) Initial DiI in ovo injection at stage HH10. (c-f) Rostral, Merged confocal sagittal images of DiI-labeled cells and *NeuroD* expressing cells from medial (c) to lateral (f) planes. In c and d DiI-labeled cells are detected in the CVG (arrows), whereas in e and f are detected in the geniculate placode (arrows). Asterisk in e and f mark geniculate cells unlabeled with DiI. (B) *Delta1* and *NeuroD* expression stained by in situ hybridization. (a) Dorsolateral view of HH11 otic cup. Scattered cells expressing *Delta1* in the anterior region of

the otic cup (arrowhead) and at the geniculate placode (arrow). (b) Dorsolateral view of HH14 cup. *NeuroD* cells are detected in the otic epithelium, the CVG (arrowhead) and more laterally in the geniculate placode (g) that is moving lateral and ventral. (c) Schematic representation of the DiI experiment and the results of the injection after 24 hours of incubation. Abbreviations: A, anterior; L, lateral; D, dorsal; r, rhombomere; CVG, Cochleovestibular ganglion; nt, neural tube; p, petrosal placode; g, geniculate placode; t, trigeminal placode. Scale bars in Ab and Bb =200μm, in Ac and Ba =100μm.

We propose that at stage HH9 appears a *Sox3* positive territory capable of generating neurons that comprises the geniculate placode and the anterior region of the otic placode. This common field segregates in two regions as the otic and geniculate placodes move away from each other.

3.2.2. Fate mapping the proneural and non-neural domains

In order to study the dynamics of growth of the anterior and posterior regions of the otic cup, we performed a fate map study of the otic cup by single injections of the red fluorescent lipophylic cell membrane tracer DiI. Injections are represented by dividing the otic cup into a clock wheel where anterior was 3 o'clock, lateral 6 o'clock, and posterior at 9 o'clock (FIG. 21A). We followed a similar procedure used by Fekete and colleagues (Brigande et al., 2000a), but performing the injections at earlier stages (HH12) and not limiting to the otic rim. By stage HH12, the otic primordium was still flat and thus, in a dorsal view, injections at 12, 3, 6 and 9 positions were medial, anterior, lateral and posterior, respectively (n=58; FIG. 21A). 65% of the injections performed between positions 1 and 4 resulted in DiI labeled progeny cells in the anteroventral epithelium and CVG (light red, FIG. 21A and B), while in 100% of the injections at positions 6-11 (excluded from the ridge), the resulting labeled cells occupied the posterior domain without labeling delaminating neuroblasts (FIG. 21A and B, blue). On the other hand, in 70% of the injections at positions 4-6, the labeled cells extended dorsoventrally along the interface between anterior and posterior domains and also resulted in labeled ganglionar neuroblasts, suggesting that at these positions injections were at the limit between both regions (FIG. 21A and B, green). When labeling was performed in the posterior ridge of the otic placode, at positions 7-10, most of the cells (70%) developed into posterior-dorsal positions (FIG. 21A and B, black).

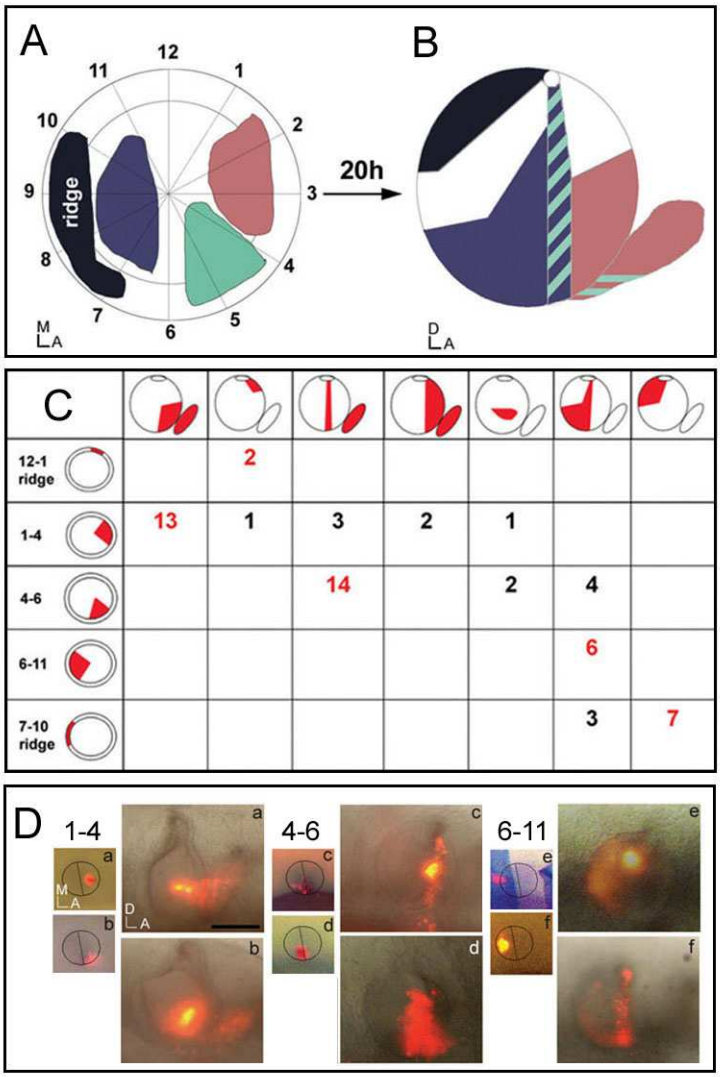

Figure 21. Fate map analysis of anterior and posterior domains from otic cup to otic vesicle stage. (A) Schematic drawing of an early otic cup (HH12) from a dorsal view. The cup is divided into 12 clock positions and the different initial DiI injected areas are represented in different colors. (B) Drawing of the otic vesicle in which final positions of DiI labeled cells are represented. (C) Table displaying a detailed analysis of the resulting labeled progeny examined. Rows indicate the position of initial injections. Numbers in columns indicate the occurrence of events with the shape and position similar to the schematic drawings of HH16-18 otic vesicles shown in the upper row. The most common condition is highlighted in red. (D) Representative examples of labeled cells originating from an anterior injection (1-4), lateral injection (4-6) and posterior injection (6-11). Abbreviations: A, anterior; D, dorsal; M, medial. Scale bar in Da = 200 μm.

Table (FIG. 21C) displays a detailed analysis of the shape and distribution of labeled progeny cells. Rows indicate the position of initial injections. Numbers in columns indicate the occurrence of events with similar shape and position to the schematic drawings shown in the upper row. Although the growth pattern of the otic cup is not extensively described here, our data indicated that the lateral wall predominantly developed from posterior epithelium, while the antero-ventral epithelium was derived from the most anterior placodal tissue. The dorsal otic pore was mainly generated by the posterior tissue, although labeling of the anterior medial rim at position 12-1 resulted in dorsal anterior progeny labeled cells (n=2/2). Several examples of the DiI labeled group of cells observed after 20 hours of incubation are shown in Figure 6D. Anterior labeled cells in the otic epithelium seemed to expand less than posterior cells. This probably is due in part to gross delamination of DiI labeled anterior cells into the CVG. It is also worth noting that labeled cells tended to remain contiguous and with little dispersion or splitting of the original group of labeled cells.

3.2.3. Proneural and non-neural cells undergo limited cell mixing

In order to study the degree of cellular exchange between the proneural and the posterior domains of the otic cup, we labeled otic cups of stage HH12 with two fluorescent dyes and followed their development until otic vesicle stage. The anterior and posterior regions were injected respectively with DiI (red vital dye) and with DiO (green vital dye). Labeled cells and their progeny were examined after 24h-incubation, when embryos reached stage HH16-17. At the stage of injection, gene expression patterns are clearly restricted (see Figure 17). Only non-overlapping initial injections that grew until touching each other were used for this analysis. Frequently, injections showed a sharp antero-posterior DiI/DiO interface at the lateral aspect of the otic vesicle (OV), running dorso-ventrally from the otic pore to the ventral aspect of the vesicle (n=10/13). Analysis by confocal microscopy revealed DiI and DiO labeled cells touching each other without mixing (n=10/15; FIG. 22A-A'). In a coronal view, a certain superposition of both dyes (yellow color) was observed in a 2-3 cells width, suggesting that at the lateral border cells labeled with the different dyes could intercalate (FIG. 22B-B', see inset in FIG. 24M). DiI labeled cells migrating away from the otic vesicle correspond to delaminating neuroblasts (arrowhead in Fig. 22A' and B'). When initial injections were performed only in the rim, we found that both labels mapped to the dorsal otic pore, as previously described by Brigande et al. (2000a) and shown above in the fate map analysis (FIG. 22C-C').

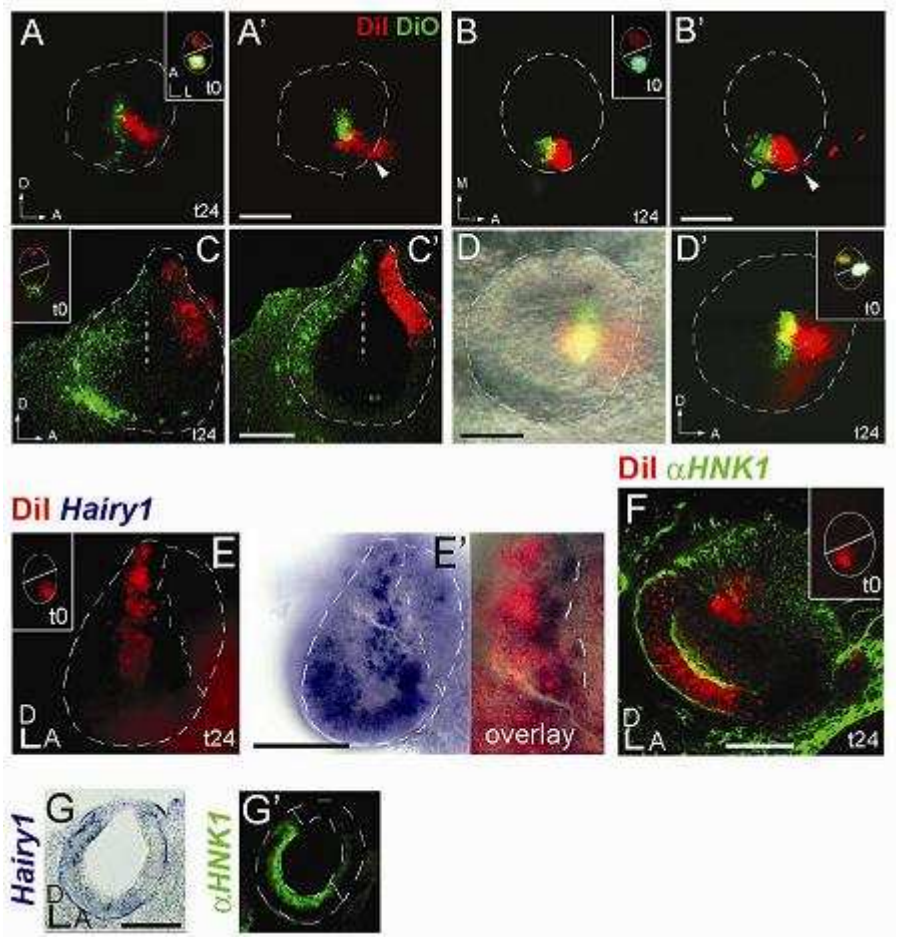

Figure 22. **Restricted cell intermingling between proneural cells and non-neural cells.** (A-D') Double labeling with DiI (red) and DiO (green) in ovo at stage HH12. (A-A') Merged confocal sagittal sections showing both dyes touching each other without mixing. (B-B') Merged confocal coronal images of an otic vesicle showing anterior DiI and posterior DiO labeled cells. Some cell intermingling was observed. Arrowhead points to DiI labeled neuroblasts coming out from the proneural domain (A'-B'). (C-C') Merged confocal sagittal sections showing that when DiI and DiO initial injections were performed at the ridge the labeled cells did not touch and reached the otic vesicle pore. (D-D') Mixing of DiI and DiO labeled cells was observed when the initial non-overlapping injections were not entirely in separate domains. Insets in A, B, C and D' depict the initial anterior injection (DiI) and posterior injection (DiO) in the otic cup shown dorsally. (E-E') Example of the growth of a posterior injection with DiI in a HH17 otic vesicle compared to the expression limits of *Hairy1* gene. (F) merged confocal para-sagittal sections showing another example of the growth of a posterior injection, now compared to HNK1 staining. (G-G') Para-sagittal section showing the expression profile of *Hairy1* transcript and immunostained for HNK1 epitope. Abbreviations: A, anterior; D, dorsal; M, medial; L, lateral; t0, initial time; t24, 24 hours of incubation. Scale bar in A' and B' =50 μm; C', D, E', F and G =100 μm.

In those cases in which DiI and DiO showed gross overlap, the initial injections, although separated, partially shared the anterior or posterior domains, indicating that cells intermingled when were labeled within a given domain (n=3/3; FIG. 22D-D').

To study whether labeled cells were contained within gene expression domains, the position of DiI labeled cells was analyzed in relation to the expression of anterior or posterior genes. Embryos with initial injections at the presumptive boundary (initial positions 4-6) were rejected. *FGF10* was used as an anterior marker and *Hairy1* as a posterior one. From the injections analyzed with the *FGF10* probe, we observed that almost all anterior DiI labeled cells stopped at the posterior edge of the *FGF10* expression limit (n=8/10; data not shown). Posterior labeled cells analyzed with *Hairy1* also indicated that DiI cells were contained in the *Hairy1* expression domain (n=6/8; FIG. 22E-E'). We also analyzed the expansion of posterior DiI labeled cells in relation to HNK1 epitope, which is restricted in the non-neural domain overlapping with *Hairy1* expression (FIG. 22F and G-G'). HNK1 is a sugar residue carried by many neural recognition molecules, including N-CAM, L1 and integrins and others. Again we observed that most cells respected the HNK1 limits (n=8/9). In summary, the data indicates that the expansion of cells was limited to the gene expression domains. Analysis was restricted to the lateral aspect of the otic vesicle where the limits of the AP domains were easily recognized. In the medial wall, however, the anterior-posterior boundary transforms into a dorso-ventral boundary (see Alsina et al., 2004 and Figure 17 for the position of the proneural domain throughout the formation of the otic vesicle).

3.2.4. Preliminary studies in otic neuroblast delamination

Epithelial neuroblasts delaminate from the ventral aspect of the otic cup/vesicle to form the CVG, where they differentiate and innervate back the vestibular and choclear sensory organs (Noden and van de Water, 1986; Hemond and Morest 1991b, Adam et al. 1998). In Alsina et al. (2004) it was reported that epithelial neuroblasts only delaminate along the posterior margin of the proneural domain by staining otic neuroblasts with anti-islet1/2 antibody and proneural domain with RNA probe specific for *FGF10* (Alsina et al., 2004 and Figure 23). In order to examine how otic neuroblast delamination was taking place, a real time imaging system was developed in Dr. D.Henrique laboratory. The reporter system was designed to follow migration and delamination of otic neuroblasts. The promoter region of the proneural bHLH *NeuroD*, gene known to drive progenitors to differentiate into neuronal lineage and to promote delamination of neuroblasts, has been highly studied and was therefore chosen to identify otic neuroblasts. The *NeuroD*

promoter was placed upstream of the Green Fluorescent Protein (GFP) that was used as an in vivo reporter (construct kindly given by Dr. J.Lee). HH13 embryos were elecroporated with the GFP under the control of the *NeuroD* promoter together with the Red Fluorescent Protein (RFP) that was used as a read out of the efficiency of that electroporation. Embryos were harvested after 22h hours in order to allow CVG formation. Analysis of electroporated embryos showed RFP localization all throughout the otic epithelium, while GFP protein was restricted to the proneural domain, confirming that the *NeuroD* promoter can only be activated in the proneral region. To monitor the behaviour of otic neuroblasts in vivo, otic vesicle sagittal slices were quickly prepared and visualized under confocal microscopy for periods up to 12h. Fluorescence images were recorded in a confocal Zeiss LSM510 microscope spanning 16 z stacks with 31.35μm step size at 2.09 min intervals up to 12h. The individual fluorescent cells were identified manually, and the stacks with higher fluorescent signal were followed throughout the time-lapse series to create the movies. Analysis of the fluorescent profiles of single cells revealed that otic neuroblasts were having a typical epithelial morphology, making contact with the basal lamina and the lumen of the otic epithelium. However, prior to delamination the shape of the neuroblast changed to became a rounder cell located in the basal aspect of the otic epithelium. Once rounder, the neuroblast detached from the otic epithelium to the CVG were it moved around (FIG. 23). Nevertheless, this technique presented few limitations. For instance, it was easy to lose the otic vesicle orientation after sectioning the electroporated vesicles with the McILTwain Tissue Chopper. Besides, it was hard to follow one neuroblast within the otic epithelium since the neuroblast density within the neurogenic placode was high. Moreover, in 12h movies only delamination of neuroblasts that were placed along the neural/non-neural limit could be detect. For this reason we could not analyze the migration profile of neuroblasts from the neurogenic domain to their delamination site. All in all, we could follow how otic neuroblasts delaminate from the otic placode. Recently, Graham et al., (2007) have illustrated that delamination of cells from neurogenic placodes does not involve an epithelial-to-mesenchymal transition instead; neuroblasts release neurogenic placodes through breach in the underlying basal lamina.

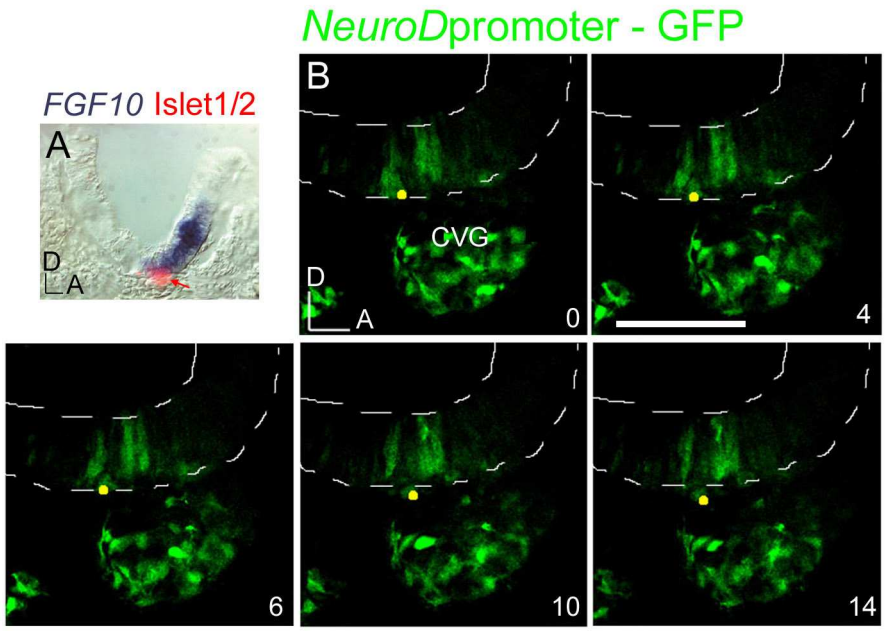

Figure 23. Real time imaging of NeuroD reporter at the single cell level. (A) Sagittal section of an otic cup showing *FGF10* expression in the neurogenic domain and islet1/2-positive neuroblasts. Notice that delamination site is at the posterior edge of proneural domain. Adapted from Alsina et al., (2004). (B) Single cell labelled with yellow dot expressing GFP under the control of NeuroD promoter (neuronal determination marker) was tracked from the otic epithelium to the CVG. Abbreviations: A, anterior; D, dorsal; CVG, cochleovestibular ganglion; Scale bar = 50μm.

3.3. Role Of Notch Pathway On Early Otic Patterning

3.3.1. Expression profile of Notch family members in the early steps of inner ear development

Ligands and targets of Notch signaling pathway are differentially expressed in the otic placode/cup

Notch is one of the main signalling pathways that mediate communication between different populations of cells. Through the lateral induction mechanism of action Notch can amplify one territory with a common identity that differs from the adjacent territory (Daudet and Lewis, 2005). Lateral induction can restrict Notch pathway activity along the boundary cells between adjacent territories as occurs, for example, at the *Drosophila* wing margin (Bray, 1998; de Celis and Bray, 1997) in the mouse and zebrafish interrhonbomeric boundaries (Amoyel et al., 2005; Cheng et al., 2004; Baek et al., 2006; Riley et al., 2004) and in the mouse ZLI (Baek et al., 2006). During inner ear development, Notch signaling has been implicated in the specification of the sensory territory (Kiernan et al., 2006; Chen et al., 2002; Kiernan et al., 2001; Tsai et al., 2001;

Daudet et al., 2007; Daudet and Lewis, 2005). In order to know if Notch signaling could be involved in early neural/non-neural otic regionalization, we mapped the expression of Notch signaling elements at early stages of otic cup regionalization.

At the core of the Notch signalling pathway is the transmembrane Notch receptor in one cell, interacting with the transmembrane ligand in a neighbouring cell. The best known Notch ligands belong to Delta-Serrate-Lag2 (DSL) family. When Notch signalling is initiated, NICD makes the switch from CSL-mediated repression to NICD/CSL/MAM activation, triggering transcription of the Notch target genes (Bray, 1998; Castro et al., 2005; Bailey and Posakony, 1995; Nellesen, 1999; Cave et al., 2005; Lamar and Kintner, 2005; Ong et al., 2005; reviewed in Mumm and Kopan, 2000). The best-characterized Notch targets are the bHLH (basic Helix Loop Helix) transcription repressors of the Hes (Hairy- Enhancer of Split) and Hrt (hes related type) family genes. This core signalling pathway is evolutionary conserved in the metazoan phyla. However, the number of paralogues of each element of the core pathway differs in the different animal models studied

Receptors:

In chick, only two Notch receptors are coded in the genome, *Notch1* and *Notch2*. *Notch1* was expressed in the entire otic placode (HH11) and cup (HH14) in a homogenous manner, as seen in a dorsal view in Figure 24G and H, an as observed in transverse section (FIG. 24N and FIG. 25A; also shown in Adam et al., 1998). Conversely, *Notch2* mRNA was expressed at low levels in the ectoderm surrounding the otic cup, but not in the otic epithelium (FIG. 24O and FIG. 25B).

Ligands:

One *Delta* gene (*Delta1*) and two *Serrate* genes (*Serrate1* and *Serrate2*) have been annotated in the chicken genome. At HH11, the Notch ligand *Delta1* was detected in scattered cells in the antero-lateral edge and by stage HH14 *Delta1* expressing cells extended throughout the entire proneural domain (compare FIG. 24C and D; also shown in Adam et al., 1998). Note that the otic ridge was devoid of *Delta1* except for the anterior-lateral stripe, where the otic and geniculate placodes were continuous (arrow in FIG. 24C). The Notch ligand *Serrate1* was expressed complementary to *Delta1* at early otic placode/cup stages. At HH11, *Serrate1* started to be detected in the otic ectoderm and it was already regionalized in all cells of the non-neural region (FIG. 24I and FIG. 25C). In addition, it was also observed in few cells of the geniculate placode (FIG. 24I, arrow). As development proceeded, expression of *Serrate1* was enhanced in the non-neural domain of the otic cup (FIG. 24J; also

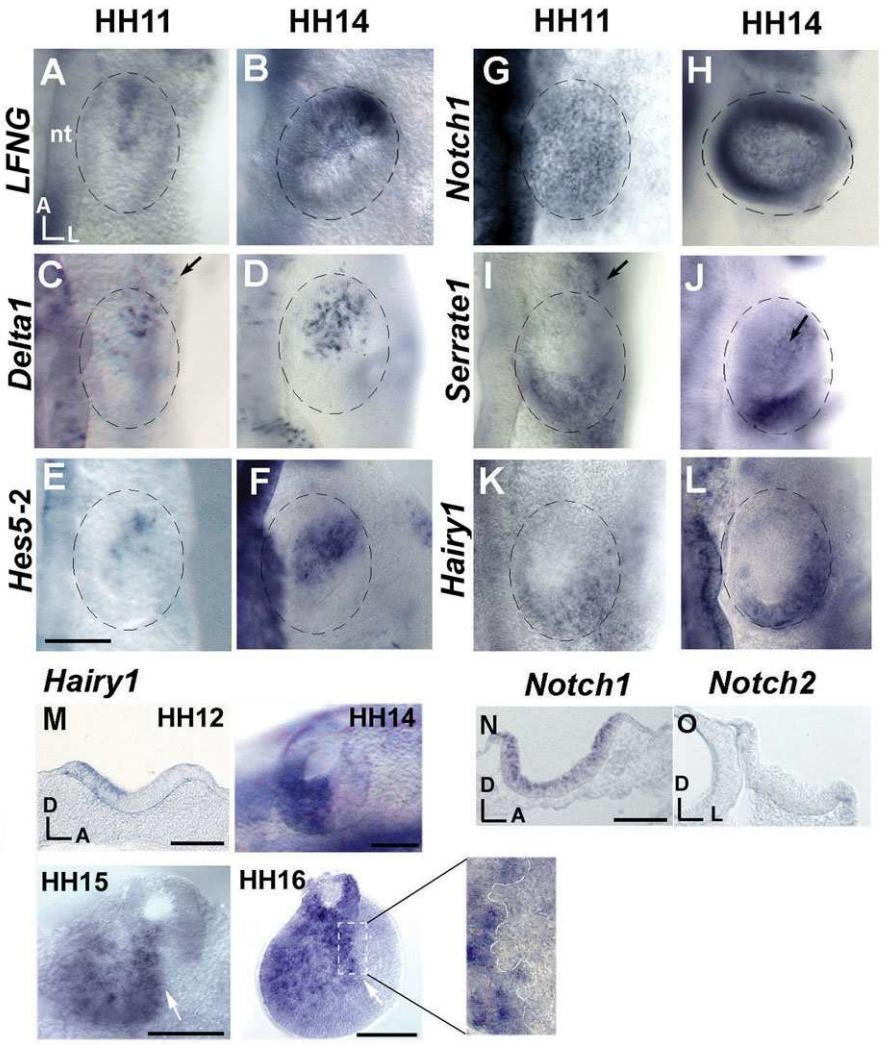

Figure 24. **Regionalized expression of the ligands and targets of Notch pathway in the proneural and non-neural domains**. Dorsal views of HH11 and HH14 otic cups. (A, C, E) Expression of *LFNG*, *Delta1* and *Hes5-2* at stage HH11. All genes were expressed in the proneural domain. Arrow in C indicates *Delta1* expressing cells from the geniculate placode. (B, D, F) Expression of the same genes in HH14 otic cups, showing that the expression of all genes has increased and has occupied the entire proneural domain. (G, I, K) Expression of *Notch1*, *Serrate1* and *Hairy1* at stage HH11. Arrow in I indicates very faint *Serrate1* labeling outside the otic placode. (H, J, L) Expression of the same genes at HH14. In J, arrow indicates scattered expression in the proneural territory. (M) The expression profile of *Hairy1* from otic placode to otic vesicle. Notice high levels of *Hairy1* mRNA on the lateral aspect of the otic vesicle (arrows). Inset in M shows *Hairy1* expressing cells intercalated with non-expressing cells along the lateral AP border. (N) Para-sagittal section of an HH13 otic cup showing luminal localization of Notch1 transcripts. (O) Transverse section of an HH11 otic cup showing faint expression of Notch2 outside the otic epithelium. Abbreviations: A, anterior; D, dorsal; L, lateral; nt, neural tube. Scale bars in E, M and N =100 μm.

shown in Cole et al., 2000) and new expression started in scattered cells within the proneural otic region (FIG. 24J, arrow). *Serrate1* expression in the anterior domain was delayed with respect to *Delta1* and to non-neural *Serrate1* expression. Moreover, *Serrate2* transcripts were not present in the otic territory by HH11 (FIG. 25D). Later on, when *Serrate1* was also distinguished in the proneural domain, *Serrrate2* started to be expressed in a scattered manner within the proneural domain.

Target genes:

In mammals, there are 5 different *Enhancer of Split* subfamily genes (*Hes2*, *Hes3*, *Hes5*, *Hes6* and *Hes7*). Hes3, Hes5 and Hes6 have a role in neural development, but only *Hes5* unequivocally responds to Notch signaling (Nishimura et al., 1998; Ohtsuka et al., 1999; de la Pompa et al., 1997; Koyano-Nakagawa et al., 2000; Bae et al., 2000). In chicken, *Hes5* and *Hes6* have been noted to the genome. The chicken genome contains three genes encoding proteins with strong homology to the mammalian HES5 protein, *Hes5-1*, *Hes5-2* and *Hes5-3* (Fior and Henrique, 2005). In the otic cup, *Hes5-1*, *Hes5-2* and *Hes5-3* were expressed in groups of cells within the proneural domain (*Hes5-2* expression profile shown in FIG. 24E and F, FIG. 25E). Close attention to the early expression of *Delta1* and *Hes5-2* in the otic placode showed that *Delta1* expression initiated in single spaced cells (FIG. 28H), while H*es5-2* was expressed in the same territory but in groups of cells (FIG. 28I). Double fluorescent in situ hybridization revealed that *Delta1* and *Hes5-2* transcripts were indeed expressed in adjacent cells within the proneural epithelium (FIG. 28J and K). Besides, two homologues of the mammalian *Hes6* are present in the chick genome, *Hes6-1* and *Hes6-2*. *Hes6-2* was expressed in group of cells in the proneural territory within the *Hes5-2* expression domain from otic placode to late otic cup (FIG. 25F), but its expression was lost at otocyst stage.

On the other hand, the expression of the mammalian *Hes1*, belonging to the Hairy subfamily of genes, is not regulated by Notch signaling (Hatakeyama et al., 2004). However, *Hes1* maintenance could be Notch-dependent (Hatakeyama et al., 2004; Jarriault et al., 1995; Ohtsuaka et al., 1999). In chick, two different *Hes1* homologues exist, *Hairy1* and *Hairy2* (Palmeirim et al., 1997; Jouve et al., 2000). *Hairy1* was uniformly expressed in the non-neural territory from very early stages and was complementary to *Hes5* (FIG. 24K-L). At 13 ss (HH11) *Hairy1* started to be expressed in the otic ectoderm already regionalized in the posterior domain. From lateral views, it was evident that *Hairy1* expression spanned throughout the posterior territory and the major lateral epithelium (FIG. 24M and FIG. 25G). Interestingly, high levels of *Hairy1* expression were found at the interface between proneural and non-neural territories at late otic cup stage

(arrows in FIG. 24M). Analysis at high magnification of the anterior *Hairy1* expression limit showed that the border of *Hairy1* was not perfectly straight, suggesting again the intercalation of cells between anterior and posterior domains (white line in FIG. 8M). On the contrary, *Hairy2* was not detected at otic placode stage, while was expressed throughout the otic cup from anterior to posterior (FIG. 27H)

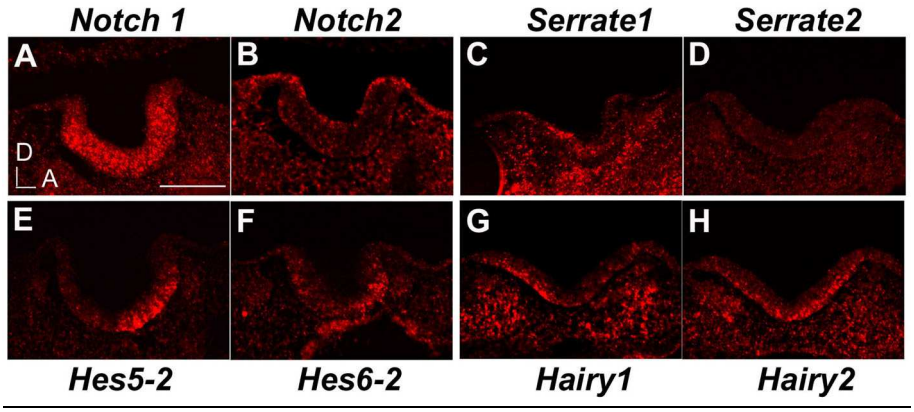

Figure 25. **Regionalized expression of Notch pathway receptors, ligands and targets in the proneural and non-neural domains**. Para-sagittal section showing the expression profile of *Notch1* (A), *Notch2* (B), *Serrate1* (C), *Serrate2* (D), *Hes5-2* (E), *Hes6-2* (F), *Hairy1* (G) and *Hairy2* (H) at stage HH11-12. In situ hybridization was developed with tyramide-Cy3 fluorochrome. Abbreviations: A, anterior; D, dorsal. Scale bars in A =100 μm.

Modulators:

Fringe genes are glycosyl-transferases known to regulate the activity of Notch through glycosylation. Fringe proteins glycosilate Notch extracellular EGF repeats in the Golgi apparatus (Moloney et al., 2000; Bruckner et al., 2000; Munro and Freeman, 2000). As a consequence, Fringe modifies the affinity between Notch and its ligands Delta and Serrate (Fleming et al., 1997; Panin et al., 1997). There are three vertebrate homologous, *Lunatic* (*LFNG*), *Radical* (*RFNG*) and *Manic Fringe* (*MFNG*). However, the ability of Fringe molecules to suppress Notch activation varies between the different Notch and Fringe molecules. *LFNG* expression in the otic territory started at HH11 in cells detected in the anterior-lateral domain (FIG. 24A). By stage HH14, its expression expanded medially, and *LFNG* was observed in all cells of the anterior otic cup (FIG. 24B, also shown in Cole et al., 2000). Although we did not detect *Radical Fringe* nor *Manic Fringe* transcripts in the otic cup, *Manic Fringe* has been reported to be expressed in the otocyst in both zabrafish and mammals (Johnston et al., 1997; Qiu et al., 2004).

In summary, these experiments show that several members of the Notch signaling pathway are regionalized in the otic cup and map to the proneural and non-neural regions.

3.3.2. Functional analysis of the Notch pathway: Loss of function.

3.3.2.1. Gamma-secretase inhibitor treatment (DAPT)

Notch blockade induces the expansion of Lmx1 and Irx1 expression into the proneural domain

To explore the function of Notch pathway in early otic development, activity of the Notch pathway was blocked in chick embryo explants exposed to the γ-secretase inhibitor (N-[N-(3,5-diuorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, DAPT inhibitor) (Dovey et al., 2001; Geling et al., 2002). The activity of the γ-secretase is specifically required for cleavage of the intracellular domain of Notch (NICD) that leads to gene activation. Experiments were performed in embryos at HH9-10 (6-10 somites), before the establishment of anterior-posterior regionalization. Notch inhibition had two different effects on the otic placode: on the one hand induced the expansion of non-neural genes into the proneural domain and, on the other, resulted in the overproduction of neuronal precursors.

The effects of Notch inhibition on posterior genes are illustrated in Figure 26. The expression of the Notch target gene, *Hairy1,* was inhibited after DAPT treatment indicating that DAPT inhibitor was effective in inhibiting Notch signaling (n=8/8; FIG. 26A-A'). The effects on *Serrate1* expression were more complex. Surprisingly, the posterior expression of *Serrate1* was unaffected by DAPT, whereas the anterior expression of *Serrate1* was reduced or abolished (n=10/10; FIG. 26B-B', arrow). This suggests multiple roles for *Serrate1*, some of them not totally dependent on Notch activity.

However, the most striking observation from these experiments was that *Lmx1* and *Irx1* expanded into the anterior domain after DAPT incubation. After DAPT treatment, *Lmx1* expression was detected throughout the anterior proneural domain and in the anterior-lateral ridge from where it is normally excluded (n=12/15; compare control and DAPT in FIG. 26C-C'). Sagittal sections in Figure 26E'-F´ show that the expression of *Lmx1* gene invaded the proneural domain of the otic cup overlapping

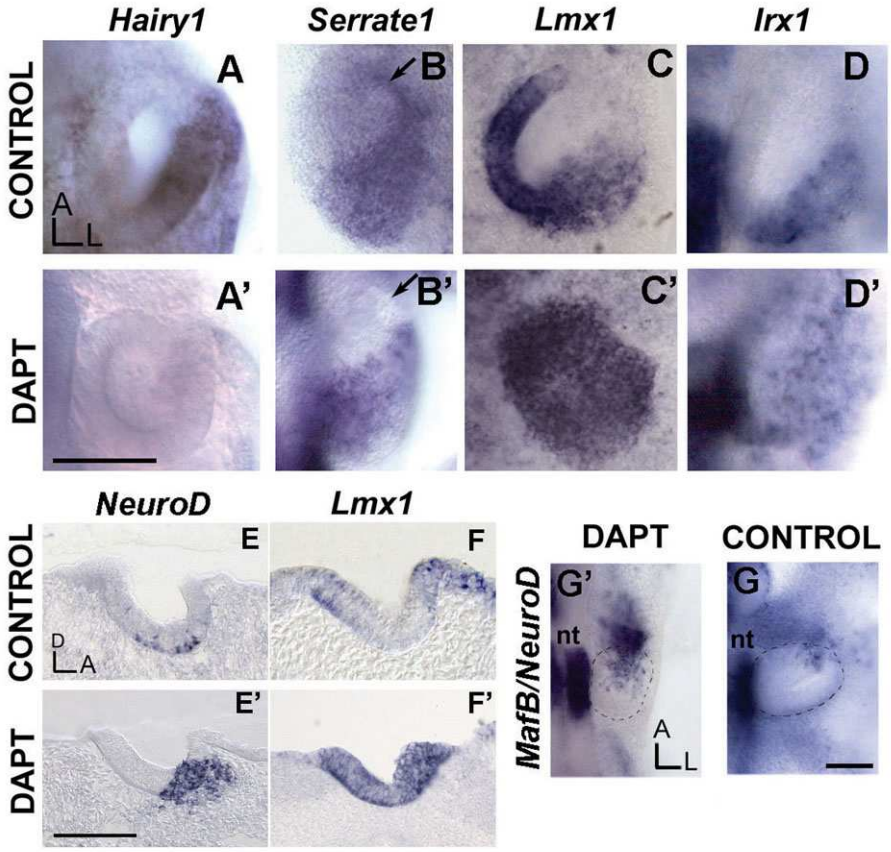

Figure 26. Blockade of Notch signaling disrupts posterior regionalization of *Lmx1* and *Irx1*. Effects of inhibiting the Notch signaling by incubation of HH9 explanted embryos with 100 μM DAPT. Dorsal view of otic cups after 24 hours of incubation. (A, A') *Hairy1* expression was inhibited after DAPT treatment. (B, B') *Serrate1* expression in the non-neural territory was not disturbed but expression in the proneural domain was suppressed (arrows). (C, C') *Lmx1* expression is detected throughout the entire otic cup after DAPT treatment. (D, D') A similar effect is observed for *Irx1* expression. Sagittal sections of Control (E, F) and DAPT treated (E', F') embryos stained with *NeuroD* and *Lmx1*. Note high expression of *NeuroD* cells and *Lmx1* cells in the proneural domain. (G, G') *MafB* and *NeuroD* double staining in control and DAPT treated embryos, showing no effects on hindbrain patterning after DAPT treatment. Abbreviations: A, anterior; L, lateral; D, dorsal; nt, neural tube. Scale bars in A', E', G =100 μm.

with the expression of *NeuroD*, a gene expressed in the proneural domain. DAPT treatment also induced the anterior expansion of the other major posterior gene *Irx1*, although it was not homogeneous and it always exhibited a higher expression in the posterior domain (n=25/33; FIG. 26D-D').

It has been described that disruption of hindbrain patterning leads to defects in otic regionalization (Kwak et al., 2002; Lecaudey et al., 2007) and also that Notch is involved

in hindbrain segmentation (Cheng et al., 2004). Therefore it was possible that the effects of DAPT were secondary to the disruption of neural tube AP regionalization. To test this possibility we examined the expression of *MafB,* a transcription factor expressed in rhombomeres 5 and 6 (Eichmann et al., 1997). After DAPT treatment, the expression of *MafB* was unaffected, suggesting that DAPT did not disrupt hindbrain patterning (n=6/6; FIG. 26G-G', *MafB* and *NeuroD* transcripts detected both in blue).

Occasionally, DAPT-treated otic cups showed a smaller size and hampered invagination, suggesting that a reduction in cell proliferation may have occurred in those experiments. Since in some tissues Notch signaling maintains the self-renewal potential or induces cell differentiation, we tested the effects of Notch blockade on cell proliferation by assaying for BrdU incorporation. Explants (HH9-10) were incubated with BrdU for the last 2 hours of the culture period and assayed for BrdU incorporation by immunofluorescence. Although the difference was not statistically significant, BrdU uptake after DAPT treatment tended to be below control values (control:DAPT, 12.8:10.2 (cell/au), n=12:n=18, $p \leq 0.08$). This suggests that the effects of DAPT on patterning were not related to changes in cell proliferation.

In summary, these experiments show that Notch activity is required for restricting the expression of *Lmx1* and *Irx1* to the non-neural domain, and that the non-neural expression of *Serrate1* is Notch-independent.

Effects of Notch on Lmx1 and Irx1 are not due to increased cell mixing

The expansion of *Lmx1* and *Irx1* to the anterior domain could be caused by the migration of cells with posterior identity towards the anterior compartment, and/or by the altered regulation of the expression of those genes. To further study this problem, explants were labeled with DiI in the posterior domain and then incubated with DAPT. Initial injections were performed at HH10. Figure 27 shows an example of posterior injections after incubation in control conditions (FIG. 27A) or with DAPT (FIG. 27A'). None of the injections showed DiI-labeled cells invading the anterior domain after incubation for 24 hours with DAPT (n=4/4 control, n=6/6 DAPT). In these experiments, in situ hybridization against *Irx1* transcripts confirmed that DAPT treatment was effective in expanding *Irx1* towards the anterior domain (FIG. 27B-B').

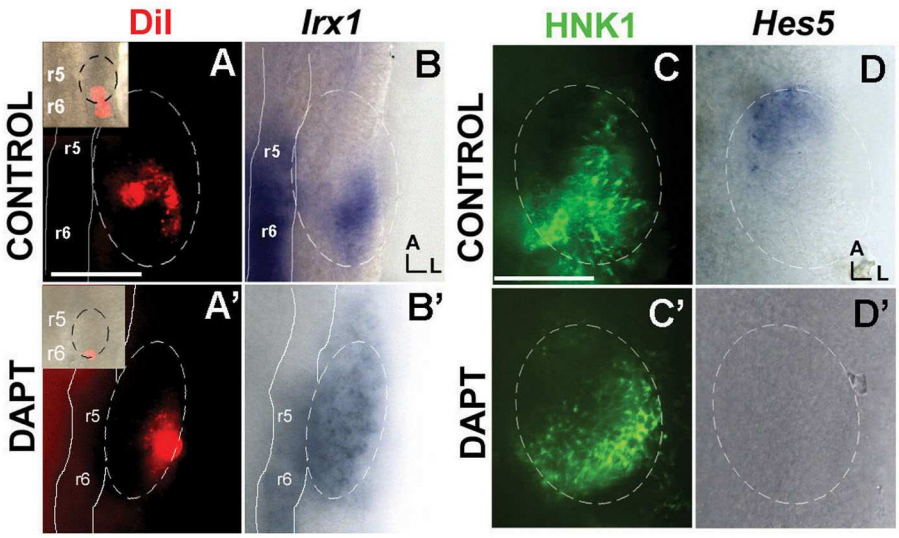

Figure 27. Notch blockade does not affect cell migration. (A-B') DAPT treatment was combined with DiI injection to reveal whether posterior cells invade the anterior domain. (A) DiI labeled cells in control conditions. (A´) After culturing embryos with DAPT inhibitor DiI remained in the posterior territory. (B, B') *Irx1* expression in the posterior domain in control conditions and expansion of *Irx1* expression after DAPT treatment. Inset in A and A' shows the initial DiI injection. HNK1 epitope staining was compared in control (C) and DAPT treated embryos (C') and no invasion of the anterior domain was observed after DAPT treatment. (D, D') Inhibition of *Hes5* expression in the proneural domain in the same embryos indicates effectiveness of the DAPT treatment. Abbreviations: A, anterior; L, lateral; r, rhombomere. Scale bars in A and C = 100 μm.

