Proneural gene requirements and progenitor dynamics in sensory organ development

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Abstract

The inner ear is the sensory organ for hearing and balance. Its functional unit is the sensory patch that comprises: i) hair cells, which are the mechano-transducers sensing the stimuli and are embedded in the supporting cell layer, and ii) sensory neurons, which conduct these stimuli to the hindbrain. The generation of hair cells and neurons occurs in the otic placode early during embryonic development. Cell fate specification relies on expression of proneural genes and is concomitant with organ growth and morphogenesis. We used zebrafish embryos and combined live imaging and genetic tools to investigate: i) the location of the different progenitor pools, ii) the potentialities they exhibit, and iii) the dynamic behavior of these cells in generating the different fates. We have generated progenitor maps for the different cell fates by lineage tracing and focused our analysis on the behavioral changes of progenitors upon depletion of a proneural gene and the spatial and temporal aspects of cell fate specification.

Resum

L'oïda interna és l'òrgan sensorial responsable de l'audició i l'equilibri. La seva unitat funcional és el parxe sensorial que contèn: i) les cèl·lules ciliades, que són els mecano-transductors que detecten, i ii) les neurones sensorials, que envien aquests estímuls al cervell posterior. La generació de cèl·lules ciliades i de neurones te lloc a la placoda òtica molt aviat durant el desenvolupament embrionari . L'especificació del destí cel·lular es basa en l'expressió dels gens proneurals i és concomitant amb el creixement de l'òrgan i la seva morfogènesi. Hem utilitzat embrions de peix zebra i combinat imatges en viu amb eines genètiques per investigar: i) la ubicació dels diferents grups de progenitors, ii) les potencialitats que presenten, i iii) el comportament dinàmic d'aquestes cèl·lules en la generació dels diferents destins. Hem generat mapes progenitors pels diferents destins cel·lulars a partir d'experiments de llinatge i hem centrat la nostra anàlisi en els canvis de comportament dels progenitors després de la inactivació d'un gen proneural i els aspectes espacials i temporals de l'especificació de destí cel·lular.

Preface

In this thesis I document our efforts in understanding how cell fate specification of different cell types is coordinated in space and time. The inner ear primordium, a simple ectodermal placode gives rise to sensory hair cells and sensory neurons, which together constitute the functional unit of the organ, the sensory patch. Proneural genes play a mayor role in temporally and spatially controlling these processes. However, while the proneural genes important in these processes are well known, little information is available about their spatio-temporal expression.

The first part of the results entitled **Cell Lineage Analysis Reveals Three Different Progenitor Pools for Neurosensory Elements in the Otic Vesicle** is a joint project with Dora Sapède, former postdoc in the lab. In this project we characterize the different progenitor pools in the zebrafish otic vesicle by in situ hybridization of proneural genes for hair cell and neuronal fates. We describe the phenotypes in hair cell specification upon inhibition of neurogenesis by blocking *neurog1* -the main proneural implicated in otic neurogenesis-. Specifically we show that neuroblast progenitors not able to undergo neurogenesis switch their fate to hair cells of the posterior macula. By photoconversion of single cells we crudely map the different progenitors: unipotent progenitors giving rise only to either hair cells or neurons. And bipotent progenitors giving rise to both fates. We also analyze the phenotypes upon inhibition of *neuroD*, another proneural gene that is a known downstream target of *neurog1* in the inner ear and find that *neuroD* expression is necessary for the development of hair cells from this bipotent progenitor pool, suggesting that it has a pivotal role in cell fate specification of sensory hair cells and neurons of the zebrafish inner ear.

In the second part of the results entitled **Cellular dynamics of neurosensory progenitors during development** we took a step forward to analyze and visualize the cell behavior of these progenitors during development. In this project we apply long term high resolution in vivo SPIM imaging to record the development of the otic vesicle. We analyze this data to reconstruct the cell

lineages of hair cells and neurons. We characterize the spatial and temporal cellular dynamics in sensory patch formation and we also investigate these parameters in *neurog1*-depleted embryos. We show that supernumerary hair cells in these fish do not come from more active progenitors but from an enlarged progenitor domain. On the other hand, we describe the temporal and spatial aspects of otic neurogenesis. Neuroblasts are specified within the otic epithelium upon which they delaminate and accumulate ventral to the otic structure to form the statoacoustic ganglion. We show how the delamination domain is established and that time and place of birth determine neuroblast allocation within the ganglion. From this tracking data we generate progenitor maps for hair cells and neurons in the early otic structure. The usefulness of such progenitor maps is explained in the discussion.

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Index

1 INTRODUCTION

1.1 Structure and function of the inner ear

1.1.1 Auditory and vestibular components of the inner ear

The ear is the sensory organ devoted to the perception of sound and motion. In humans and other terrestrial vertebrates the ear consists of three main morphological domains: the outer ear to which sound waves enter, the middle ear where these airborne waves are amplified and pass from the airy medium to the aqueous medium of the body, and the inner ear where the perception of sound and motion takes place (Figure 1A; (Purves et al., 2004)). These are common structures, although fish lack an outer and middle ear and comprise only the inner ear structure (Abbas et al., 2010). The two sensory modalities of motion and sound are perceived in two different regions of the inner ear: the vestibular domain can detect rotational and linear accelerations and gravity, and the auditory domain permits perception of sound within a given frequency range, depending on the species. We humans have the so-called organ of Corti, a long spiral shaped tubular structure responsible of hearing. Birds and amphibians hear with the basilar papilla, an elongated rod shaped tube, and the hearing specialized aquatic vertebrates like the zebrafish sense sound via the saccular macula and the lagena (Figure 1B; (Abbas et al., 2010; Purves et al., 2004)).

While the auditory domain has evolved into these different endorgans, the vestibular apparatus is highly conserved among vertebrates. It comprises the semicircular canals and the otolithic organs. The semicircular canals, also referred to as the membranous labyrinth, contain the three cristae (anterior, lateral and posterior cristae) for sensing angular acceleration. The otolithic organs comprise the anterior (utricular) and the posterior (saccular) macula (Figure 1B; (Purves et al., 2004)). The otolithic organs contain the otoconia (“earstones”) which due to their high mass are particularly important in sensing gravity (Purves et al., 2004). In zebrafish there is a third otolithic organ, the lagena, which together with the saccular macula mediates the sense of hearing (Abbas et al., 2010). Additionally, teleost fish posses another small macula, the

macula neglecta, which might be primarily involved in velocity sensing (Casper and Mann, 2007).