If the effects of Notch blockade were related to cell migration, we would also expect that the HNK1 epitope present in posterior cells would also loose its posterior restriction after DAPT treatment. Figure 27C-C' shows that this was not the case and, after DAPT treatment, HNK1 labeled cells were still present in the posterior domain and did not invade the anterior domain (n=24/24), suggesting that those cells carrying the HNK1 epitope remained restricted to their normal territory. DAPT activity in this experiment was assessed by *Hes5-2* (hereafter referred as *Hes5*) expression (FIG. 27D-D'). Altogether, these data reinforce the notion that most probably the anterior expansion of *Lmx1* and *Irx1* was not due to migration of *Lmx1* and *Irx1*-positive cells from posterior to anterior, but to up-regulation of their expression in the anterior domain.

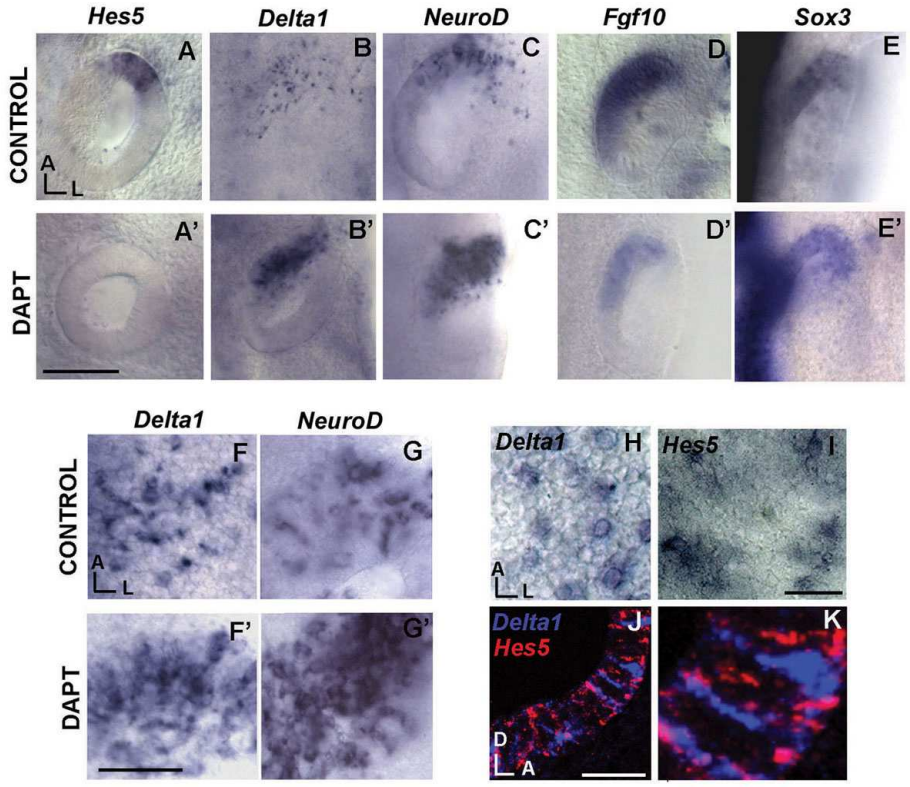

Figure 28. Blockade of Notch signaling induces the overproduction of neuronal precursors. Effects of inhibiting the Notch signaling by incubation of HH9 explanted embryos with 100 µM DAPT. Dorsal view of otic cups after 24 hours of incubation. (A, A') *Hes5* expression was abolished after DAPT treatment. (B-C') Compared to control explants, the number of neuronal cells (*Delta1* and *NeuroD* expressing cells) was dramatically increased. (D, D') The domain of *FGF10* expression was not affected by DAPT treatment, but decreased levels of *FGF10* expression were observed. (E-E') *Sox3* expression did not change after DAPT treatment. High magnification images of *Delta1* (F, F') and *NeuroD* (G, G') expressing cells in control embryos and embryos after DAPT treatment. (H) High magnification image of otic epithelium at stage HH12 with single *Delta1* expressing cells. (I) At the same stage, *Hes5* was expressed in clusters of cells. (J) Sagittal section of an otic cup showing *Delta1*-expressing cells (blue) and *Hes5* expressing cells (red) detected by double fluorescent ISH. (K) High magnification image of *Delta1* cells adjacent to *Hes5* cells. Abbreviations: A, anterior; D, dorsal; L, lateral. Scale bars in A' = 100 µm, I = 20 µm, F' and J = 50 µm.

Blockade of Notch signaling increases the number of neuronal precursors without affecting the size of the proneural domain

In the proneural domain, Notch signaling inhibition by DAPT resulted in the abolition of the expression of *Hes5,* a direct target of Notch signaling activation (n=12/12; FIG. 28A-A'), In parallel, the density of *Delta1* and *NeuroD* positive cells highly increased in the proneural domain (n=11/14 for *Delta1,* n=15/24 for *NeuroD*; FIG. 28B-B'and 28C-C'). The

increase in the number of *Delta1*-positive cells was 3.9-fold (control 5.6±3.7; 21.6±5.7 (cells/au); p≤0.001; compare magnifications FIG. 28F-F') and 3.3-fold for *NeuroD*-positive cells (control 8.4±5.7, DAPT 27.9±8.1 (cell/au); p≤0.001; FIG. 28G-G'). The observed effects on neurogenesis fit with the expected role of Notch in regulating the number of neuronal precursors by lateral inhibition (de la Pompa et al., 1997).

In summary, these experiments indicate that as suggested in previous work in zebrafish (Haddon et al., 1998), Notch is required in the proneural domain for inhibiting neuronal fate through a classical mechanism of lateral inhibition. Note that despite the increase in the number of neuronal precursors after DAPT, they were always restricted to the proneural domain of the otic placode. The establishment of the proneural compartment was not affected, and this was also revealed by assessing *FGF10* (n=14/14) and *Sox3* (n=11/12) expression (FIG. 28D-D' and FIG. 28E-E' respectively). The levels of *FGF10* expression after DAPT treatment were consistently lower than in control conditions (n=12/14; FIG. 28D-D'), suggesting that there is some link between Notch signaling and FGF function.

3.3.2.2. Electroporation of a dominant negative form of mastermind-like1

Blockade of Notch with dominant-negative Mastermind-like1 upregulated Lmx1

In order to analyze the cell-autonomous effects of Notch signaling in cell fate and cell affinity during otic development, we took advantage of the electroporation technique to locally block the Notch pathway. A GFP-tagged dominant-negative form of the human Mastermind-like1 (DN-MAML1; gift from Dr. J.Aster) was electroporated into the otic placode at stage HH9-10. This approach also allowed us to exclude possible effects of DAPT inhibitor on surrounding tissues. MAML1 binds to the ankyrin repeat domain of Notch receptors, forming a DNA-binding complex with NICD and CSL transcription factor to activate Notch target genes. The truncated form of MAML1 (only aminoacids 1-302) binds to the CSL:NICD complex creating a complex that is incapable of Notch signaling (Weng et al., 2003). The effectiveness of DN-MAML1 construct in chick was analyzed on *Hairy1* expression as our previous experiments with DAPT inhibition indicated that maintenance of *Hairy1* expression was Notch-dependent. *Hairy1* down-regulation in the non-neural domain was observed in electroporated cells, indicating that in ovo electroporation of the DN-MAML1 was efficiently blocking Notch pathway (n=104/124 cells (84%) from 7 different embryos; FIG. 29I-J'). Electroporated cells were detected by immunohistochemistry against

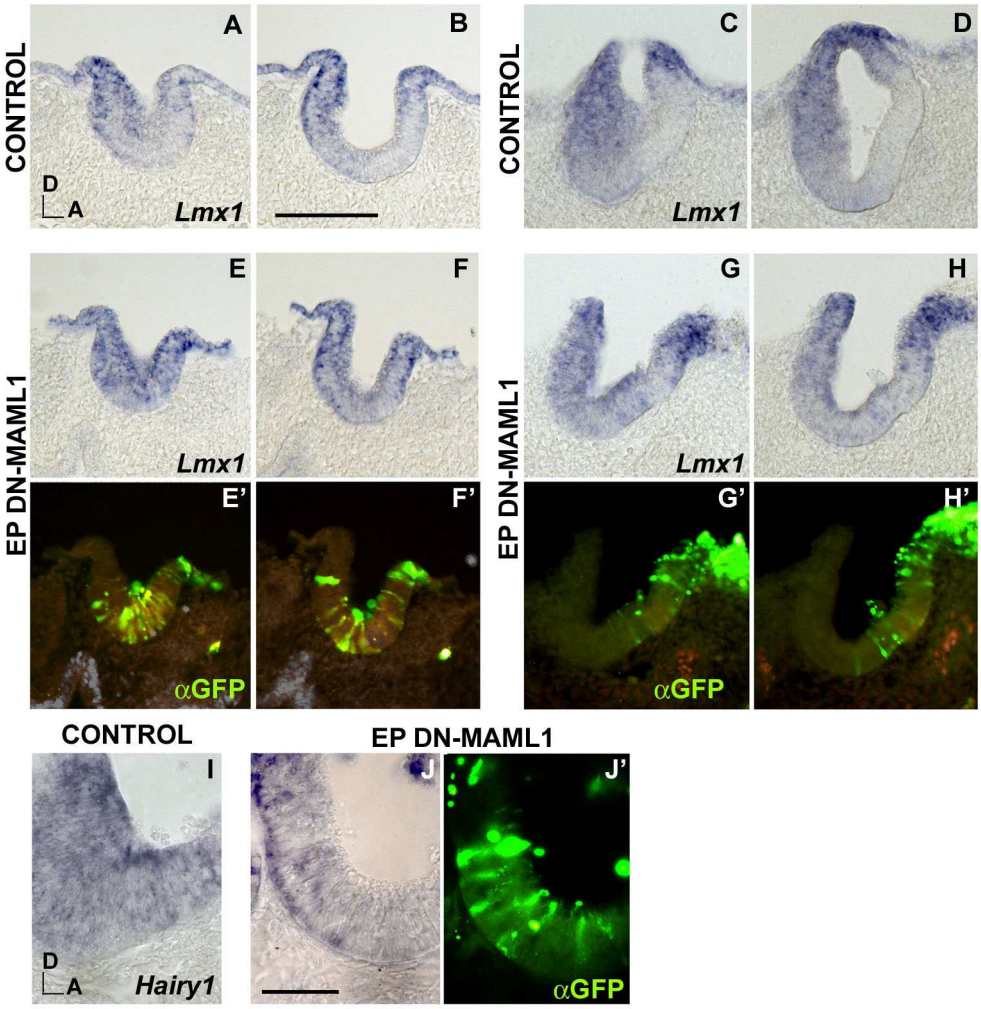

Figure 29. *Lmx1* expression was enhanced in the proneural domain after blocking Notch pathway. (A-D) *Lmx1* expression profile in the non-electroporated otic vesicle of two different embryos. (E-H) *Lmx1* transcripts in the DN-MAML1-electroporated otic vesicle. (E'-H' and J') Localization of the electroporated cells by immunohistochemistry using anti-GFP antibody. (I-J') High magnification of *Hairy1* expression in the non-neural domain of control otic vesicle (I) and electroporated with DN-MAML1 (J). Abbreviations: A, anterior; D, dorsal. Scale bars in B = 100 μm; J = 20 μm.

GFP epitope (green cells in FIG. 29). The effects of DN-MAML1 on *Lmx1* were analyzed by examining the extension of *Lmx1* expression domain. Consistent with our previous observations using DAPT inhibitor (described above), *Lmx1* was expanded into the proneural domain after Notch blockade (n=5/6; FIG. 29A-H'). Furthermore, we examined whether the electroporated cells ectopically expressed *Lmx1* in the proneural domain. We found that most of the cells positive to DN-MAML1 did also express *Lmx1*, indicating that the inhibition of the Notch pathway upregulates *Lmx1* in a cell-autonomous manner

(88/140 cells (63%) from 5 different embryos). In the hindbrain, it has been reported that electroporated cells in which Notch signaling was specifically blocked with a dominant-negative Su(H) (or CSL) preferentially distributed in non-boundary regions (Cheng et al., 2004). In our experiments, we could not detect a preferential location of DN-MAML1 electroporated cells. In summary, these experiments indicated that Notch signaling can regulate the transcription of *Lmx1* in a cell-autonomous manner, independently of surrounding tissues.

3.3.2.3. Mouse RBP-Jkappa mutant embryos

To further analyze the effects of Notch signaling in cell fate and regionalization during otic development, we decide to analyze the RBP-Jkappa (Jk) mutant mice generated by Dr. J.L. de la Pompa. RBP-Jk protein of this mutant is disrupted in the exon 7 (Oka et al., 1995), which contains an integrase motif that has been shown to be important for the sequence-specific DNA-binding capacity of RBP-Jk by site directed mutagenesis analysis (Chung et al., 1994). When NICD translocates to the nucleus binds to the RBP-Jk transcription factor (also called CBF-1) and to the Mastermind co-activator, forming a ternary complex that activates Notch target genes expression. The mutated form of RBP-Jk prevents the binding of the ternary complex to DNA, thus blocking Notch signalling activation. Although RBP-Jk mutants did not show any difference in otic placode size, the otic vesicles presented a reduction of the 18% in size, indicating a role of Notch pathway in otic growth/morphogenesis (n_{wt}=6, $n_{RBP\text{-}jk}$=12; FIG. 30). It has been reported in RBP-Jk mutant embryos that *Hes5* is downregulated in the neural tube and geniculate placode, while *Mash1* and *Dll1* are upregulated, suggesting that activation of Notch signalling negatively regulates the formation of neurons in the neural tube of the mouse (de la Pompa et al., 1997). However, the effects on otic neurogenesis and patterning were not explored. E8.75 embryos were sent to us and we observed that the RBP-Jk mutant embryos were expressing *NeuroD* (n=2/4; FIG. 30H-J), while it was not detectable in the wild-type littermates (n=0/4; FIG. 30E-G). This observation suggests that, as it occurs in the neural tube, Notch pathway negatively regulates otic neurogenesis and, as a result, we observed precocious generation of early differentiating neurons.

Furthermore, we analyze the expression profile of two non-neural markers, *Lmx1* and *Irx1*, at otic vesicle stage (E9.5) in the absence of Notch signalling. Although blocking Notch pathway in chick embryos resulted in *Lmx1* and *Irx1* expansion in the proneural domain, none of the otic vesicles analysed from the RBP-Jk mutants were showing

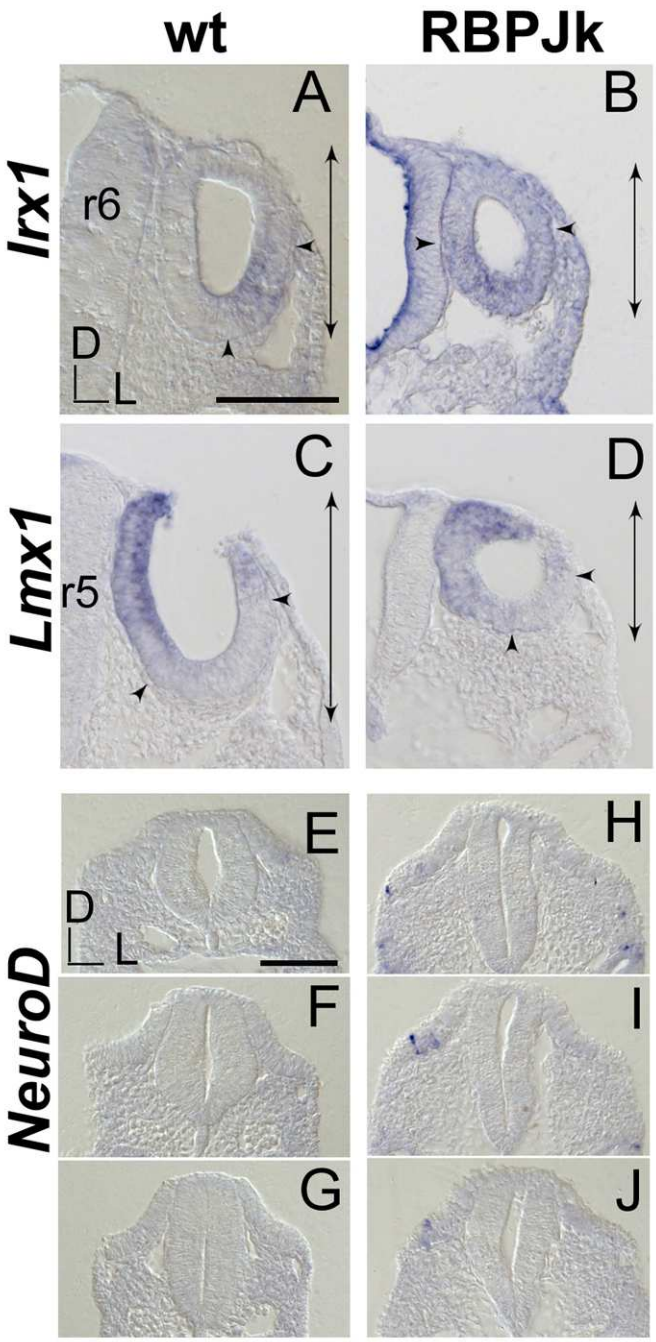

Figure 30. Blocking Notch pathway in mouse did not lead to upregulation of *Lmx1* and *Irx1* in the neurogenic domain. Comparison of *Irx1* and *Lmx1* expression on transversal sections of wild-type 9.5 dpc otic vesicle (A and C) and RBPJk 9.5dpc mutant embryos (B and D). Comparison of *NeuroD* expression on transversal sections of wild-type 8.5 dpc otic placode (E-G) and RBPJk mutant embryos (H-J). Abbreviations: D, dorsal; L, lateral; r, rhombomere. Scale bar in in A and E = 100 μm.

Lmx1 and *Irx1* transcripts all throughout the otic epithelium. However, a slight increase of the otic epithelium expressing *Irx1* (5%; n_{wt}=2, $n_{RBP\text{-}jk}$=6; FIG. 30A-B) and *Lmx1* (11%; n_{wt}=4, $n_{RBP\text{-}jk}$=6; FIG. 30C-D) was shown in RBP-Jk mutants in comparison with wt mouse embryos.

3.3.3. Functional analysis of the Notch pathway: Gain of function

3.3.3.1. Electroporation of the Notch1 intracellular domain

Notch signaling pathway regulates the expression of Lmx1 and Irx1 genes

To further analyze if Notch signaling pathway can regulate the expression of the non-neural genes, *Lmx1* and *Irx1*, the intracellular domain of Notch (NICD) was electroporated into the otic placode at stage HH9-10 (construct gift from Dr. J.L. de la Pompa). Interaction of Notch with either of its ligands results in a series of proteolytic events, liberating the intracellular domain of the Notch1 receptor (NICD) into the cytoplasm, to be then translocated to the nucleus and used to activate downstream genes (de la Pompa et al., 1997; Fortini, 2001). No effects on otic closure were seen in electroporated otic vesicles compared to control vesicles, indicating that overactivation of Notch pathway did not interfere with otic formation and early morphogenesis.

As control, the construct was first electroporated into the caudal neural tube of HH11-12 stage embryos where neurogenesis is initiating (Diez del Corral et al., 2002). We observed an upregulation of *Hes5* in the electroporated side of the neural tube already after 4-6 hours of incubation (n=8/8), indicating that in ovo electroporation of the NICD was indeed capable of overactivating Notch downstream genes (FIG. 31 A-B''). We then analyzed the effects of constitutive activation of Notch pathway on the otic field. Effects of Notch activation are shown in Figure 31 and Figure 32 by a series of transverse sections. Electroporated cells were detected by immunohistochemistry against myc epitope (green cells in FIG. 31A', B'', D', F', H' and FIG 32B', D', F'). In order to analyze the effects of constitutive Notch activation on Notch direct target genes (*Hes5* and *Hairy1* genes), electroporated embryos were incubated for only 6 hours. When the effects on *NeuroD*, *Lmx1* and *Irx1* gene expression were analyzed, electroporated embryos were incubated for 12 hours. NICD caused an increase of *Hes5* cells in the anterior domain and, furthermore, was also able to overactivate *Hes5* expression in the posterior domain (FIG. 31C-D') (n=5/5). In addition to increase the number of *Hes5* cells, NICD was able to reduce the number of *NeuroD* expressing cells generated in the proneural domain (FIG. 31E-F'). However, we found

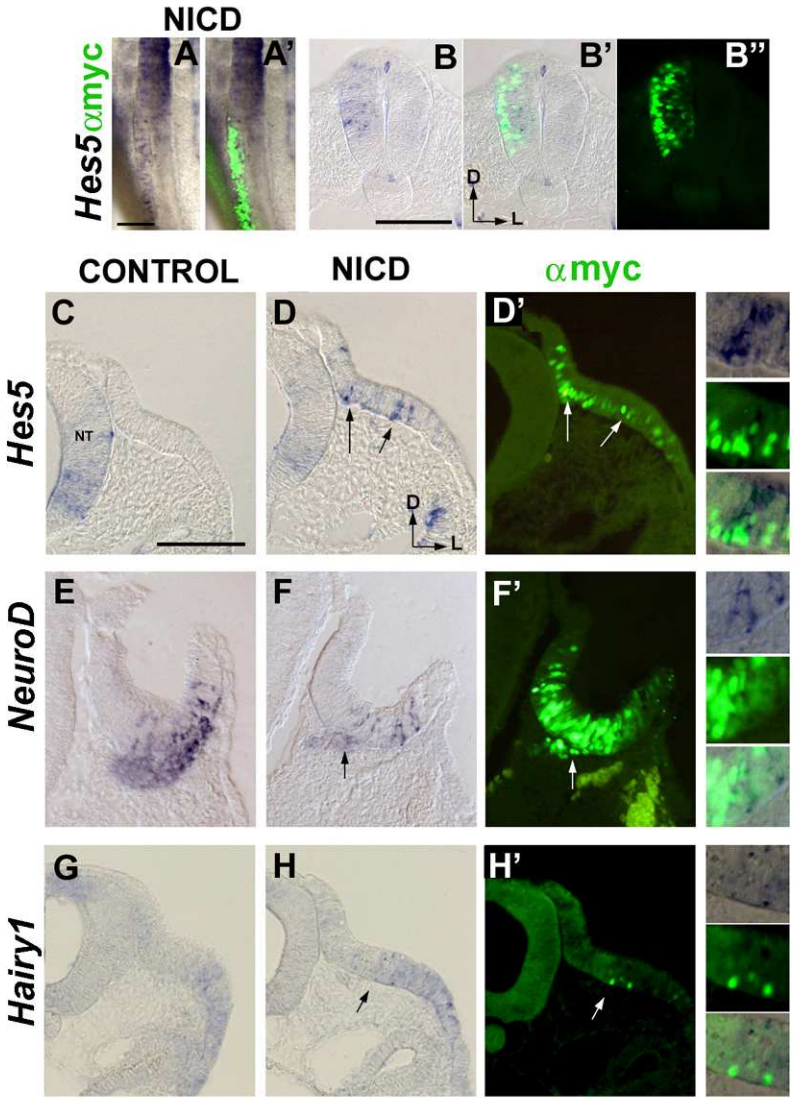

Figure 31. Effects of constitutive activation of Notch pathway during early steps of inner development. Electroporated cells are detected by immunohystochemistry against anti-myc tag shown in A', B', B'', D', F' and H'. (A-B'') NICD electroporated in the neural tub promoted *Hes5* expression. (A-A') Dorsal view of caudal neural tube from HH12 embryos showing ectopic *Hes5* expression. (B-B'') Cryostat transverse sections showing ectopic *Hes5* expression in the caudal neural tube. (C-H') The otic placode was electroporated with NICD and changes in gene expression was assayed by in situ hybridization. (C-D') Cryostat transverse sections showing *Hes5* expression in the non-electroporated vesicle (C) and electroporated vesicle (D). Arrows point to cells with increased expression of *Hes5*. (E-F')Transverse sections of control (E) and electroporated (F) otic vesicles. Arrow points a reduction of *NeuroD* positive cells in the CVG. (G-H') Transverse sections of control (G) and electroporated (H) otic vesicles showing no strong *Hairy1* activation in the electroporated cells. Abbreviations: D, dorsal; L, lateral; NT, neural tube. Scale bars in A, B and C = 100 μm.

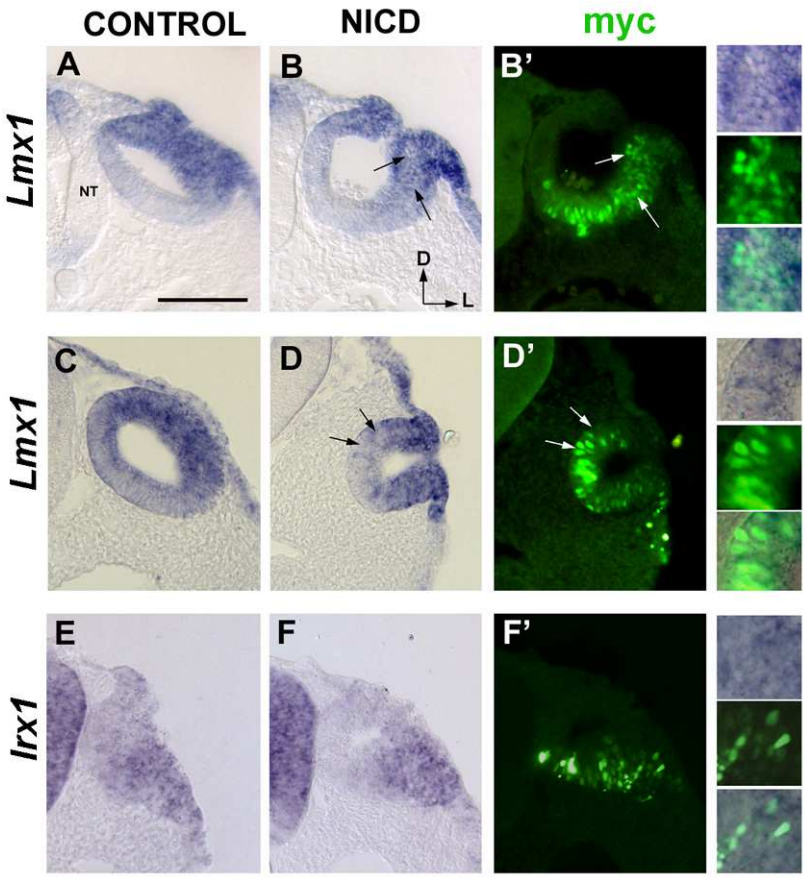

Figure 32. Effects of constitutive activation of Notch pathway during early steps of inner development. The otic placode was electroporated with the NICD fragment and changes in gene expression assayed by in situ hybridization. Electroporated cells are detected by immunohistochemistry against anti-myc tag shown in (B',D',F'). (A-D') Two representative examples of down-regulation of *Lmx1* expression in cells with the NICD construct. (F-F'') Transverse sections showing no major effects on *Irx1* expression after NICD electroporation. Abbreviations: D, dorsal; L, lateral; NT, neural tube. Scale bars in A = 100μm.

some cells with the NICD construct, visualized by the myc epitope, that were able to express *NeuroD* (FIG. 31F-F'; see arrow). This may be due by the fact that, at the time of Notch pathway activation, those cells had already initiated their neuronal determination program. When the effects on *Hairy1* expression was analyzed, NICD electroporation did not significantly increase the number of *Hairy1* expressing cells as it should be expected if *Hairy1* was directly regulated by Notch1 (n=4/4, FIG. 31G-H'). However, suppression of Notch pathway did indeed blocked *Hairy1* expression, suggesting that Notch pathway can be involved in the maintenance of *Hairy1* expression but not in its induction. The effects of NICD on *Lmx1* and *Irx1*, two genes expanded after DAPT treatment, were analyzed in

the non-neural domain. Two examples of the effects of NICD electroporation on *Lmx1* expression are shown in Figure 32A-B' and C-D'. Most of the electroporated embryos showed a decrease in the levels of *Lmx1* transcripts, indicating that constitutive activation of Notch1 pathway can lead to a down-regulation of *Lmx1* expression (n=7/9, FIG. 32A-B' and FIG. 32C-D'). In the case of *Irx1*, results were more ambiguous. *Irx1* expression is low and differences between electroporated and non-electroporated cells were not strong. The decrease on *Irx1* expression seemed to be general and not always restricted to the electroporated cells (FIG. 32E-F'; n=4/5 embryos). As expected, no induction of *Lmx1* (n=8/8) nor *Irx1* (n=8/8) was observed in the proneural domain.In summary, Notch signalling was found to regulate two different processes during early otic development. On one hand, Notch was involved in regulating the number of neuronal precursors by a mechanism of lateral inhibition; on the other hand Notch signalling was regulating otic regionalization. Moreover, our experiments indicated that Notch signalling was not involved in the specification of a neural territory. For this reason, we seek to go further on to investigate the genes and signalling pathways involved in setting up a proneural field.

3.4. **Role Of Sox3 On Otic Proneural Domain Establishment.**

3.4.1. Sox3 is required for the establishment of the neural territory.

Throughout evolution, the expression of the *SoxB1* genes (*Sox1*, *Sox2* and *Sox3*), directly correlates first, with ectodermal cells that are competent to acquire a neural fate and second, with the commitment of cells to a neural fate (Rex *et al.*, 1997; Pevny and Placzek, 2005). The restricted expression of *Sox3* in the proneural/neurogenic domain complementary to *Lmx1* and *Tbx1,* known to negatively regulate neurogenesis (Vitelli et al., 2003; Raft et al., 2004; Xu et al., 2007) led us to postulate that Sox3 may be responsible for the acquisition of a neural fate in the inner ear. Results from our group have addressed whether Sox3 is responsible for giving proneural character to the inner ear by missexpressing the chick *Sox3* cDNA in the non-neural otic region prior to the formation of the otic placode (5-9 ss). Sox3 was able to induce ectopic neural markers in the non-neural domain. Some of the ectopic neuronal precursors transit to *NeuroD* gene expressing cells but did not show a fully differentiated program. The results suggested that Sox3 was sufficient to give neural character to the otic field and to drive cells to a neuronal determination state. Instead, was inhibiting final differentiation (manuscript in preparation and S Khatri PhD

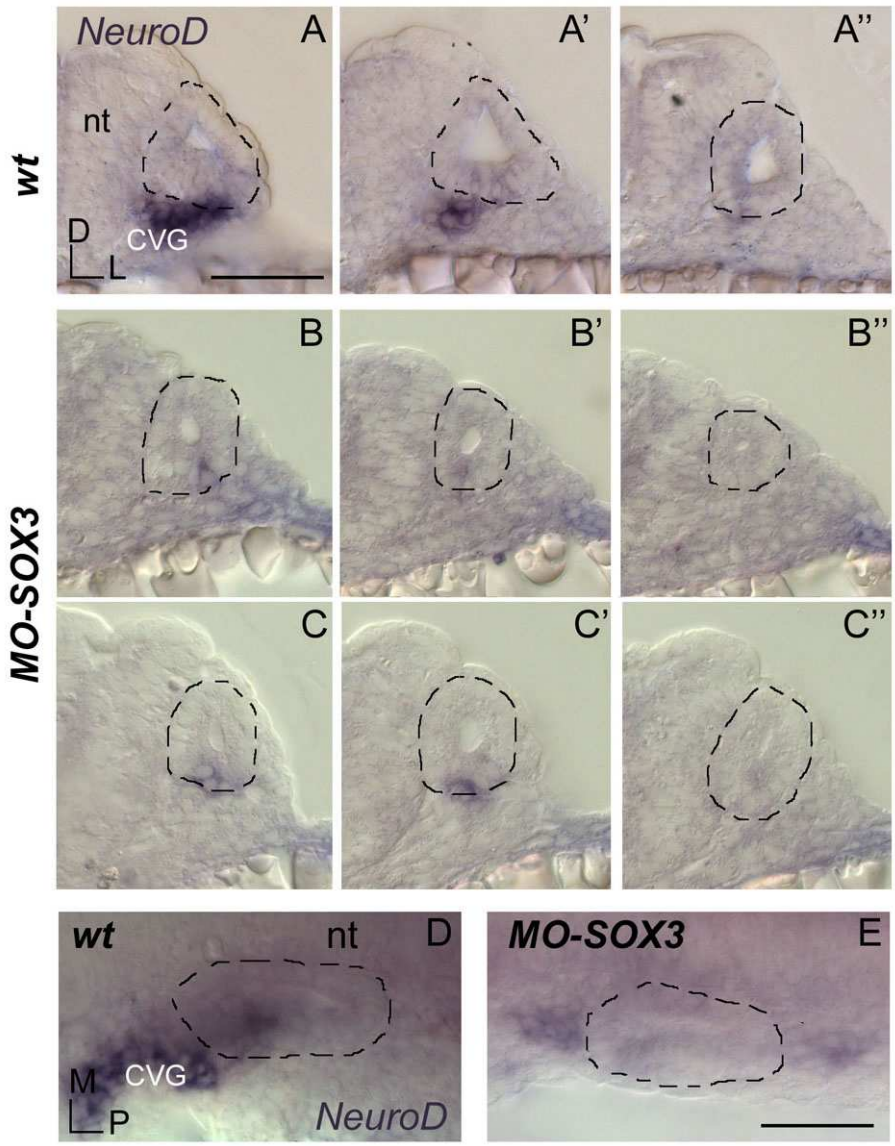

Figure 33. Sox3 is required in zebrafish for otic neurogenesis. In situ hybridization specific for *NeuroD* on wild-type 24hpf zebrafish embryos and MO-Sox3 injected embryos. (A-A'') Transversal sections of wild-type otic vesicle showing *NeuroD*-expressing cells in the otic epithelium and in the CVG. (B-C'') Transversal sections of otic vesicle of MO-Sox3 injected embryos showing a decrease or completely absent *NeuroD* transcripts. (D) Dorsal view of a wild-type otic vesicle. (C) Dorsal view of an otic vesicle of MO-Sox3 injected embryo. Abbreviations: D, dorsal; P, posterior; L, lateral; M, media; nt, neural tube; CVG, cochleovestibular ganglion. Scale bars in A and E = 50 μm.

Thesis). To further analyze the role of Sox3 in neurogenesis, in collaboration with Dr. P.Scotting laboratory we took advantage of the morpholino technique in zebrafish to

inhibit *Sox3* translation. In zebrafish, *Sox3* is expressed in the entire otic-epibranchial primordium (Nikaido, et al, 2007) as also reported in chick (Ishii et al., 2001; Abu-elmagd et al., 2001). Slightly later, by 5-ss stage, the prospective otic placode cells, still expressing *Sox3,* have been segregated from the lateral epibranchial domain. Unlike in chick, *Sox3* is restricted ventrally when the placode cavitates to from the otic vesicle, but is expressed in the entire otic territory during otic placode stage. *Sox3* expression is not longer expressed after 25hpf (Nikaido et al, 2007). When two morpholinos specific for *Sox3* were injected at the 1-2 cell stage (2.5ng MO1-Sox3 and 2.5ng MO2-Sox3) two main different phenotypes were observed, a loss of otic vesicles (35.5%, n=32/90 of the embryos analyzed) or smaller and rounder otic vesicles (64.5%, n=58/90 of the embryos analyzed).. A reduction of the 41% was detected in otic vesicle size at stage 24hpf, indicating a role of Sox3 in otic growth/morphogenesis (n=20/20 otic vesicles analyzed on sections; FIG. 33A-C''). Interestingly, stronger effects on neurogenesis were found and 67% of the otic vesicles analyzed presented an altered *NeuroD* expression profile (37%, n=10/27, with complete loss of *NeuroD* transcripts; and 30%, n=8/27, with severe reduction of *NeuroD* expression; FIG. 33B-C''). The results reinforced our previous gain of function experiments indicating a direct role of Sox3 in promoting neural character in the inner ear.

3.5. Role Of FGF Signals On Otic Proneural Domain Establishment

3.5.1. FGFs are responsible for early otic neurogenesis.

Several FGF molecules have been involved in the early and late development of the inner ear. In particular, during otic induction a cascade of FGF signaling from several surrounding tissues dictate to the ectoderm adjacent to rhombomeres 4 to 6 to adopt an otic fate. Studies from many laboratories, allow now to draw a picture in which FGF8, FGF19 and FGF3, first from endomesoderm and later from the neural tub, are involved in chick otic induction (Brand, 2002; Phillips et al., 2001; Leger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003; Mahmood et al., 1995; McKay et al., 1996; Wright and Mansour, 2003; Ohuchi et al., 2000; Mansour et al., 1993; Alvarez et al., 2003; Ladher et al., 2000; Martin and Groves, 2006; Represa et al., 1991; Vendrell et al., 2000; Ladher et al., 2005; Zelarayan et al., 2007). Relevant for otic neural development, it has been shown that FGF signaling is able to activate *SoxB1* genes during the induction of the neural plate, the lens and the epibranchial placodes (Streit et al., 2000; Takemoto et al, 2006; Faber et al., 2001; Hayashie et al., 2004; Reifers et al., 1998; Sun et al., 2007; Nikaido et al., 2007). With this in mind, we wanted to rule out whether FGFs, in addition to

its role in otic induction, were responsible for setting up *Sox3* in the otic field and thus, involved in otic neural induction. For this purpose, chick embryos were cultured for 6 hours with 25 μM SU5402, an FGF Receptor1 inhibitor that does not inhibit tyrosine phosphorylation of IGFR, EGFR and PDGFR (Mohammadi et al., 1997). SU5402 could inhibit *Pea3* expression in the ectoderm, caudal hindbrain and presomitic mesoderm (PSM), confirming its ability of blocking FGF signaling (n=9/9 embryos analyzed; data not shown). As previously reported (Groves and Bronner-Fraser, 2000), embryos from otic induction stage (from 3 to 6 somites) treated with the SU5402 did not develop otic tissue, revealed by undetectable expression of *Pax2* (n=24/24 otic ectoderms analyzed; FIG. 34A-B') and *Sox3* (n=14/16 otic ectoderm analyzed; FIG. 34C-D') and no thickening of the ectoderm (n=40/40 ectoderms analyzed; FIG. 34A-D'). The expression of *Pax2* in the isthmus and presomitic mesoderm (n=9/12 embryos analyzed; FIG. 34A and B), as well as *Sox3* expression in the neural tube (n=8/8 embryos analyzed; FIG. 34C and D) was unaltered. In order to discard that the effects observed after blocking FGF signaling pathway for 6 hours were due to a delay in inner ear development, we increased the duration of the culture. After 24 hours, embryos showed severe defects on somitogenesis and brain morphology (n=15/21 embryos analyzed; data not shown). Moreover, the ectoderm adjacent to the hindbrain was devoid of *Pax*2 expression and did not invaginate to form a cup (n=44/44 head ectoderms analyzed; FIG. 34E and F). The results indicated, indeed, that FGF signals are required for otic induction at 3-6 ss. When embryos were collected from 7-8ss stage, once the otic induction has occurred and *Sox3* expression is getting regionalized anteriorly, no effects on *Pax2* expression was observed after blocking FGF signaling (n=8/8 otic placodes analyzed; FIG. 34G-H') and the ectodermal thickening occurred normally (n=60/60 otic placodes analyzed; FIG. 34G' and H'). However, within 6 hours SU5402 was able to inhibit *Sox3* expression in the otic ectoderm in 100% of the cases (n=10/10 otic placodes analyzed; FIG. 34I-J'), while it was unaltered in the neural tube (n=5/5 embryos analyzed), suggesting that FGFs can directly regulate *Sox3* expression in the pre-otic ectoderm. Moreover, this data indicates that either sustained FGF signaling or a later FGF signaling is required for *Sox3* expression, independently of *Pax2*. The expression of *Sox3* after 24 hours of culture was absent in the otic cup (n=8/10 otic cups analyzed; data not shown). Again, *Sox3* expression was unaltered in the neural tube (n=5/5 embryos analyzed; data not shown). Interestingly, *Sox3* expression was also absent in the epibranchial placodes where, in zebrafish, requirement of FGF signaling for the induction of the epibranchial *Sox3* expression has been recently reported (Nikaido et al., 2007; Sun et al., 2007).

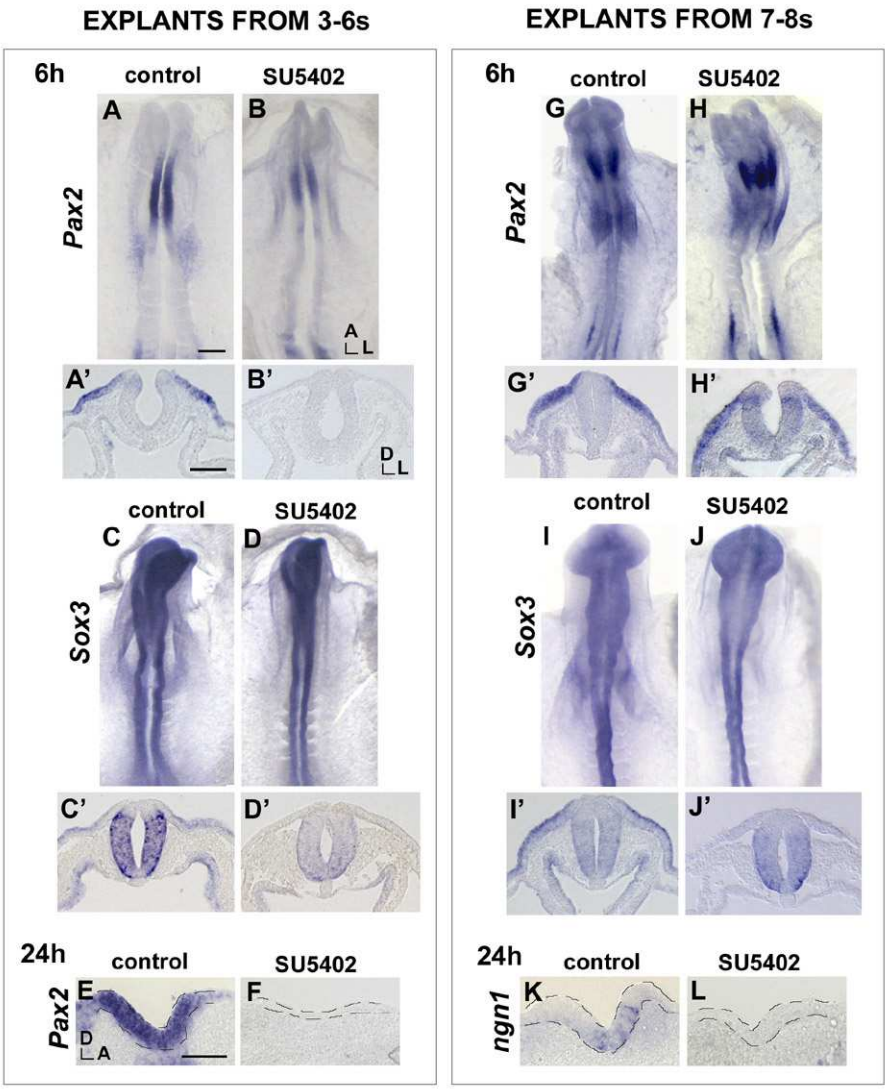

Figure 34. Blockade of FGF signaling disrupts otic neurogenesis. Effects of inhibiting FGF signaling by incubation explanted embryos with 50 μM SU5402. *Pax2* expression profile on whole-mount embryos (A, B, G and H) and transversal sections (A', B', G' and H') of DMSO or SU5402-treated embryos after 6 hours of incubation. *Sox3* expression profile on whole-mount embryos (C, D, I and J) and transversal sections (C', D', I' and J') of DMSO or SU5402-treated embryos after 6 hours of incubation. *Pax2* expression profile on transversal sections of DMSO (E) or SU5402-treated (F) embryos after 24 hours of incubation. *Neurog1* expression profile on transversal sections of DMSO (K) or SU5402-treated (L) embryos after 24 hours of incubation. Abbreviations: A, anterior; L, lateral; D, dorsal; s, somite; h hours. Scale bars in A' and E = 100 μm, in A = 200 μm.