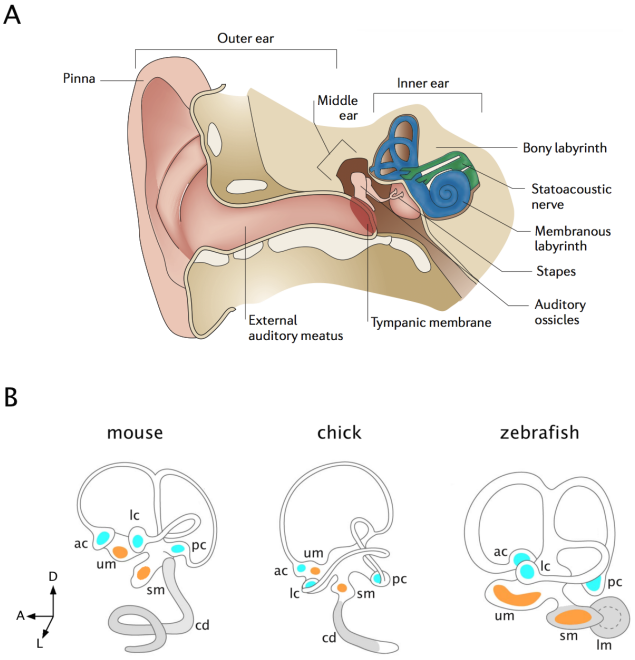


Figure 1. The structure of the ear.
A) Diagrammatic cross-section through the human head illustrating the outer, middle and inner ear. Soundwaves enter in the outer ear. In the middle ear these are amplified by the three auditory ossicles as they pass from the air to aqueous medium of the body. Perception of sound and motion occurs in the inner ear in the membranous labyrinth (blue), which is innervated by the VIIIth (statoacoustic) cranial nerve (green). Adapted from (Kelley, 2006). **B**) Morphology of the inner ear in three vertebrate species: mouse, chicken and zebrafish. The dorsal vestibular part is formed by three semicircular canals harboring the cristae (blue) and the utricular and saccular maculae (orange). The ventral, auditory part of the inner ear (grey) is highly variable in morphology and complexity in different vertebrates. In the mouse, the cochlear duct, a coiled structure, contains a finely patterned sensory organ, the organ of Corti. In chicken, the auditory organ, the basilar papilla, is also contained in the cochlear duct. In zebrafish, there is no ventral cochlear duct and the auditory function is carried by the saccular and lagenar maculae. ac/lc/pc: anterior/lateral/posterior crista; cd: cochlear duct; lm: lagenar macula; sm: saccular macula; um: utriclular macula. The embryonic axes are indicated at the bottom. Adapted from (Schneider-Maunoury and Pujades, 2007).

1.1.2 The functional unit of the ear: the sensory patch and its cellular components

The cellular basis for conversion of mechanical stimuli to electric impulses is the same in all the auditory and vestibular endorgans (see BOX 1). In each of the functional domains the otherwise monolayered epithelium is pseudo-stratified with an apically

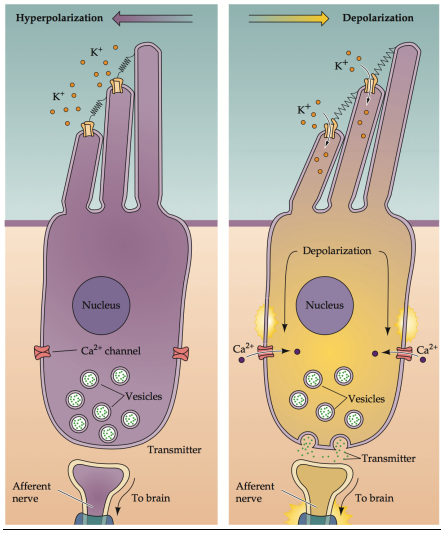
situated layer of mechanosensory hair cells embedded in a layer of supporting cells. The hair cells face with their apical side the fluid filled cavities of the inner ear. Projecting from the apical surface of each hair cell is a stiff hair bundle, consisting of rows of interconnected stereocilia tethered to a single kinocilium. Stereocilia are densely packed with actin filaments and form parallel rows that increase progressively in height, creating a staircase-like structure; adjacent to the tallest row of stereocilia is the kinocilium, Deflection of the hair bundle toward the kinocilium is thought to tension tip links and open mechanosensitive channels, depolarizing the hair cell. Conversely, deflection away from the kinocilium is thought to relieve tip-link tension and close mechanosensitive channels (see BOX 1; (Hudspeth and Corey, 1977; Purves et al., 2004)). These received stimuli are then transduced to the bipolar sensory neurons of the statoacoustic ganglion (SAG), which contact the hair cells at their basal site and will convey the sensory information to the brainstem.

The arrangement of sensory hair cells embedded in a layer of supporting cells and contacted by neurons of the SAG is called the **sensory patch** and constitutes the functional unit of the inner ear. The neurons that contact the hair cells can be afferents transducing the stimuli to the relay centers of the brain or efferents serving for example in adaptation. In the human cochlea the row of inner hair cells is mainly contacted by afferents whereas the three rows of outer hair cells are primarily contacted by efferents. A remarkable feature of the human cochlea is its tonotopic organization: sound waves are decomposed in the cochlear duct such that the base senses low frequencies and the apex senses high frequencies, and this tonotopic organization is reflected even in the neuronal populations in the higher order brain centers up to the auditory cortex (Purves et al., 2004).

Interestingly, all the cellular components of the sensory patch arise from a common structure, the otic placode –an ectodermal thickening arising adjacent to the hindbrain. Therefore, to acquire the extraordinary organization of the different sensory epithelia within the adult inner ear structure, embryonic development has to ensure that cell specification and morphogenesis are tightly regulated. At the end, the orientation of hair bundles within these epithelia is key for the organ especially in the detection of motion in all directions.

BOX 1: Structure and function of mechanosensory hair cells

Hair cells are highly polarized cells and they display at their apical surface the hair bundle: one kinocilium and several sterocilia. These cellular components are the actual mechanotransducers. The stereocilia are arranged around the kinocilium in a bilateral symmetric fashion and these cellular protrusions are interconnected by different links, most notably the so-called tip links (Flock et al., 1982). At their tips these protrusions contain gated K+ channels. The fluid of the inner ear cavities, the endolymph, is K+ rich and Na+ poor, in contrast to the perilymph at the basal site of the sensory epithelium which is K+ poor and Na+ rich. The generated electrical gradient across the epithelium drives K+ inside the hair cell upon opening of the channels and this in turn leads to opening of voltage gated calcium channels and depolarization of the hair cell. Opening of the K+ channels occurs upon deflection of the hair bundle towards the kinocilium (Hudspeth and Corey, 1977). In resting state, some K+ channels are open constituting the resting potential and upon deflection of the bundle in the opposite direction the channels close and the cell is hyperpolarized (Purves et al., 2004). Upon depolarization of the hair cell, signal transduction to the sensory afferent neuron occurs at the so-called ribbon synapse. This is a specific synapse found in systems that require a tonic release of neurotransmitter, such as the auditory, vestibular and also the visual system. The ribbon synapse contains a large number of specific presynaptic vesicles tethered to the plasma membrane such that upon stimulation neurotransmitters can be released quickly and continuously (Zanazzi and Matthews, 2009).



Mechanoelectrical transduction mediated by hair cells
After (Lewis and Hudspeth, 1983); Adapted from (Purves et al., 2004).

1.2 Progenitors and Cell Specification

The development of multicellular organisms requires the progressive specification of groups of multipotent cells to a number of final fates. In this process of specification, progenitor cells progressively limit their potential to generate all the cells of the body. The latest step in this specification process is terminal differentiation: the acquisition of the distinct morphology that defines a specific fate. The lineage of a cell describes the sequence of divisions and decisions that have generated this cell.

In the human body there are more that 200 different cell types present, which all have the same origin: the fertilized egg. In order to generate an organism, or even just an organ, the behavior of progenitors must be tightly controlled, since this happens in the

tissue context concomitant with growth and morphogenesis. First, it has to be ensured that competent progenitors are specified at the right time in the adequate number and that homeostasis of the progenitor pool is regulated for as long as specification has to take place. Next, the generation of fates from this competent pool of cells has to be regulated, again spatially and temporally.

Patterning and **cell fate specification** by proneural genes are tightly connected and are of great importance in these processes. In patterning, a developing tissue acquires regional identities in response to morphogens. This involves a change in the cellular state, the internal gene regulatory network that the cell displays. Upon cell division this state can be inherited leading to two identical daughters. Alternatively, asymmetric cell division can be the trigger for generation of one daughter cell that is fate restricted due to unequal distribution of cytoplasmic determinants upon division (Paridaen and Huttner, 2014).