We then went to address whether neurogenesis was impaired in the otic placodes in which *Sox3* expression was suppressed. Explanted embryos were cultured for longer

periods, until neurogenesis is taking place. In most of the embryos, no expression of *Neurog1* was detected compared to control otic cups (n=5/8 otic cups analyzed; FIG. 34K and L), in the others a decreased number of *Neurog1*-positive cells was detected in the otic cups (n=3/8 otic cups analyzed; data not shown). This data reinforced the notion that Sox3 was required for the acquisition of neural fate. However, one should bear in mind that in the chick otic cup, FGF10 regulates the determination of neuronal cells (Alsina et al., 2004). Thus, the absence of neurogenesis could be due either to the lack of *Sox3*, either to the inhibition of FGF10 signaling or to the sum of both effects. Interestingly, when embryos were collected from 9-12 ss stage and incubated with SU5402 inhibitor during 6 or 24 hours, only 30% of the otic cups analyzed showed *Sox3* downregulation (n=6/20 otic cups; data not shown), indicating a role of FGF signals in *Sox3* induction but not in its maintenance.

The otic cups treated for 24 hours with SU5402 were often smaller and less invaginated inferring also a role of FGFs on inner ear morphogenesis and/or growth as it comes to occur during limb development (Capdevila and Izpisua-Belmonte, 2003).

Altogether, our results suggest that FGFs are necessary, in addition to otic induction, for the induction of *Sox3* gene, and for establishing the neurogenic domain.

3.5.2. *FGF8* is expressed in the correct place and at the correct time.

Our results so far indicated that *Sox3* expression was enhanced in the anterior otic region at 7-8 somite stage and that *Sox3* expression is depended on FGF signaling. Thus, we carried out an analysis to find which FGF signaling molecules could be also regionalized along the antero-posterior axis accounting for the regionalized expression of *Sox3*. This was achieved by exploring the expression pattern of several FGF molecules involved in otic induction (FGF3, 8, and 19) from 5 to 10 somite stage. At 5-6 ss, *FGF3* and *FGF19* were expressed in the caudal hindbrain (FIG. 35A); *FGF3* and *FGF8* in the future pharyngeal endoderm underlying the pre-otic territory (FIG. 35A); and *FGF3* and *FGF19* within the head mesoderm at the level of the pre-otic field (FIG. 35A). However, none of them presented a clear anteroposterior gradient at the level of rhombomeres 4 to 6 nor were detected in the cranial ectoderm. By 7-8 ss, *FGF3* expression from the hindbrain was restricted to the r4 and r5, while *FGF19* transcripts were detected in the posterior ventral hindbrain (FIG. 35B). *FGF3*, *FGF8* and also *FGF19* were expressed in the endoderm at the level of the presumptive otic region (FIG. 35B). In addition, *FGF19* was still localized in the head mesoderm underlying the pre-otic territory, while *FGF3* was downregulated from this territory (FIG. 35B). Again, none of them presented an

anteroposterior gradient similar to that of *Sox3* expression profile. Interestingly, *FGF8* started to be expressed in scattered cells in the ectoderm from the level of the midbrain-hindbrain boundary to r4 (arrow in FIG. 35B). No changes on the distribution of *FGF3* (data not shown) and *FGF8* (FIG. 35C and D) were observed at stage 9-10 ss compared to 7-8 ss. However, *FGF19* was downregulated in the hindbrain and levels of *FGF3* and *FGF19* transcripts detected in the mesoderm were lower (data not shown). To better analyze the expression pattern of *FGF8* in the ectoderm, we removed the underlying mesoendoderm from 5 to 9 ss. The results showed that, at 7-10 ss *FGF8* expression pattern included the anterior pre-otic territory and the presumptive geniculate placode resembling the expression profile of *Sox3* at 9 ss (FIG. 35C and B compared to FIG. 19b"). In parallel, we also explored the expression pattern of *FGF10,* since it also plays a role in otic neuronal development (Alsina et al., 2004). *FGF10* was observed in the otic ectoderm overlapping with the expression profile of *FGF8* and *Sox3*, whereas it was not distinguished in the epibranchial placodes or in other surrounding tissues. Moreover, *FGF10* onset of expression was at 10 ss, discarding its involvement in restricting *Sox3* in the more anterior part of the otic region (FIG. 35G and H). At 13 somite stage, *FGF8* was down-regulated form the otic territory, while its expression in the epibranchial placodes stayed behind. Meanwhile, otic *FGF10* transcripts were enhanced, suggesting that FGF8 might be inducing *FGF10* in the otic field (FIG. 35E and I). Strikingly, although FGF molecules are only detected in the anterior domain of the pre-otic ectoderm and otic placode, FGFR1 (FIG. 35F and J) and FGFR4 (Lunn et al., 2006) are expressed throughout the otic territory, suggesting that FGF molecules from the otic ectoderm and surrounding tissues may be paracrine effectors of the posterior otic territory or that not all FGFReceptors are activated.

With this in mind, several experiments were carried out. First, embryos from 9-10 ss, just before the onset of *FGF10* expression in the otic ectoderm, were cultured with SU5402 for 6 hours. Strikingly, after blocking FGF signaling no *FGF10* transcripts were detected in 75% of the otic regions analyzed (n= 12/16; FIG. 36A-B'), while the rest were showing a decrease in *FGF10* expression. This result, gave strength to our hypothesis of a FGF8-FGF10 cascade in the otic field. In parallel, embryos from 6-7ss, once the cranial ectoderm has received the otic inductive signals and just before

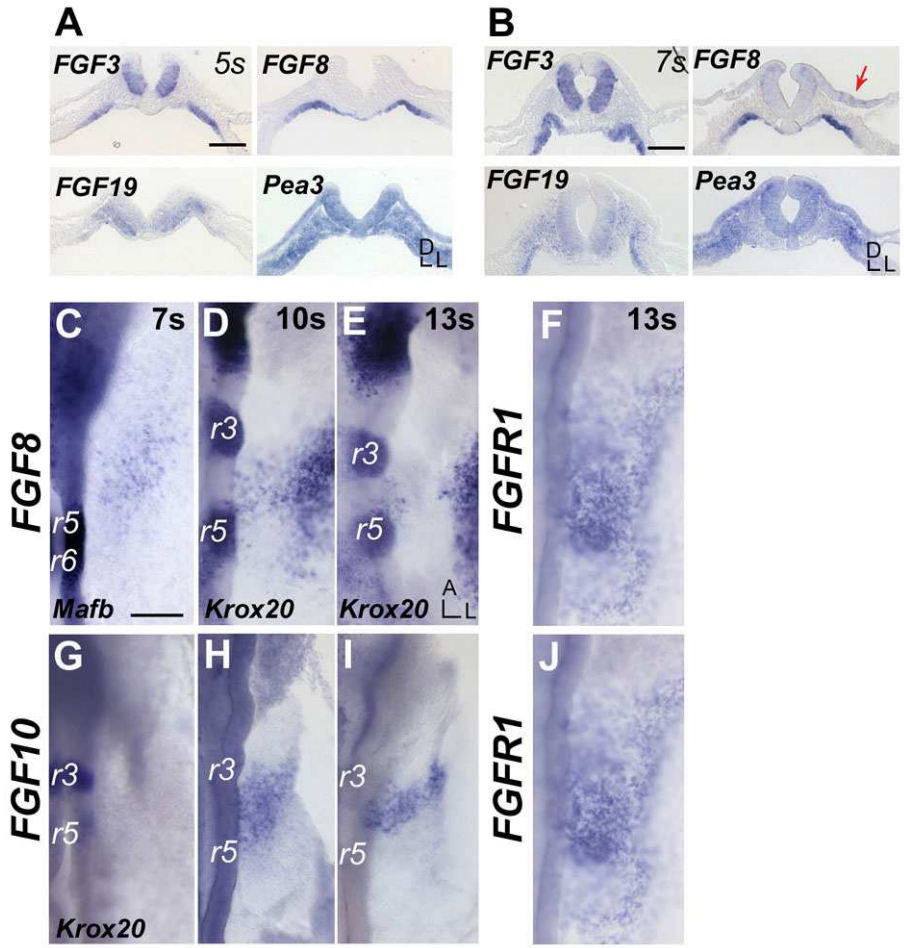

Figure 35. *FGF8* is expressed in the correct place and at the correct time to regulate *Sox3* regionalization. (A-B) Transversal sections of 5 ss (A) and 7 ss (B) embryos at the level of r5 showing the localization of *FGF3*, *FGF8*, *FGF19* and *Pea3* transcripts. (C-J) Dorsal views of otic region from 7 to 13 ss showing the expression of *FGF8 and MafB* (C), *FGF8* and *Krox20* (D and E), *FGF10* and *Krox20* (G), *FGF10* (H and I) and *FGFR1* (F and J). Abbreviations: A, anterior; L, lateral; D, dorsal; r, rhombomere; s, somites. Scales bars in A, B and C =100 μm.

the onset of expression of *FGF8*, were cultured with SU5402. Under this conditions, *FGF8* expression was also lost in the otic ectoderm and epibranchial placodes (n=8/8 otic placodes analyzed; FIG. 36C-D'), inferring a role of other FGFs, such as FGF3 and FGF19 from the neural tube and/or from the underlying mesendoderm, on activating *FGF8* expression. However, *FGF8* expression was not affected in the midbrain-hindbrain boundary (n=8/8).

All in all, we can say that *FGF8* transcripts were localized in the preotic ectoderm foreshadowing *Sox3* expression and were downregulated concomitant to the onset of *FGF10* expression in the otic placode. Moreover, we showed evidences that *FGF8* and *FGF10* expression in the otic territory is FGF-dependent. In summary, these results pointed *FGF8* as a good candidate for *Sox3* regionalization and *FGF10* induction in the otic ectoderm.

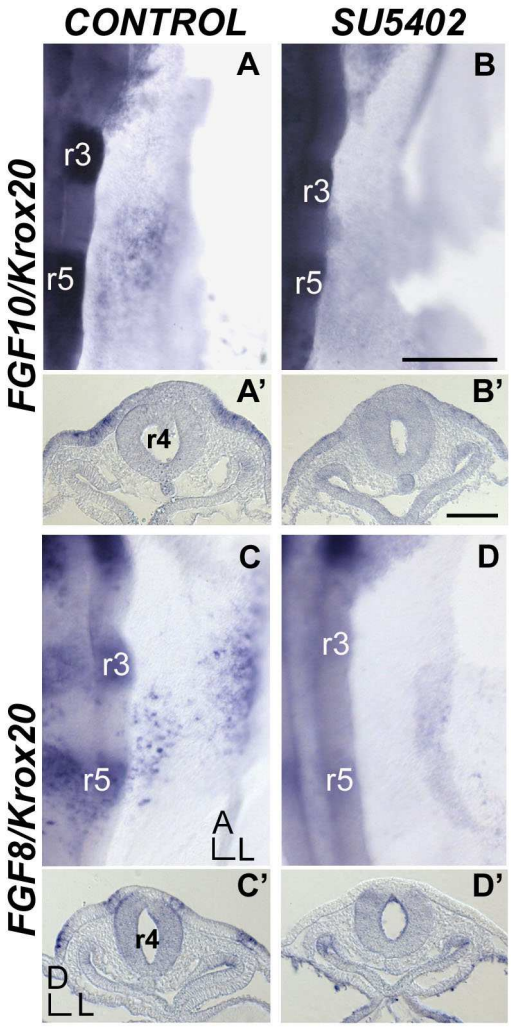

Figure 36. Otic expression of *FGF10* and *FGF8* is FGF-dependent. (A-D) Dorsal views of embryos treated either with DMSO or 50µm SU5402 showing the expression of *FGF10* (A and B) and *FGF8* (C and D). (A'-D') Transversal sections of embryos treated with DMSO or 50µm SU5402 showing the expression of *FGF10* (A' and B') and *FGF8* (C' and D'). Abbreviations: A, anterior; L, lateral; D, dorsal; r, rhombomere. Scale bars in B and B' = 100 µm.

3.5.3. FGF8/FGF10 positive loop involved in giving neural competence to the otic region and driving neuroblast to differentiation.

In other systems a mutual relationship between FGF8 and FGF10 has been described, for example in limb development FGF8 is able to induce *FGF10* expression (reviewed in Martin, 2001). Likewise, Zelarayan and co-workers showed ectopic expression of *FGF8* in r3 in a transgenic mouse line which expresses *FGF10* under the control of an EphA4 enhancer (Zelarayan et al., 2007). Furthermore, we have previously shown that *FGF8* and *FGF10* expression in the otic region was FGF-dependent. Since *FGF8* expression foreshadows the otic neurogenic domain and precedes *FGF10* expression in otic territory one could hypothesize a role of FGF8 in inducing *FGF10* expression. We therefore set out to determine whether exogenous FGF8 was able to induce *FGF10* by culturing embryos with FGF8-soaked heparin beads (1 mg/ml, mFGFb, R&D). FGF8 beads were placed in the lateral ectoderm from the level of the new-forming somite to the r3. In 4 hours, FGF8 could induce *Sprouty2* expression in the ectoderm in a 5-10 cells distance, confirming its ability of activating FGF signaling (n=3/4 embryos analyzed; FIG. 37A-B'). The first otic *FGF10*-positive cells are detected at 10-11 ss. When the culture started at 9-10 ss, only the 20% of the embryos analyzed showed and upregulation of *FGF10* expression (n=2/10; data not shown). However, when embryos were collected at 11-12 ss all the FGF8-socked beads were surrounded by ectopic *FGF10* expression (n=3/3; FIG. 37 D-D' and F-F'). None of the embryos treated with PBS-socked beads showed ectopic *FGF10* expression (n=6/6; FIG39). The results demonstrated that mFGF8b could induce *FGF10* in the cranial ectoderm.

3.5.4. FGF8 is sufficient for establishing the proneural otic domain.

When FGF signaling was inhibited during 6h or 24h we observed a suppression of *Sox3* expression. In addition, we showed *FGF8* as a good candidate for establishing *Sox3* in the more anterior domain of the otic territory. We therefore wanted to confirm whether *FGF8* was able to regulate otic *Sox3* expression. For this purpose we explored the effects of exogenous FGF8 in the posterior domain by culturing embryos with FGF8-soaked heparin beads (1 mg/ml; mFGF8b, R&D). FGF8 beads were placed in the lateral head ectoderm from the level of the first somite to the r3 in

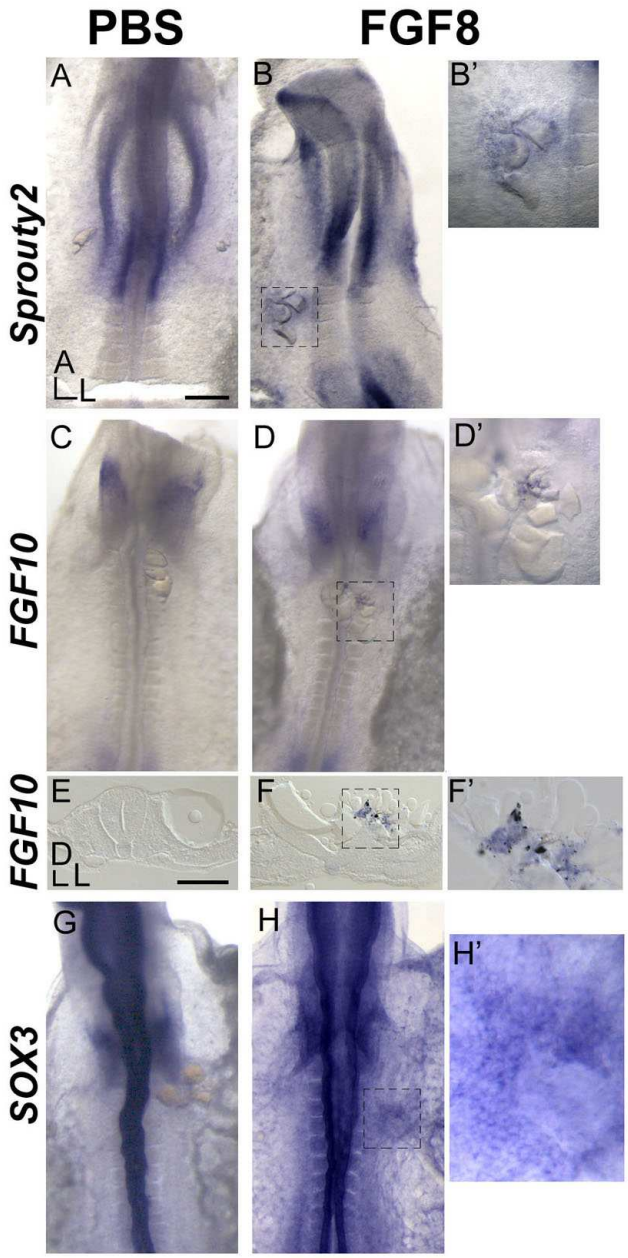

Figure 37. FGF8b induces *FGF10* and *Sox3* expression. Dorsal view of embryos treated with PBS or FGF8-coated beads showing expression of *Sprouty2* (A and B), *FGF10* (C and D) and *Sox3* (G and H). (E and F) Transversal sections of embryos treated with PBS or FGF8-coated beads showing the expression of *FGF10*. (B', D', F' and H') high magnifications of squares shown in B, D, F and D respectively. Abbreviations: A, anterior; L, lateral; D, dorsal. Scale bars in A = 200 μm and E = 100 μm.

embryos form 5-8ss. We then looked into the effects of ectopic FGF8 on *Sox3* expression. After 3 hours (FIG. 37H-H') we observed ectopic expression of *Sox3* in the ectoderm and endoderm at the level of the anterior hindbrain or the first somite in 23.5% of the embryos analyzed (n=4/17 embryos analyzed) while ectopic *Sox3* expression was never observed when PBS soaked beads were used (n=12/12; FIG. 37G). The results suggested that FGF8 was able to induce *Sox3* expression, but that may require additional factors.

3.5.5. FGFs are necessary for otic AP regionalization

Additionally, we examined whether FGFs, direct or indirect, were required for the expression pattern of the non-neural genes *Lmx1* and *Tbx1*. Embryos from 7-8 ss were cultured for 17h with SU5402. In most of the cases, an ectopic expression of *Lmx1* in the proneural domain was distinguished when blocking FGF signaling (n=14/19 otic cups analyzed; FIG. 38A and B). Besides, *Lmx1* transcripts were not fully detected in the Isthmus, further demonstrating the involvement of FGFs in the Midbrain-Hinbrain boundary; and ectopically observed in the head ectoderm. Unpublished results from our laboratory showed a cell-autonomous downregulation of *Lmx1* when Sox3 was missexpressed in the non-neural domain. Since it has been published that FGFs can drive Sox3 expression in other placodes such as epibranchials (Sun et al., 2007; Nikaido et al., 2007) we could hypothesize that FGFs through the activity of Sox3 are mediating *Lmx1* restriction to the posterior otic domain. However, we can not discard a synergistic effect of FGF signals and Sox3 transcription factor on *Lmx1* expression.

In parallel, we also analyzed the expression pattern of *Tbx1* after culturing embryos with SU5402 inhibitor for 17 hours. Surprisingly, expression of *Tbx1* was abolished (n=14/17 otic cups analyzed; FIG. 38C and D), or decreased (n=3/17 otic cups analyzed; data not shown) in the otic cup, while maintained unaltered in the periotic mesoderm. The same effect could be achieved by blocking FGF signaling for 6 hours (n=8/10 otic ectoderm analyzed; data not shown). These results propose a role of FGFs in inducing *Tbx1* expression in the otic field. Given that there are no FGFs molecules being expressed in the caudal preotic domain at the time of *Tbx1* appearance, we would expect that FGFs from the surrounding environment including mesoderm, neural tube or otic neurogenic domain, would be the ones involved in otic *Tbx1* gene regulation. Taken together, we can postulate that FGFs through the activity of Sox3 are necessary and sufficient for establishing the proneural domain of the inner ear. As a result *Lmx1* is excluded from the neurogenic domain getting restricted to the non-neural domain.

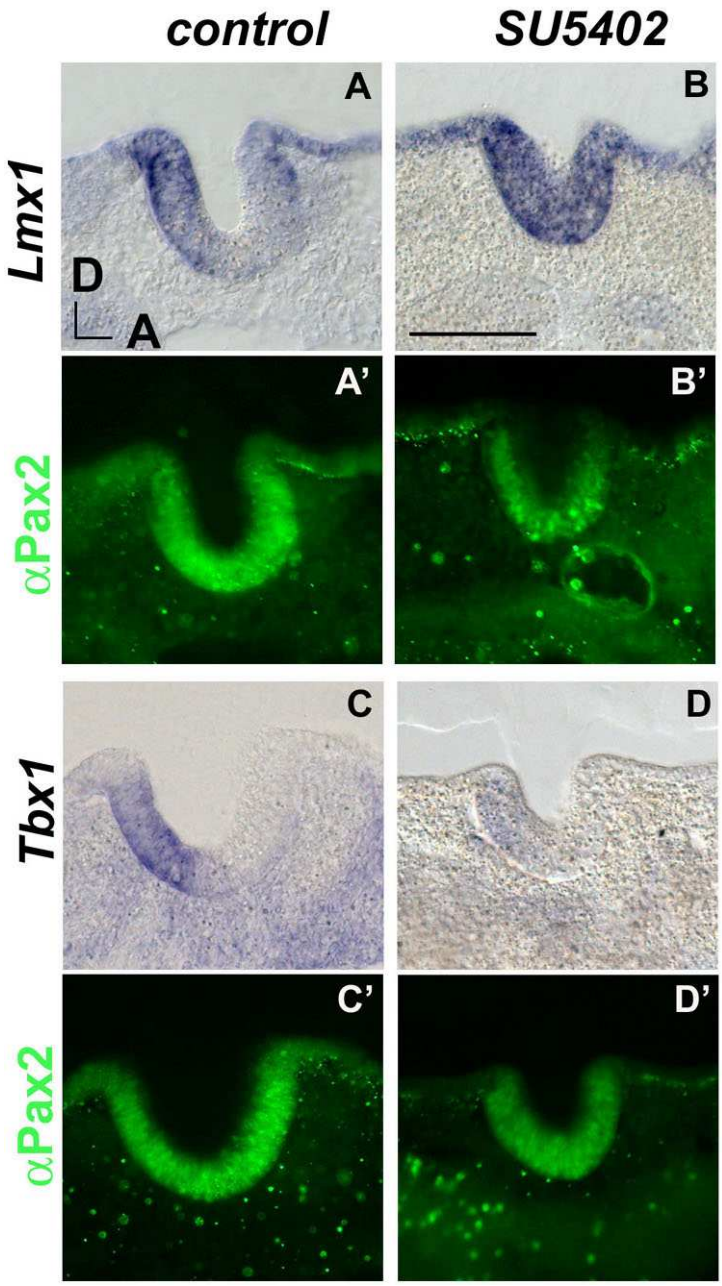

Figure 38. FGFs are involved in early inner ear AP regionalization. (A, B, C, D) Para-sagittal sections of embryos treated with DMSO or 50μM SU5402 showing the expression of Lmx1 (A and B) and Tbx1 (C and D). (A', B', C', D') Immunohystochemistry showing Pax2 expression in the same para-sagittal sections shown in A, B, C and D respectively. Abbreviations: D, dorsal; A, anterior. Scale bars in B = 100 μm.

DISCUSSION

4. DISCUSSION

In the present work, we have studied the early events of otic regionalization and the role of Notch and FGF signaling in this process. Patterning events have mainly been analyzed at otic vesicle stage. However, here we show that the first event of inner ear regionalization, the establishment of a proneural domain, occurs at the onset of the otic placode formation. Two main populations of cells are incorporated into the otic placode, an anterior group with high levels of *Sox3* and a more caudal expressing *Tbx*1 and other non-neural markers. At otic cup, limited cell mixing takes place between anterior and posterior cells as revealed by fate map analysis. We demonstrated that Notch pathway plays a new role during inner ear development. In addition of being involved in neuroblast and hair-cell fate decisions, as well as in the establishment of the sensory patches, we demonstrated the involvement of Notch in early otic neural/non-neural regionalization by stabilizing gene expression patterns. However, Notch signaling pathway was dispensable for the specification of a proneural territory. The latter role depends on FGF signaling and the activity of the Sry-related gene *Sox3*. We reported *Sox3* as the first gene expressed in a common proneural territory shared between epibranchial and otic placodes and demonstrated the requirement of FGF signals in inducing Sox3 in the proneural domain. We therefore propose that proneural character is acquired in the anterior territory by the action of localized ectodermal FGF8-FGF10 signaling that enhances Sox3 function. FGF signals through Sox3 activity would be essential for the specification of the proneural domain versus a non-neural territory, while Notch would be involved in refining this early regionalization.

Regionalization into proneural and non-neural territories takes place at the onset of otic formation

Several genes are regionally expressed at otic cup/vesicle stages, and their role in the development of specific parts of the adult inner ear has been studied in recent years (Fekete, 1999; Cantos et al., 2000; Baker and Bronner-Fraser, 2001). In a previous work, we showed that the otic placode/cup is patterned into a neurogenic domain and a caudal territory which is devoid of neurogenic capacity, and that this appears to precede other regional asymmetry (Alsina et al., 2004). We have now studied when we can detect the first signs of the patterning into a proneural and non-neural territory. Here we present new data on the origin of otic regionalization and the transition between the pre-otic ectoderm and otic cup. During otic induction, ectodermal *Sox3* and *Lmx1* expression domains

overlapped within the *Pax2*-positve domain, while *Tbx1* was already confined to the more posterior *Pax2* expression domain. Slightly later, the pre-otic field was patterned into an anterior region expressing high levels of *Sox3*, and a caudal region expressing *Lmx1* and *Tbx1*. This leads to the interesting question of whether *Sox3*, *Lmx1* and *Tbx1* are co-expressed in the same cells at earlier stages and then gene expression is restricted to regional subdomains, or else different intermingled populations of cells already exist but become sorted out after placode formation. Extensive cell movements takes place before otic formation and the otic placode is populated by cells that share common progenitors with neural fold and ectodermal cells (Streit, 2002). Thus, one could imagine that the different populations of cells exist before otic placode formation, some with neural potential expressing *Sox3* and some others expressing *Lmx1* and/or *Tbx1* and devoid of neural potential. Then, cell movements would concentrate and allocate them to their final positions. In contrast, cells could be initially naïve or express several transcription factors to then be regulated to finally express a set of transcription factors and become restricted along the AP axis. Expression patterns of *Sox3* and *Lmx1* do overlap in different degrees before the formation of the otic placode and it is not until this stage that *Lmx1* is strictly banned from the proneural domain. Contrastingly, *Tbx1* expression is restricted to the more caudal region of the presumptive otic territory since its expression switch on. The fact that Notch inhibition results in a loss of this restriction without need for cell movements (see bellow) indicates that differential gene expression patterns most likely result from differential gene regulation. However, this does not exclude that other mechanisms may still be regulating cell sorting at this early stages. We therefore propose a role of Notch pathway in the stabilization of early otic gene regionalization. However, final regionalization of the otic placode is probably not fixed until the otic cup stage. Otocyst rotations and recent work on otic cup rotations in the AP axis suggest that, in spite of the regionalized expression of various genes in the AP axis, the AP regionalization is not totally fixed at 16 somites, and re-specification may take place after otic placode formation (Wu et al., 1998; Bok et al., 2005). Once patterning is fixed, segregation of territories could control local cell fate decisions or govern morphogenetic processes and otic growth.

Finally, it has been reported that *Sox3* transcription factor is implicated in preplacodal and placodal development (Wood and Episkopou, 1999; Nikaido et al., 2007; Sun et al., 2007). In addition, *SoxB1* genes correlate with ectodermal cells that are competent to acquire a neural fate (Rex *et al.*, 1997; Pevny and Placzek, 2005). Contrastingly, *Tbx1* is known to negatively regulate neurogenesis (Vitelli et al., 2003; Raft et al., 2004; Xu et al.,

2007). Thus, the observation that at preotic stages *Sox3* high expression band preceded the otic neurogenic domain and the geniculate placode, and its complementarity to *Tbx1* expression domain, suggested that the proneural character is acquired in the otic field earlier than it was described.

Shared proneural domain between otic and geniculate placodes

The otic placode is a neurogenic placode but in contrast to the epibranchial placodes that develop next to it, only a subdomain has neural potential. By stages HH9-10, *Sox3* is expressed in the most anterior domain of the presumptive otic ectoderm, a region that foreshadows the domain of *Delta1* and *Neurog1* expression. During epibranchial placode development, *Sox3* expression precedes neurogenesis and has been postulated to play a role in allowing the placodal thickened ectoderm to undergo neurogenesis (Rex et al., 1997; Abu-Elmagd et al., 2001). Cell fate analysis of the *Sox3* expression domain at HH10 was preformed by Ishii and colleagues (2001). The more lateral *Sox3*-positive domain was labeled with DiO, and the medial with DiI. Embryos were incubated until they reached HH13 and processed for *in situ* hybridization specific for *Sox3*. The observation was that the geniculate placode and otic cup arose from the same *Sox3* area. They analysed the fate of two different populations of cells within the same gene expression domain, and compared them with *Sox3* expression pattern. Instead we followed the fate of a group of cells of the *Sox3* area labeled with DiI in comparison to *NeuroD*, a neuronal determination marker. Our experiments indicate that the proneural domain is continuous and that neuroblasts generated anterior to the otic placode will migrate laterally to the branchial arches while cells located more caudally will generate neuroblasts that will delaminate ventrally and give rise to the CVG. Furthermore, in Ishii studies, Sox3 function was associated with placodal fate, while our work suggests that Sox3 is required for neural placodal fate. Hemmond and Morest (1991) described what they called the otic crest, a ridge of epithelium surrounding the placode in which cells migrate and become continuous spatially with those derived from the epibranchial placodes of cranial nerves VII, IX, and X. How do cells know whether to become otic or geniculate? Both placodal cells share the expression of *Pax2* and *Sox3* but not *Lmx1*, suggesting that this transcription factor could be involved in the recruitment of the proneural domain into the otic field. It could also be considered that otic *Sox3*-positive cells could be differentially attracted by secreted signals emanating from the midline, while epibranchial *Sox3*-positive cells by BMP7 secreted from the pharyngeal endoderm, since it has been described that BMP7 is required for epibranchial induction (Begbie et al., 1999).

Alternatively, final otic or geniculate could be purely a stochastic phenomenon depending on the rostrocaudal position of birth. Another interesting consideration is that using replication-defective retroviruses, (Satoh and Fekete, 2005) showed that the perithympanic organ (the middle ear of the birds) and the geniculate ganglion share common progenitors, which points towards this close relation between otic and facial territories. These authors, however, did not report a lineage connection between otic and facial neurons. At HH11 neuronal specification is starting, therefore small differences on the timing of the DiI and retroviral infections could account for this difference.
Overall, our analysis on the dynamics of the otic proneural domain provides evidence for the existence of an initial common proneural domain that is shared between the otic and the geniculate placode.

Restricted cell intermingling between otic proneural and non-neural domains

The two functional domains of the otic cup, proneural and non-neural, differ in their developmental potential and underlying mechanisms must ensure that both domains keep their differential identities throughout development. Restricted cell mixing between different populations of cells has been found to be instrumental in maintaining adjacent territories with different identities during development and for the control of cell proliferation. For example, during neural tube formation, the caudal stem zone region behaves as a coherent cellular domain that maintains an undifferentiated cell state that involves continued cell cycling, providing a continuous pool of cells to the newly forming neural tube (Mathis et al., 2001). During the segmentation of the hindbrain and the formation of the *zona limitans intrathalamica*, restricted regional gene expression is accompanied by cell lineage restriction (for reviews see Lumsden and Krumlauf, 1996; Pasini and Wilkinson, 2002; Lumsden, 2004; Kiecker and Lumsden, 2005). In inner ear development, otocyst compartmentalization is believed to be important for allocating sensory organs and the endolymphatic duct (Brigande et al., 2000a; Brigande et al., 2000b; Fekete and Wu, 2002). The complementary expression patterns of the chick otic proneural and non-neural regions led us to study the degree of cell mixing between these two cell populations. The results show that cells in the otic epithelium exhibit a coherent and ordered pattern of expansion with no invasion of posterior labeled cells to the proneural territory or vice versa, demonstrating a new cell lineage boundary in the ventrolateral aspect of the otic vesicle. Moreover, cells labeled with vital dyes remained contained within gene expression boundaries. We therefore infer that the anterolateral region of the otic placode maintains cohesive during development giving rise to otic

neuroblasts, and postulate that this boundary of gene expression and cell mixing would be representing the neuroblast delamination site. Our studies also suggest that Notch activity probably is not regulating the cohesion of the domains since posterior cells did not invade the proneural domain after Notch blockade. In nascent neural tube, the cohesiveness of the stem zone seems to require FGFs (Mathis et al., 2001), but it is not mediated by Notch (Akai et al., 2005). *FGF10* is regionally expressed in the proneural domain of the chick otic placode and vesicle and it has been shown to be required for neuron production (Alsina et al., 2004). Although suggestive, we know nothing about its role in maintaining cohesion of the proneural domain. Similarly, little is known on the differential expression of adhesion molecules that could restrict cell mixing at these early stages of placode development. Most information relies on late otic vesicle or otocyst stages, where they have been studied in relation to sensory development and hair-cell (reviewed in Kelley, 2003). We know that at early stages of otic vesicle (HH17-19), BEN and HNK1 show complementary expression patterns that are restricted to the two functional domains (FIG. 39). Therefore, the asymmetric expression of similar cell-adhesion molecules may underlie differential cell affinity and the observed restricted cell movement.

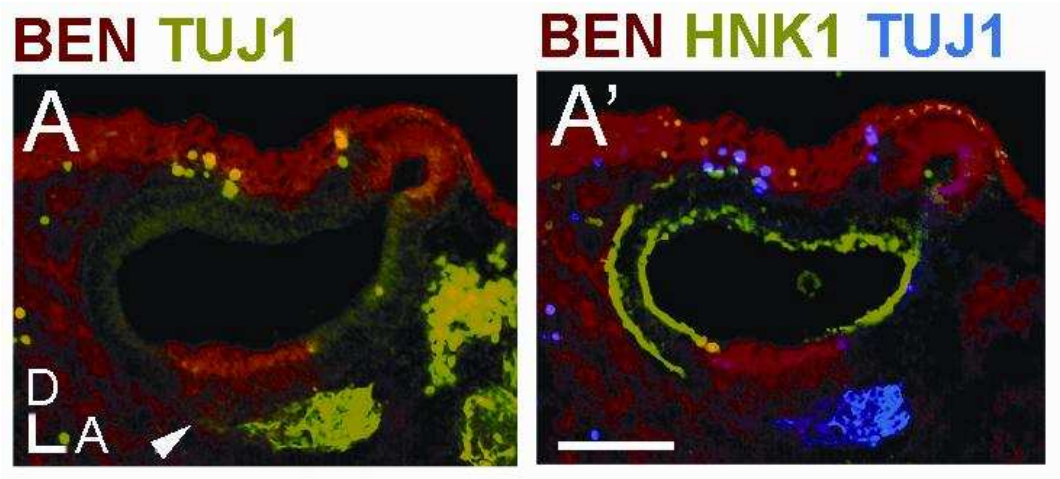

Figure 39. (A-A') 10μm para-sagittal alternate sections were stained for BEN and TUJ1 or HNK1 epitopes. Double immunostaining for BEN (red) and TUJ1 (green) is shown in A, while overlay of BEN (red) and HNK1 (green) from alternate sections with TUJ1 (shown in the blue channel) are shown in A'. BEN and HNK1 showed a complementary pattern in a HH18 otic vesicle and the origin of the cochleo-vestibular ganglion (false blue, TUJ1) was detected at the interface of both stainings (arrowhead in A). Abbreviations: A, anterior; D, dorsal. Scale bar in A' = 100 μm.

Other cell adhesion molecules may also be responsible for the restriction of cell mixing. For example, differential expression of α3 and α6 integrins in the developing mouse inner ear has been described (Davies and Holley, 2002). On the other hand, an immense amount of work has been done in order to elucidate whether otic neurons and hair-cells originate from a common progenitor (Fekete et al., 1998; Satoh and Fekete, 2005; Fritzsch *et al.*, 2000, Ma *et al.*, 2000; Koundakjian et al., ARO 2007, Abstract 962). Based on this data, it has been hypothesized that a population that expresses both *Neurog1* and *Sox2* can give rise to neurons and, later, to hair-cells/supporting cells (Fritzsch et al., 2006). In our fate map analysis, we initially labeled a group of cells probably with different characters. Hence, we could not explore whether there is lineage relationship between different otic progenitors. Further single cell fate mapping experiments would be needed to unveil this question.

Notch signaling pathway is activated differentially in the proneural and non-neural domain

The Notch signaling pathway has a wide array of functions that depend on its ligands, co-factors and modulators (Panin and Irvine, 1998; Kadesch, 2004; Schweisguth, 2004). Advances in understanding the regulation of Notch signaling have led to the discovery of new functions of Notch. Our studies show that different members of the Notch signaling pathway are differentially expressed in the proneural and non-neural domains of the otic placode.

Delta1 and *Serrate1,* two Notch ligands, as well as different members of the HES family of proteins, *Hes5-2*, *Hes6-2* and *Hairy1*, were expressed in a complementary pattern at otic placode/cup stages, suggesting that Notch can be differentially activated in both territories. In the retina and in the spinal cord there is also a clear complementarity between the expression of *Jagged1* in the non-neural ciliary margins of the retina and floorplate and of *Delta1* in the neurogenic domains (Lindsell et al., 1996; Bao and Cepko, 1997). In the proneural domain *Delta1* and *Hes5-2* presented mutually exclusive expression pattern at the cellular level. The expression of *Hes5-2* was blocked when Notch pathway was inhibited indicating that *Hes5-2* was a Notch target gene. As a result of the lateral inhibition, increased numbers of *Delta1* expressing cells were detected all through the proneural domain, which is in agreement with previous work in zebrafish (Haddon et al., 1998) and recent data from chick (Daudet et al., 2007). However, the proneural territory was neither expanded nor abolished after Notch singaling blockade. In contrast, in zebrafish, as it takes place in *Drosophila*, it has been shown that the early

establishment of a proneurosensory territory and the appearance of the proneural gene Atoh1 depend on Notch activity (Millimaki et al., 2007).

On the contrary, *Hairy1* and *Serrate1* were coexpressed in non-neural domain cells. The results showed that *Hairy1* expression was suppressed after Notch blockade, indicating that it was dependent on Notch signaling. One could hypothesize that posterior expression of *Serrate1* and *Hairy1* is related to patterning. *Hairy* and *Enhancer of Split* homologs (*Hes/Her*) act as patterning genes in the zebrafish midbrain-hindbrain boundary, mouse olfactory placode and the inter-proneural stripes of the *Xenopus* and zebrafish neuroectoderm by repressing neural fate (Cau et al., 2000; Geling et al., 2003; Bae et al., 2005). In those model systems, *Hairy1* activity is Notch independent. In the otic placode, maintenance of *Hairy1* expression was Notch dependent, but was not clearly activated by NICD indicating that most probably its initial establishment is not Notch dependent. On the other hand, suppression of *Hairy1* expression did not expand neurogenesis to the posterior region suggesting that repression of neurogenesis may require additional signals in the ear. In the mouse, *Hes1* (*Hairy1*) and *Hes3* regulate maintenance of the isthmic organizer (Hirata et al., 2001) and, in recent works, it has been proposed that high levels of *Hes1* expression are required for boundary formation in the developing central nervous system (Davies and Holley, 2002; Baek et al., 2006). In the otic vesicle high levels of *Hairy1* are consistently found along the lateral wall of the AP boundary. Experiments to determine the function of this gene in the inner ear described for the first time are being carried out.

Which is the role of Serrate during early otic development? Recently it has been shown that *Serrate1* is involved in the specification of the sensory patches. Inner ears from mice in which *Jagged1* has either been specifically deleted at the early otocyst stage using a *FoxG1*-dependent *Cre* expressing line, or made hypomorphic, present absence of most of the vestibular organs, with the exception of the saccular maculae. Moreover, a reduced number of mis-patterned hair cells are restricted to the apical region of the cochlear duct (Kiernan et al., 2001; Tsai et al., 2001; Kiernan et al., 2006). However, in those studies the effects on neurogenesis due to the loss of Serrate1 were not analyzed. Similarly, deletion of *Rbp-Jk* leads to a complete absence of all vestibular epithelia as well as to a nearly complete loss of all cochlear hair cells (Yamamoto and Kelley, unpublished), suggesting that Serrate1 activates Notch singaling to specify prosensory patches. Finally, inhibition of γ-secretase activity, a component of the Notch signalling pathway, inhibits prosensory formation in the chick otocyst (Daudet et al., 2007). In order to have a role in prosensory patch specification, Serrate1 should act through a lateral induction

mechanism. During lateral induction, a positive feed-back promotes that Serrate1 ligand activates its expression in the neighbouring cell through Notch receptor activation. Our data indicates that *Serrate1* expression is regulated differently in the proneural and non-neural territory. In the proneural region was found to be Notch dependent, suggesting that early expression of *Serrate1* in the proneural domain reported was foreshadowing sensory specification. Surprisingly, posterior *Serrate1* was not affected by blocking Notch pathway. Daudet et al. (2007) also showed that posterior *Serrate1* expression was Notch-independent. However, they revealed that *Serrate1* maintenance was requiring activation of Notch, suggesting that a more complex context is regulating sensory specification. A more detailed cell tracing study is required to test whether posterior *Serrate1* expression is contributing to the sensory patches or the early caudal expression of Serrate1 only acts as a prepatterning gene to specify the non-neural territory. At otic placode/cup stage *LFNG* was expressed in the proneural domain overlapping with *Delta1* expression domain and containing anterior *Serrate1* patch. By otocyst stage it has been reported to be excluded from the neurogenic domain, and confined to the presumptive sensory organs accompanied by *Bmp4* and *Serrate1* expression (Cole et al., 2000; Satoh and Fekete, 2005). In *Drosophila,* cell-autonomous modification of Notch by Fringe protein favors the interaction with Delta1 over Serrate1 ligand. During otic proneurosensory development, when neurogenesis is taking place in the proneural domain, *LFNG* could balance the Notch-Delta signaling, leading to activation of *Hes5-2*. Later in development, it is possible that the Notch-Serrate1 signaling in the anterior region could favor sensory fate. All in all, we demonstrated that otic neurogenesis is regulated by Delta1-Notch1 lateral inhibition. Moreover, we suggested that anterior *Serrate1* foreshadowed the otic sensory patches, while the posterior expression of Serrate1 is Notch independent.