The transcriptional cascades leading to the generation of distinct fates have been extensively studied. For example in the spinal cord signals from the dorsal roofplate (Wnt/BMP) and ventral floorplate (Shh) establish opposing dorsoventral gradients, which govern the process of dorsoventral patterning. Growth and morphogenesis present a challenge to the process of patterning. Cell proliferation and morphogenesis of both, the signal-emitting and signal-receiving cells, during this process necessarily leads to a higher level of noise in the signaling gradient. Lately it has been proposed that spinal cord progenitor cells have the capacity to respond to signals, by sorting and thus generate discrete expression domains, which are important in the later steps of specification (Xiong et al., 2013).

These progenitor domains, which are established by the patterning process, are characterized by the spatially restricted expression of patterning genes (Guillemot, 2007). Within these domains, there is expression of proneural proteins in a subset of cells and they in turn induce expression of specific neuronal differentiation genes. These factors coordinately regulate cell specification and differentiation (Figure 2). Proneural proteins are expressed before any sign of neuronal differentiation and they are required and sufficient to drive neuronal differentiation (Bertrand et al., 2002): they commit progenitors to a given fate and initiate their specific differentiation program, activating specific sets of target genes depending on spatial and temporal cues.

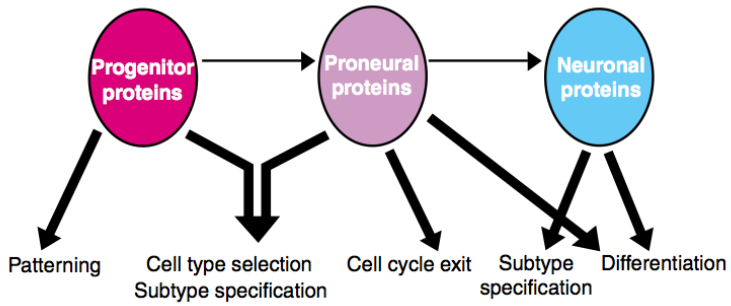


Figure 2. Cell Specification in the central nervous system.
The differentiation of multipotent progenitor cells into specific classes of postmitotic neurons and glia involves transcriptional cascades in which patterning proteins induce proneural proteins, which in turn induce, often directly, neuronal homeodomain proteins (thin arrows). These factors regulate different phases of neural development (thick arrows). Subtype specification is initiated in dividing progenitors coordinately by progenitor proteins and proneural proteins and further promoted by neuronal proteins after cell cycle exit. Adapted from (Guillemot, 2007).

SoxB1 genes promote the self-renewal and multipotency of progenitors. In order to promote neurogenesis, proneural proteins must trigger the inhibition of SoxB1 gene expression and one mechanism is by the activation of SoxB1 antagonists, such as *Sox21*. However, this process needs to be highly regulated, in order not to lead to premature depletion of the progenitor pool (Guillemot, 2007). A mechanism for controlling progenitor pool homeostasis is by regulating the way in which cells divide. In the chick spinal cord three kinds of divisions take place: "self expanding divisions" give rise to two progenitors, "self replacing divisions" give rise to one progenitor and one fate restricted daughter cell and "self-consuming divisions" give rise to two fate restricted daughter cells. Shh signaling in this context has a role in maintaining the stem cell character of the progenitors (Saade et al., 2013).

The exact mechanism, by which patterning proteins, proneural proteins and extrinsic signals work together to drive cell fate specification from multipotent progenitors is not completely unveiled. However, these factors may work on multiple levels in a combinatorial manner and conjunctly promote changes in the internal gene regulatory network of a cell, that allows for progression along the lineage (Guillemot, 2007). An important feature of proneural genes is their expression around the time of terminal division of a progenitor. Distinct fates are often generated in a sequential manner, and so controlling the terminal division might affect the cell fates that can be produced. In fact, studies from the retina suggest that progenitors have distinct competences to

generate a small number of fates that rely on temporal cues. Retinal progenitors generate the set of fates that correspond to their developmental time even when placed in early or late environments. This suggests that there are intrinsic changes in states of competence, which change over time (Cepko, 2014; Guillemot, 2007).

1.2.1 Proneural genes in the inner ear – the drivers of sensory and neuronal fates

Proneural proteins are transcription factors of the basic helix-loop-helix (bHLH) family of genes (Figure 3) and they were first discovered in the Drosophila sensory bristle (Bertrand et al., 2002; Ghysen and Dambly-Chaudière, 1988). Four related genes, achaete (*ac*), scute (*sc*), lethal of scute (*lsc*) and asense (*ase*), form the so-called *Acheate-Scute* complex. Based on sequence homology, additional bHLH genes were identified and grouped according to their conserved sequence identity into distinct gene families (Figure 3A; (Bertrand et al., 2002)).

In Drosophila the proneural genes of the *asc* family are involved in cutaneous sense organ development while atonal (*ato*) guides photoreceptor and chordotonal organ development. Of the chordotonal organs, the Johnstons organ shows the highest similarity to the vertebrate ear in terms of function. A highly polarized sensory neuron mediates both mechano-reception and signal transduction (Abelló and Alsina, 2007; Fritzsch and Beisel, 2001). In vertebrates, these two functionalities are distributed to two distinct cell types, each of which depends on proneural gene functions of distinct atonal homologs. Both cell types, sensory hair cells and neurons, are produced from a single placode early during embryonic development (Haddon and Lewis, 1996). Cranial placodes are vertebrate innovations and so is the capacity to generate peripheral neurons (Patthey et al., 2014).

In evolution proneural genes have undergone multiplication and diversification both at the level of the protein and of their DNA binding sites (Fritzsch et al., 2010). In the vertebrate inner ear context the atonal homologs *Atoh1*, *Neurogenin1* (*Neurog1*), *NeuroD* and *NeuroD4* are of mayor importance (Jarman et al., 1993).

By definition proneural gene expression precedes and coincides with the selection of neurosensory progenitor cells. Proneural proteins confer to the progenitors the ability to

differentiate into neural elements, and in this their function is both necessary and sufficient (Alsina et al., 2009; Bertrand et al., 2002; Raft and Groves, 2015).