Notch signaling is required for the down-regulation of Lmx1 in the proneural territory

In addition to Notch involvement in otic neuronal production through lateral inhibition, another effect was detected after Notch blockade. *Lmx1* and *Irx1* lost their restriction to the posterior non-neural domain and were up-regulated in the proneural domain where is usually excluded. DiI experiments and HNK1 expression analysis combined with Notch blockade, together with electroporation of a Dominant negative form of the mastermind-like 1 indicated that Notch primarily regulates the regionalized expression of *Lmx1* and not the sorting of neural and non-neural cells. This data is further supported by the decrease of *Lmx1* expression in cells in which NICD has been overexpressed. However,

the molecular mechanisms are still unknown. *Lmx1* gene belongs to the subfamily of LIM homeodomain (LIM-HD) proteins, which are key transcription factors in regulating developmental processes. In limb development, *Lmx1* is fundamental for establishing dorsal identity, and in the isthmus and roof plate *Lmx1* has been shown to promote organizer activities by regulating the expression of secreted signaling molecules (Vogel et al., 1995; Adams et al., 2000). The expression of *Lmx1* in the ear has been reported, as well as its regulation by dorsal hindbrain signals (Giraldez, 1998). However, the exact role of *Lmx1* in the ear is still unknown. Here, we show that *Lmx1* is regionally expressed in the non-neural domain and that Notch activity is required for excluding its expression from the proneural territory. During the development of the *Drosophila* wing disc, *apterous* a member of the LIM-HD transcription factors regulates the expression of Notch ligand *Serrate1* in the dorsal compartment. However, in the inner ear expansion of *Lmx1* expression into the proneural domain is not accompanied by *Serrate1* expansion.

Irx1 also belongs to a family of transcription factors implicated in several functions during development, including organizer formation, neural plate specification and patterning, sensory placode formation, and heart chamber specification (for review see Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002). In the eye disc, IRO-C complex represses the expression of *Fringe* and juxtaposition of IRO-C expressing and non-expressing cells generates a straight border that promotes growth and serves as a pattern-organizing border in the eye disc (Cavodeassi et al., 1999). In spite of these studies in *Drosophila*, a relationship between Notch signaling and *Lmx1* and *Irx1* function during vertebrate patterning is still unknown. Thus, it is difficult to describe the molecular mechanism by which Notch, *Lmx1* and *Irx1* could be interacting in the otic placode. One of the targets of Notch could be a repressor expressed in the entire proneural domain (*Delta1* and *Hes5-2* cells) and thus, different from *Hes5-2.* This repressor would be involved in repressing *Lmx1* and *Irx1* in the anterior domain. This function is probably not related to the classical function of Notch in lateral inhibition during neurogenesis. Nevertheless, other signals must be regulating early otic patterning because the establishment and the size of the proneural domain were not dependent on Notch activity. Our data does not favor the idea that one of the functions of *Lmx1* and *Irx1* in non-neural territory is to repress neurogenesis, as can proceed in the proneural domain in the presence of *Lmx1* and *Irx1.* Another possibility could be that the interaction between *Irx-Lmx* domains with a *LFNG* positive domain directs growth of the otic cup, as occurs in the eye and wing disc of *Drosophila*.

Nevertheless, different observations were taken when mouse was used as a developmental model. Although the domain devoid of *Lmx1* and *Irx1* expression was smaller in RBPJk mutant embryos, a complete expansion of *Lmx1* or *Irx1* was never detected. The above mentioned differences can be achieved by species-specific requirement of Notch in otic *Lmx1* and *Irx1* gene regulation.

The role of Sox3 in otic neural fate specification

During neural induction, *Sox1-3* genes are correlated with uncommitted ectodermal cells that develop into neuroectoderm in response to inductive signals (Uwanogho et al., 1995; Rex et al., 1997; Pevny et al., 1998; Kamachi et al., 1998 lens). The fact that *Sox3* expression in the ectoderm foreshadowed the otic proneural domain and otic *Delta1* expression was Sox3-dependent (Khatri et al., unpublished data) suggests that *Sox3* is required for otic neural specification. This finding coincides with recent data reported by Dee et al (2007) where *Sox3* knock-down by morpholino injection at 1-2 cell stage caused a dramatic loss of neurogenesis in zebrafish CNS, as well as in the epibranchial and otic placodes. However, some otic vesicles (30%) presented unaltered neurogenesis. Combinatorial codes of transcription factors, in particular factors of the Sox, Pax and POU family proteins, seem to be the major mechanism employed in genetic switches for cell differentiation (Kamachi, et al., 2000). In neural stem cells, combined action of Sox2 and Group III POU factors (e.g., Brn2) activates neural stem cell specific gene *Nestin* (Tanaka et al., 2004). Alteration of only one component in the dimer causes an overt change of the target sites and hence the genes under their regulation, and may be sufficient to elicit a different type of cell differentiation (Kondoh et al., 2004). Interestingly, Tanaka et al., (2004) demonstrated that Brn2 in combination with all group B1 Sox proteins, Sox1, Sox2, and Sox3, activated the expression of *Nestin*, confirming analogous activities of these factors. Hence, the lack of phenotype in 30% of the zebrafish mutant for Sox3 could account by the continued presence of other SoxB1 family members such as Sox2, which is expressed in the zebrafish otic placode (Dee et al., 2007). Mice deleted for *Sox3* are affected by hypopituitarism (Rizzoti et al., 2004), as are human patients carrying *SOX3* mutations (Laumonnier et al., 2002), reflecting the important role of the protein in the CNS. The mutant mice, and a subset of the human patients, are also affected by craniofacial defects. However, the role of human and mouse Sox3 in otic neural development has not been addressed. In order to avoid SoxB1 compensation it would be interesting to develop SoxB1 compound mutants. Nevertheless, targeted inactivation of Sox2 in mouse results in peri-implantation lethality of homozygous embryos (Avilion et al.,

2003). Thus, genetic demonstration that Sox3 gene is required to specify otic neural fate awaits the generation and characterization of conditional SoxB1 alleles. Jo, de fet, el que deia és que com que al només treure Sox3, els altres soxb1 poden suplir la funció de soox3 (ja que poden unir al mateix partner) i per això no veiem el fenotip sempre. Per tant, la amnera de treure tota la funció de sox3 és treure sox1+sox2+sox3. pero com que sox2 es moren en implantacio, s'han de fer dobles o triples mutants condicionals per evitar la lethalitat de sox2.

Koster and colleagues obtained ectopic otic vesicles expressing *Pax2* and *Eya1* after injecting *Sox3* in *medaka* trunk ectoderm (Koster et al., 2000). Accordingly, 36% of the Sox3 morphants analyzed presented a loss of otic vesicle formation reinforcing the idea that Sox3 may also be required for otic-epibranchial placode formation. As mentioned, SoxB1 genes use different co-factors to activate several sets of genes (Kamachi et al., 2000). During lens placode development, Sox2 protein interacts with Pax6 to specify lens. It is reasonable to think that Sox3 together with Pax2 may be necessary for otic specification. Subsequently, Sox3 in the proneural field would be required for neural commitment. In chick however, ectopic expression of Sox3 was not capable of inducing ectopic otic vesicles as in *medaka* fish (data not shown).

In summary, we propose that Sox3 drives also a crucial role during inner ear development. Sox3 is required not only for otic neural fate determination but in teleosts is also required for otic formation and morphogenesis, the molecular mechanisms for the differential actions of Sox3 still to be determined.

FGF signaling is required for Sox3 expression in the inner ear

It has been described that FGF signals are necessary for otic induction, neurogenesis progression and patterning (induction: Brand, 2002; Phillips et al., 2001; Leger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003; Mahmood et al., 1995; McKay et al., 1996; Wright and Mansour, 2003; Ohuchi et al., 2000; Mansour et al., 1993; Alvarez et al., 2003; Ladher et al., 2000; Martin and Groves, 2006; Represa et al., 1991; Vendrell et al., 2000; Ladher et al., 2005; Zelarayan et al., 2007; neurogenesis progression: Alsina et al., 2004; Adamaska et al. 2001; patterning: Pauley et al., 2003; Ohuchi et al., 2005; Hatch et al., 2007; Mansour et al., 1993; Pirvola et al., 2002; Pirvola et al., 2000; Zelarayan et al., 2007). Streit and coworkers showed that FGF8-coated beads could induce *Sox3* expression within 3 hours when placed in the extra-embryonic epiblast (Streit et al., 2000). In addition, *Ace* -/- mutant embryos showed no *Sox3* expression within the otic-epibranchial placode, while *Sox3* transcripts were recovered after

implanting a FGF8-soaked bead (Nikaido et al., 2007). Our findings determined that FGF signals were required for otic neurogenesis and otic *Sox3* expression. As above mentioned, we have also demonstrated Sox3 requirement for otic neurogenesis. Thus, we propose that FGF signaling promotes anterior otic cells to become competent to undergo neural identity through the activity of the transcription factor *Sox3*. Takemoto and co-workers have demonstrated that FGF8 regulates *Sox2* expression in the posterior neural plate by activating enhancer N-1 (Takemoto et al., 2005). Accordingly, we suggest that FGF signaling can also interact with the promoter region of *Sox3* since the effects were shown already in 6 hours.

Our experiments could uncouple crucial steps for inner ear development, including inner ear induction, otic neurogenesis and otic cup formation. Thus, as proposed by Groves and colleagues (Martin and Groves, 2005; Kil et al., 2005), our data also points towards a multi-step process driving inner ear development were sequential activity of different FGF molecules from the otic ectoderm itself and surrounding tissues would be fundamental at different time points.

Although our work demonstrated that FGF signaling is important for initiating *Sox3* expression in the otic territory, FGF molecules were not required for *Sox3* maintenance since the ability of SU5402 inhibitor of blocking *Sox3* expression in the otic placode was hardly discernible when the treatment started at 9-10-somite stage. Contradictory, it has been shown in zebrafish that FGF signals were required for both *Sox3* expression initiation and maintenance, since *Sox3* expression from the otic-epibranchial placode recovered 3-6 hours following SU5402 removal (Sun et al., 2007). Otic cups treated with SU5402 were smaller and less invaginated, suggesting that, as it was described in *FGF8 and FGF3* zebrafish or mice mutant embryos, FGF signalling is also required for otic cup-vesicle morphogenesis and/or growth (Reifers et al., 1998; Leger and Brand, 2002; Phillips et al. 2001), As above mentioned depletion of Sox3 in zebrafish also caused reduction or loss of otic vesicles, suggesting a similar disruption to that seen when FGF signalling is abolished. Since Sox3 is regulated by FGF signals, we can hypothesize that FGF effects on otic cup/vesicle morphogenesis could also be mediated by Sox3.

It has been described that disruption of hindbrain patterning leads to defects in otic regionalization (Kwak et al., 2002; Lecaudey et al., 2007) and also that FGFs are essential for caudal hindbrain patterning. Nevertheless, the effects of SU5402 on otic neurogenesis were not secondary to the disruption of neural tube AP regionalization, since the caudal hindbrain is already patterned by the stage the experiments were

performed (Schneider-Maunoury et al, 1993; Schneider-Maunoury et al, 1997; Voiculescu et al, 2001).

FGF8 is a candidate for initial regulation of Sox3

It has been reported the ability of FGF8 in enhancing *Sox3* expression either in the extra-embryonic epiblast of the chick and in the otic-epibranchial placode ectoderm of Ace mutant embryos (Streit et al., 2000; Nikaido et al., 2007). The expression of several FGF molecules along the AP axis from 5 to 10 somites was analyzed to reveal which FGFs could account for restricting Sox3 to the proneural territory. We described that *FGF8* was expressed in the pre-otic ectoderm at the level of r4-5 prior to *Sox3* regionalized expression in the proneural domain. Moreover, our studies demonstrated that FGF8 was able to induce *Sox3* expression in the cranial and trunk ectoderm in 5-8 ss embryos within 3h. Hence, we postulate that FGF8 have a role on *Sox3* establishment in the anterior region of the otic territory. Thus, a series of FGF signaling cascades activated from the surrounding tissues (mesoendoderm and neural tube) would be required for otic placode induction. However, local FGF signaling would be required before the onset of placode formation to restrict and enhance Sox3 to the proneural domain. Our results are in accordance with previous studies. Graft experiments at different axial levels performed by Noden and Van de Water (1986) revealed that presumptive otic placode ectoderm could ectopically generate otic vesicles without the ability to form neurons. Again, Groves and Bronner-Fraser (2000) observed that quail anterior epiblast grafted in the presumptive otic region of host of 3-10ss could start to express *Pax2* and *Sox3*, while grafts performed at 11-21 ss, only expressed *Sox3* but not *Pax2*, suggesting that *Pax2*-inducing signals are lost before *Sox3*-inducing signals. Thus, depending on the time/length of exposure of signals the appearance of some molecular markers, as *Pax2* and *Sox3* can be dissociated. Microsurgical manipulation studies showed that otic cups at 16-somite stage, when rotated in the AP axis, but not ML, downregulated *LFNG* expression from the initial proneural domain and induced *LFNG* expression in the initial non-neural domain, indicating that the proneural domain can be respecified at otic cup stage (Bok et al. 2005). Moreover, rotation of the AP axis of the neural tube at 16-somite stage did not alter the expression profile of *LFNG* and *NeuroD* in the inner ear, suggesting that the signals capable of respecifying the proneural domain did not come from the neural tube (Bok et al., 2005) but from the lateral ectoderm or underlying mesoderm. However, as *LFNG* expression is very dynamic from 16-somite stage to otic

vesicle stage and results were not shown on sections, one should be caution on the results of the respecification of the proneural domain.

In summary, our data suggests that at the onset of otic placode formation, intrinsic FGF8 delineates the proneural domain in the anterior domain by enhancing *Sox3* expression. Once established, *Sox3* expression is maintained by signals different from FGFs and probably extrinsic from the otic territory.

FGF8-FGF10 cascade regulating otic neurogenesis

In other systems, such as in limb development, FGF8 is responsible for the maintenance of *FGF10* expression (reviewed in Martin, 1998). In addition, Zelarayan and co-workers showed ectopic expression of *FGF8* in r3 in a transgenic mouse line which expresses *FGF10* under the control of an EphA4 enhancer (Zelarayan et al., 2007). Besides, the expression of mesodermal *FGF10* is reduced in *FGF3/FGF8* double mutants (Ladher et al., 2005). Thus, is not surprising that during otic development a similar mutual relationship between several FGF molecules takes place. We showed evidences that expression of *FGF8* and *FGF10* in the otic territory is FGF-dependent. In addition, we demonstrated that FGF8-coated beads could induce *FGF10* in the cranial and trunk ectoderm. Altogether, a picture of otic development that is emerging in which initially FGF signals from the endomesoderm such as FGF19/FGF8 and FGF3 from the neural tube are responsible for otic induction and for the appearance of *FGF8* in the anterior pre-otic ectoderm. Subsequently, FGF8 would be inducing *FGF10* and *Sox3*. The role of FGF10 is not to maintain *Sox3* expression as *Sox3* does not depend of FGF signaling after 9-10 somite stage but to drive progenitors to neuronal commitment and express *Neurog1* as reported in Alsina et al., (2004). The question why *FGF8* expression appears restricted at the level of the ectoderm next to the rhombomere 4 still remains unresolved. Although FGF3 and FGF19 are expressed in the mesoderm underlying the pre-otic territory, their expression is not restricted at the level of r4. Thus, other molecules present at the anterior pre-otic ectoderm may cooperate with FGF in order to activate FGF8 expression, such as Wnt signals (note that Wnt8c is expressed in the rhombomere 4 of the hindbrain) or alternatively, posterior signals or transcription factors may inhibit posterior FGF8 expression.

Canonical Wnt signalling plays a crucial role in mediating a placode-epidermis fate decision within the pre-otic field, with cells receiving high levels of Wnt signaling differentiating as otic placode, while cells receiving little or no Wnt signaling differentiating as epidermis (Ohyama et al., 2006). However, Wnt signalling could be having other roles

on otic neural fate. In this regard, it has been shown that dorsal activity of Wnt pathway is responsible for otic dorsal/non-neural fates. Blockade of Wnt signaling leads to expansion of the neurogenic domain to dorsal positions (Riccomagno et al., 2005). This is in accordance with results during neural plate induction, in which Wnt and FGF signaling pathways have antagonistic functions, and low levels of Wnt signaling are required for neural fate (Wilson, 2000). However, it remains to be tested the role of Wnt signalling on *Sox3* or *FGF8* expression during the commitment of the neural fate in the pre-otic ectoderm. During osteoblast differentiation, Manzukhani et al., (2005) found that Sox2 was able to inhibit β-catenin signaling with no requirement of the Sox2 DNA-binding domain. Instead, Sox2 interfered with Wnt responsive genes by sequestering nuclear β-catenin and preventing its binding to TCF/LEF factors. Consistent with this, Zorn et al., (1999) also showed that xSox17a/b and xSox3 bind to β-catenin and excludes TCFs from interacting with β-catenin. They propose that cells expressing relatively high levels of Sox proteins would not transcribe β-catenin/TCF target genes in response to a Wnt signal because β-catenin would be sequestered and rendered unavailable for interaction with TCF. Alternatively, nearby cells expressing lower levels of Sox proteins would respond. Thus, the relative levels of TCF and Sox proteins would determine how a cell responds to a Wnt signal. Interestingly, in murine neural progenitor cells Sox1 also binds to β-catenin and suppresses β-catenin-mediated TCF/LEF signaling, thus potentially attenuating the Wnt signaling pathway (Kan et al., 2004). β-catenin is a potent signal for maintaining neural progenitor cells in a proliferative state (Chen and Walsh, 2002), and interactions between the Sox proteins and β-catenin may be important for maintaining a balance between proliferation and differentiation of neural progenitor cells (Kan et al., 2004). Conversely, it has been reported a cooperative action of Wnt and FGF signaling on activating the Sox2 N1 enhancer in the caudal stem zone plus conserved domains for Lef1 and FGF pathway in this enhancer (Takemoto et al., 2005). Again, Lef1 binding sites were found in the *Sox3* promoter and Wnt8b-mediated Lef1 activation was required for hypothalamus neurogenesis (Lee et al., 2006). Altogether, it would be interesting to explore how regulation of Wnt signaling levels, in conjunction with FGF signaling, may be dictating whether cells acquire a Sox and neural fate.

In summary, we propose a model in which a first wave of FGF molecules, in cooperation with other factors, initiate local *FGF8* expression in the pre-otic ectoderm to then FGF8 enhance Sox3 and induce *FGF10* expression in the anterior otic cup.

Differential requirements of FGF signals and Sox3 Transcription Factor in regulating Lmx1 and Tbx1

Lmx1 (German et al., 1992) is a member of the vertebrate LIM homeobox genes. LIM domain genes seem to function as regulators of cell fate in an variety of different tissues and organisms (Freyd et al., 1990; Karlsson et al., 1990; Taira et al., 1994; Tsuchida etal., 1994; Lundgren et al., 1995; Shawlot and Behringer, 1995; Way and Chalre, 1998). The expression of *Lmx1* in the ear has been reported to be complementary to the proneural domain (Giraldez, 1998; Cole et al., 2000; Alsina et al., 2004) and its regional profile regulated both by inner ear Notch activity and dorsal hindbrain signals (Giraldez, 1998). However, the exact role of *Lmx1* in the ear is still unknown. We showed that *Lmx1* restriction to the posterior domain was already lost after 6 hrs of FGF signaling blockade. Thus, FGF signaling can directly inhibit *Lmx1* expression in the proneural domain. We therefore postulate that *Lmx1* restriction to the non-neural domain of the otic territory is a complex process that requires the convergence of signals from the dorsal hindbrain (Giraldez, 1998) and Notch pathway and FGF signals from the inner ear.

Tbx1 is a member of the gene family encoding transcription factors with a conserved DNA-binding T-box domain. Haploinsufficiency of *Tbx1* has been associated with DiGeorge syndrome (DGS). Most DGS patients have hearing impairment, sometimes of sensorineural type (Yagi et al. 2003; Digilio et al., 1999; Reyes et al., 1999; Swillen et al., 1999). During otocyst development, *Tbx1* is expressed complementary to the neurogenic domain and in the periotic mesenchyme (Raft et al., 2004; Vitelli et al., 2003). Tbx1 in the otic vesicle is required for sensory organ formation (Arnold et al., 2006; Raft et al., 2004; Xu et al., 2007), regulates cell contribution to the otocyst and maintains proliferation (Xu et al., 2007). Besides, *Tbx1* mutation is associated with a considerable expansion of the neurogenic domain, inferring a role of Tbx1 in delimiting the neurogenic region in the otocyst (Arnold et al., 2006; Raft et al., 2004; Vitelli et al., 2003; Xu et al., 2007). It is not know which genes regulate *Tbx1* expression in the otic territory. Several studies have shown that *Tbx1* is a target of Shh signalling in the pharyngeal arches (Garg et al., 2001; Yamagishi et al., 2003) and periotic mesenchyme (Riccomagno et al., 2002) but diverse experimental data do not support this relationship in the ear. Here, we provide evidence that otic *Tbx1* expression is FGF dependent. Our data is also supported by different work in mouse and chick. In Eya1$^{-/-}$ and Eya1$^{bor/bor}$ mutant embryos, which are characterized by lacking otic *FGF3* expression, *Tbx1* regionalization is altered leading to more ventral otocyst expression and either absent (Friedmann et al., 2005; Mansour et al., 1993; Xu et al., 1999). Moreover, in vitamin A-deficient (VAD) quails, which the lack of endogenous

retinoic acid is accompanied by a loss of *FGF3* and *FGF19* from the cranial paraxial mesoderm but not from the neural tube, *Tbx1* expression patterns were disrupted early in development and expression was subsequently lost in all tissues (Roberts et al., 2005). Furthermore, *Kreisler* mutant embryos which have reduced levels of *FGF3* in the hindbrain and severe otic patterning defects, did not present alterations in the expression domain of *FGF3* and *Tbx1* in the otocyst (unpublished; CP). Given that there are no FGF molecules being expressed in the non-neural domain, and that VAD quails present FGF molecules in the neural tube, we would expect that other FGF molecules from the surrounding environment, mesoderm, endoderm or neurogenic domain, would be the ones involved in otic *Tbx1* gene regulation. Interestingly, *FGF10* and *FGF8* have been shown to have *Tbx1*-dependent domains of expression in the heart and pharynx (Vitelli et al., 2002; Hu et al., 2004). Strikingly, *FGF10* expression in the inner ear of *Tbx1* mutants is lost, while *FGF3* expression domain is expanded, inferring a role of Tbx1 in regulating inner ear *FGF3* and *FGF10* (Vitelli et al., 2003; Arnold et al., 2006; Raft et al., 2004). Nevertheless, Vitelli et al., (2003) hypothesize that the loss of epithelial *FGF10* expression could be a consequence of a failure of a subpopulation of otic epithelial cells to proliferate and expand in the absence of *Tbx1*, rather than evidence of a genetic relationship between the two genes. One explanation could be that paracrine effects of FGF signals in the non-neural domain would restrict *Tbx1* expression complementary to the neurogenic domain. Additionally, *Tbx1* in the posterior would position the posterior limit of the neurogenic domain, explaining the enlargement of the neurogenic domain in *Tbx1*$^{-/-}$ mutant embryos. However, when we blocked FGF Receptor activity with SU5402, the downregulation of *Tbx1* was accompanied by a loss of otic neural character but not with an expansion of the neurogenic domain. Interestingly, in Eya1$^{-/-}$, Eya1$^{bor/bor}$ and conditionally activated β-catenin mutant embryos, which lack otic *Tbx1* expression, the amount of cells committed to neural fate was also reduced or absent (Friedmann et al., 2005; Mansour et al., 1993; Xu et al., 1999; Ohyama et al., 2006). All this data suggest again, as previously shown by blocking Notch pathway, that establishment of the proneural domain and the non-neural domain can be uncoupled. Strikingly, SU5402 could not downregulate *Tbx1* periotic expression, suggesting a tight tissue-specific regulation.

A general overview

Sensory organs are responsible for the perception of our environment. Among them, the inner ear is essential for providing information on sound and balance inputs. During the last two decades, increasing amounts of work on the development of the inner ear have provided data on the cellular and molecular mechanisms that govern the formation of the otic primordium, as well as how hair-cells develop. However, less is known on how a proneurosensory region is specified and how this is coupled with cell-fate decisions and morphogenesis. The results of my doctoral research work shed light on the early events of otic regionalization, the establishment of a proneural territory. Early patterning into a proneural and a non-neural domain is associated with expression of patterning genes in the pre-otic ectoderm and subsequent limited cell mixing between the two regions at otic cup stage. The transition from the pre-otic territory to a patterned otic cup requires the activity of Notch and FGF molecules, which down-regulate *Lmx1* in the proneural domain. In addition, we propose that otic neural induction is initiated before the otic placode formation. Strikingly, we show that intrinsic molecules such as FGF8 from the pre-otic ectoderm can also have a role in early otic AP regionalization. We therefore hypothesize that ectodermal FGF8 enhances *Sox3* expression in the more rostral part of the presumptive otic region. As a result, the enhaced *Sox3*-positive domain is responsive to neural inducing signals directing the switch that enables uncommitted otic ectoderm to develop into neuroectoderm. Once committed, proneural genes are expressed and Delta1-Notch1 lateral inhibition controls neurogenesis. This designs a dual role of Notch and FGF signaling in early otic development: first, as part of a mechanism that regulates regional patterning of the otic placode by regulating the expression of patterning genes and, second, as part of the mechanism controlling neural specification and neuronal production by FGF and Notch pathway respectively.

MATERIAL AND METHODS

5. MATERIAL AND METHODS

5.1. Embryos And Staging

Most of the experiments carried out in this thesis work were performed in chick embryos. However, in collaboration with other laboratories comparative analysis of our results were done by using other model organisms such as mouse and zebrafish.

Chick

Fertilized hens´ eggs (Granja Gibert, Tarragona, Spain) were incubated in a humidified atmosphere at 38ºC for designated times (Covatutto incubators). Embryos were staged according to the (Hamburger and Hamilton, 1992) (see also Table I). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; pH7.4) at 4ºC for 24 hours.

Mouse

The mouse line used in this study has been described previously: *RBP-Jk* mutant (Oka et al., 1995). Noon of the day on which the vaginal plug was detected was considered to be 0.5 days of gestation (E0.5), although some variation was observed in developmental stage within litters at the given embryonic ages. Embryos were dissected free of maternal residual tissue and fixed overnight in 4% PFA dissolved in PBS at 4ºC.

Zebrafish

Embryos derived from AB line (ZFIN) were used throughout theses studies. The eggs were spawned synchronously at down of the next morning, and embryos were developed in fish tank water containing methylene blue at 28ºC, staged according to morphological features as described by (Kimmel et al., 1995) and fixed over-night at 4ºC with 4% PFA in PBS.

	Otic Induction	**Otic Placode**		**Otic Cup**		**Otic Vesicle***	
	stage	*stage*	*somites*	*stage*	*somites*	*stage*	*somites*
chick	late gastrula	HH 10-11	9-12 ss	HH 12-15	13-24 ss	HH 16-18	25-36ss
mouse	late gastrula	E8-8.5	1-9ss	E8.75	10ss	E9-10.5	15-35 ss
zebrafish	late gastrula	14-16 hpf	10-14 ss	np	np	18-42hpf	18-30 ss

TABLE 1. Otic development stages in different species. (*) Before morphogenesis of semicircular canals and cochlea.

5.2. In Vitro Embryonic Assays

Two culture conditions were employed during this work. In the organotypic explants the embryo is partially dissected and cultured in semi-suspension conditions, while in explants with matrigel embryos are explanted without removing any tissue or organ. The latter allow to culture embryos of younger ages.

5.2.1. Organotypic explants without collagen

-Dissect embryos corresponding to stage HH9-10 before the second-third somite

-Separate embryos according to stage and transfer into four-well culture plates in 199 medium.

- Transfer the embryos (4 embryo/well) in 199 medium and place them dorsal up.
- Remove 199 medium.
- Add the final solution of control or experimental culturing media. Make sure that the embryos are covered with the medium (250-300µl/well). If embryos do not have enough medium the development of the DV axis will be affected.
- Incubation is carried out at 37.5°C in a water-saturated atmosphere containing 5% CO_2.

Culturing Media

- 5% feotal bovine serum (FBS)
- 1x antibiotic antimycotic solution
- 4mM L-Glutamine
- D-MEM medium (Dulbecco's modified Eagle medium)
- Dimetyl sulphoxide (DMSO) or 100 µM DAPT

⇨ **DAPT Inhibitor Treatment**

In order to assess the role of Notch in early ear development, Notch pathway was blocked by inhibiting the γ-secretase specific for the Notch pathway in these organotypic explants. DAPT inhibitor (Calbiochem, 565770), was shown to effectively block the γ-secretase in vivo in chick and zebrafish (Geling et al., 2004; Dovey et al., 2001). DAPT inhibitor was used at a concentration of 100 µM. At lower concentrations (20 µM) Notch pathway inhibition had the same effects: increasing the number of *NeuroD* cells (n=5/7) and expansion of *Lmx1* (n=12/18). However, at higher concentration, the expressivity of the effects was higher, suggesting that 20 µM was not a saturating concentration.

⇨ **Density of *NeuroD* and *Delta1* expressing cells measurement**

The number of cells expressing *NeuroD* and *Delta1* in control and DAPT treated organotypic explants were counted manually from 40x microphotographs (*Dl1* control n=12, *Dl1* DAPT n=9, *NeuroD* control n=15, *NeuroD* DAPT n=15) and expressed as number of *NeuroD* expressing cells per arbitrary unit of surface area (200x200 pixels). Student´s t-test was used for statistics.

5.2.2. Whole-embryo explants in BD matrigel matrix

- Keep Matrigel (Invitrogen, 354234) on ice at least 2 hours before use (e.g.: during the dissecting period).
- Add 10µl of Martigel per well making a round-shaped layer.
- Keep Matrigel at Room Temperature (RT) until it solidifies (10 minutes is enough, in 1 hour gets dry).
- Transfer the embryos (one embryo/well) in 199 medium. Embryos are dissected around the area pellucida with a bit of area opaca.
- Place them dorsal up on top of the Matrigel layer.
- Remove 199 medium.
- Keep the embryos for 5 minutes (min) at RT without medium to attach them to the Matrigel layer.
- Add the final solution of control or experimental culturing media. Make sure that the embryos are covered with the medium (250-300µl/well). If embryos do not have enough medium the development of the DV axis will be affected.
- Incubation is carried out at 37.5°C in a water-saturated atmosphere containing 5% CO_2.

Culturing Media

- 2% FBS
- 1x Antibiotic antmycotic
- 4mM L-Glutamine
- D-MEM medium

⇨ **SU5402 inhibitor treatment**

SU5402 was shown to effectively block FGF receptors in vivo in Mohammadi et al., (1997). Embryos corresponding to stage HH8-11 were dissected in PBS, separated by stage and transferred into four-well culture plates in 199 medium. SU5402 inhibitor (Calbiochem, 572630) was used at a concentration were either *Pea3* or *Sprouty2* expression was abolished (25µM).

⇨ **FGF8 beads treatment**

Embryos corresponding to stage HH8-11 were dissected in PBS, separated by stage and transferred into four-well culture in 199 medium. Heparin acrylic beads were kept with either 1mg/ml mouse FGF8b (R & D Systems, 423-F8) or PBS in eppendorfs at 4 ºC for 2 hours before use. Mouse FGF8b was used at 1mg/ml.

5.3. Preparation Of Cryostat Sections

Embryos stained for analysis of gene or protein expression were cryosectioned at 16-20µm using a Leica cryostat.

- After fixing the embryos in 4% PFA in PBS, wash 2 x 5 min in PBT.
- Transfer to 15% sucrose in PBS at 4ºC until the embryo sinks (30 minutes aprox.).
- Incubate in 15% sucrose / 7.5% gelatine in PBS at 38ºC for 30 minutes.
- Place embryos embedded in 15% sucrose / 7.5% gelatine in PBS in cryomold under scope to obtain the desired section orientation.
- Cool isopentan at -80ºC.
- Dip the block into pre-colled isopentan for 1 min.
- Keep the blocks at -20ºC until use.
- Before sectioning keep blocks into cryostat chamber for 15 minutes.
- Collect sections in Superfrost
- Dry sections for 30 min at 38ºC and keep at -20ºC in cryoboxes until use or mount in mowiol.

5.4. Assays Of Cell Proliferation By Brdu

Explants treated in control and DAPT inhibitor conditions were incubated with 10 µg/µl 5-Bromo-2´-deoxyuridine (BrdU, Roche, 280879) for 2 hours prior to fixation.

BrdU detection:

- After in situ hybridization, incubate embryos in 2N HCl for 30 minutes.
- Wash three times in Sodium Borate pH 8.9.

- Process embryos for immunohistochemistry (see section 6 in Material and Methods).

BrdU analysis:

Positive cells were counted manually in an area of 200x200 pixels (resolution of 150 pixels/inch). 18 squares have been measured in DAPT treated otic cups and 12 squares for control conditions. Student´s t-test was used for statistics.

5.5. Detection Of Gene Expression

5.5.1. Antisense rna probe synthesis

⇨ **In vitro transcription by DNA linearization**

In this method the DNA sequence to be transcribed is cloned into a vector and flanked by T7, T3 or SP6 RNA-polymerases sequence. TABLE 2 shows the constructs used as templates for in vitro transcription reactions.

DNA Linearization (V_T= 100µl), 2 hours at 37ºC. Add:

10-15 µg of DNA

1x Restriction Enzyme buffer

15U Restriction Enzyme

H_2O final volume

Protein degradation 30 min at 37ºC. Add:

500µg/ml + 0.5% SDS (final concentration)

DNA purification:

Add 1x volume of Phenol and centrifuge 5 min at 13 rpm at 4ºC. Transfer the aqueous solution (upper) to another eppendorf.

Add 1x volume 50% Phenol/ 50% Chloroform and centrifuge 5 min at 13 rpm at 4ºC. Transfer the aqueous solution (upper) to another eppendorf.

Add 2.5x volume 100% Ethanol and precipitate DNA 30 min at -20ºC.

Centrifuge 15 min at 13 rpm at 4ºC. Remove supernatant and add 1ml 70% Ethanol.

Centrifuge 15 min at 13 rpm at 4ºC. Remove supernatant and keep at 37ºC for 5 minutes.

Redissolve linearized DNA in 10µl H_2O.

Gene	Vector	Linearization site and RNA-Polymerase	Ref.	Origin
Chick				
Delta1	Blue Script KSII	EcoRI/T3	(Henrique et al., 1995a)	D. Henrique
FGF10	pGEM-Teasy	NcoI/ SP6	(Ohuchi et al., 1997)	T. Scimmang
FGF19		NotI/T7		R.K. Ladher
FGF3		NotI/T3	EST	
FGF8	Blue Script SK-	EcoRI/T7		G. Martin
FGFR1	EcoRI BS	XhoI/T3		K. Storey
Hairy1	Blue Script KSII+	HindIII/ T7	(Palmeirim et al., 1997)	D. Henrique
Hairy2		HindIII/T7		O. Pourquie
Hes5-1		NotI/T3	(Fior and Henrique, 2005)	D. Henrique
Hes5-2		NotI/T3	(Fior and Henrique, 2005)	D. Henrique
Hes5-3		NotI/T3	(Fior and Henrique, 2005)	D. Henrique
Hes6-2	EcoRV (400pb) Blue Script KSII-	HindIII/ T7	(Fior and Henrique, 2005)	D. Henrique
Irx1		Not/T3	(EST ChEST433E21)	
Krox20	Blue Script KS-	StuI/T7		P. Charnay
Lmx1b		EcoRI/T3	(Giraldez, 1998b)	
Lunatic Fringe		ClaI/T3	(Laufer et al., 1997)	
MafB		NotI/T3	(Eichmann et al., 1997a)	
NeuroD	Blue Script KS+	EcoRI/T3	(Laufer et al., 1997)	
Neurog1		SacI/T7		A.Graham
NeuroM	EcoRI (1.6 bp) Blue Script SK	HindIII/T3		D. Henrique
Notch1	Blue Script KSII	BamHI/T3	(Henrique et al., 1995a)	D. Henrique
Notch2	EcoRV (800pb) Blue Script KSII+	SalI/T7	(Henrique et al., 1995a)	D. Henrique
Pax2		XbaI/T3		T. Schimmang
Pea3	Blue Script SK	NotI/T7		K McCabe
Radical Fringe		ClaI/T3		D. Henrique
Serrate1	Blue Script KSII	HindIII/ T7	(Henrique et al., 1995a)	D. Henrique
Serrate2		EcoRI/T3		
Sox3	Blue Script SK+	SalI/T7	(Rex et al., 1997)	
Sprouty2		SalI/ T7		
Tbx1		NotI/T3	EST, MRC Geneservice	
Mouse				
Irx1	Sac1/Xho1 (1kb) pBK-CMV	XhoI/T7		Christoffels
Lmx1	(full lenght) pGEM-T	NdeI/T7		S. Retaux
NeuroD	(1.074kb) Blue Script KS	EcoRV/T7		
Zebrafsh				
NeuroD	pCNB-SPORT 6.1	EcoRI/T7		

TABLE 2. Constructs used as templates for in vitro transcription reactions.

In Vitro Transcription (V_T= 20µl) 2 hours at 37ºC. Add:

- 1µg linearized DNA
- 1x Transcription buffer
- 1mM DIG-UTP mix
- 40U RNAsine
- 40U RNA-polymerase
- H_2O final volume

DNA degradation 15 min at 37ºC. Add:

- 20U DNAse I -RNAse free

RNA precipitation 30 min at -20ºC. Add:

100µl H_2O

10µl LiCl 4M

300µl 100% Ethanol

RNA purification.

Centrifuge 10 min at 13rpm at 4ºC. Discard supernatant and add 500µl 70% Ethanol.

Centrifuge min at 13rpm at 4ºC. Discard supernatant, dry for 5 min at 37ºC and redissolve with 20µl H_2O.

⇨ **In vitro transcription by PCR**

In this strategy the SP6 promoter sequence is added to DNA sequence to be transcribed by PCR.

Forward primer: specific for the gene to be investigated.

Reverse primer: contains both a sequence specific for your gene and the sequence of the SP6 RNA-polymerase. TABLE 3 shows oligonucleotide sequences used for obtaining RNA probes specific for *Delta1*, *Hairy1* and *Serrate1*.

PCR reaction (V_T= 25µl). Add:

200µM dNTPs

5U Taq-polymerase

1x Buffer (with $MgCl_2$)

5pmols reverse primer

5pmols forward primer

0.01-0.1µg DNA template

H_2O final volume

PCR program:

2 x (94ºC 30 sec, 62ºC 1 min 30 sec, 72ºC 1 min 30 sec)

23 x (94ºC 30 sec, 68ºC 3 min)

4ºC

In Vitro Transcription (V_T= 20µl) 2 hours at 37ºC. Add:

1µg PCR product

1x Transcription buffer

1mM DIG-UTP mix

40U RNAsine

40U SP6 RNA-polymerase

DNA degradation 15 min at 37ºC. Add:

20U DNAse I -RNAse free

RNA precipitation 30 min at -20ºC. Add:

100µl H_2O

10µl LiCl

300µl 100% Ethanol

RNA purification.

Centrifuge 10 min at 13rpm at 4ºC. Discard supernatant and add 500µl 70% Ethanol.

Centrifuge min at 13rpm at 4ºC. Discard supernatant, dry for 5 min at 37ºC and redissolve with 20µl H_2O.

	Oligonucleotide Sequence	TM	Secondary Structures
Delta1			
5' primer	5' CGACCTCACCACAGAAAACC 3'	64.5ºC	none
3' primer	**5' CATTTAGGTGACACTATAGAA**ATTCTTGCATGGCTTGTGGT 3'	76.6ºC	weak
Serrate1			
5' primer	5' GGCGGGAATACCTTCAATTT 3'	63.6ºC	very weak
3' primer	**5' CATTTAGGTGACACTATAGAA**CGGGTGTGGAATGCACTTAT 3'	76.5ºC	moderate
Hairy1			
5' primer	5' GACGAGGTTCCTGTCCACCT 3'	65.5ºC	moderate
3' primer	**5' CATTTAGGTGACACTATAGAA**ACCTCCATGCTGTGGACACT 3'	77.2ºC	moderate

TABLE 3. Oligonucleotide sequences used for obtaining RNA probes specific *Delta1*, *Hairy1* and *Serrate1*.

5.5.2. Whole-mount in situ hybridization (ISH) in chick and mouse

Whole-mount in situ hybridization was carried out with Digoxigenin-labeled (DIG-labeled) RNA probes and alkaline-phosphatase-coupled anti-DIG antibody (anti-DIG-AP), which was then detected with NBT/BCIP according to Nieto et al., 1996.

- Dissect the embryos from desired stages.
- Fix the embryos in 4% PFA in PBS o/n at 4ºC.
- Wash 2 x 5 min in PBS-0.1% Tween-20 (PBT).

Pre-treatments

-Dehydratation/rehydratation:

- 10 min 50% Methanol (MeOH), 50% in PBT.
- 10 min 100% MeOH.
- 10 min 50% MeOH, 50% PBT.

-Wash 2 x 5 min in PBT.

-Incubate without rocking with Proteinase K (10mg/ml, 1/1000 in PBT)

5 min	< 4ss
7 min	5-10ss
10 min	HH11-14
15min	HH15-18

-Rinse 5 min in PBT.

-Fix again the embryos 40 min in 0.25% Glutaraldehyde in 4% PFA.

-Wash 2 x 10 min in PBT.

Hybridization

-Prehybridize embryos with Hybridization buffer for 1 hour at 70ºC.

Hybridization buffer:

- 50% Formamide (FAD)
- 5x SSC pH 4.5
- 1% Sodium dodecyl sulphate (SDS)
- 50 µl/ml yeast RNA
- 0.05 mg/ml heparin

-Hybridize the embryos with Hybridization buffer o/n at 70ºC (RNA probe dilution: 1/500)

Washes

-Wash at 70ºC 3 x 20 min in Wash1.

-Wash at 65ºC 3 x 20 min in Wash 2

-Wash at RT 2 x 10 min in TBS-0.1% Tween-20 (TBST)

Wash1:

50% FAD

2x SSC pH 4.5

1% SDS

Wash2:

50% FAD

5x SSC pH 4.5

Immunohistochemistry

-Incubate the embryos for 1 hour at RT with blocking solution (Heat inactivated and filtered 10% Goat Serum in TBST)

-Incubate the embryos with antibody anti-DIG-AP (1/2000) for 4 hours at RT.

-Wash 6 x 5 min at RT in TBST and keep it o/n at 4ºC.

- Equilibrate the embryos in NTMT 3 x 10 min at RT

NTMT:

- 100mM NaCl
- 100mM TRIS pH 9.5
- 50mM $MgCl_2$
- 0.1% Tween-20

-To develop the reactions incubate samples with 35µl BCIP + 45µl NBT in 10ml NTMT in the dark and at RT.

-Stop the reaction with NTMT and do several washes with PBT o/n.

-Fix ISH with 4% PFA in PBS for 30 min at RT.

5.5.3. Whole-mount in situ hybridization (ISH) in zebrafish

Whole-mount in situ hybridization was carried out with Digoxigenin-labeled (DIG-labeled) RNA probes and anti-DIG-AP antibody, which was then detected with NBT/BCIP according to Nieto et al., 1996.

- Dissect the embryos from desired stages.

- Fix the embryos in 4% PFA in PBS o/n at 4ºC.

- Wash 2 x 5 min in PBT.

Pre-treatments

-Dehydratation/rehydratation:

- 10 min 100% MeOH.
- 1 hr 100% MeOH -20ºC.
- 10 min 75% MeOH, 25% PBT.
- 10 min 50% MeOH, 50% PBT.
- 10 min 25% MeOH, 75% PBT.

-Wash 2 x 5 min in PBT.

-Bleaching with H_20_2 only necessary in embryos older that 36hpf.

-Incubate without rocking with Proteinase K.

5 min	10hpf
10 min	24hpf

-Rinse 2 x 5 min in PBT.

-Fix again the embryos 20 min in 4% PFA.

-Wash 2 x 5 min in PBT.

Hybridization

-Prehybridize embryos with Hybridization buffer for 1 hour at 58ºC.

Hybridization buffer:

- 50% FAD
- 5x SSC pH 4.5
- 1% SDS
- 500 µg/ml yeast RNA
- 0.05 mg/ml heparin
- 0.1% Tween-20

-Hybridize the embryos with Hybridization buffer o/n at 58ºC (RNA probe dilution: 1/500)

Washes

-10 min in 75% FAD, 2X SSC at 58ºC.

-10 min in 50% FAD, 2X SSC at 58ºC.

-10 min in 25% FAD, 2X SSC at 58ºC.

-10 min in 2X SSC at 58ºC.

-2 x 30 min 0.2X SSC at 58ºC.

-Wash at RT 2 x 5 min in MAB-01% Tween-20 (MABT).

Immunohistochemistry

-Incubate the embryos for 1 hour at RT with Blocking solution (MAB + 2% Blocking reagent + 20% heat inactivated Goat Serum in TBST).

-Incubate the embryos with antibody anti-DIG-AP (1/2000) o/n at 4ºC.

-Wash 5 x 20 min in MABT.

- Equilibrate the embryos in Alkaline Phospatase buffer 3 x 10 min at RT.

AP buffer:

- 100mM NaCl
- 100mM TRIS pH 9.5
- 50mM $MgCl_2$
- 0.1% tween-20
- 0.1% triton-X100

-To develop the reactions incubate samples with 35µl BCIP + 45µl NBT in 10ml AP buffer in the dark and at RT.
-Stop the reaction with PBT and do several washes with PBT o/n.
-Fix ISH with 4% PFA in PBS for 30 min at RT.

5.5.4. Double/single fluorescent in situ hybridization (fISH) on cryostat sections

Double/Single fluorescent in situ hybridization was carried out on cryostat sections by the Tyramide Signal Amplification method (TSA-Plus system; Perkin-Elmer Life Sciences) as described in (Yamagata et al., 2002). RNA probes used were Digoxigenin-labeled (DIG) or Fluorescein-labeled (FLUO) and were detected by Peroxidase (POD) enzyme-coupled anti-DIG or anti-FLUO antibody.

Pretreatments

- Defrost slides at RT for 15 min
- incubate 10 min at 65ºC
- Cool down 10 min at RT
- Fix in 4% PFA in PBS for 10 min at RT (in the fume hood). Use 500µl PFA per slide
- Transfer slides into a wash vessel and rinse 5 min in PBS
- Digest proteins 6 min at RT in Proteinase K without rocking

Proteinase K solution:

- 50mM TRIS pH7.4
- 5mM EDTA pH8
- Proteinase K (10mg/ml; 1/10000)

- Rinse 2 min in PBS.

- Fix again in 4% PFA n PBS for 5 min at RT (in the fume hood). Add 500µl PFA per slide.
- Place slides into a wash vessel and rinse 3 x 3 min in PBS.
- Incubate in Acetylation buffer for 10 min at RT.

Acetylation buffer (300ml):

- 4ml Triethanolamine
- 0.525 ml HCl
- 0.75 ml Acetic anhydride
- H_2O final volume

- Wash 3 x 3 min in PBS.
- Permeablize with PBS-1% tritonX100 for 30 min at RT.
- Rinse well 5 x 3 min in PBS to remove all TritonX100.
- Inactivate endogenous peroxodases by incubating 30 min at RT in 0.3% H_2O_2 in PBS.
- Wash 3 x 10 min in PBS.

Hybridization

- Prehybridize samples 30 min at RT with Hybridization buffer in a humidified chamber. Add 200µl per slide.
- Hybridize with RNA probes (for doubles fISH incubate simultaneously DIG-labeled and Fluo-labeled RNA probes; RNA probe dilution: 1/200-1
/500) o/n in a humidified chamber (50% FAD, 5x SSC) at 65ºC. Use 100-200µl per slide and gently lower a glass coverslip over this, avoiding bubbles.

Hybridization buffer:

- 50% FAD
- 5x SSC
- 5x denhart's solution
- (50X: 1% Ficoll, 1% polyuinyl pyrrolidone, 1% BSA)
- 0.25mg/mL yeast tRNA
- 0.25mg/mL heparin
- 0.5 mg/ml salmon sperm DNA (ssDNA)

Washes

- Remove each coverslip by gently squirting a stream of 5x SSC onto the slide, just above the coverslip.
- Place each slide in a wash vessel full of 0.2X SSC preheated at 65ºC. Replace solution and put it in 65ºC incubator. Wash 3 hours with 4 changes of solution.
- Let the last wash to cool down at RT for 15 minutes.
- Wash 2 x 5 min in TBS.

Immunohistochemistry

- Block slides with TNB 1 hour at RT in a humidified chamber adding 200 µl TNB per slide.

TNB

- 0.1M TRIS-HCl, pH7.5
- 0.15M NaCl
- 0.5% Blocking reagent

- Replace with the antibody diluted in TNB (anti-DIG-POD: 1/2000; anti-FLUO-POD: 1/4000) and incubate o/n at 4ºC in a humidified chamber adding 200 µl of antibody solution per slide.
- Transfer each slide into a wash vessel and rinse 3 x 10 min with TNT.

TNT

- 0.1M TRIS-HCl pH 7.4
- 0.15M NaCl
- 0.05% tween-20

- Make up TSA staining solution by adding Cy3-tyramide stock solution in Fluorophore Tyramide working solution (1/100). Apply staining solution (200µl per slide) in the dark for the appropriate time (see TABLE 4 for incubation periods).
- Place slides into a wash vessel full of TBS. If only one probe is to be detected, rinse in TBS, air dry and mount in mowiol.

Double detection of DIG and FLUO-labeled RNA probes

- Incubate 40 min with 0.3% H_20_2 in TBS (to kill the peroxidase enzyme on the first antibody).
- Rinse 2 x 3 min in TBS.

Immunohistochemistry

- Replace with the second antibody diluted in TNB (anti-DIG-POD: 1/2000, anti-FLUO-POD: 1/4000) and incubate o/n at 4ºC in a humidified chamber adding 200 µl of antibody solution per slide.
- Place each slide into a wash vessel and rinse 3 x 10 min in TNT.
- Make up TSA staining solution by adding Cy5-tyramide stock solution in Fluorophore Tyramide working solution (1/100). Apply staining solution (200µl per slide) in the dark for the appropriate time (see TABLE 4 for incubation periods).
- Stop the reaction by transferring slides into a wash vessel full of TBS and wash 3 x 10 min.
- Mount slides in mowiol and remember keeping them in the dark.

Gene	DIG/FLUO	Cy3/Cy5 incubation (min)
Hes5-2	FLUO	10
Hes6-2	DIG	50
Hairy1	DIG	40
Hairy2	DIG	30
Notch1	DIG	30
Notch2	DIG	20
Serrate1	DIG	40
Serrate2	DIG	30
Delta1	DIG	10
LFNG	FLUO	10
Lmx1	DIG	10

TABLE 4. Cy3 and Cy5 incubation periods

5.6. Immunochemistry

Immunochemistry procedure in whole-mount and cryostat sections was performed as Alsina et al. 2004.

- Fix the embryos with 4% PFA in PBS at 4ºC (o/n after dissection, 30 minutes after ISH).
- Wash the embryos 3 x 10 min in PBS-1% Tween-20 (PBT1%).
- Incubate for 1 hour at RT in blocking solution (heat inactivated and filtered 10% Goat Serum + filtered 3% Bovine Serum Albumin (BSA) / PBT1%.

- Incubate with the primary antibody in blocking solution o/n at 4ºC (see TABLE 5 for antibody dilutions).
- Wash 10 x 15 min in PBT1%.
- Incubate the embryos with the secondary antibody in blocking solution o/n at 4ºC (see TABLE 5 for antibody dilutions).
- Wash 10 x 15 min in PBT1%.
- Fix again the embryos in 4% PFA in PBS for 30 min at RT.

	Polyclonal /Monoclonal	Firm	Dilution
Primary Antibodies			
HNK1	monoclonal	Becton Dickinson	50/50
BEN	monoclonal	DSHB	1/200
beta-tubulin III	rabbit polyclonal	Covance	1/400
BrdU	monoclonal	Roche	1/200
GFP	rabbit polyclonal	Molecular Probes	1/500
Pax2	rabbit polyclonal	Zyned Laboratories	1/100
c-myc	monoclonal	DSHB	1/10
Secondary Antibodies			
mouse IgG - alexa 488	goat polyclonal	Molecular Probes	1/400
mouse IgG - alexa 594	goat polyclonal	Molecular Probes	1/400
rabbit IgG - alexa 488	goat polyclonal	Molecular Probes	1/400
rabbit IgG - alexa 594	goat polyclonal	Molecular Probes	1/400

TABLE 5. Antibodies used.

5.7. Fate Map Studies Of The Otic Cup

5.7.1. Double labeling with the vital dyes dDiI and DiO

Double injections of CM-DiI (Molecular Probes, C-7000) and DiO (Molecular Probes, D-275) vital dyes (1μg/μl in DMF) were performed in ovo in the otic placode at stages HH12 in the anterior (proneural) domain and in the posterior domain respectively. Minute amounts of the lipophilic vital dyes were iontophoretically microinjected. Size and location of injections at time zero were visualized and recorded (1/4-1/5 of the width of the placode area) with fluorescence scope (LEICA, MZ FL III). Embryos with overlapping DiI and DiO-labeled initial injections were discarded, the rest were not harvested until stages HH16-17, when they were dissected and fixed with 4% paraformaldehyde/PBS at 4ºC and o/n. The growth of the labeled cells was analyzed by conventional fluorescence microscopy (LEICA DMR) and confocal fluorescence microscopy (LEICA DM IRBE).

5.7.2. DiI labeling and in situ hybridization or immunochemistry

Anterior or posterior CM-DiI injections were generated as above and resulting progeny imaged with conventional fluorescence microscopy after 20 hours of incubation. Embryos were then processed for in situ hybridization with either *FGF10* mRNA (used as an anterior marker) or *Hairy1* mRNA (used as a posterior marker), or immunochemistry using an antibody against HNK1 as a posterior marker. The growth of the resulting progeny in relation to the gene expression domains was analyzed by conventional fluorescence microscopy (LEICA DMR) and confocal fluorescence microscopy (Leica DM IRBE).

5.8. Overexpression Experiments In Ovo By Electroporation

The constructs of interest (see TABLE 6 for constructs used) were electroporated into the otic territory of 9-10HH embryos. A small hole was made into the vitelline membrane to expose the otic placode. The cathode platinium electrode was placed next to the otic territory and anode electrode underneath the embryo. The desired vector (3 μg) mixed with fast green (0.4 μg/μl) was electroporated by injection onto the otic placode by gentle air pressure through a fine micropipette. Square pulses (4 pulses of 10 V) were generated by an electroporator Square CUY-21 (BEX Co., LTd, Tokiwasaiensu, Japan). Medium-199 was added immediately after each electroporation. Eggs were sealed and incubated for 20 hours. Embryos were collected and fixed overnight in 4% PFA at 4ºC for further analysis.

Construct	Plasmid	Given by
DN-MAML1	EGFP-N1-tagged	Dr. J.Aster
NICD	pCS2-MT-N1IC myc tagged	Dr. J.L. de la Pompa
pNeuroD-GFP	pCS2-Myc tag NeuroDpromoter-GFP	Dr. J.Lee
RFP	pCAGGS-IRES-RFP	Dr. D. Henrique

TABLE 6. Constucts used for in ovo electroporation

5.9. Injection Of Morpholinos In Dr. Scotting Laboratory

Morpholinos specific for *SOX3* were injected as described in Dee et al., 2007. Briefly, antisense morpholino oligonucleotides (MOs) designed to target the 5' region of *sox3* were obtained from Genetools (Philomath, OR). The MO sequences were: *SOX3*MO1 5'-GGTGCCAAGCACTCGAAAGAAAACG-3', and *SOX3*MO2 5'-CCATCATGTTATACATTCTTAAAAG-3'. Embryos were injected with a mixture of *SOX3*MO1 (2.5 ng) and *SOX3*MO2 (2.5 ng) in 0.5 nl volume at the 1-2 cell stage. Embryos were incubated at 28ºC in water until they reach stage 24hpf for gene expression analysis.

5.10. Time-Lapse Movies In Dr. Dominguez Henrique Laboratory

⇨ **Embryo slice preparation and culture**

HH13 embryos were coelectroporated with a vector carrying the GFP under the control of the *NeuroD* promoter together with the Red Fluorescent Protein (RFP) and incubated for an additional 22h.

Electroporated embryos were screened under a fluorescent dissecting stereomicroscope (Leica MZFLIII) and selected the ones that showed fluorescence in the otic vesicle.

Selected embryos were sectioned into 100 mm slices using a McILTwain Tissue Chopper (Mickle Laboratory Engineering). The slices were always handled with a pipette with medium HAM'S F12 SUP [HAM'S F12 supplemented with Fungizone 1%+ Glutamine 2%+ piruvateNa 1%+ Penicillin/streptomycin (Gibco)1%] and transferred to a Petri dish containing the same medium.

The slices that presented better fluorescent intensity and isolated cells were selected under the inverted microscope and then harvested in a Petri dish with medium HAM'S F12 SUP AG [HAM'S F12 SUP supplemented with agarose1% pre-heated at 40ºC]. Slices are embedded in this pre-heated medium with agarose and then transferred to a new Petri dish with a central hole surrounded by solidified medium (HAM'S F12 SUP AG). The slices must be well spread in the central bottom of the Petri dish. After solidification of the agarose ~5ml of medium HAM'S F12 SUP was added, followed by mineral oil until all surface of the Petri dish was covered in order to avoid evaporation of the medium.

⇨ **Slice imaging**

Slices were imaged in confocal Zeiss LSM510 microscope, with a 20x objective (Plan-NeoFluor; NA0.5) in a 37ºC humid chamber. 16 optical sections (z stacks) with 31.35 mm step size were imaged at 2.09 min intervals up to 12h.

The fluorescent images were subjected to the data analysis as follows: The individual fluorescent cells were identified manually, and the stack with higher fluorescent signal was followed manually throughout the time-lapse series to create the movies. The movies were then assembled using Image J software. ImageJ was also used to convert image sequences to QuickTime movies.

5.11. Photography And Imaging

Whole or sectioned embryos were photographed using LEICA DMR conventional fluorescence microscope fitted with Leica DFC 300FX camera or a LEICA, MZ FL III fluorescence scope fitted with Leica DFC 300FX camera and LEICA DM IRBE confocal microscope. Images were captured with Leica IM50 v4.0 and analysed with Adobe photoshop v7.0.1.

5.12. Measurement Of Relative Size Of Otic Vesicles

To compare the relative sizes of otic vesicles between wild type zebrafish embryos / SOX3-Morphants embryos and wild type mouse embryos / RBPjk mutant embryos, we analysed on transversal sections the average of sections that were containing the otic vesicle in each different condition and calculated the increment. To compare the extension of the expression domains of both *Lmx1* and *Irx1* in wild type mouse embryos and RBPjk mutant embryos, we analyse on otic vesicle transversal sections the amount of sections containing *Lmx1* or *Irx1* non-expressing domain.

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APPENDIX

7. APPENDIX

7.1 Alsina et al., 2004

(see attached file)

7.2 Abelló et al., 2007

(see attached file)

Original article

Electronic version:

Alsina B, Abelló G, Ulloa E, Henrique D, Pujades C, Giraldez F.

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FGF SIGNALLING IS REQUIRED FOR DETERMINATION OF OTIC NEUROBLASTS IN THE CHICK EMBRYO

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Short running title: FGF signaling and otic neurogenesis

ABSTRACT

The interplay between intrinsic and extrinsic factors is essential for the transit into different cell states during development. We have analyzed the expression and function of *FGF10* and FGF-signaling during the early stages of the development of otic neurons of the cochleo-vestibular ganglion (CVG). *FGF10* is expressed in a highly restricted domain overlapping the presumptive neurogenic domain of the chick otic placode. A detailed study of the expression pattern of *FGF10*, proneural and neurogenic genes revealed the following temporal sequence in the onset of gene expression: *FGF10>Ngn1/Delta1/Hes5>NeuroD/NeuroM*. FGF10 and FGF receptor inhibition cause mirror images on cell-determination and cell proliferation. Ectopic expression of FGF10 *in vivo* promotes an increase in *NeuroD* or *NeuroM* expression, but not *Delta1*. BrdU incorporation experiments showed that the increase in *NeuroD*-expressing cells is not due to an increase in cell-proliferation. These effects occur only within the neurogenic domain of the otic vesicle. Inhibition of FGF receptor signaling in otic explants causes a severe reduction in *NeuroD* and *Delta1* expression with no change in non-neural genes like *Lmx1.* FGF signaling inhibition does not interfere with *NeuroD* expression within the CVG or neuroblast delamination. We suggest that FGF10-mediated signaling in the otic epithelium is required for neuronal precursors to withdraw from cell division and for their determination.

INTRODUCTION

The vertebrate inner ear is a complex sensory organ responsible for the sensations of sound and balance, as well as a variety of reflexes. The inner ear derives from the otic placode that is formed early in development in the ectoderm adjacent to the hindbrain, and develops later into the otic vesicle. The otic vesicle undergoes complex morphogenenesis resulting in a highly organized apparatus named the ear labyrinth that holds the ear sensory organs. What is striking in ear development is that the apparently homogeneous otic placode -formed by no more than few thousand cells - contains the cues and information to generate different cell types, including the innervating neurons, with a precise and exquisite topology (Swanson et al., 1998).

Otic neurons connect sensory mechano-receptors of the ear, the hair-cells, with their targets in the central nervous system. The generation of otic neurons is a sequential process, which includes first, the specification of otic precursors in the otic epithelium, secondly, the delamination of epithelial neuroblasts to form the cochleo-vestibular ganglion (CVG), thirdly, the proliferative expansion of ganglionar neuroblasts, and finally the differentiation of neurons that innervate back the vestibular and cochlear (auditory) sensory organs (reviewed in Alsina et al., 2003). The first visible output of otic neurogenesis is the delamination of otic neuroblasts from the otic vesicle and the formation of the CVG, but cell fate specification starts much earlier in otic development, at the otic placode stage (Adam et al., 1998)

Vertebrate proneural genes are basic helix-loop-helix (bHLH) proteins with homology to *Drosophila* proneural genes. *Neurogenins* have conserved the neuronal determination functions of the *Drosophila* counterparts, whereas *NeuroD* is required for neuronal differentiation and survival (Cau et al., 2002; Bertrand et al., 2002). Early expressed proneural genes are involved in the selection of progenitor cells that become competent to acquire defined cell fates and commit to differentiation (Cau et al., 2002). Accordingly, the inactivation of *Neurogenin1* or *NeuroD* causes a reduction in the output of otic neurons (Ma et al., 1998; 2000; Liu et al. 2000; Kim et al., 2001).

The activity of the proneural genes is influenced by cell extrinsic signals. Secreted factors mediate crucial steps in development like cell growth and survival, bias between self-renewal and differentiation, or choices between different cell-fates (Edlund and Jessell, 1999; Vaccarino, 1999). Fibroblast growth factors (FGFs) play multiple roles in cell communication during development, and are attractive candidates for regulation of critical steps in neurogenesis (Vaccarino, 1999). Interestingly, FGF signaling has been shown to be required for neural induction in the chick embryo (Wilson et al., 2001), induction of posterior neuronal precursors in the neural tube (Henrique et al, 1997), early differentiation of retinal ganglion cells (McCabe et al., 1999), and for crucial steps in olfactory development and regeneration (Schwob, 2002).

Our study was prompted by the observation that *FGF10* is expressed in the presumptive neural-sensory epithelium of the otic vesicle (Pirvola et al., 2000, in mouse, and our results in the chick – see below). The present work was aimed at studying the function of *FGF10* and FGF-signaling in early otic neurogenesis. We carried out a detailed analysis of the expression of *FGF10* and several proneural and neurogenic genes on the chick otic vesicle, to then study the effects of gain- and loss-of-function of FGF10 on the generation of otic neurons. The results show that *FGF10* expression defines an early regional domain that anticipates proneural and neurogenic gene expression in the otic placode. The sequence in the onset of gene expression is *FGF10/Lfng>Ngn1/Delta1/Hes5>NeuroD/M*. Over-expression of *FGF10*, FGF10 delivery with microbeads *in ovo*, or the addition of FGF10 to otic explants promote the expression of neuronal differentiation genes like *NeuroD* or *NeuroM*, but not *Delta1*. On the contrary, FGF-receptor blockade produces a reduction of the expression of *NeuroD* and *Delta1*. Combined analysis of cell proliferation and gene expression show that the increase in *NeuroD* expression is neither associated with the recruitment of neural precursors, nor with the proliferative expansion of neuroblasts, but with the acceleration of the transit towards the state of neuronal determination.

Materials and Methods

Embryos

Fertilized hens´ eggs (Granja Gibert, Tarragona, Spain) were incubated at 38ºC for designated times and embryos were staged according to Hamburger and Hamilton (1951). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS; pH7.4) at 4ºC for 24 hours.

Organotypic explants

Otic placode explants were done as reported by Giraldez (1998). Briefly, transverse sections of chick embryos were aseptically isolated and microdissected. Embryos were sectioned behind the rhombo-mesencephalic limit and before the second-third somite, and the heart removed. The explant was formed by the neuroectoderm, the adjacent ectoderm and the pharyngeal endoderm. Incubation was carried out in Dulbecco's modified Eagle medium DMEM (Gibco) at 37.5 ^{o}C in atmosphere of 5%CO_2. For organotypic cultures of otic vesicles and CVG, otic vesicles were dissected from embryos corresponding to stage 17-18, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37^{o}C in a water-saturated atmosphere containing 5% CO_2 as described (León et al., 1995). Additions were 1-5% fetal bovine serum (Bio Whittaker Europe), 100 ng/ml human recombinant FGF10 (R&D), and 5-50 μM SU5402 (Calbiochem).

Whole-mount in situ hybridization and immunochemistry

Whole-mount in situ hybridization was carried out according to Nieto et al. (1993). Details of *FGF10* probe are given in (Bellusci et al., 1997), *Delta1* (Henrique et al.,1995), *neurogenin1* (Bebgie et al, 2002), *NeuroD*, *NeuroM* and *Lunatic Fringe* (Laufer et al.,1997), The chick *HES5* probe will be described elsewhere. Whole-mount immunohistochemistry after in situ hybridization was used to detect several antigens. Embryos were blocked at room temperature with 5% Blocking Reagent (Roche in Maleic acid buffer), 5% Goat Serum in PBT(0.1% Tween) for 90 minutes, incubated overnight with the primary antibody (2% blocking reagent, 5% goat serum, RT), ten-

times washed with the same solution and incubated with secondary antibodies overnight. Embryos were rinsed several times in PBT before mounting in Mowiol. Anti-GFP polyclonal antibody (Molecular Probes; 1:500), anti-Tuj1 monoclonal antibody (Covance; 1:200), anti-pH3 polyclonal antibody (Upstate Biotechnology; 1:200) have been used as primary antibodies, while goat anti-rabbit Alexa549 and goat anti-mouse Alexa 488 (Molecular Probes;1:200) have been used as secondary antibodies.

In some experiments a semi-quantitative estimation of the intensity of expression was done by averaging the density histogram of single expression domains. Photographs of paired otic vesicles were acquired with a CCD camera, processed in Adobe Photoshop and expression domains manually outlined. Background was measured in a non-labeled portion of ectoderm and substracted from measurements.

BrdU experiments

Explants and otic vesicles were incubated with 10 μg/ml 5-Bromo-2´-deoxyuridine (Aldrich) added to the culture medium 2 hours prior to fixation. After in situ hybridization procedure, explants and otic vesicles were incubated in 2N HCl for 30 minutes, three times washed in Sodium Borate pH 8.9 and processed for immunohistochemistry as described above. BrdU mAb BMC9318 antibody (Roche) was used in whole-mount at 1:200 dilution.

Cryostat and vibratome sectionning

For cryostat sectioning, embryos were fixed in 4% paraformaldehyde, dehydrated in 15% sucrose and embedded in 30%gelatin/15%sucrose. Blocks were frozen in isopentane to improve tissue preservation and then sectioned at 10 μm thickness onto Superfrost Plus Slides (Fisher,Pittsburg, PA) and stored at –20ºC.

For immunohistochemistry on frozen sections, the following protocol was used. Sections were blocked in 10% goat serum, 3%BSA for 1 hour and then incubated with primary antibodies in blocking solution at 4ºC overnight. Then, ten washes with PBT (15 minutes each) were applied before incubating with secondary antibodies for 2 hours at room temperature. Sections were then extensively washed in PBT prior to

mount in Mowiol. Anti-Islet 1/2 (From the Developmental Studies Hybridoma Bank; 1:200), anti-Tuj1 (Covance;1:400), anti-pH3 (Upstate Biotechnology;1:400) were used as primary antibodies. Same secondary antibodies were used as described before but diluted 1:400.

Bead implantation

Bead implantation was carried out on stage 10-12 embryos. Heparin coated acrylic beads (Sigma) were washed in PBS and soaked in human recombinant FGF10 (R&D, 1 mg/ml) for two hours at 4 ^{o}C, plus another 30 minutes at room temperature. Beads were implanted through a window opened in the egg and using Fast Green (3 mg/ml, Sigma) for better contrast of the embryo. A slit was made through the vitelline membrane and through the ectoderm immediately anterior or posterior to the otic placode. Eggs were sealed and incubated during 20-24 hours, when they were collected and fixed overnight in 4% paraformaldehyde/PBS at 4ºC. Results were obtained from implantations that resulted in beads located within one bead diameter from the otic vesicle.

In Ovo Electroporation.

In ovo electroporation was used to obtain ectopic expression of *FGF10* in the otic placode of stage 10-13. The full coding sequence of *FGF10* (gift from Hideyo Ohuchi, University of Tokushima, Japan) was subcloned in to the bicistronic vector pCAGGS-IRES-GFP (Bekman,E. & Henrique,D., unpublished). A small hole was made into the vitelline membrane to expose the otic placode. Platinum electrodes (0.5 mm diameter) were placed 5 mm apart, sandwiching the embryo. Vector (3-5 μg/μl) mixed with Fast Green (0.4 μg/μl) was electroporated by injection onto the otic placode/cup by a gentle air pressure through a fine micropipette. Square pulses (ten 25 V pulses 25-ms pulse leght, 10 Hz) were generated by an electroporator Square CUY-21 (BEX Co., LTD, Tokiwasaiensu, Japan). Cold medium (M-199) was added before and immediately after each electroporation. Eggs were sealed and incubated for 24 hours. Embryos were then examined under the fluorescent microscope for green fluorescence signals.

Embryos with good GFP staining in the otic vesicle were collected and fixed overnight in 4% paraformaldehyde/PBS at 4ºC for further analysis.

RESULTS

Expression of FGF10 during development of the otic vesicle.

In the chick, the otic placode is visible at stage 10 as a thickening of the ectoderm adjacent to rhombomeres 5 and 6. By stage 12, the otic placode invaginates to form the otic cup and then closes up and pinches off from the ectoderm, forming the otic vesicle by stage 17. We shall make reference to the otic placode (stage 10), early otic cup (stages 12-14) and late otic cup (stages 15-16). The earliest expression of *FGF10* was observed in the otic placode, at stage 11-12, restricted to the most anterior and medial region of the otic placode (Fig. 1A). Figure 1B shows a low magnification photomicrograph that illustrates the highly regionalized pattern of expression of *FGF10* in chick embryos between stages 12 and 16. Expression of *FGF10* was also detected in the endoderm of the fourth visceral pouch (not shown) and in the primordium of the olfactory placode (Olp in Fig. 1B). The regionalized expression of *FGF10* in the otic cup recalls that of *Delta1* (see Adam et al., 1998 and results below), being one of the earliest signs of regionalization of the otic placode. At the otic cup stage, *FGF10* expression extended to the anterior-medial half of the otic cup, keeping always within the ridge of the otic cup (Figure 1C and D). Dorsal views (Fig. 1C and E) illustrate the division of the otic cup into two territories by an axis running from anterior-lateral to posterior-medial, at about 45 degrees respect to the anterior-posterior axis of the embryo. The restriction of *FGF10* expression to the anterior domain was also evident in the otic cup and early otic vesicle from a lateral view (Figure 1D and F). At the stage of otic vesicle (Figure 1G-H), *FGF10* transcripts were detected anterior and medial, excluding the dorsal-most aspect of the otic vesicle. *FGF10* covered a spherical triangle that was better seen in oblique views (see Fig. 1H' and the diagram in Fig. 1).

At stage 18-19, the *FGF10* domain transformed into an anterior-medial band, that run through the equator of the otic vesicle (Figure 1I,J). No *FGF10* expressing cells were observed in the cochleo-vestibular sensory neurons, in contrast to what has been described in the mouse (Pirvola et al., 2001). A three-dimensional schematic drawing illustrating the dynamics of *FGF10* expression and the transition from the otic placode to the otic vesicle is shown in Fig. 1 (right panel diagrams). In summary, *FGF10* expression pattern was very dynamic, first subdividing the placode/cup into an anterior and posterior domain and later on becoming regionalized into a ventral band. Probably, this reflects the displacement of a coherent domain during the morphogenetic events that drive the transformation of the otic placode into the otic vesicle, rather than switching on and off the expression of FGF10 (see Brigande et al., 2000 and discussion).

FGF10 and the early anterior-posterior regionalisation of the otic placode.

As mentioned above, the early expression of *FGF10* is one of the earliest signs of regionalization of the otic placode. It was interesting to test whether other genes were also regionalized along the same domains that *FGF10*. Simultaneous staining of *FGF10* and HNK1 expression revealed complementary domains in the early otic cup, indicating the existence of two territories with different properties (Fig. 2A and B). HNK1 is a sugar residue carried by several recognition molecules (see Discussion). At stage 13, *Lmx1*, encoding a LIM-domain protein, was expressed in the otic ridge, but it was detected only at the posterior region of the otic cup (Fig. 2C). At otic cup and otic vesicle stages, *Lmx1* was absent from the neural-sensory domain in a manner complementary to *FGF10* (Giraldez, 1998, and results not shown). *Lunatic Fringe* (*Lfng*) was also expressed in the anterior territory, and excluded from the otic ridge (Fig. 2D). Genes detected in the anterior domain (*FGF10*, *Lfng*, and other genes described below) did not extend beyond the boundary *FGF10*/HNK1. This is illustrated by the double detection of HNK1 domain (green anti-HNK1 antibody staining) and

Delta1 (blue, in situ hybridization) in a stage-14 otic cup. Figure 2E and F show that the HNK1-expressing domain was complementary to that of *Delta1*. Other genes that were later restricted along the medial-lateral plane were not regionalized at this stage. This was the case of *Dlx5* and *Pax2* (results not shown). The early expression of FGF10 suggests that the otic placode is first patterned along the anterior-posterior axis, and this patterning precedes dorsal-ventral or medial-lateral regionalization.

We further examined the relationship between the AP expression boundary and otic neuron generation. Fig. 2G illustrates delaminating neuroblasts that were identified by means of the Islet1/2 antibody (red), along with *FGF10* expression detected by in situ hybridization (blue) on the same preparation. Neuroblasts delaminated only from a narrow stripe situated along the posterior boundary of the *FGF10* domain, as confirmed by serial para-saggittal sections running from lateral to medial (results not shown) The schematic drawing to the right of Fig. 2H illustrates the sites of delamination of otic neuroblasts. This observation suggests that neuroblasts delaminate only from a sub-domain of the neurogenic domain and not from the whole neurogenic domain as frequently assumed, and suggests a potential role of the AP boundary in the process.

FGF10 is expressed in the proneural-sensory territory (proNS) of the otic placode: early expression of proneural genes.

The expression pattern of *FGF10* recalls that of some proneural and neurogenic genes in the otic vesicle (Adam et al., 1998; Cole et al., 2000; Begbie and Graham, 2003), suggesting a possible relation between *FGF10* and otic neurogenesis. To further study this possibility we decided to carry out a systematic study of the expression profiles of proneural genes and compare them with *FGF10* expression pattern. Figure 3A shows a dorsal view of stage 14-15 otic cups, where the expression of *FGF10* is compared to that of *Neurogenin1* (*Ngn1*), *Delta1*, *Hes5*, *NeuroD* and *NeuroM*. Early expression of these genes was always within the domain of *FGF10*. *Ngn*1 is a neuronal determination gene in cranial sensory ganglia as observed by loss of function studies,

in which the CVG is also missing (Ma etal al., 1998). *Ngn*1 expression was detected at stage 11 chick embryos, when individual *Delta1* positive cells were also present (results not shown). The vertebrate *Hes* genes, homologues of *Drosophila Hairy* and *Enhancer of split*, are targets of Notch and function as proneural repressors in the CNS (de la Pompa et al, 1997, see Bertrand et al, 2002), but nothing is known about the expression of these genes during early otic neurogenesis. *Hes5* was expressed concomitantly with *Delta1* and *Ngn*1 in a scattered fashion (Fig. 3Ad). *Lfng* was also expressed in a restricted manner in the anterior domain but only after stage 12 (Fig. 2D). *Lfng* was initially expressed in the otic placode in a broad unrestricted manner from stage 10 (not shown), but then refined to the pattern shown in Fig. 2D. As illustrated in Fig. 3A, all proneural genes studied were restricted to the same anterior-medial domain as *FGF10*. We call this expression domain the *pro-Neural-Sensory domain* (*proNS),* as it foreshadows the neurogenic and sensory domains of the otic vesicle (see Adam et al., 1998 and Cole et al, 2001). The sequence of the onset of expression of the complete gene collection studied was: *FGF10* (stage11) > *Ngn*1, *Delta1, Hes5* (stage11^{+}, 14 somites) > *NeuroD*, *NeuroM* (stage 12).

Figure 3B shows in more detail the expression profile of *Delta1*, *NeuroD* and *NeuroM* during the transit between the otic placode and the otic vesicle. *Delta1* was expressed from otic placode to otic vesicle following a similar pattern as the one described above for *FGF10* (Fig. 3Ba-c, upper row). *Delta1* expressing cells were always confined to the otic epithelium, as previously reported by Adam et al. (1998). *NeuroD* was first detected at stage 12, also in scattered cells (not shown), and at otic cup stage *NeuroD* was intensely expressed in the proneural-sensory domain (Fig. 3Bd). By stage 16, *NeuroD* positive cells populated the CVG (Fig. 3Be), and by stage E18-19 NeuroD expression diminished in the otic epithelium (always ventral), but now positive neuroblasts were found in most cells of the CVG (Fig. 3Bf). This indicates that *NeuroD* is expressed in epithelial neuroblasts and as the CVG is formed, the otic epithelium looses these cells as they translocate to the CVG. *NeuroM*, another neuronal

differentiation factor closely related to *NeuroD*, was also found in the neural-sensory domain of the otic epithelium at otic cup stages (Fig. 3Bg-h), and follows a similar regional and temporal pattern of expression. However at stage 18-19 (Fig 3Bi), *NeuroM* expression was only detected in the distal-most domain of the CVG and no *NeuroM* positive cells were seen within the epithelium. Since it is known that vestibular neurons are generated earlier and placed more distal than cochlear neurons (D'Amico-Martel 1982; Hemond and Morest 1991), it is tempting to suggest that *NeuroM* may be critical for specification of vestibular *vs.* cochlear identities.

Following what is known about proneural genes in vertebrates (Bertrand et al., 2002), *Ngn1* and *Dl1* expression in the otic epithelium probably reflect the step of selection of progenitors and their commitment to neural fate, whereas *NeuroD/M* indicates the acquisition of the state of full determination, and the migration of otic neuroblasts to the CVG. In the following experiments we shall use *NeuroD* as the output of cell determination, and *Dl1* as the reflection of specification of progenitors and the initiation of neuronal commitment.

Overexpression of FGF10 induces an increase of NeuroD expression.

In order to examine the role of *FGF10* in otic neurogenesis we studied the effects on gene expression of local delivery of recombinant FGF10 protein with heparan microbeads. FGF10-soaked beads were implanted in stage 11-12 embryos under the ectoderm and anterior to the otic placode of one side, the other being the control, (Fig. 4A). Ectopic FGF10 induced the expression of *NeuroD* or *NeuroM* within their normal expression domains (Fig. 4Ba-d, upper and middle rows; 12/17 experiments). On average, the increase in the intensity of *NeuroD* expression was of 1.5-fold respect to the control side. FGF10-beads implanted posterior to otic placode did not show ectopic expression of *NeuroD* or *Delta1* (results not shown). Examination of the sections showed no ectopic or aberrant sites of delamination in otic vesicles exposed to FGF10 beads.

In order to explore whether the increase of *NeuroD*-expressing cells was caused by recruitment of progenitor cells, we also examined the effects of FGF10 over-expression on *Delta1*. *Delta1* expression did not change with FGF10 delivered with micro-beads (Fig. 4Be,f; 9/9 experiments). These results indicate that *FGF10* does not induce neuronal fate, and as discussed below, the effects of FGF10 on *NeuroD* expression may arise either from the expansion of the *NeuroD* expressing cell population, or from the accelerated transition from precursors to neuroblasts.

Further confirmation of the effects of *FGF10* on *NeuroD* was obtained with electroporation experiments. *FGF10* was subcloned in a pCAGGS bicistronic vector using GFP as a reporter (Fig. 5A, upper panel) and electroporated into the otic placode/cup at stage 10-12, allowing embryos to develop for 24h. Expression of GFP was restricted to the otic vesicle (Fig. 5A, lower pannel), and co-localized with the sites of *FGF10* overexpression (data not shown). Figure 5B, shows two examples of electroporated embryos that were assayed for *NeuroD* expression (a,c control, b,d electroporated). Over-expression of *FGF10* caused an increase in *NeuroD* expression in the anterior domain of the otic vesicle (proNS). Although *FGF10* was intensely expressed all throughout the otic vesicle (Fig. 5A), it did not induce the ectopic expression of *NeuroD*, indicating that this took place only within a neural competent domain. Vibratome sections in Fig. 5C show the induction of *NeuroD* in the electroporated side (Fig. 5Cb) as compared to the non-electroporated (Fig. 5Ca, parasaggittal sections). Again, *Delta1* expression did not appear to change after *FGF10* overexpression (Fig. 5Cc,d, coronal sections).

Inhibition of FGF10 reduces NeuroD expression in the otic cup.

To further analyze the effects of *FGF10* we made use of otic explants grown in culture, which allow a more precise and quantitative control of concentrations of added factors and inhibitors (Fig. 6). Explants containing the otic placode were dissected at stage 11-12 and grown in culture for 16-18 hours in the presence of 1-5% FCS either alone

(control), with 200 ng/ml FGF10 (FGF10), or in the presence of 5-50 μM of the FGFR inhibitor SU5402 (SU5402). Then they were assayed for gene expression by in situ hybridization and BrdU incorporation. A diagram of the experiment and an example of a batch of explants is shown in Fig. 6A. SU5402 belongs to a class of FGF receptor inhibitors that inhibit the tyrosine-kinase activity of the FGFR1 receptor by interacting with its catalytic domain (Mohammadi et al., 1997). Half-inhibitory concentrations of SU5402 range from 2 to 30 μM (Mohammadi et al., 1997; MacCabe et al., 1999). Treatment of explants with 50 μM SU5402 produced a dramatic inhibition of both *NeuroD* and *Delta1* expression when compared either to FGF10-treated or control explants (Fig. 6B, n=11/14 for *NeuroD* and 5/5 for *Delta1*), whereas both genes were still expressed in the neural tube. SU5402 did not inhibit the expression of *Lmx1* (Fig. 6Bj,kl) or cell proliferation in the explants (Fig. 6Bc,f,j), indicating that the loss of *NeuroD* and *Delta1* was specific and not caused by a general retardation of growth. A semi-quantitative analysis of results is shown in Fig. 6C. *FGF10* induced *NeuroD* in the otic epithelium (Fig. 6C, upper histogram), but not *Delta1* (Fig. 6C, lower histogram), and SU5402 reduced drastically *NeuroD* and *Delta1* expression. Therefore, inhibition of FGF-signaling leads to an inhibition of expression of both *NeuroD* and *Delta1* in otic explants.

FGF10 does not induce proliferation of epithelial neuroblasts but accelerates the transition to neural determination.

Knowing that neuronal precursors are able to proliferate in the CVG (D´Amico Martel 1982, Adam et al., 1998, Begbie et al, 2003) and that FGFs promotes cell proliferation in many systems, one possible explanation for the increase of *NeuroD* caused by *FGF10* and its reduction by SU5402 is that FGF10 is required for proliferation of *NeuroD* expressing neuroblasts *within* the otic epithelium. To test this possibility, we studied the effects of exposure to recombinant FGF10 and suppression of FGF-signal

on both *NeuroD* and cell proliferation. To better understand the effects, we wanted to have access to a broad window of states of commitment of the neuroblast population, both in the epithelium and the CVG. With this in mind, we performed experiments with otic vesicles of stage 17-18, that show the coexistence of newly generated epithelial neuroblasts (first expressing *NeuroD*), those that delaminate, and also those ganglionar neuroblasts that populate the CVG (that continue to express *NeuroD* as well as other markers, see Alsina et al., 2003). Otic vesicles were grown in culture for 16-18 hours until they developed to equivalent stage 20-22 (see diagram in Fig. 7A). Note that the primordium of CVG was removed before culture (Fig. 7A) and that the epithelial (proNS) and gaglionar (CVG) domains of *NeuroD* expression were well distinguished after 16-18h in culture. Figure 7B shows that the increase of *NeuroD* by FGF10 (compare Fig. 7Ba and b, n=16/19) and its suppression by SU5402 recapitulated the experiments described above (n=17/18). The relationship between FGF10, *NeuroD* expression and cell-proliferation was studied in these experiments by double-labeling for BrdU incorporation (green) and *NeuroD* expression (black), and analyzing otic vesicles with confocal microscopy (Fig.7d-f). Figure 7 displays overlay confocal sections of otic vesicles from control (d), FGF10 (e) and SU5402 (f) treated otic vesicles, dotted lines indicating the otic epithelium positive to *NeuroD*, the proNS domain. In control (Fig. 7Bd, control), BrdU positive cells were intermingled with *NeuroD* expressing cells that appear in black. However, treatment with FGF10 reduced BrdU positive cells within the *NeuroD* expressing domain, cell proliferation that remained high in the adjacent region (Fig. 7Be). On the other hand, FGF10 and SU5402 showed mirrored effects on cell proliferation and *NeuroD* expression (compare Fig. 7Be, FGF10, and 7Bf, SU5402). SU5402 treated otic vesicles showed low *NeuroD* expression and many BrdU positive cells within the *NeuroD* domain, whereas FGF10 exhibited the opposite. This suggests that FGF10 may regulate the transition to *NeuroD* by withdrawing progenitors from cell-division cycle.

Figure 7C shows a detail of the CVG from one otic vesicle that was treated with SU5402 and assayed for *NeuroD* and BrdU incorporation. As shown, in spite the drastic inhibition of *NeuroD* in the epithelium of the otic vesicle, neuroblasts of the CVG continued to express *NeuroD* (Fig.7Ca) and to proliferate (Fig.7Cb). This implies that once cells express *NeuroD* they are no further dependent on FGF signaling to support its expression, to delaminate and to proliferate within the CVG. *NeuroD* expressing neuroblasts are fully determined. On the other hand, close examination of preparations like in Fig. 7B showed no coincidence of *NeuroD* expressing cells with BrdU, implying that neuroblasts remain in cell-division arrest while in the epithelium.

Discussion

The interplay between intrinsic and extrinsic mechanisms is believed to be crucial for ensuring the correct number and identity of neurons in particular regions of the embryo (Edlund and Jessell, 1999; Alsina et al., 2003). The present work shows the ability of FGF10 to regulate the expression of *NeuroD/M* by otic neuronal precursors. These genes are neural differentiation genes whose onset announces full commitment to the neuronal fate, that is, neuronal determination. We propose a model where FGF signaling shifts otic epithelial precursos towards neuronal commitment and keep them in relative quiescence while in the epithelium. *NeuroD* expression allows epithelial neuroblasts to delaminate and only after leaving the otic vesicle they undergo transit-amplification and differentiation. The results show also that *FGF10* defines an early domain in the otic placode that anticipates the neural-sensory competent region, whose regional and temporal gene expression pattern we studied in detail. We shall discuss first briefly the topic of regional compartimentalisation of the otic placode, and ~~to~~ then proceed into the question of neural commitment and the role of FGF10.

Is there an early compartmentalisation of the otic placode?

Establishment of compartments and boundaries is thought to be important for patterning and cell diversification (Dahman & Basler, 1999). The remarkable regionalization of gene expression in the otic vesicle has suggested that this principle may operate during otic development to ensure the coupling between the complex three-dimensional topology of the inner ear, and the positioning and diversity of cell types (Fekete & Wu, 2002). The complementary expression of *FGF10* with other markers such as *Lmx1* and HNK-1 that are also early regionalized suggests that there is a very early subdivision of the otic placode into two domains: one anterior-medial, which is neural-sensory competent, and another posterior-lateral domain, which is not. How this basic two-dimensional patterning is transformed into the three-dimensional regionalization of the otic vesicle is most interesting and needs to be addressed. The functional role of the early expression of *FGF10* associated with the proneural domain is intriguing since it does not occur in any other neurogenic placode with the exception of the nasal placode**.** FGF10 does not seem to be responsible for patterning the neural-sensory domain, but on the contrary a result of it, since ectopic expression of *FGF10* in posterior or dorsal domains is not able to re-pattern the otic placode and ectopically induce neural fate.

The FGF10/proneural expression domain evolves highly dynamically throughout the formation of the otic vesicle, in such a way that it resolves into a ventral medial band that is equatorial to the otic vesicle (see diagram of Fig. 1). The generation of this domain may be explained by the dynamics of growth of the otic vesicle. In order to transform from a circle into a spheroid, the otic placode expands along the dorso-ventral axis, because of radial-meridional growth (Alsina et al., preliminary results). Time-lapse analysis of otic cup closure, as well as a fate map of otic rim cells generated by Fekete and colleagues, revealed that the otic cup grows along the dorso-ventral axis (Brigande et al., 2000). This growth dynamics would displace the initial

anterior-medial domain of FGF10 to more ventral positions, resulting in the observed pattern of the otic vesicle.

In addition to differences in gene expression profiles, differences in cell adhesion molecules exist in the otic placode, as reflected by expression of the HNK1 epitope, present in many adhesion molecules, in the posterior domain of the otic pit. The HNK-1 carbohydrate structure, a sulfated glucuronyl-lactosaminyl residue carried by many neural recognition molecules including NCAM, L1, ependymin and integrins, is involved in cell interactions during development. The recent observation that the cell adhesion molecule BEN1 is restricted to the pro-neural sensory domain since stage 11 (Goodyear et al., 2001), and that of a Eph-ephrin interface where neuroblast delamination occurs (Raf et al., ARO 730 2002), is probably part of this early regional patterning of the placode.

Our observations on the delamination of otic neuroblasts indicate that it takes place only at a particular sub-domain of the proneural-sensory region, at the posterior edge of the *FGF10* domain. This is somehow surprising because it is frequently assumed that delamination occurs from the medial domain. The boundary between neural and non-neural competent regions, as well as the above mentioned differences in cell adhesion properties, may dictate the site of delamination of neural cells that are generated within the whole neurogenic domain. How neuroblast generation and delamination are coupled at a particular site is very intriguing. Expression of *Lunatic Fringe*, that codes for a molecule that regulates cell boundary formation, coinciding with *FGF10* reinforces the idea of a boundary formation in the AP axis. The role of *Lunatic Fringe* in boundary formation and morphogenesis in vertebrates has been documented for somitogenesis and tooth development (Sato et al. 2002; Mustonen et al. 2002), and also suggested in the ear for patterning the sensory organs (Cole et al., 2000).

The sequence otic neuron generation.

The present experiments deepen further in the characterization of the gene expression pattern throughout the generation of otic neurons (Adam et al. 1998; Cole et al., 2001). The diagram in Fig. 8 illustrates a descriptive model of otic neuron generation and the proposed role of *FGF10*. Summarizing our results and collecting previous information, we can identify particular cell states throughout the process of otic neurogenesis, which are characterized by specific combinations of gene and protein expression patterns (see Fig. 8). The first visible output of otic neurogenesis is the delamination of cells that populate the CVG, but neuronal cell fate specification starts in the otic placode, as indicated by the expression in the otic placode of both *Neurogenin*1 and *Delta* (Delta1) (Adam et al., 1998; Ma et al., 1998, and our present results). We can assume that the early expression of *Ngn1* and *Delta1* corresponds to the specification of neuronal precursors from a *multipotent progenitor cell* (MP_e**,** Fig. 8) It is believed that both neurons and sensory cells derive from a common progenitor (an epithelial), although direct evidence is not available (see Lang and Fekete, 2001 for a discussion). *NeuroD* and *NeuroM* are expressed in the epithelium of the otic cup, in delaminating neuroblasts and in the CVG (see results Fig. 3), and the *knock out* mice for *NeuroD* display defects of delamination of neuroblasts but not in their generation (Liu et al. 2000; Kim et al., 2001). Therefore a second identifiable cell state in the neuronal lineage is the *epithelial neuroblast* (Nb_e, Fig. 8). Epithelial neuroblasts are still epithelial cells that are committed to the neuronal fate. They are positive to *NeuroD/M*, but not yet to Islet or Tuj1 (see below), and exhibit a low if any proliferative activity. After determination, epithelial neuroblasts delaminate and condense into the CVG. Neuronal cells populating the CVG express Islet1/2, Tuj1 and cell proliferation markers (Adam et al., 1998, Camarero et al., 2003), as well as *NeuroD* and *NeuroM*. This cell state can be called *ganglionar neuroblast* (Nb_g), and it constitutes a transit-amplifying population of cells, which are determined as neurons but still proliferative.

From the data summarized above, we can describe the generation of otic neurons by stating that *Delta1* reflects the activity of the neuronal determination function of *Ngn1*.

The onset of neural differentiation genes *NeuroD/M* can be taken as the out-reading of full commitment to the neural fate, that is the acquisition of a state of cell determination and the generation of epithelial neuroblasts. Epithelial neuroblasts delaminate and transform into ganglionar neuroblasts that differentiate only after an unknown number of cell divisions. With this in mind, we pursue further to discuss the effects of increasing or decreasing FGF signaling during otic development.

FGF10 accelerates the transit towards irreversible commitment

Intrigued by the observation of a regionalized expression of *FGF10* in the neuro-sensory region of the inner ear, in this paper we have analysed the role of *FGF10* in early stages of otic neurogenesis. Briefly, the increase in *FGF10* activity by different means promoted the expression of neuronal differentiation genes *NeuroD/NeuroM,* whereas *FGF10* suppression interfered with neuroblast generation.

Our interpretation of these results is that *FGF10* shifts multipotent precursors towards a state of full commitment (epithelial neuroblasts) characterized by the expression of *NeuroD/M*. Once this state is reached, cells delaminate and continue their development independently of FGF10. This role of FGF signaling is rather novel, and analogous to those recently proposed for FGF8/FGFR4 in muscle (Marics et al., 2002), and that of *FGF1* and FGF receptors in the onset of retinal ganglion cell differentiation in the chick (McCabe et al., 1999). The *FGF10* induced increase of neuronal differentiation genes *NeuroD, NeuroM* is not associated with proliferation of epithelial neuroblasts, which nevertheless have the capacity to proliferate once migrated to the CVG. Neuronal precursors expressing *NeuroD* do not normally divide within the otic epithelium and FGF10 does not increase their cell proliferation rate. On the contrary, FGF10 drives epithelial cells out of cell-cycle reducing the cell division rate of neural progenitors. This is most strikingly demonstrated by the mirrored effects of FGF10 and SU5402 on cell proliferation and *NeuroD* expression in otic explants. But it is interesting to note that *NeuroD* expressing cells do divide once in the CVG, where the proliferative activity of

neural precursors has been extensively documented (Adam et al., 1998; Begbie et al., 2002; Camarero et al., 2003). This implies that *NeuroD* expressing cells within the epithelium are actually epithelial neuroblasts - neural determined cells with the capacity to divide, but that remain arrested until they become ganglionar neuroblasts). The fact that the generation of *Delta1* positive cells is little affected by exposure to FGF10, while it is suppressed by SU5402 in early otic placodes could be explained by the transient nature of *Delta1* expression. FGF signaling would be required for epithelial precursors to express *Delta1*, however exposure of FGF10 would shift the entire population to the next differentiation state, the *NeuroD/NeuroM* epithelial neuroblast.

What maintains these cells in quiescence or at a low proliferation rate until they reach the CVG? We do not know, but one interesting possibility would be that they are silenced under the influence of FGF10, and it is not until they enter an FGF10-free environment that they undergo transit-amplifcation. In this connection, it has been recently shown that IGF-1 and IGFR1 are expressed in the otic vesicle and ganglion, and that IGF-1 is an essential requirement for cell survival and proliferation of ganglionar neuroblasts. IGF-1 is expressed in the otic epithelium but nevertheless it is not required for *NeuroD* expression (Camarero et al., 2003).

Our results emphasize that FGF signaling is a key element in the regulation of initial stages of neuronal fate determination. The more general emerging picture is that extracellular signaling factors regulate defined stages of the generation of otic neurons, from early commitment to late survival and differentiation, as an example of interplay between intrinsic and extrinsic factors in the regulation of neurogenesis

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Figures

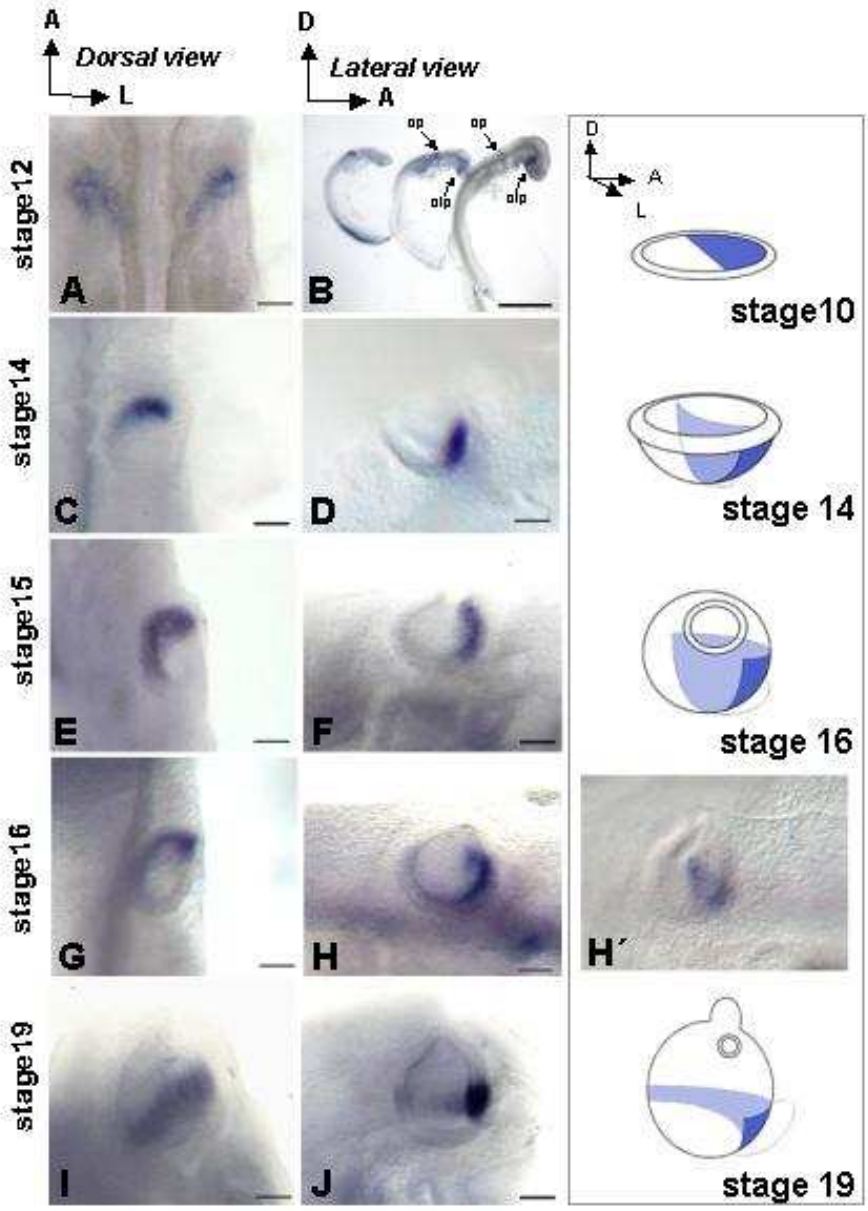

Figure 1. Early expression profile of FGF10 during otic development. A, *FGF10* expression in a stage 11 embryo was found restricted to an antero-medial domain. B, Embryos of stages 12, 14 and 16 in which *FGF10* was detected in the otic placode/vesicle (op), the endoderm of the fourth visceral pouch and the primordium of the olfactory placode (olp). C and D show dorsal and lateral views of stage 13 embryos with *FGF10* expression in the antero-medial domain. E and G illustrate the expansion of this domain to more posterior positions in stages 15 and 16 embryos. F and H, lateral views showing how as the otic cup invaginated and closed up from stage 15 to stage 16, *FGF10* was excluded from the dorsal region and detected in an antero-ventral position. H´ is an oblique view of a stage 16 embryo showing *FGF10* expression profile with a triangular shape, and its narrow angle pointing to posterior and medial positions . I and J show the expression of *FGF10* in a stage 19 embryo. The otic vesicle was closed and the *FGF10* domain restricted to a narrow band running medial and equatorial. In the right panels, three-dimension schematic drawings show the idealised expression profile of *FGF10*. In B scale bar is 1 mm, and 250 μm for the other photographs.

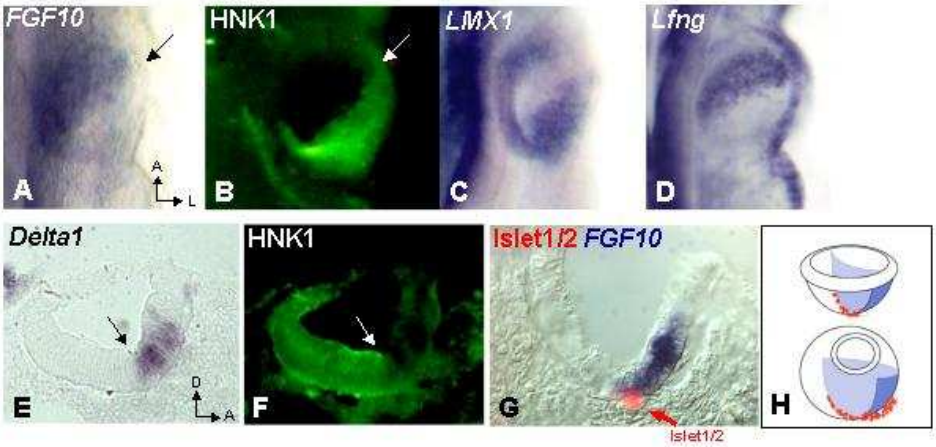

Figure 2. Early AP regionalization of the otic cup. A and B, Early expression of *FGF10* and HNK1 epitope were detected in complementary domains in a stage 13 embryo, subdividing the cup into an anterior and a posterior region. HNK1 is a carbohydrate moiety bound to many cell adhesion and recognition molecules. C, *Lmx1*, a gene from the LIM-domain family, was also expressed complementary to the posterior domain in a stage 13 embryo and also expressed in the otic ridge, while D, *Lunatic Fringe* is detected in the anterior domain but excluded from the ridge. Note the bisection of the cup in an anterior and posterior domain by an axis running 45º from the AP axis of the embryo. E and F, Saggital cryostat section with double detection of HNK1 and *Delta1*, that were also expressed in complementary domains in a stage 15 embryo. G, Immunostaining of earliest delaminating neuroblasts with Islet1/2 epitope (red) in a stage 15 embryo shows delamination in the posterior border of *FGF10* expressing cells (blue). H, Neuroblasts delaminating at the boundary between the anterior *FGF10* domain and the posterior *FG10*-negative domain are represented in a three-dimension drawing. A-D are dorsal views, anterior to the top and lateral to the right. E-G Lateral views with dorsal to the top and anterior to the right. CVG: cochleo-vestibular ganglion.

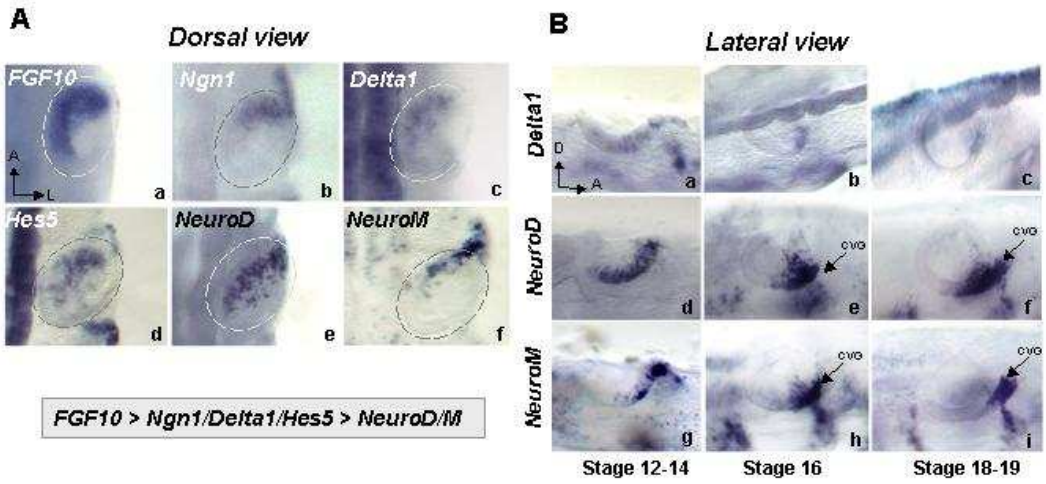

Figure 3. FGF10 is expressed in the proneural-sensory domain. A, Dorsal views showing the expression of *FGF10* (a), *Neurogenin1 (Ngn1)* (b), *Delta1* (c), *Hes5* (d), *NeuroD* (e) and *NeuroM* (f) in otic cups of stage 14 embryos. All proneural and neurogenic genes are expressed in an anterior region within the *FGF10* expression domain. The otic cup is enhanced by dotted circles. Note neuroblasts from the VII placode expressing *NeuroD* and *NeuroM* anterior to the otic cup. In the otic cup, the proneural differentiation gene *NeuroM* (f) was expressed in fewer cells in comparison to *NeuroD* (f). The sequence of gene activation is represented in the bottom line. Restricted expression of *FGF10* was detected before the detection of the first *Delta1* and *Ngn1* expressing cells. B, Lateral views of *Delta1*, *NeuroD* and *NeuroM* expression profiles in early otic cup (stage 12-14), late otic cup (stage16), and otic vesicle stage (stages 17-19). *Delta1* was always detected in scattered cells in the anterior epithelial wall of the otic cup. In later stages its expression was also restricted to a antero-ventral domain. *Delta1* was not found in the CVG in later stages suggesting that it is expressed only in neuronal precursor cells. *NeuroD* and *NeuroM* were detected in the otic epithelium as well as in neuroblasts populating the CVG, indicating that these genes are expressed in neuronal determined cells. At later stages, *NeuroD* was detected in the otic epithelium and in the CVG, while *NeuroM* was only found in the distal neuroblasts of the CVG. CVG: cochleo-vestibular ganglion. A: anterior, L: lateral, D: dorsal.

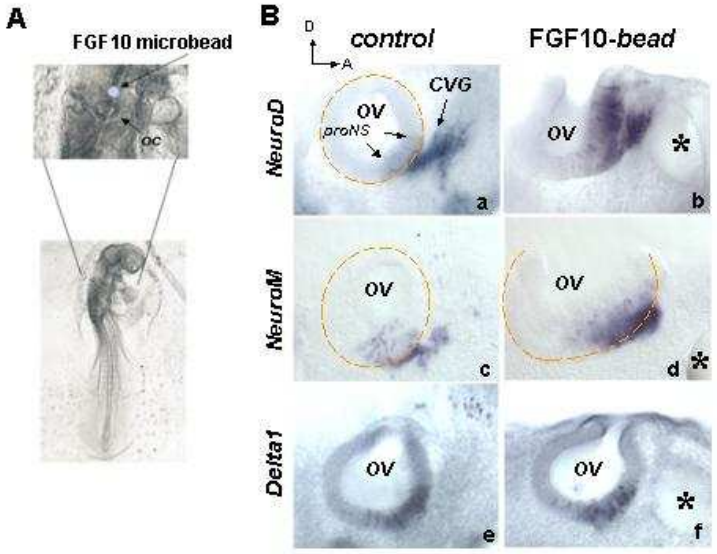

Figure 4. Induction of NeuroD and NeuroM expresssion by local implantation of FGF10 coupled beads. A, Recombinant FGF10 was coupled to acrylic heparin-beads and implanted under the ectoderm anterior to the otic placode/cup (stages 10-12). Embryos were incubated for 24 hours and processed for *NeuroD, NeuroM and Delta1* expression as markers of the neuronal determination pathway. B, a, c and e show the otic vesicle of the untreated side and b, d and f show otic vesicles with implanted FGF10 beads. Asterisks show bead position. As observed in top and middle rows, exposure to increased levels of FGF10 in the anterior domain induced the expression of *NeuroD* and *NeuroM*. No change was detected when *Delta1* assayed for expression when compared between untreated (e) and and FGF10 exposed otic vesicles (f). CVG: cochleo-vestibular ganglion. A: anterior, P: posterior, D: dorsal, V: ventral.

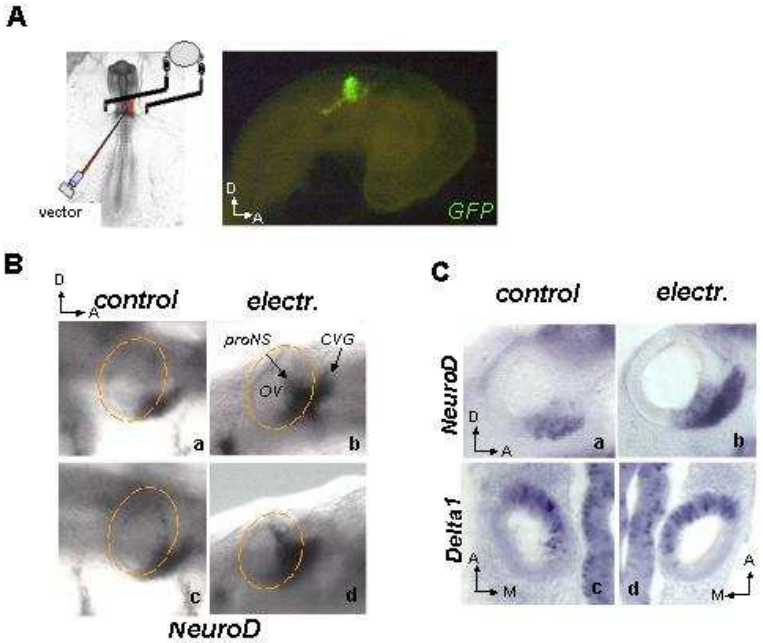

Figure 5. Electroporation of the FGF10 cDNA induced NeuroD but not Delta1. A, FGF10 was subcloned into a pCAGGS-IRES-GFP vector (upper panel), DNA microinjected into the otic placode/cup (stages 10-13), in ovo electroporated and left for 24 hours. Embryos with GFP expression restricted to the otic vesicle were screened and processed for NeuroD and Delta1 expression. The lower panel of A shows a representative embryo with GFP expression in the otic vesicle. B, Two examples of electroporated embryos in which the expression of NeuroD was assessed. Lateral views of unelectroporated vesicles (a,c), electroporated otic vesicles (b,d). C, Vibratome para-saggittal section of an electroporated embryo processed for NeuroD showing a dramatic increase of NeuroD expression in the electroporated side as well as the absence of ectopic expression in the posterior domain (compare a and b). No morphological defects in otic development were observed after electroporation. Coronal sections of experiments processed for Delta1 expression are shown in c and d. No change was observed between the electroporated (d) and non-electroporated vesicle (c). OV: otic vesicle, CVG: cochleo-vestibular ganglion, proNS: proneural-sensory

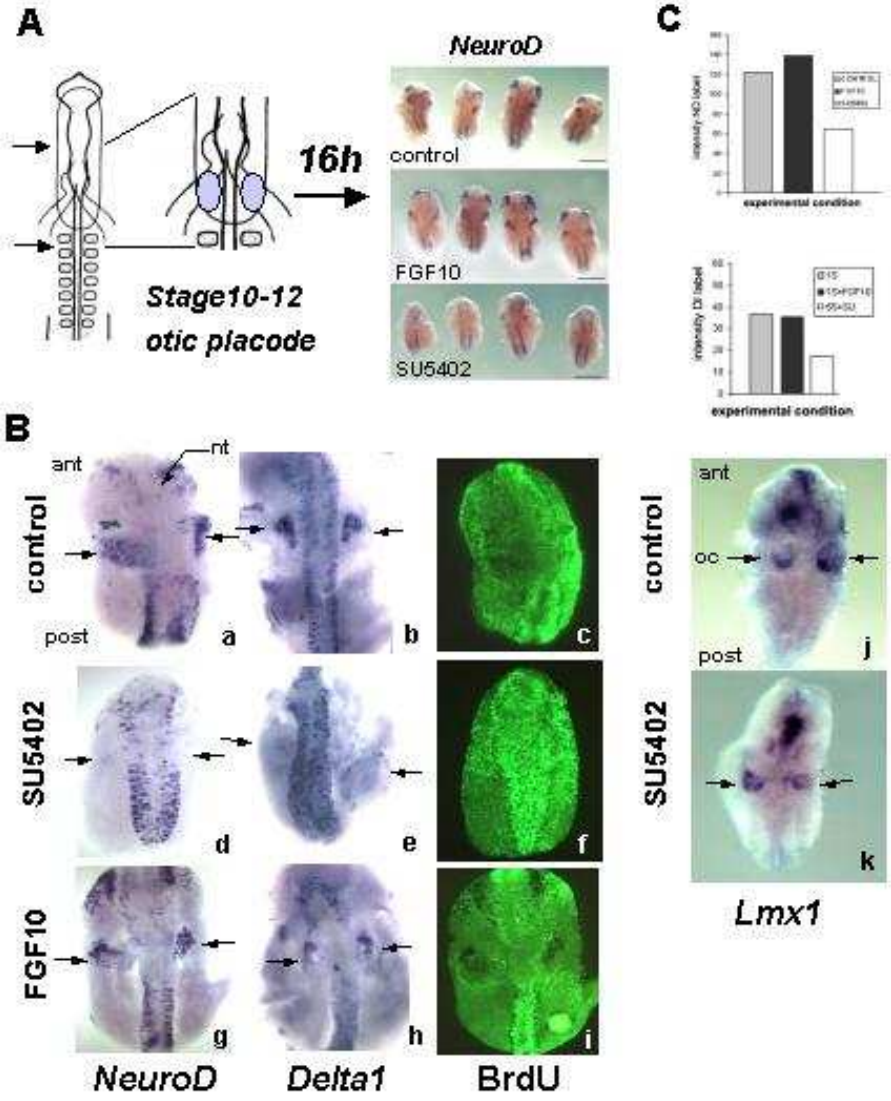

Figure 8. Inhibition of FGF signalling suppresses NeuroD and Delta1 expression but not Lmx1. A, In order to address the effects of FGF10 in neuroblast generation, otic explants were incubated in 5% FCS alone (control) or 200 ng/ml of recombinant FGF10 (FGF10) or 50 µm of SU5402 (SU5402), an FGF receptor inhibitor. The drawing represents a stage 10 embryo in which the most anterior part of the head and posterior to the 1st somite was excised and cultured for 16 hours, fixed and processed for *NeuroD* and *Delta1* expression. A representative experiment with *NeuroD* expression in control, FGF10 or SU5402 explants is shown in the right panel. Note small increment on *NeuroD* expression in the otic cups after FGF10 treatment, while its complete suppression after incubation with the inhibitor. B, Effects on *NeuroD*, *Delta1* expression or BrdU incorporation after each different treatment is shown at a higher magnification. Suppression of FGF-signalling abolished the expression of *NeuroD* (compare a and d) and *Delta1* (compare b and e). FGF10 was able to induce *NeuroD* (g) but not *Delta1* (i). No major effects on cell proliferation were detected in SU5402 treated explants, showing that loss of expression was not due to general growth retardation or toxic effects. *Lmx1* expression (j and k) was not affected by SU5402, indicating that suppression of *NeuroD* and *Delta1* was specific. (C) Semi-quantitative analysis of the intensity of *NeuroD* and *Delta1* labelling in the otic cup. As mentioned before FGF10 was able to increase the level of *NeuroD* expression but not *Delta1*, and SU5402 drastically suppressed the expression of both genes. (C) Semi-quantitative analysis of the intensity of *NeuroD* and *Delta1* labelling in the otic cup. As mentioned before FGF10 was able to increase the level of *NeuroD* expression but not *Delta1*, and SU5402 drastically suppressed the expression of both genes.

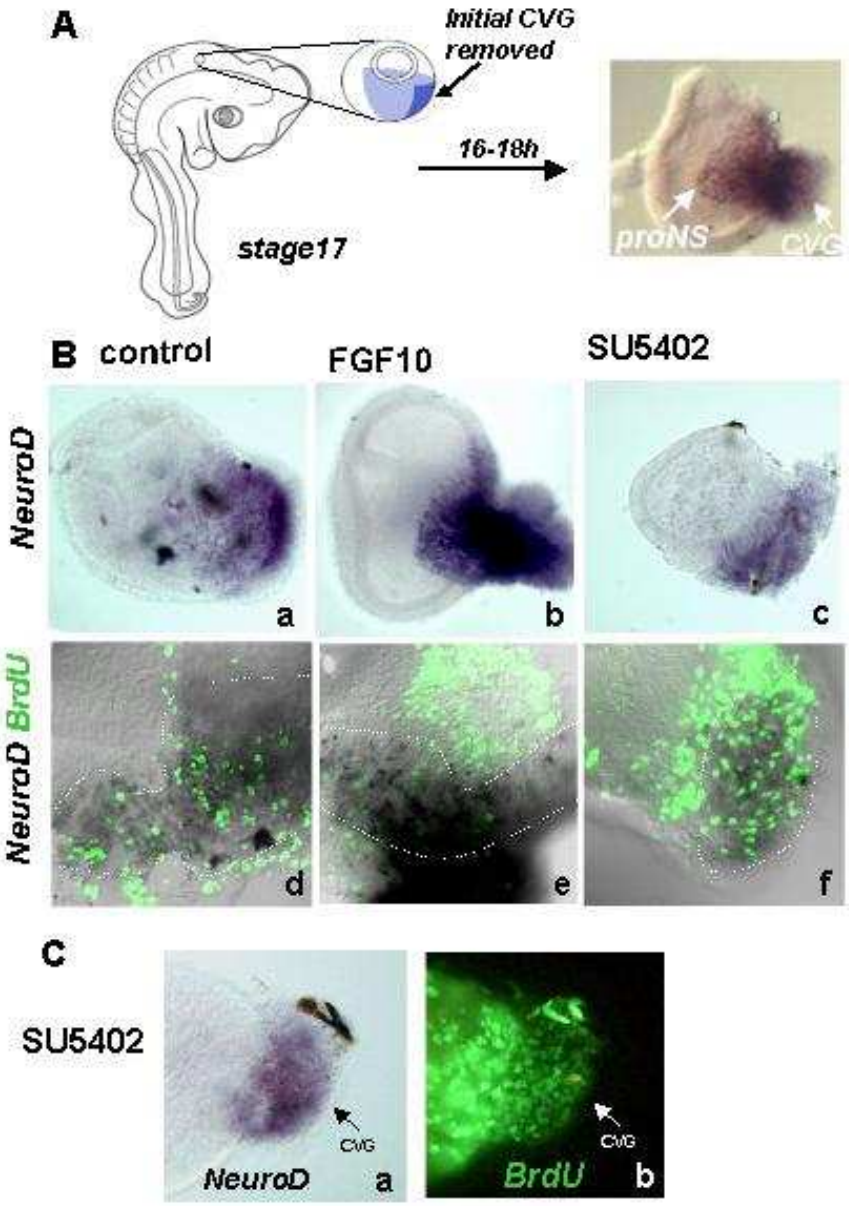

Figure 7. FGF10 reduces proliferation in the proneural-sensory (proNS) region of the otic vesicle. A, Otic vesicles were isolated from stage 17 embryos and CVG removed. Explants were incubated for 16-18 hours with FCS, FGF10 or SU5402. Right panel shows an incubated vesicle processed for *NeuroD* expression. Note expression of *NeuroD* in neuroblasts within the otic epithelium, before delamination (proNS), and in the CVG. B, Induction of *NeuroD* was very clear in FGF-10 treated otic vesicles (b) in comparison to control vesicles (a). Again, SU5402 was able to reduce *NeuroD* expression in the otic vesicle (c). Overlay confocal sections of a FGF10 treated control showing *NeuroD* expressing cells (black) and BrdU labelled cells (green). Three confocal sections of otic vesicles processed for *NeuroD* (black) and BrdU (green) in control (d), FGF10 (e) or SU5402 (f) conditions. In control vesicles BrdU positive cells were intermingled with *NeuroD* expressing cells in the proNS region. After FGF10 treatment, reduction of BrdU occurred in the proNS region, with no apparent effect outside this region. SU5402, in contrast, enhanced or maintained BrdU incorporation in the *NeuroD* positive, proNS domain. proNS: proneural-sensory. C, Expression of *NeuroD* persisted in the CVG (i), indicating that these cells were determined and *NeuroD* expression had become independent from FGF signalling. High levels of BrdU labelled cells were detected in the CVG of SU5402 treated CVG (j), indicating that in the ganglion expansion of neuroblasts was also independent on FGF signalling. pNS: proneural-sensory, CVG: cochleo-vestibular ganglion

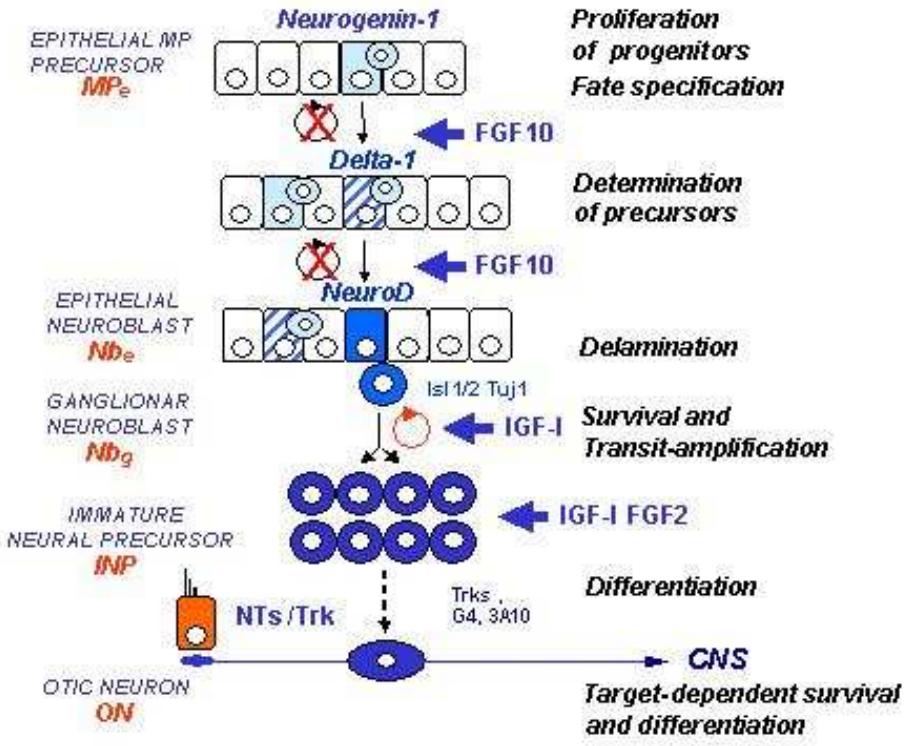

Figure 8. Early otic neurogenesis. States of cell commitment and differentiation of the neural lineage are summarized along with molecular markers. Transition states are represented by arrows. The early expression domain of *Ngn1* and *Delta1* corresponds to the proneural-sensory domain, that is, the epithelium that will generate otic neurons and sensory organs. It is believed that both neurons and sensory cells derive from a common progenitor, an epithelial *multipotent progenitor cell*, MP_e. A second identifiable cell state in the neural lineage is the *epithelial neuroblast* , Nb_e, that emerges after neural cell fate determination. Epithelial neuroblasts are still epithelial cells that are committed to the neural fate. They are positive to *NeuroD/M*, but not yet to Islet or Tuj1 and exhibit a low if any proliferative activity. After determination, epithelial neuroblasts delaminate and condense into the CVG. Neural cells populating the CVG express Islet1/2, Tuj1 and cell proliferation markers as well as *NeuroD* and *NeuroM*. This cell state can be called *ganglionar neuroblast*, Nb_g, and it constitutes a transit-amplifying population of cells, which are determined as neurons but still proliferative. *FGF10* shifts multipotent precursors towards a state of full commitment (epithelial neuroblasts) characterised by the expression of *NeuroD/M*. Once this state is reached, cells delaminate and continue their development independently of FGF10. Other growth factors as IGF-I (Camarero et al., 2003) and FGF2 (Hossain et al., 1996) are critical then for transit amplification and differentiation.

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Early regionalization of the otic placode and its regulation by the Notch signaling pathway

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Keywords: proneural, Lmx1, Notch signaling, patterning, Sox3, DAPT, neurogenesis, otic placode, Hairy1, chick, DiI

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Abstract

Otic neuronal precursors are the first cells to be specified and do so in the anterior domain of the otic placode, the *proneural* domain. In the present study, we have explored the early events of otic proneural regionalization in relation to the activity of the Notch signaling pathway. The proneural domain was characterized by the expression of *Sox3, Fgf10* and members of the Notch pathway such as *Delta1*, *Hes5* and *Lunatic Fringe*. The complementary non-neural domain expressed two patterning genes, *Lmx1b* and *Iroquois1*, and the members of the Notch pathway, *Serrate1* and *Hairy1*. Fate map studies and double injections with DiI/DiO showed that labeled cells remained confined to anterior or posterior territories with limited cell intermingling. To explore whether Notch signaling pathway plays a role in the initial regionalization of the otic placode, Notch activity was blocked by a γ-secretase inhibitor (DAPT). Notch blockade induced the expansion of non-neural genes, *Lmx1* and *Iroquois1*, into the proneural domain. Combined gene expression and DiI experiments showed that these effects were not due to migration of non-neural cells into the proneural domain, suggesting that Notch activity regulates the expression of non-neural genes. This was further confirmed by the electroporation of a dominant-negative form of the Mastermind-like1 gene that caused the up-regulation of *Lmx1* within the proneural domain. In addition, Notch pathway was involved in neuronal precursor selection, probably by a classical mechanism of lateral inhibition. We propose that the regionalization of the otic domain into a proneural and a non-neural territory is a very early event in otic development, and that Notch signaling activity is required to exclude the expression of non-neural genes from the proneural territory.

Introduction

The neurogenic placodes are specialized ectodermal regions found in the embryonic vertebrate head, which contribute extensively to the cranial sense organs (Begbie and Graham, 2001). Some placodes such as the epibranchial and trigeminal give rise only to sensory neurons, while others like the otic and olfactory produce other cell types in addition to sensory neurons (D'Amico-Martel, 1982; Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001; Schlosser, 2005). The inner ear originates from the otic placode, an oval thickening of the ectoderm adjacent to the hindbrain. The original flat otic epithelium progressively invaginates to then pinch off and form the otic vesicle (Bancroft and Bellairs, 1977; Alvarez and Navascues, 1990; Torres and Giraldez, 1998). Among the different cell types of the inner ear, sensory neurons are the first cells to be specified (Adam et al., 1998). Studies of early otic neurogenesis have shown that neuroblasts originate from the anterior and medial domain of the otic placode/cup (the *proneural* domain), while the posterior and lateral region is not neurogenic (Adam et al., 1998; Alsina et al., 2003; Alsina et al., 2004).

Several genes have been reported to be expressed asymmetrically during otic cup and otic vesicle stages, their mutation or deletion causing major developmental defects (reviewed in Torres and Giraldez, 1998; Cantos et al., 2000; Baker and Bronner-Fraser, 2001; Fekete and Wu, 2002; Riley and Phillips, 2003). Most studies on otic patterning have focused on otocyst stages with less attention to the earliest events of otic regionalization and the establishment of the proneural territory. Although several otic genes can modulate neurogenesis, only the mouse *Tbx1* gene has been reported to directly regulate the extension of the proneural domain of the otic vesicle (Raft et al., 2004). Segregation of the original otic vesicle territory into distinct gene expression regions or compartments has been hypothesized to be instrumental in specifying the location and identity of different parts of the inner ear (Brigande et al., 2000b). Fate map studies of the otic rim in chick identified two boundaries of lineage restriction near the dorsal pole (Brigande et al., 2000a; Brigande et al., 2000b; Fekete and Wu, 2002). Furthermore, viral infections of chick otic cups showed that the majority of clones were restricted to a single anatomical sensory subdivision, indicating limited clonal dispersion in the ear (Satoh and Fekete, 2005). Prior to the formation of the otic placode, cell migration is not restricted and extensive cell movements throughout the otic field have been reported in chick (Streit, 2002). In other species, for example in *Xenopus*, extensive cell migration throughout the developing ear was shown (Kil and Collazo, 2001) and in mouse, recent data also points towards cell migration from dorsal to ventral structures (Riccomagno et al., 2005). The question of how the dynamics of cell movements and cell mixing relate to patterning of the otic placode is far from being understood.

Notch signaling pathway is involved in many developmental processes, such as cell fate specification, cell proliferation, patterning and boundary formation (reviewed in Louvi and Artavanis-Tsakonas, 2006; Bray, 2006). The transmembrane Notch receptor is activated upon binding to membrane-bound Delta or Serrate ligands present in adjacent cells. The function of the Notch signaling pathway can be viewed as a switch, regulating an on/off state of cell choices. In lateral inhibition, Notch activity individualizes a cell from the adjacent cells. In other cases, by lateral induction or more complex interactions, a whole territory becomes different from the adjacent region and Notch, instead, promotes a territory with a new developmental fate (Lewis, 1998; Artavanis-Tsakonas et al., 1999).

In the inner ear, defective Notch signaling in zebrafish or mammals results in overproduction of sensory neurons and hair-cells, indicating that Notch signaling mediates crucial events in otic cell fate specification by a mechanism of lateral

inhibition (Lanford et al., 1999; Zine et al., 2000; Kiernan et al., 2001). This role of Notch signaling is mediated by the Notch ligand *Delta1* and *Jagged2* that are expressed in a salt and pepper pattern (Adam et al., 1998). In contrast, *Jagged1* (*Serrate1* in chick) is broadly expressed in the entire prosensory patch and thus, it has been proposed to be involved in the specification of sensory versus non-sensory epithelium within the ear, limiting the territories (Adam et al., 1998). In support of this idea, complete inhibition of Notch signaling in the zebrafish otocyst results in an increase in the size of the initial sensory patch (Haddon et al., 1998). In chick, Notch1 activation, but not its inactivation, increases prosensory patch formation (Daudet and Lewis, 2005). Finally, *Jagged1* has been shown to be required for the specification of the prosensory patch and maintenance of a sensory progenitor state (Kiernan et al., 2001; Brooker et al., 2006; Kiernan et al., 2006). Whilst, all this work supports the involvement of the Notch pathway in sensory specification, little is known on the possible role of Notch in early otic proneural patterning.

In the present study, we explored the first steps in the establishment of the proneural domain of the otic placode. With this in mind, we studied the spatial and temporal expression profiles of several potential patterning genes, including those coding for elements of the Notch pathway. The results show that the anterior proneural region is characterized by the expression of *Sox3*, *Fgf10* and *LFng*, whereas *Iroquois1 (Irx1)* and *Lmx1b* are restricted to the complementary posterior domain. Expansion of DiI labeled cells was confined to the proneural or non-neural territories. Posterior injections expanded dorsally and laterally, while anterior injections remained ventral and labeled new delaminating neuroblasts. DiI/DiO injections revealed that proneural and non-neural territories exhibited limited cell intermingling. Different components of the Notch pathway showed complementary expression patterns between proneural and non-neural domains, suggesting a possible role of Notch in early otic regionalization. The inhibition of the Notch signaling pathway revealed that Notch is required for restricting the expression of *Lmx1b* and *Irx1* to the non-neural domain, and for neuroblast selection in the proneural domain. We suggest that the regionalization of the otic placode into proneural and non-neural territories may be the first event in otic patterning, and that it requires the functional integrity of the Notch pathway for its stabilization.

Results

The otic cup is regionalized into a proneural and a non-neural domain

Otic neurogenesis in the chick starts at the otic placode/cup transition (HH11, 13ss) as revealed by the expression of *Neurogenin1* and *Delta1* (Henrique et al., 1995; Adam et al., 1998; Alsina et al., 2004). Expression of these genes is detected only in a subdomain of the otic cup, suggesting that specification of the proneural region has already taken place by otic cup stage. Previous work showed that *Fgf10* and *Lunatic fringe (LFng*) are confined to the proneural domain, and *Lmx1* and the HNK1 epitope to the non-neural (Giraldez, 1998; Cole et al., 2000; Alsina et al., 2004). The experiments that follow analyzed the expression of other genes with a similar regional expression pattern.

The *Sox3* gene (Sry like HMG-box) belongs to a large family of transcription factors, expressed at early stages of neural plate induction and placode development (Rex et al., 1997). *Sox3* expression precedes the expression of neurogenic genes and is downregulated by neuronal differentiation genes (Abu-Elmagd et al., 2001; Bylund et al., 2003). In the otic cup of HH13 stage embryos, *Sox3* was predominantly expressed

in the anterior proneural domain, in a similar manner to *Fgf10* and *LFng* (Fig. 1A, B and C).

Two other genes involved in neural patterning in *Drosophila* and vertebrates were studied during otic proneural regionalization. The *Iroquois* (*IRO* in *Drosophila* and *Irx* in vertebrates) genes are a family of homeodomain proteins within the TALE class (Burglin, 1997) that are involved in neural prepatterning (Gomez-Skarmeta and Modolell, 2002; Gomez-Skarmeta et al., 2003). In the otic cup, expression of *Irx1* was restricted to the posterior domain, in a complementary manner to *Sox3* and the proneural region (compare Fig. 1D with 1A-C). A second gene analyzed was *Lmx1b* (hereafter *Lmx1*), which is a transcription factor of the LIM family, homologous to the *Drosophila apterous* gene. *Apterous*/*Lmx1* are involved in the specification of the dorsal limb character in *Drosophila* and vertebrates (Blair et al., 1994; Riddle et al., 1995; Blair, 1995; Vogel et al., 1995b), and in roof plate and isthmus development in mouse (Adams et al., 2000; Chizhikov and Millen, 2004). In the otic cup, *Lmx1* was detected in the posterior domain and in the otic ridge all around the border between otic and non-otic ectoderm (Fig. 1E), except at the anterior-lateral position (red arrowhead in Fig. 1E). Several genes of the Notch pathway were expressed in the non-neural territory, complementary to the expression of the Notch modulator, *LFng* (see Fig. 4). As an example, *Hairy1* (*Hes1* in mammals), a member of the *Hairy* and *Enhancer of Split* family of genes, was expressed in the posterior domain of the otic cup (Fig. 1F). Para-sagittal sections in Figure 1A'-F' show complementary expression patterns and illustrate that all genes were confined to the otic epithelium and not expressed in the surrounding mesenchyma.

The proneural/non-neural boundary viewed dorsally at otic cup stage is 45 degrees tilted with respect to the anterior-posterior (AP) embryonic axis (Figure 1A-F). The proneural domain is, strictly speaking, the anterior-medial aspect of the otic placode, and the non-neural domain is posterior-lateral. However, for convenience they will be referred to as anterior (proneural) and posterior (non-neural) domains. As shown in Figure 1G (modified from Alsina et al., 2004), the proneural domain (blue), is initially a flat triangle. As the otic placode invaginates and the lateral wall grows, the proneural domain ends up in the antero-ventral aspect of the otic vesicle. *Lmx1* expression is excluded from the proneural domain and, in the medial wall, is always dorsal to the proneural region.

The complementary gene expression patterns are better illustrated by the double fluorescent in situ hybridization for *LFng* and *Lmx1* from otic cup (Fig. 1H) to otic vesicle (Fig. 1I). *LFng* probe was detected with a tyramide-Cy3 fluorochrome (red) and *Lmx1* with a tyramide-Cy5 fluorochrome (blue). Taken together, the results show that at early cup stages, patterning genes and members of the Notch signaling pathway are differentially expressed between the neural and non-neural regions.

Fate mapping of proneural and non-neural domains

In order to study the dynamics of early otic domains and otic growth we performed a fate map study of anterior and posterior regions by single injections of the red fluorescent lipophylic cell membrane tracer DiI (see methods). Injections are represented by dividing the otic cup into a clock wheel where anterior was 3 o'clock, lateral 6 o'clock, and posterior at 9 o'clock (Fig. 2A). We followed a similar procedure used by Fekete and colleagues (Brigande et al., 2000a), but performing the injections at earlier stages (HH12) and not limiting to the otic rim. By stage HH12, the otic primordium was still flat and thus, in a dorsal view, injections at 12, 3, 6 and 9 positions were medial, anterior, lateral and posterior, respectively (Fig. 2A; n=58). 65% of the injections performed between positions 1 and 4 resulted in DiI labeled progeny cells in

the anteroventral epithelium and CVG (light red, Fig. 2A and B), while in 100% of the injections at positions 6-11 (excluded from the ridge), the resulting labeled cells occupied the posterior domain without labeling delaminating neuroblasts (Fig. 2A and B, blue). On the other hand, in 70% of the injections at positions 4-6, the labeled cells extended dorso-ventrally along the interface between anterior and posterior domains and also resulted in labeled ganglionar neuroblasts, suggesting that at these positions injections were at the limit between both regions (Fig. 2A and B, green). When labeling was performed in the posterior ridge of the otic placode, at positions 7-10, most of the cells (70%) developed into posterior-dorsal positions (Fig. 2A and B, black).

Table (Fig. 2C) displays a detailed analysis of the shape and distribution of labeled progeny cells. Rows indicate the position of initial injections. Numbers in columns indicate the occurrence of events with similar shape and position to the schematic drawings shown in the upper row. Although the growth pattern of the otic cup is not extensively described here, our data indicated that the lateral wall predominantly developed from posterior epithelium, while the antero-ventral epithelium was derived from the most anterior placodal tissue. The dorsal otic pore was mainly generated by the posterior tissue, although labeling of the anterior medial rim at position 12-1 resulted in dorsal anterior progeny labeled cells (2/2). Several examples of the DiI labeled group of cells observed after 20 hours of incubation are shown in Figure 2D. Anterior labeled cells in the otic epithelium seemed to expand less than posterior cells. This probably is due in part to gross delamination of DiI labeled anterior cells into the CVG. It is also worth noting that labeled cells tended to remain contiguous and with little dispersion or splitting of the original group of labeled cells.

Proneural and non-neural cells undergo limited cell mixing

In order to study the degree of cellular exchange between the proneural and the posterior domains of the otic cup, we labeled otic cups of stage HH12 with two fluorescent dyes and followed their development until otic vesicle stage. The anterior and posterior regions were injected respectively with DiI (red vital dye) and with DiO (green vital dye). Labeled cells and their progeny were examined after 24h-incubation, when embryos reached stage HH16-17. At the stage of injection, gene expression patterns are clearly restricted (see Figure 1). Only non-overlapping initial injections that grew until touching each other were used for this analysis. Frequently, injections showed a sharp antero-posterior DiI/DiO interface at the lateral aspect of the otic vesicle (OV), running dorso-ventrally from the otic pore to the ventral aspect of the vesicle (n=10/13). Analysis by confocal microscopy revealed DiI and DiO labeled cells touching each other without mixing (n=10/15, Fig. 3A-A'). In a coronal view, a certain superposition of both dyes (yellow color) was observed in a 2-3 cells width, suggesting that at the lateral border cells labeled with the different dyes could intercalate (Fig. 3B-B', see inset in Fig. 4M). DiI labeled cells migrating away from the otic vesicle correspond to delaminating neuroblasts (arrowhead in Fig. 3A' and B'). When initial injections were performed only in the rim, we found that both labels mapped to the dorsal otic pore, as previously described by Brigande et al. (2000a) and shown above in the fate map analysis (Fig. 3C-C'). In those cases in which DiI and DiO showed gross overlap, the initial injections, although separated, partially shared the anterior or posterior domains, indicating that cells intermingled when were labeled within a given domain (n=3/3, Fig. 3D-D').

To study whether labeled cells were contained within gene expression domains, the position of DiI labeled cells was analyzed in relation to the expression of anterior or posterior genes. Embryos with initial injections at the presumptive boundary (initial positions 4-6) were rejected. *Fgf10* was used as an anterior marker and *Hairy1* as a posterior one. From the injections analyzed with the *Fgf10* probe, we observed that almost all anterior DiI labeled cells stopped at the posterior edge of the *Fgf10*

expression limit (n=8/10, data not shown). Posterior labeled cells analyzed with *Hairy1* also indicated that DiI cells were contained in the *Hairy1* expression domain (n=6/8) (Fig. 3F-F'). We also analyzed the expansion of posterior DiI labeled cells in relation to HNK1 epitope, which is restricted in the non-neural domain overlapping with *Hairy1* expression (Fig. 3E-E' and G). HNK1 is a sugar residue carried by many neural recognition molecules, including N-CAM, L1 and integrins and others. Again we observed that most cells respected the HNK1 limits (n=8/9). In summary, the data indicates that the expansion of cells was limited to the gene expression domains. Analysis was restricted to the lateral aspect of the otic vesicle where the limits of the AP domains were easily recognized. In the medial wall, however, the anterior-posterior boundary transforms into a dorso-ventral boundary (see Alsina et al., 2004 and Figure 1 for the position of the proneural domain throughout the formation of the otic vesicle).

Ligands and targets of Notch signaling pathway are differentially expressed in the otic placode/cup

During inner ear development, Notch signaling has been implicated in neuron and hair cell development, as well as in the specification of the sensory territory. The experiments that follow were designed to map the expression of Notch signaling elements at the early stages of otic cup regionalization. We found that, at early stages, several members of the pathway were regionally expressed overlapping the proneural or non-neural domains. *LFng*, a Notch modulator, was expressed in all cells of the anterior territory, its expression starting in the anterior-lateral domain (HH11, Fig. 4A). By stage HH14, its expression expanded medially (Fig. 4B). *Delta1* was detected in scattered cells in the antero-lateral edge (HH11) and by stage HH14 *Delta1* expressing cells extended throughout the entire proneural domain (compare Fig. 4C and D). Note that the otic ridge was devoid of *Delta1* except for the anterior-lateral stripe, where the otic and geniculate placodes were continuous (arrow in Fig. 4C). *Hes5-2* belongs to the *Hairy* and *Enhancer of Split* family of genes (HES) and is expressed upon Notch activation (Fior and Henrique, 2005). In the otic cup, *Hes5-2* (here referred to as *Hes5*) was expressed in groups of cells within the proneural domain (Fig. 4E, high magnification in Fig. 7I).
In chick, only two Notch receptors are coded in the genome, *Notch1* and *Notch2*. *Notch1* was expressed in the entire otic placode and cup in a homogenous manner, as seen in a dorsal view in Figure 4G and H. As observed in transverse section, expression of *Notch1* was concentrated in the apical-luminal side of the epithelium (section in Fig. 4N). Conversely, *Notch2* mRNA was expressed at low levels in the ectoderm surrounding the otic cup, but not in the otic epithelium (Fig. 4O). Some Notch signaling elements were expressed in the non-neural domain. The Notch ligand *Serrate1* was expressed complementary to *Delta1* at early otic placode/cup stages. At HH11, *Serrate1* was detected in all cells of the non-neural region (Fig. 4I) and also in few cells of the geniculate placode (Fig. 4I, arrow). As development proceeded, expression of *Serrate1* was enhanced in the non-neural domain but, in addition, new expression started in scattered cells within the proneural otic region (Fig. 4J, arrow). *Serrate1* expression in the anterior domain was delayed with respect to *Delta1* and to non-neural *Serrate1* expression. Another member of the HES family of proteins, *Hairy1*, was present in the posterior domain at very low levels in HH11 otic placode. *Hairy1* was uniformly expressed in the non-neural territory from very early stages and was complementary to *Hes5* (Fig. 4K-L). From lateral views, it was evident that *Hairy1* expression spanned throughout the posterior territory and the major lateral epithelium (Fig. 4M). Interestingly, high levels of *Hairy1* expression were found at the interface between proneural and non-neural territories at late otic cup stage (arrows in Fig. 4M). Analysis at high magnification of the anterior *Hairy1* expression limit showed that the border of *Hairy1* was not perfectly straight, suggesting again the intercalation of cells between anterior and posterior domains (white line in Fig. 4M). In summary, these

experiments show that several members of the Notch signaling pathway are regionalized in the otic cup and map to the proneural and non-neural regions.

Notch blockade induces the expansion of Lmx1 and Irx1 expression into the proneural domain

To explore the function of Notch pathway in early otic development, activity of the Notch pathway was blocked in chick embryo explants exposed to the γ-secretase inhibitor (N-[N-(3,5-diuorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, DAPT inhibitor) (Dovey et al., 2001; Geling et al., 2002). The activity of the γ-secretase is specifically required for cleavage of the intracellular domain of Notch (NICD) that leads to gene activation. Experiments were performed in embryos at HH9-10 (6-10 somites), before the establishment of anterior-posterior regionalization. Notch inhibition had two different effects on the otic placode: on the one hand induced the expansion of non-neural genes into the proneural domain and, on the other, resulted in the overproduction of neuronal precursors.
The effects of Notch inhibition on posterior genes are illustrated in Fig. 5. The expression of the Notch target gene, *Hairy1,* was inhibited after DAPT treatment indicating that DAPT inhibitor was effective in inhibiting Notch signaling (n=8/8, Fig. 5A-A'). The effects on *Serrate1* expression were more complex. Surprisingly, the posterior expression of *Serrate1* was unaffected by DAPT, whereas the anterior expression of *Serrate1* was reduced or abolished (n=10/10, Fig. 5B-B', arrow). This suggests multiple roles for *Serrate1*, some of them not totally dependent on Notch activity.
However, the most striking observation from these experiments was that *Lmx1* and *Irx1* expanded into the anterior domain after DAPT incubation. After DAPT treatment, *Lmx1* expression was detected throughout the anterior proneural domain and in the anterior-lateral ridge from where it is normally excluded (n=12/15, compare control and DAPT in Fig. 5C-C'). Sagittal sections in Figure 5E'-F´ show that the expression of *Lmx1* gene invaded the proneural domain of the otic cup overlapping with the expression of *NeuroD*, a gene expressed in the proneural domain. DAPT treatment also induced the anterior expansion of the other major posterior gene *Irx1*, although it was not homogeneous and it always exhibited a higher expression in the posterior domain (n=25/33, Fig. 5D-D').

It has been described that disruption of hindbrain patterning leads to defects in otic regionalization (Kwak et al., 2002; Lecaudey et al., 2007) and also that Notch is involved in hindbrain segmentation (Cheng et al., 2004). Therefore it was possible that the effects of DAPT were secondary to the disruption of neural tube AP regionalization. To test this possibility we examined the expression of *MafB,* a transcription factor expressed in rhombomeres 5 and 6 (Eichmann et al., 1997). After DAPT treatment, the expression of *MafB* was unaffected, suggesting that DAPT did not disrupt hindbrain patterning (n=6/6, Fig. 5G-G', *MafB* and *NeuroD* transcripts detected both in blue).

Occasionally, DAPT-treated otic cups showed a smaller size and hampered invagination, suggesting that a reduction in cell proliferation may have occurred in those experiments. Since in some tissues Notch signaling maintains the self-renewal potential or induces cell differentiation, we tested the effects of Notch blockade on cell proliferation by assaying for BrdU incorporation. Explants (HH9-10) were incubated with BrdU for the last 2 hours of the culture period and assayed for BrdU incorporation by immunofluorescence. Although the difference was not statistically significant, BrdU uptake after DAPT treatment tended to be below control values (control:DAPT, 12.8:10.2 (cell/ua), n=12:n=18, $p \leq 0.08$). This suggests that the effects of DAPT on patterning were not related to changes in cell proliferation.

In summary, these experiments show that Notch activity is required for restricting the expression of *Lmx1* and *Irx1* to the non-neural domain, and that the non-neural expression of *Serrate1* is Notch-independent.

Effects of Notch on Lmx1 and Irx1 are not due to increased cell mixing

The expansion of *Lmx1* and *Irx1* to the anterior domain could be caused by the migration of cells with posterior identity towards the anterior compartment, and/or by the altered regulation of the expression of those genes. To further study this problem, explants were labeled with DiI in the posterior domain and then incubated with DAPT. Initial injections were performed at HH10. Figure 6 shows an example of posterior injections after incubation in control conditions (Fig. 6A) or with DAPT (Fig. 6A'). None of the injections showed DiI-labeled cells invading the anterior domain after incubation for 24 hours with DAPT (n=4/4 control, n=6/6 DAPT). In these experiments, in situ hybridization against *Irx1* transcripts confirmed that DAPT treatment was effective in expanding *Irx1* towards the anterior domain (Fig. 6B-B').

If the effects of Notch blockade were related to cell migration, we would also expect that the HNK1 epitope present in posterior cells would also loose its posterior restriction after DAPT treatment. Figure 6C-C' shows that this was not the case and, after DAPT treatment, HNK1 labeled cells were still present in the posterior domain and did not invade the anterior domain (n=24/24), suggesting that those cells carrying the HNK1 epitope remained restricted to their normal territory. DAPT activity in this experiment was assessed by *Hes5* expression (Fig. 6D-D'). Altogether, these data reinforce the notion that most probably the anterior expansion of *Lmx1* and *Irx1* was not due to migration of *Lmx1* and *Irx1*-positive cells from posterior to anterior, but to up-regulation of their expression in the anterior domain.

Blockade of Notch with dominant-negative MAML1 upregulated Lmx1

In order to analyze the cell-autonomous effects of Notch signaling in cell fate and cell affinity during otic development, we took advantage of the electroporation technique to locally block the Notch pathway. A GFP-tagged dominant-negative form of the human Mastermind-like1 (DN-MAML1) was electroporated into the otic placode at stage HH9-10. This approach also allowed us to exclude possible effects of DAPT inhibitor on surrounding tissues. MAML1 binds to the ankyrin repeat domain of Notch receptors, forming a DNA-binding complex with NICD and CSL transcription factor to activate Notch target genes. The truncated form of MAML1 (only aminoacids 1-302) binds to the CSL:NICD complex creating a complex that is incapable of Notch signaling (Weng et al., 2003). The effects of DN-MAML1 on *Lmx1* were analyzed by examining the extension of *Lmx1* expression domain (Fig. 6E-E'). Consistent with our previous observations using DAPT inhibitor (described above), *Lmx1* was expanded into the proneural domain after Notch blockade (n=5/6, Fig. 6E-E'). We examined whether the electroporated cells ectopically expressed *Lmx1* in the proneural domain. We found that most of the cells positive to DN-MAML1 did also express *Lmx1*, indicating that the inhibition of the Notch pathway upregulates *Lmx1* in a cell-autonomous manner (88/140 cells (63%) from 5 different embryos). The effectiveness of DN-MAML1 construct in chick was analyzed on *Hairy1* expression, observing *Hairy1* down-regulation in the non-neural domain (104/124 cells (84%) from 7 different embryos). In the hindbrain, it has been reported that electroporated cells in which Notch signaling was specifically blocked with a dominant-negative Su(H) (or CSL) preferentially distributed in non-boundary regions (Cheng et al., 2004). In our experiments, we could not detect a preferential location of DN-MAML1 electroporated cells. In summary, these experiments indicated that Notch signaling can regulate the transcription of *Lmx1* in a cell-autonomous manner, independently of surrounding tissues.

Blockade of Notch signaling increases the number of neuronal precursors without affecting the size of the proneural domain

In the proneural domain, Notch signaling inhibition by DAPT resulted in the abolition of the expression of *Hes5,* a direct target of Notch signaling activation (Fig. 7A-A'; n=12/12), In parallel, the density of *Delta1* and *NeuroD* positive cells highly increased in the proneural domain (n=11/14 for *Delta1,* and n=15/24 for *NeuroD*) (Fig. 7B-B'and 7C-C'). The increase in the number of *Delta1*-positive cells was 3.9-fold (control 5.6±3.7; DAPT 21.6±5.7 (cells/ua); p≤0,001, compare magnifications Fig. 7F-F') and 3.3-fold for *NeuroD*-positive cells (control 8.4±5.7, DAPT 27.9±8.1 (cell/ua); p≤0.001; Fig. 7G-G'). The observed effects on neurogenesis fit with the expected role of Notch in regulating the number of neuronal precursors by lateral inhibition. Close attention to the early expression of *Delta1* in the otic placode showed that *Delta1* expression initiated in single spaced cells (Fig. 7H), while H*es5* was expressed in the same territory but in groups of cells (Fig. 7I). Double fluorescent in situ hybridization revealed that *Delta1* and *Hes5* transcripts were indeed expressed in adjacent cells within the proneural epithelium (Fig. 7J and K). In summary, these experiments indicate that as suggested in previous work (Haddon et al., 1998), Notch is required in the proneural domain for inhibiting neuronal fate through a classical mechanism of lateral inhibition.
Note that despite the increase in the number of neuronal precursors after DAPT, they were always restricted to the proneural domain of the otic placode. The establishment of the proneural compartment was not affected, and this was also revealed by assessing *Fgf10* (n=14/14) and *Sox3* (n=11/12) expression (Fig. 7D-D' and Fig. 7E-E' respectively). The levels of *Fgf10* expression after DAPT treatment were consistently lower than in control conditions (n=12/14, Fig. 7D-D'), suggesting that there is some link between Notch signaling and FGF function.

Discussion

We have studied the early events of otic regionalization and the role of Notch signaling in this process. The results show that: 1) The otic cup is regionalized into a proneural and a non-neural territory at early stage of otic development; 2) There is a restriction to cell mixing between these two domains; 3) Notch signaling elements are differentially expressed in these two domains; and 4) Notch signaling is required for excluding non-neural genes *Lmx1* and *Irx1* from the proneural domain, as well as for neuron selection in the proneural compartment. We propose an early role for Notch signaling in the early regionalization of otic placode.

Restricted cell intermingling between otic proneural and non-neural domains

The two functional domains of the otic cup, proneural and non-neural, differ in their developmental potential and underlying mechanisms must ensure that both domains keep their differential identities throughout development. Restricted cell mixing between different populations of cells has been found to be instrumental in maintaining adjacent territories with different identities during development and for the control of cell proliferation. For example, during neural tube formation, the caudal stem zone region behaves as a coherent cellular domain that maintains an undifferentiated cell state that involves continued cell cycling, providing a continuous pool of cells to the newly forming neural tube (Mathis et al., 2001). During the segmentation of the hindbrain and the formation of the *zona limitans intrathalamica*, restricted regional gene expression is accompanied by cell lineage restriction (for reviews see Lumsden and Krumlauf, 1996;

Pasini and Wilkinson, 2002; Lumsden, 2004; Kiecker and Lumsden, 2005). In inner ear development, compartmentalization is believed to be important for allocating sensory organs and the endolymphatic duct (Brigande et al., 2000a; Brigande et al., 2000b; Fekete and Wu, 2002). The complementary expression patterns of the chick otic proneural and non-neural regions led us to study the degree of cell mixing between these two cell populations. The results show that cells in the otic epithelium exhibit a coherent and ordered pattern of expansion with no invasion of posterior labeled cells to the proneural territory or vice versa. Moreover, cells labeled with vital dyes remained contained within gene expression boundaries. Our studies also suggest that Notch activity probably is not regulating the cohesion of the domains since posterior cells did not invade the proneural domain after Notch blockade. In nascent neural tube, the cohesiveness of the stem zone seems to require FGFs (Mathis et al., 2001), but it is not mediated by Notch (Akai et al., 2005). *Fgf10* is regionally expressed in the proneural domain of the chick otic placode and vesicle and it has been shown to be required for neuron production (Alsina et al., 2004). Although suggestive, we know nothing about its role in maintaining cohesion of the proneural domain. Similarly, little is known on the differential expression of adhesion molecules that could restrict cell mixing at these early stages of placode development. Most information relies on late otic vesicle or otocyst stages, where they have been studied in relation to sensory development and hair-cell (reviewed in Kelley, 2003). We know that at early stages of otic vesicle (HH17-19), BEN and HNK1 show complementary expression patterns that are restricted to the two functional domains (see Suppl. Fig.1). Therefore, the asymmetric expression of similar cell-adhesion molecules may underlie differential cell affinity and the observed restricted cell movement. Other cell adhesion molecules may also be responsible for the restriction of cell mixing. For example, differential expression of α3 and α6 integrins in the developing mouse inner ear has been described (Davies and Holley, 2002).

Notch signaling pathway is activated differentially in the proneural and non-neural domain

The Notch signaling pathway has a wide array of functions that depend on its ligands, co-factors and modulators (Panin and Irvine, 1998; Kadesch, 2004; Schweisguth, 2004). Advances in understanding the regulation of Notch signaling have led to the discovery of new functions of Notch. Our studies show that different members of the Notch signaling pathway are differentially expressed in the proneural and non-neural domains of the otic placode and that patterning genes characteristically expressed in the posterior domain require the integrity of the Notch signal to remain confined to this region.

Delta1 and *Serrate1,* two Notch ligands, as well as two members of the HES family of proteins, *Hes5* and *Hairy1*, were expressed in a complementary pattern at otic placode/cup stages, suggesting that Notch can be differentially activated in both territories. The expression of *Serrate1* was very dynamic. The early expression of *Serrate1* in the non-neural territory was not dependent on Notch activity, suggesting that this expression is probably related to patterning. In the retina and in the spinal cord there is also a clear complementarity between the expression of *Jagged1* in the non-neural ciliary margins of the retina and floorplate and of *Delta1* in the neurogenic domains (Lindsell et al., 1996; Bao and Cepko, 1997). In the otic cup, *Serrate1* expression progressively appears in the proneural region. (Daudet and Lewis, 2005) showed that *Serrate1* is involved in the specification of the sensory patches. At otocyst stage, presumptive sensory organs, as identified by *Bmp4* expression, arise within the broad *LFng* and *Serrate1* positive domain (Cole et al., 2000; Satoh and Fekete, 2005). The early expression of *Serrate1* in the proneural domain reported here could foreshadow the early specification of a sensory territory. A more detailed fate map study is required to test that this *Serrate1* expression is contributing to the sensory

patches. In *Drosophila,* cell-autonomous modification of Notch by Fringe protein favors the interaction with Delta1 over Serrate1 ligand. During otic proneurosensory development, when neurogenesis is taking place in the proneural domain, *LFng* could balance the Notch-Delta signaling, leading to activation of *Hes5*. Later in development, it is possible that the Notch-Serrate1 signaling in the anterior region could favor sensory fate. Blockade of Notch increased the number of *Delta1* positive cells in the anterior domain while suppressing the incipient expression of *Serrate1* in the proneural domain. This suggests that the increase of *Delta1* cells in the proneural region could be at the expense of *Hes5* and *Serrate1* cells.

Hairy and *Enhancer of Split* homologs (*Hes/Her*) act as patterning genes in the zebrafish midbrain-hindbrain boundary, mouse olfactory placode and the inter-proneural stripes of the *Xenopus* and zebrafish neuroectoderm by repressing neural fate (Cau et al., 2000; Geling et al., 2003; Bae et al., 2005). In those model systems, *Hairy1* activity is Notch independent. However, in the otic placode, *Hairy1* expression was suppressed after Notch blockade, indicating that it is dependent on Notch signaling. Moreover, suppression of *Hairy1* expression did not expand neurogenesis to the posterior region suggesting that repression of neurogenesis may require additional signals in the ear. In the mouse, *Hes1* (*Hairy1*) and *Hes3* regulate maintenance of the isthmic organizer (Hirata et al., 2001) and, in recent work, it has been proposed that high levels of *Hes1* expression are required for boundary formation in the developing central nervous system (Davies and Holley, 2002; Baek et al., 2006). In the otic vesicle high levels of *Hairy1* are consistently found along the lateral wall of the AP boundary.

Notch signaling is required for the down-regulation of Lmx1 and Irx1 in the proneural territory

One major finding in this paper was the ectopic expression of *Lmx1* and *Irx1* in the proneural domain after Notch blockade. DiI experiments and HNK1 expression analysis, combined with Notch blockade, indicate that Notch regulates the regionalized expression of both genes and not the sorting of neural and non-neural cells. *Lmx1b* gene belongs to the subfamily of LIM homeodomain (LIM-HD) proteins, which are key transcription factors in regulating developmental processes. In limb development, *Lmx1b* is fundamental for establishing dorsal identity, and in the isthmus and roof plate *Lmx1b* has been shown to promote organizer activities by regulating the expression of secreted signaling molecules (Vogel et al., 1995; Adams et al., 2000). The expression of *Lmx1b* in the ear has been reported, as well as its regulation by dorsal hindbrain signals (Giraldez, 1998). However, the exact role of *Lmx1b* in the ear is still unknown. Here, we show that *Lmx1b* is regionally expressed in the non-neural domain and that Notch activity is required for excluding its expression from the proneural territory. During the development of the *Drosophila* wing disc, *apterous* a member of the LIM-HD transcription factors regulates the expression of Notch ligand *Serrate1* in the dorsal compartment. However, in the inner ear expansion of *Lmx1b* expression into the proneural domain is not accompanied by *Serrate1* expansion.
Irx1 also belongs to a family of transcription factors implicated in several functions during development, including organizer formation, neural plate specification and patterning, sensory placode formation, and heart chamber specification (for review see Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002). In the eye disc, IRO-C complex represses the expression of *Fringe* and juxtaposition of IRO-C expressing and non-expressing cells generates a straight border that promotes growth and serves as a pattern-organizing border in the eye disc (Cavodeassi et al., 1999). In spite of these studies in *Drosophila*, a relationship between Notch signaling and *Lmx1b* and *Irx1* function during vertebrate patterning is still unknown. Thus, it is difficult to describe the molecular mechanism by which Notch, *Lmx1b* and *Irx1* could be interacting in the otic placode. A Notch target expressed in the proneural domain could be a repressor

involved in the down-regulation of *Lmx1* and *Irx1* expression. This function is probably not related to the classical function of Notch in lateral inhibition during neurogenesis. Nevertheless, other signals must be regulating early otic patterning because the establishment and the size of the proneural domain were not dependent on Notch activity.

In conclusion, this paper provides one of the first comprehensive descriptions of the early events of otic regionalization. Early patterning into a proneural and a non-neural domain is associated with limited cell mixing between the two regions, the restricted expression of patterning genes and, a requirement for the activity of Notch to down-regulate *Lmx1* and *Irx1* expression in the proneural domain. This suggests a dual role for the Notch pathway in early otic development: first, as part of a mechanism that regulates regional patterning of a proneural and a non-neural domain and, second, as part of the mechanism of neuronal precursor selection.

Experimental Procedures

Embryos and staging
Fertilized hens´ eggs (Granja Gibert, Tarragona, Spain) were incubated in a humidified atmosphere at 38ºC for designated times. Embryos were staged according to the (Hamburger and Hamilton, 1992). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS; pH7.4) at 4ºC for 24 hours.

Organotypic explants and DAPT treatment
Embryos corresponding to stage HH9-10 were sectioned before the second-third somite and transferred into four-well culture plates (NUNC, Roskilde, Denmark). Incubation was carried out in Dulbecco's modified Eagle medium DMEM (Gibco) at 37.5°C in a water-saturated atmosphere containing 5% CO_2. Additions were 5% fetal bovine serum (Bio Whittaker Europe), antibiotic antimycotic solution 1x (Sigma), L-Glutamine (Bio Whittaker Europe) and DAPT, γ-secretase inhibitor, Calbiochem) in DMSO or DMSO alone in control conditions. DAPT inhibitor was used at a concentration of 100 μM. At lower concentrations (20 μM) Notch pathway inhibition had the same effects: increasing the number of *NeuroD* cells (n=5/7) and expansion of *Lmx1* (n=12/18). However, at higher concentration, the expressivity of the effects was higher, suggesting that 20 μM was not a saturating concentration. Density of *NeuroD* and *Delta1* expressing cells was measured as follows. Briefly, the number of cells expressing *NeuroD* and *Delta1* in control and DAPT treated organotypic explants were counted manually from 40x microphotographs (*Dl1* control n=12, *Dl1* DAPT n=9, *NeuroD* control n=15, *NeuroD* DAPT n=15) and expressed as number of *NeuroD* expressing cells per arbitrary unit of surface area (200x200 pixels). Student´s t-test was used for statistics.

Proliferation assay by BrdU
Explants treated in control and DAPT inhibitor conditions were incubated with 10 μg/μl 5-Bromo-2´-deoxyuridine (Aldrich) for 2 hours prior to fixation. For BrdU detection after in situ hybridization, explants were incubated in 2N HCl for 30 minutes, washed three times in Sodium Borate pH 8.9 and processed for immunohistochemistry. BrdU positive cells were counted manually in an area of 200x200 pixels (resolution of 150 pixels/inch). 18 squares have been measured in DAPT treated otic cups and 12 squares for control conditions. Student´s t-test was used for statistics.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was carried out with DIG-labeled RNA probes and alkaline-phosphatase-coupled anti-DIG antibody, which was then detected with NBT/BCIP according to Nieto et al., 1996. Probes used: *FGF10* (Ohuchi et al., 1997); *Delta1*, *Notch1*, *Notch2* and *Serrate1* (Henrique et al., 1995a); *NeuroD* and *Lunatic Fringe* (Laufer et al., 1997); *Lmx1b* (Giraldez, 1998b); *Sox3* (Rex et al., 1997); *Irx1* (EST ChEST433E21); *Hairy1* (Palmeirim et al., 1997), *MafB* (Eichmann et al., 1997a) and *Hes5-2* (Fior and Henrique, 2005). Double

fluorescent in situ hybridization was carried out on cryostat sections by the Tyramide Signal Amplification method (TSA-Plus system; Perkin-Elmer Life Sciences) as described in (Yamagata et al., 2002). *Delta1* DIG-probe/*Hes5-2* Fluo-probe and *LFng* Fluo-probe/*Lmx1* DIG-probe were hybridized at 65ºC. For double-labeling, probes were hybridized together and detected sequentially using anti-digoxigenin or anti-fluorescein antibodies conjugated to peroxidase, followed by amplification with Cy3-tyramide and Cy5-tyramide. Peroxidase-conjugated antibody was inactivated with 0.3% H_2O_2 for 40 min.

Immunochemistry

Immunochemistry procedure in whole-mount and cryostat sections was performed as Alsina et al. 2004. Antibodies used: mouse monoclonal antibody to HNK1 epitope (Becton Dickinson, 347390; 50:50), mouse monoclonal antibody to BEN (DSHB, 1:200), rabbit polyclonal antibody to β-tubulin III (TUJ1, Covance, 1:400), monoclonal antibody to BrdU (Roche, 1:200) and rabbit polyclonal antibody to GFP (Molecular Probes, A-11122, 1:500). Secondary antibodies: goat anti-mouse Alexa 488 (Molecular Probes), goat anti-rabbit Alexa 488 (Molecular Probes), goat anti-mouse Alexa 549 (Molecular Probes) were used at a 1:400 dilution.

DiI/DiO labeling

Double injections of CM-DiI and DiO vital dyes (Molecular Probes, 1μg/μl, in DMF) were performed in ovo in the otic placode at stages HH12 in the anterior (proneural) domain and in the posterior domain respectively. Minute amounts of the lipophilic vital dyes were iontophoretically microinjected. Size and location of injections at time zero were visualized and recorded (1/4-1/5 of the width of the placode area) with fluorescence scope (LEICA, MZ FL III). Embryos with overlapping DiI and DiO-labeled initial injections were discarded, the rest were not harvested until stages HH16-17, when they were dissected and fixed with 4% paraformaldehyde/PBS at 4ºC. The growth of the labeled cells was analyzed by conventional fluorescence microscopy (LEICA DMR) and confocal fluorescence microscopy (LEICA DM IRBE).

DiI labeling and in situ hybridization/immunochemistry

Anterior or posterior CM-DiI injections were generated as above and resulting progeny imaged with conventional fluorescence microscopy after 20 hours of incubation. Embryos were then processed for in situ hybridization with either *FGF10* mRNA (used as an anterior marker) or *Hairy1* mRNA (used as a posterior marker), or immunochemistry using an antibody against HNK1 as a posterior marker. The growth of the resulting progeny in relation to the gene expression domains was analyzed by conventional fluorescence microscopy (LEICA DMR) and confocal fluorescence microscopy (Leica DM IRBE).

In ovo electroporation

The dominant negative DNMAML1-GFP fusion construct in EGFP-N3 (a kind gift from J. Aster; Weng et al., 2003) was electroporated into the otic territory of 10HH embryos. A small hole was made into the vitelline membrane to expose the otic placode. The cathode platinium electrode was placed next to the otic territory and anode electrode underneath the embryo. Vector (3 μg/μl) mixed with fast green (0.4 μg/μl) was electroporated by injection onto the otic placode by gentle air pressure through a fine micropipette. Square pulses (4 pulses of 10 V) were generated by an electroporator Square CUY-21 (BEX Co., LTd, Tokiwasaiensu, Japan). Medium (M-199, Gibco) was added immediately after each electroporation. Eggs were sealed and incubated for 20 hours. Embryos were collected and fixed overnight in 4% PFA at 4ºC for further analysis.

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Figures

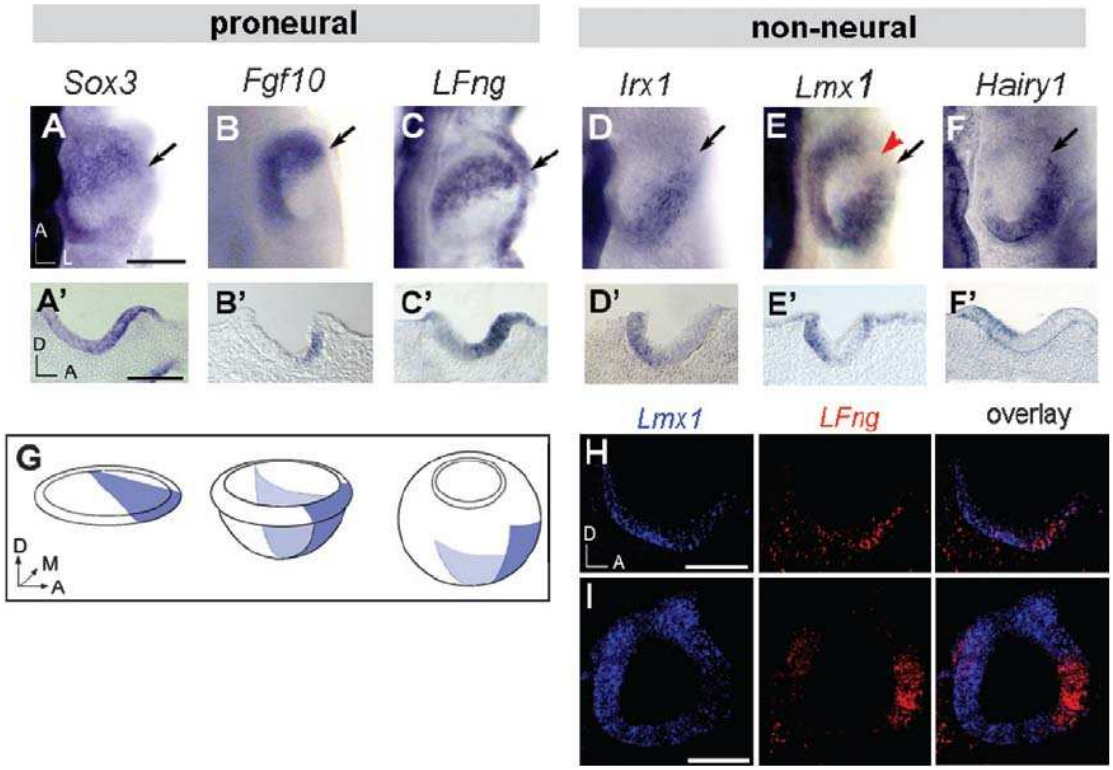


Figure 1. Regionalization of the otic cup into a proneural and a non-neural domain. (A-F) Dorsal views of HH13 otic cups showing expression of *Sox3* (A), *Fgf10* (B), *LFng* (C) in the proneural domain and complementary expression of *Irx1* (D), *Lmx1* (E) and *Hairy1* (F) in the non-neural domain. Note that *Lmx1* was also expressed in the medial and anterior otic ridge but excluded from the anterior lateral ridge (red arrowhead). Arrows indicate antero-posterior expression boundary. (A'-F') Sagittal sections of HH12-13 otic cups showing higher expression of *Sox3* (A'), *Fgf10* (B'), *LFng* (C') in the anterior proneural domain and restricted expression of *Irx1* (D'), *Lmx1* (E') and *Hairy1* (F') in the posterior non-neural territory. (G) Schematic drawing illustrating the dynamics of the proneural/non-neural domains from otic placode stage to otic vesicle viewed laterally. Blue indicates the proneural territory, while in white is depicted the non-neural region. (H-I) Double fluorescent ISH of an otic cup and otic vesicle showing complementarity of expression between *LFng* (red) and *Lmx1* (blue). A: anterior, L: lateral, D: dorsal, M: medial. Scale bars in A, A', H and I = 100 μm.

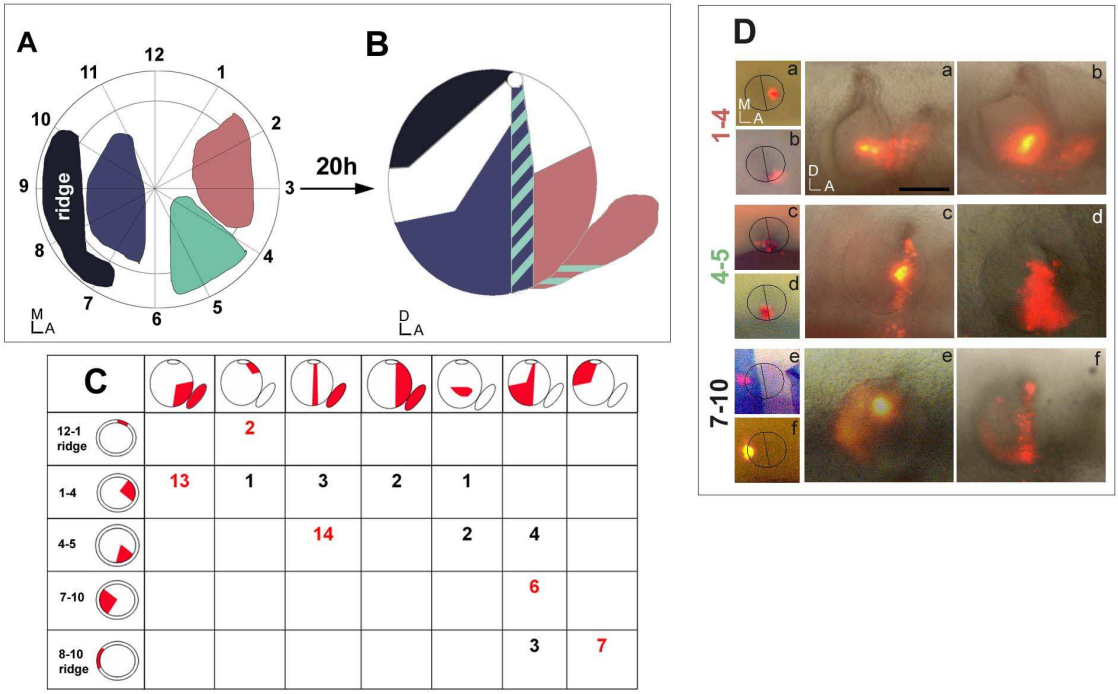


Figure 2. Fate map analysis of anterior and posterior domains from otic cup to otic vesicle stage. (A) Schematic drawing of an early otic cup (HH12) from a dorsal view. The cup is divided into 12 clock positions and the different initial DiI injected areas are represented in different colors. (B) Drawing of the otic vesicle in which final positions of DiI labeled cells are represented. (C) Table displaying a detailed analysis of the resulting labeled progeny examined. Rows indicate the position of initial injections. Numbers in columns indicate the occurrence of events with the shape and position similar to the schematic drawings of HH16-18 otic vesicles shown in the upper row. The most common condition is highlighted in red. (D) Representative examples of labeled cells originating from an anterior injection (1-4), lateral injection (4-6) and posterior injection (6-11). A: anterior, D: dorsal, M: medial. Scale bar in Da = 200 μm.

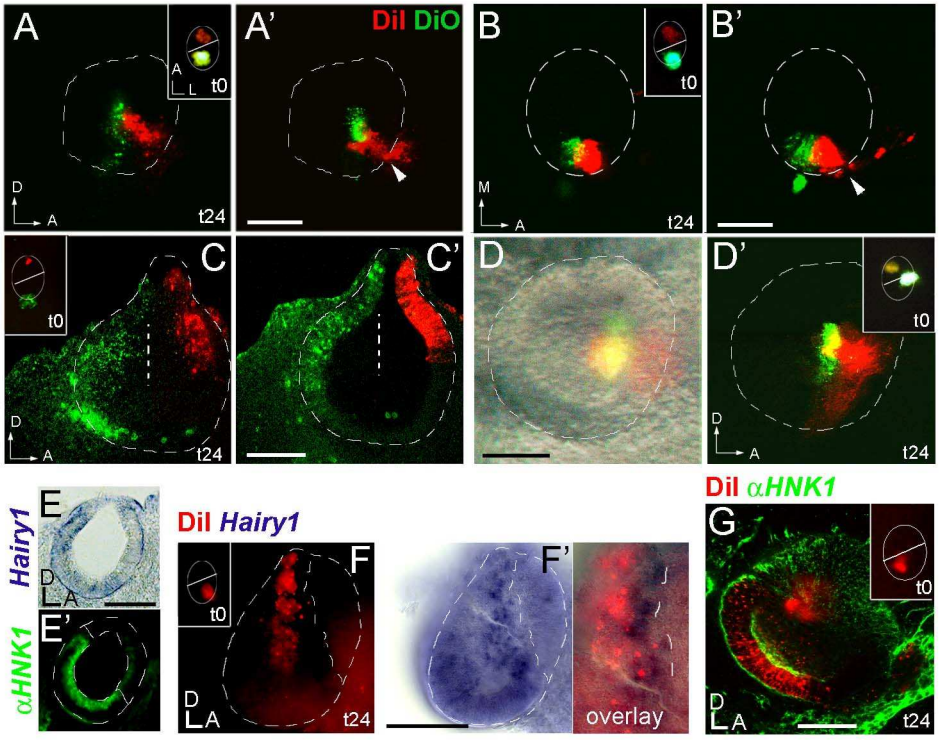


Figure 3. Restricted cell intermingling between proneural cells and non-neural cells. (A-D') Double labeling with DiI (red) and DiO (green) in ovo at stage HH12. (A-A') Merged confocal sagittal sections showing both dyes touching each other without mixing. (B-B') Merged confocal coronal images of an otic vesicle showing anterior DiI and posterior DiO labeled cells. Some cell intermingling was observed. Arrowhead points to DiI labeled neuroblasts coming out from the proneural domain (A´-B'). (C-C') Merged confocal sagittal sections showing that when DiI and DiO initial injections were performed at the ridge the labeled cells did not touch and reached the otic vesicle pore. (D-D') Mixing of DiI and DiO labeled cells was observed when the initial non-overlapping injections were not entirely in separate domains. Insets in A, B, C and D' depict the initial anterior injection (DiI) and posterior injection (DiO) in the otic cup shown dorsally. (E-E') Parasagittal section showing the expression profile of *Hairy1* transcipt and immunostained for HNK1 epitope. (F-F') Example of the growth of a posterior injection with DiI in a HH17 otic vesicle compared to the expression limits of *Hairy1* gene. (G) merged confocal parasagittal sections showing another example of the growth of a posterior injection, now compared to HNK1 staining. A: anterior, D: dorsal, M: medial. L: lateral. Scale bar in A' and B' = 50 µm; C', D, E, F' and G = 100 µm.

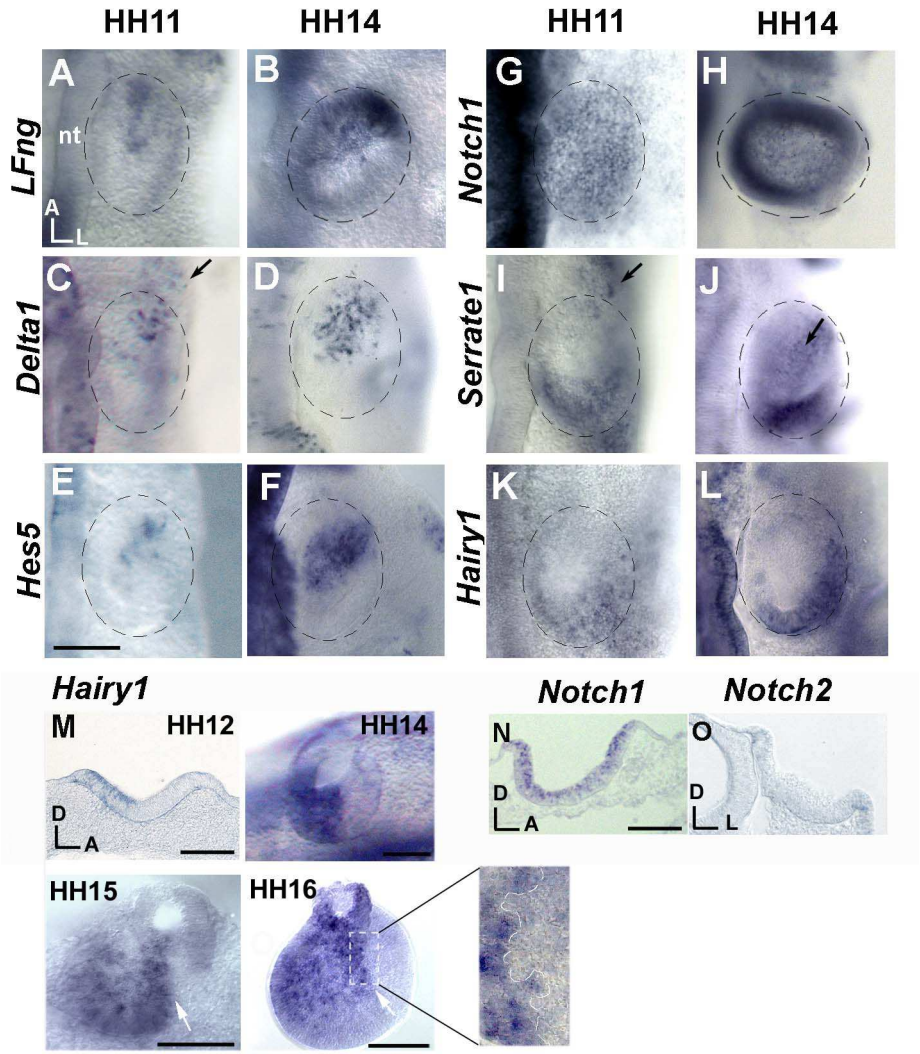


Figure 4. Regionalized expression of the ligands and targets of Notch pathway in the proneural and non-neural domains. Dorsal views of HH11 and HH14 otic cups. (A, C, E) Expression of *LFng*, *Delta1* and *Hes5* at stage HH11. All genes were expressed in the proneural domain, detected initially as a small triangle running from lateral to medial. Arrow in C indicates *Delta1* expressing cells from the geniculate placode. (B, D, F) Expression of the same genes in HH14 otic cups, showing that the expression of all genes has increased and has occupied the entire proneural domain. (G, I, K) Expression of *Notch1*, *Serrate1* and *Hairy1* at stage HH11. Arrow in I indicates very faint *Serrate1* labeling outside the otic placode. (H, J, L) Expression of the same genes at HH14. In J, arrow indicates scattered expression in the proneural territory. (M) The expression profile of *Hairy1* from otic placode to otic vesicle. Notice high levels of *Hairy1* mRNA on the lateral aspect of the otic vesicle (arrows). Inset in M shows *Hairy1* expressing cells intercalated with non-expressing cells along the lateral AP border. (N) Parasagittal section of an HH13 otic cup showing luminal localization of Notch1 transcripts. (O) Transverse section of an HH11 otic cup showing faint expression of Notch2 outside the otic epithelium. D: dorsal, A: anterior, L: lateral, nt: neural tube. Scale bars in E, M and N = 100 μm.

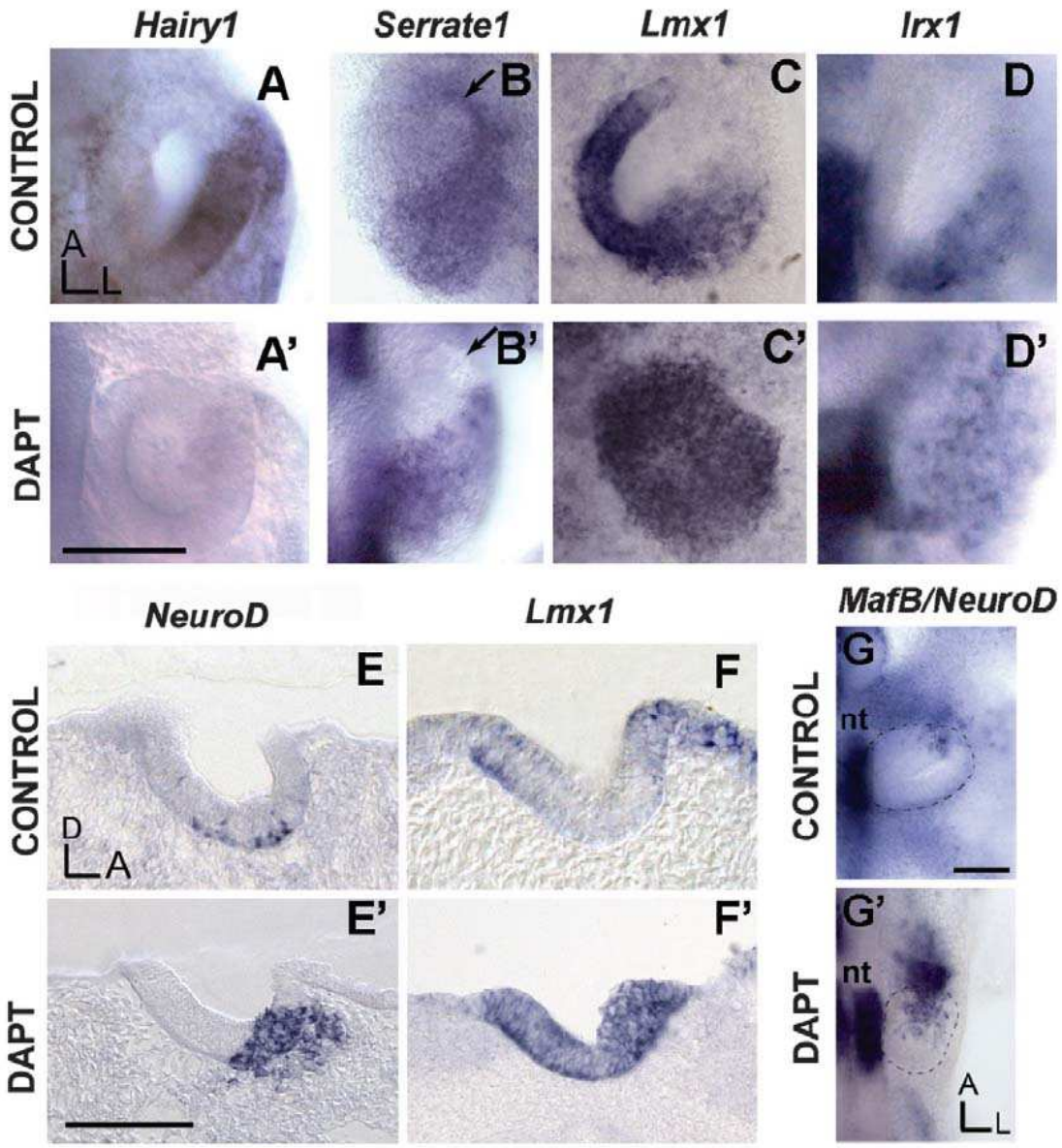


Figure 5. Blockade of Notch signaling disrupts posterior regionalization of *Lmx1* and *Irx1*. Effects of inhibiting the Notch signaling by incubation of HH9 explanted embryos with 100 µM DAPT. Dorsal view of otic cups after 24 hours of incubation. (A, A') *Hairy1* expression was inhibited after DAPT treatment. (B, B') *Serrate1* expression in the non-neural territory was not disturbed but expression in the proneural domain was suppressed (arrows). (C, C') *Lmx1* expression is detected throughout the entire otic cup after DAPT treatment. (D, D') A similar effect is observed for *Irx1* expression. Sagittal sections of Control (E, F) and DAPT treated (E', F') embryos stained with *NeuroD* and *Lmx1*. Note high expression of *NeuroD* cells and *Lmx1* cells in the proneural domain. (G, G') *MafB* and *NeuroD* double staining in control and DAPT treated embryos, showing no effects on hindbrain patterning after DAPT treatment. A: anterior, L: lateral, D: dorsal, nt: neural tube. Scale bars in A', E', G = 100 µm.

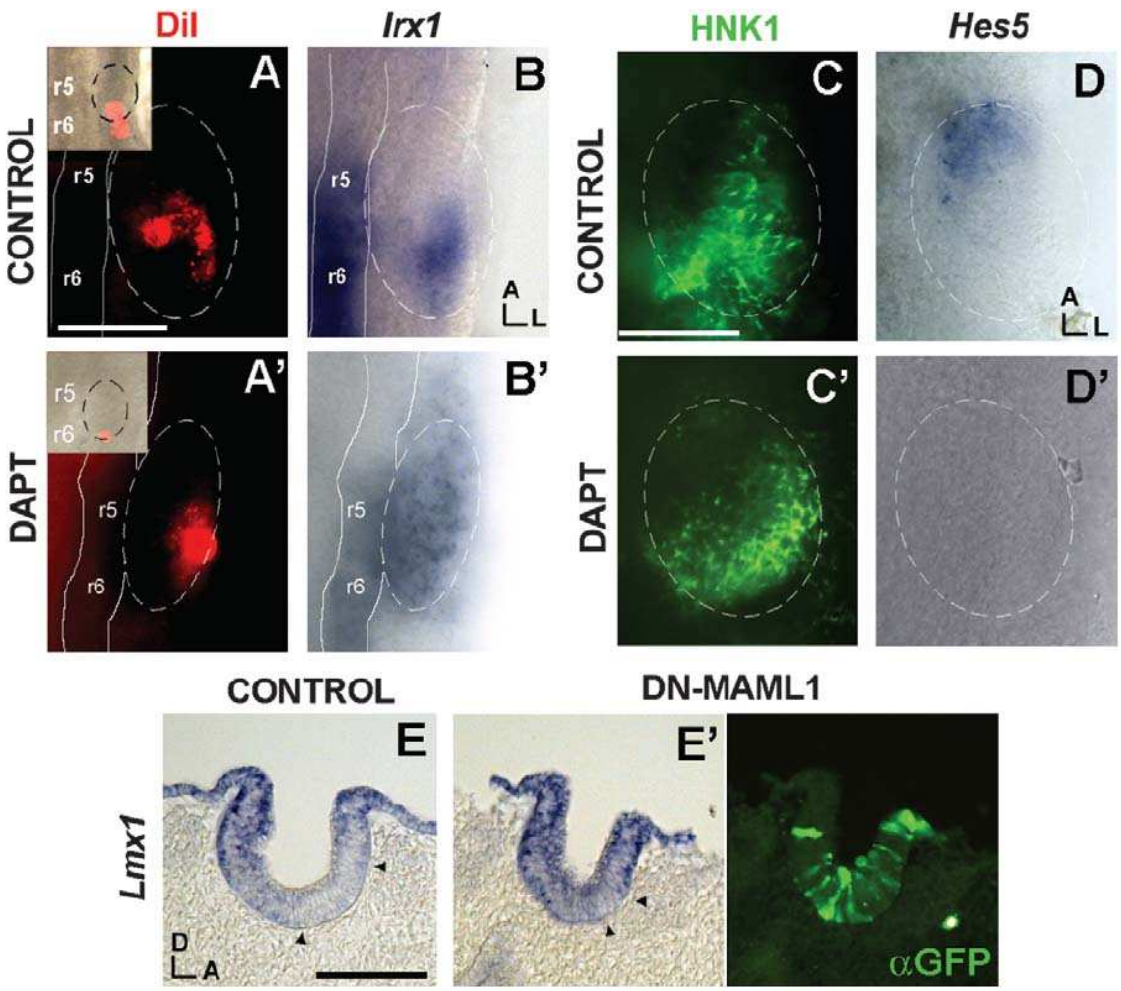


Figure 6. Notch blackade does not affect cell migration. (A-B') DAPT treatment was combined with DiI injection to reveal whether posterior cells invade the anterior domain. (A) DiI labeled cells in control conditions. (A´) After culturing embryos with DAPT inhibitor DiI remained in the posterior territory. (B, B') *Irx1* expression in the posterior domain in control conditions and expansion of *Irx1* expression after DAPT treatment. Inset in A and A' shows the initial DiI injection. HNK1 epitope staining was compared in control (C) and DAPT treated embryos (C') and no invasion of the anterior domain was observed after DAPT treatment. (D, D') Inhibition of *Hes5* expression in the proneural domain in the same embryos indicates effectiveness of the DAPT treatment. (E-E') After the electroporation of the DN-MAML1 in the otic region, *Lmx1* expression was enhanced in the proneural domain. A: anterior, L: lateral, D: dorsal, r: rhombomere. Scale bars in A, C, and E = 100 μm.

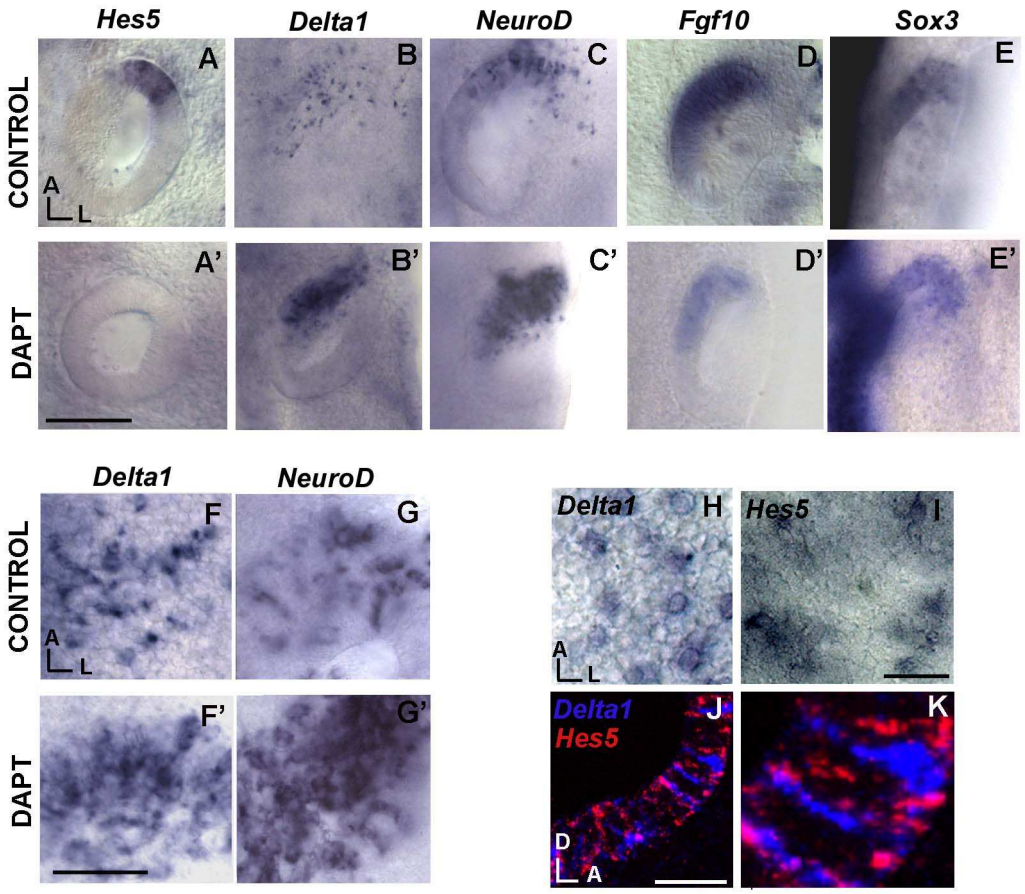
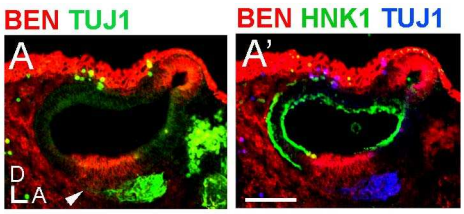


Figure 7. Blockade of Notch signaling induces the overproduction of neuronal precursors. Effects of inhibiting the Notch signaling by incubation of HH9 explanted embryos with 100 µM DAPT. Dorsal view of otic cups after 24 hours of incubation. (A, A') *Hes5* expression was abolished after DAPT treatment. (B-C') Compared to control explants, the number of neuronal cells (*Delta1* and *NeuroD* expressing cells) was dramatically increased. (D, D') The domain of *Fgf10* expression was not affected by DAPT treatment, but decreased levels of *Fgf10* expression were observed. (E-E') *Sox3* expression did not change after DAPT treatment. High magnification images of *Delta1* (F, F') and *NeuroD* (G, G') expressing cells in control embryos and embryos after DAPT treatment. (H) High magnification image of otic epithelium at stage HH12 with single *Delta1* expressing cells. (I) At the same stage, *Hes5* was expressed in clusters of cells. (J) Sagittal section of an otic cup showing *Delta1*-expressing cells (blue) and *Hes5* expressing cells (red) detected by double fluorescent ISH. (K) High magnification image of *Delta1* cells adjacent to *Hes5* cells. D: dorsal, A: anterior, L: lateral. Scale bars in A' = 100 µm, I = 20 µm, F' and J = 50 µm.



SUPPL Figure 1. (A-A') 10µm parasagittal alternate sections were stained for BEN and TUJ1 or HNK1 epitopes. Double immunostaining for BEN (red) and TUJ1 (green) is shown in A, while overlay of BEN (red) and HNK1 (green) from alternate sections with TUJ1 (shown in the blue channel) are shown in A'. BEN and HNK1 showed a complementary pattern in a HH18 otic vesicle and the origin of the cochleo-vestibular ganglion (false blue, TUJ1) was detected at the interface of both stainings (arrowhead in A). A: anterior, D: dorsal. Scale bar in A' = 100 µm.

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ANNEX

8. ANNEX

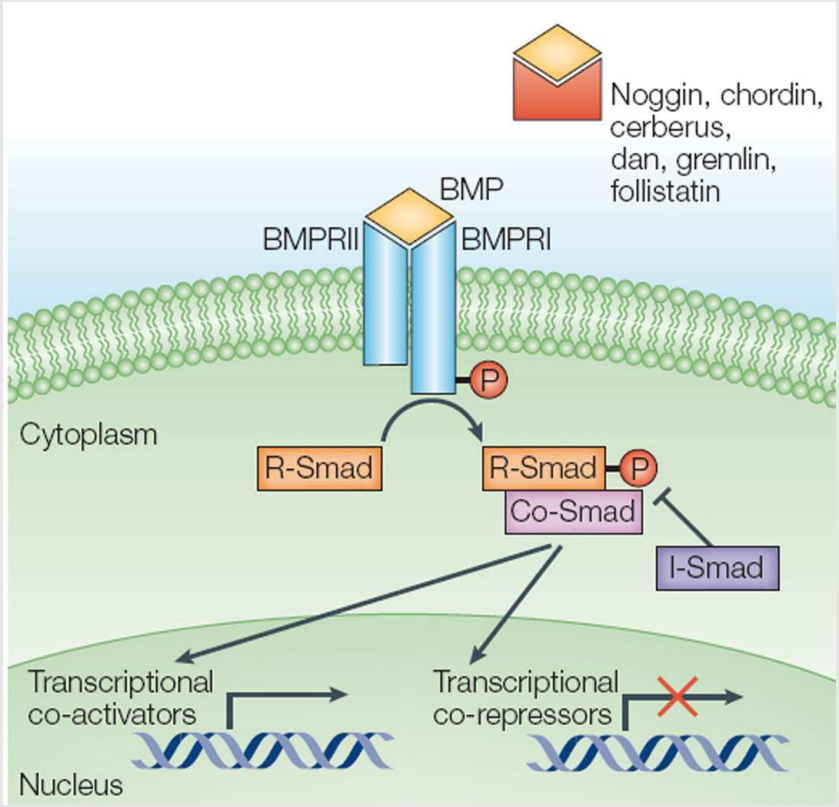
8.1 Abbreviations

As-c achaete-scute
ato atonal
AP anteroposterior
bHLH basic Helix Loop Helix
BMP Bone Morphogenetic Protein
BrdU 5-Bromo-2´-deoxyuridine
BSA Bovine serum Albumin
CNS Central Nervous System
CVG Cochleo Vestibular Ganglion
DIG Digoxigenin
dpc days post-coitus
dpf days post fertilization
DV Dorsoventral
E embryonic day
EGF Epidermal Growth Factor repeats
EP Electroporated
Espl Enhancer of Split
EST Expressed Sequence Tag
FAD Formamide
FBS Feotal Bovine Serum
FGF Fibroblast Growth Factor
fISH Fluorescent in situ Hybridization
Fluo Fluoresceine
GFP Green Fluorescent Protein
h hours
Hes Hairy and Enhancer of Split
HH Hamburguer and Hamilton
ISH in situ Hybridization
min minutes
MAM-l1 Master-minnd-like1
NICD Notch IntraCellular Domain
o/n over night
PBS phosphate buffered saline
PFA Paraformaldehyde
PNS Peripheral Nervous System
PPE Preplacodal ectoderm
RFP Red fluorescent protein
RNA Ribonucleic acid
RT Room Temperature
ss somite stage
SOP Sensory organ precursor
t time

8.2 Signalling Pathways

8.2.1 BMP signaling

Receptros. Members of the BMP family bind to two distinct type II and type I serine/threonine kinase receptors, both of which are required for signal transduction. There are three type II receptors that bind BMP ligands: type II BMP receptor (BMPR-II), type II activin receptor (ActR-II) and type IIB activin receptors (ActR-IIB). Besides, there are three types I receptors for BMPs: type IA (BMPIA or ALK3) and type IB (BMPIB or ALK6) BMP receptors and, type IA activin receptor (ActRIA or ALK2) (reviewed in Xiao et al., 2007).



FIFURE ANNEX 8.2.1 BMP pathway.

Ligands. Bone morphogenetic proteins (BMPs) are multi-functional growth factors belonging to the transforming growth factor-beta superfamily. About 20 BMP family members have been identified and characterized. All BMPs are secreted as precursor protein with a hydrophobic stretch of about 50–100 amino acids. Every BMP contains

seven cysteins, in which six of these cysteins build a cystin knot and the seventh is used for dimerization with a second monomer. BMP molecules are first synthesized as large precursors and then cleaved at a dibasic site so that the C-terminal active domain is released. Prior to secretion, BMPs consist of a signal peptide, pro-domain and mature peptide. Following cleavage of the signal peptide, the precursor protein undergoes glycosylation and dimerization. On secretion of the mature bioactive dimeric BMP by the cell, the pro-domain is cleaved. The mature BMP derives from the carboxyterminal region by proteolytical cleavage, and bind to the receptors as either heterodimers or homodimers (reviewed in Xiao et al., 2007).

***Intracellular signalling pathway and targets*.** In canonical BMP signaling pathway intracellular Smads play central roles in delivering the extracellular signals to the nucleus. Upon ligand binding, the type II receptor kinase activates the type I receptor kinase, which subsequently phosphorylates receptor-activated Smad proteins (R-Smads, Smad1, -5, -8) that act as signal transducers. After phosphorylation, the R-Smads are released from the receptor, recruit the co-mediator or common Smad (Co-Smad, Smad 4) and translocate into the nucleus to activate transcription of target genes in concert with a large and still increasing number of co-factors into the complex. The signalling of the BMPs is modulated by numerous proteins at various points containing negative feedback loops. In the extracellular compartment, secretion of antagonists, such as Cerbarus, Chordin, Dan, Gremlin and Noggin, which are known to bind only specific BMPs, act by blocking the interaction of the ligand with the receptor. Interestingly, expression of the antagonists is often regulated, directly or indirectly, by BMP signalling to refine the duration and strength of the signal. At the receptor level itself, the oligomerization mode of the receptors determines the specificity of the activation of the signaling pathway. Once the signal is transduced into the intracellular compartment, the signal can be modulated by the activation of inhibitory Smad proteins (I-Smad, Smad 6, 7), which compete with the R-Smads for binding to the activated receptor; or Smurf 1, which ubiquitinate Smads and BMP receptors, thereby targeting these proteins for proteosome- mediated degradation. In the nucleus, there is a number of co-activators needed for the activation of specific target genes and their transcription can be inhibited by corepressors. Moreover, Smad activity can be inhibited by the phosphorylation of crucial sites in the Smad protein by Ras–MAPK (mitogen-activated protein kinase) pathways, so providing an intriguing link between BMP and other signalling pathways (reviewed in Xiao et al. 2007; Liu and Niswander, 2007; Yamamoto and Oelgeschläger, 2004). To better understand the mechanisms by which BMP signalling controls vertebrate neural development, it is

important to identify the direct targets and mediators of BMP signalling. The best-studied examples are the MSX family of transcription factors, which have been identified in various experiments in different organ systems to be rapidly induced by BMP signalling. Scrutiny of the promoter region of one *Msx* gene, *Msx1*, has identified several Smad-binding sites, which suggests that *Msx1* could be a direct target of BMP signalling. Furthermore, functional analyses in the frog and chick have suggested that MSX family members can mediate several functions of BMPs, such as regulating neural induction, neural crest formation and dorsal spinal cord development (reviewed in Liu and Niswander, 2007)

8.2.2 **Wnt signaling**

Receptors. Wnt proteins released from or presented on the surface of signaling cells act on target cells by binding to the Frizzled (Fz)/LRP (low density lipoprotein receptor-related protein) forming a trimeric complex at the cell surface. Genetic and biochemical data have demonstrated that the Fz proteins are the primary receptors for the Wnts. The Frizzled family of receptors has seven transmembrane (TM)-spanning domains. Amino acid hydropathy analysis predicts a conventional 7TM structure containing one extracellular N-terminus, three extra- and three intracellular loops and an intracellular C-terminus. The large N-terminus region contains a cysteine-rich domain (CRD) that binds the receptor's cognate ligands (Nusse, 2003). In addition, putative glycosylation sites on the extracellular loops of Fz could be involved in ligand binding and have been shown to be important in the endoplasmic reticulum for receptor maturation, which is regulated in a specific manner by the protein Shisa (reviewed in Schulte and Bryja, 2007). Fz can interact with Dsh (Disheveled) directly through a C-terminal cytoplasmic motif in Fz that is required for Fz signalling. LRP is a single pass TM molecule that may also interact with a cytoplasmic component of the Wnt-signaling pathway. The cytoplasmic tail of LRP can become phosphorylated following Wnt stimulation. Recent data showed that Derailed, which is a TM tyrosine kinase belonging to the RYK subfamily, is another Wnt receptor enterily distinct from thr Frizzleds (Logan and Nusse, 2004).

Secreted Wnts may also bind members of the SFRP family. These are secreted proteins that resemble the ligand-binding domain of the Frizzled family of Wnt receptors. Alternatively, Wnts may bind WIF proteins, which are secreted molecules resembling the extracellular portion of the Derailed/RYK class of transmembrane Wnt receptors. In general, both SFRPs and WIFs are thought to function as extracellular Wnt inhibitors (Logan and Nusse, 2004)

Ligands. Wnts are (39 – 46 kDa) secreted glycoproteins. They are defined by sequence rather than by functional properties and contain a signal sequence followed by a highly conserved distribution of cysteines (reviewed by Logan and Nusse, 2004).

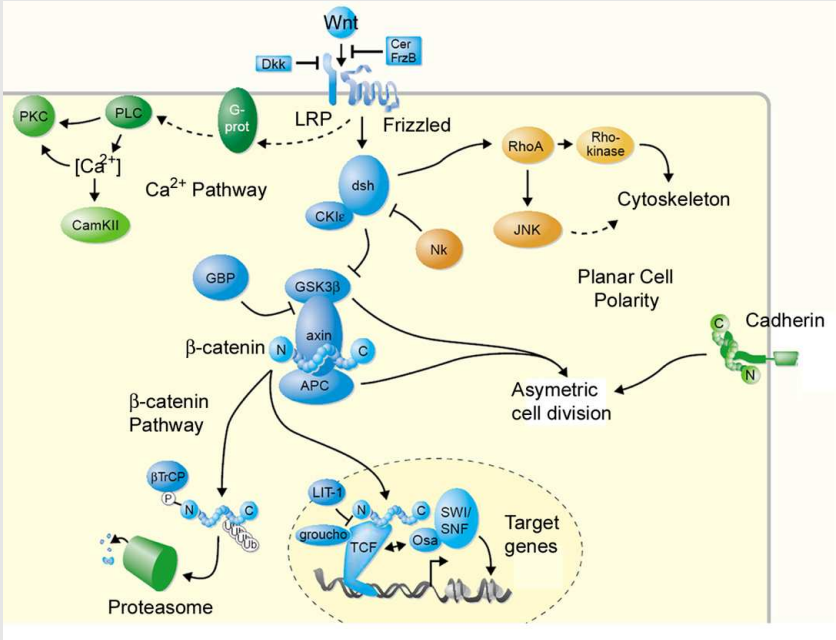


FIGURE ANNEX 8.2.2. Wnt pathway

Intracellular signalling pathways and targets. There are four signaling pathways thought to be regulated by Wnt proteins. Wnt signals are context-dependently transduced to both pathways based on the expression profile of Wnt, SFRP, WIF, Frizzled receptors, coreceptors, and the activity of intracellular Wnt signaling regulators. Wnt signals are transduced to the canonical pathway for cell fate determination, and to the noncanonical pathways for control of cell movement and tissue polarity.

Wnt/β-catenin (canonical) pathway. Among the four Wnt signaling pathways, the Wnt/β-catenin signaling pathway is best understood. The canonical Wnt pathway regulates the expression of Wnt target genes through β-catenin /TCF-LEF (T-cell factor/lymphoid enhancer factor) proteins (Moon et al., 2002; Akiyama, 2000). At least 6 of 19 Wnt proteins, including Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8, and Wnt8b, have been reported to activate this signaling pathway. TCF/LEFs form a subfamily of the high-mobility group (HMG)-box-containing superfamily of transcription factors. Strictly speaking, TCF/LEFs are not transcription factors by themselves. Although they bind to a conserved DNA sequence, the Wnt-response element (WRE: C/T-C-T-T-T-G-A/T-A/T), via their HMG domain, they must bind to other cofactors to influence transcription. Binding of beta-

catenin to an N-terminal domain of TCF/LEFs facilitates assembly of multimeric complexes containing transcriptional co-activators which can activate transcription of target genes. TCF/LEFs are not only transcriptional activators; without beta-catenin they assemble alternative complexes with transcriptional co-repressors, which act as multimeric transcriptional repressors (CtBP, groucho/TLE and others). β-catenin is an integral component of E-cadherin complexes at intercellular adherens junctions, but also recruits chromatin remodeling complexes to activate transcription in the nucleus. As a general rule nuclear beta-catenin, and therefore ultimately the canonical Wnt signalling pathway, regulates whether TCF/LEFs function in beta-catenin-free complexes as transcriptional repressors (no Wnt signalling) or in beta-catenin-containing complexes as transcriptional activators (active Wnt signalling) (reviewed by Willert and Jones, 2006; Hoppler and Kavanagh, 2007). The activity of this signalling pathway is determined by the amount of β-catenin in cytoplasm. Normally, cytoplasmic β-catenin level is kept low through continuous ubiquitin-proteasome mediated degradation of β-catenin, which is regulated by a multiprotein complex containing axin, APC (adenomtous polyposis coli), GSK-3β (glycogen synthase kinase-3β), and CK1α (casein kinase 1α). CK1α and GSK-3β mediate the degradation of β-catenin molecules by phosphorylating specific amino terminal residues, which marks the protein to be driven to degradation by the proteasome complex (Liu et al., 2002). Wnt proteins released from or presented on the cell surface initiate intracellular accumulation of β-catenin by binding to a cell surface receptor complex, Fz /LRP. After the binding of Wnt proteins to the receptor complex, cytoplasmic Disheveled, a down stream protein of the receptor complex, is phosphorylated and inhibits GSK-3β and CK1α activities through their retention at the scaffolding protein axin, resulting in the accumulation of non-phosphorylated β-catenin in cytoplasm. Non-phosphorylated β-catenin avoids degradation and is translocated into the nucleus. In the nucleus, β-catenin forms a complex with transcriptional factor, TCF. Canonical Wnt pathway down stream target genes includes c-myc and cyclin D1, TCF and CREB binding protein (Willert and Jones, 2006).

Planar cell polarity (PCP) pathway establishes asymmetric cell polarities and coordinates cell shape changes and cellular movement (Mlodzik, 2002). PCP or Disheveled-dependent pathway is transduced through Frizzled family receptors and ROR2/RYK coreceptors (Rho family GTPases and c-jun NH2- terminal kinase).

The Wnt /Ca2+ pathway by regulating cell adhesion and motility plays important roles during dorso-ventral patterning of the embryo, regulating cell migration, as well as heart development, and might play a role during tumor suppression. Ca2+-dependent pathway

is transduced through Frizzled family receptors. Some Wnt ligands like Wnt-4, Wnt-5A or Wnt-11 are able to elicit an intracellular release of calcium ions. This calcium signaling activity is sufficient to activate calcium sensitive enzymes like PKC (protein kinase C), calcium-calmodulin dependent kinase II or calcineurin. Nemo-like kinase and NFAT (nuclear factor of activated T cells) are the Ca2+-dependent effector molecules (Kühl et al., 2000).

Recently a fourth pathway has been identified that involves protein kinase A and plays a role in myogenesis (Chen et al., 2005)

A large number of Wnt targets have been identified that include members of the Wnt signal transduction pathway itself, which provide feedback control during Wnt signaling.