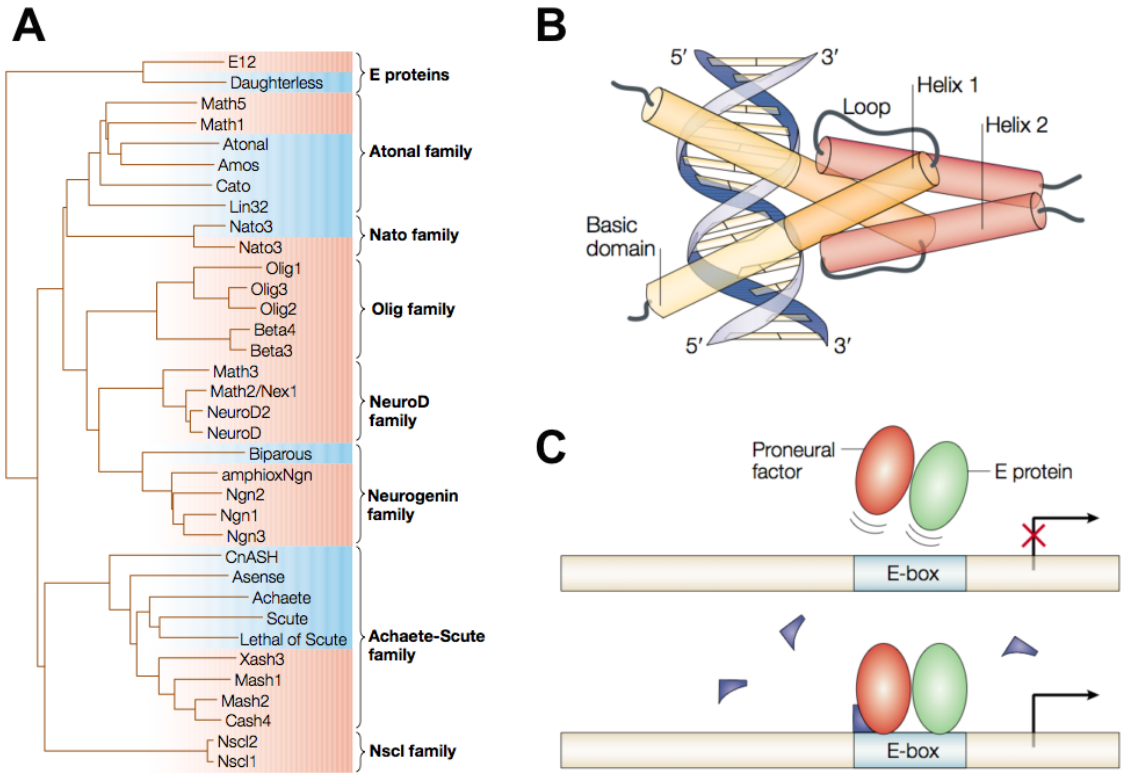


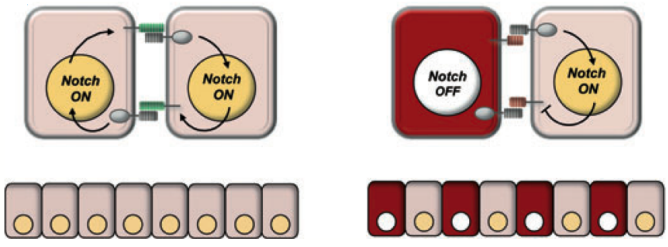
Figure 3. Structure and properties of proneural bHLH proteins.
A) Dendrogram of the sequence of the basic helix–loop–helix (bHLH) domain of invertebrate (blue) and vertebrate (red) neural bHLH proteins. Proteins have been grouped in distinct families on the basis of closer sequence similarities in the bHLH domain. **B**) Schematic representation of the structure of a bHLH dimer that is complexed to DNA. The basic region fits in the main groove of the DNA, and many residues in this region make direct contact with the E-box sequence. The two α-helices of both partners together form a four-helix bundle. **C**) Dimerization of a proneural protein with the ubiquitously expressed E-protein leads to binding of target E-box sequence and to transcription of the target gene. Modified from (Bertrand et al., 2002).

An important feature of proneural proteins in cell fate specification is that they cooperate with Notch mediated lateral inhibition to single out progenitors from the proneural field (see BOX 2). Proneural proteins bind as dimers to their target DNA sequences, the so-called E-Boxes, and they usually work as transcriptional activators (Figure 3B, C). Atonal homologs bind to DNA as heterodimers with ubiquitously expressed bHLH proteins, the so-called E-proteins (Figure 3C). Dimerization is a prerequisite for DNA binding. Some inhibitory HLH proteins like Id (inhibitor of

differentiation) proteins lack the basic motif for DNA binding and inhibit proneural protein activity by sequestering E-proteins (Bertrand et al., 2002).

BOX 2: Notch signaling

The Notch signaling pathway is a juxtacrine signaling system that involves binding of the extracellular domains of the Notch receptor on one cell to the Notch ligand on the neighboring cell (*trans* activation) or even on the same cell (*cis* activation). Receptor-ligand binding then results in a series of proteolytic cleavages that ultimately release the intracellular domain of the Notch receptor (NICD). This active form of the Notch receptor can then translocate to the nucleus, form a complex with the CSL transcription factor and MAM, and bind to the CSL target sequence. In the absence of NICD, CSL forms a transcriptional repressor complex, while formation of the ternary complex (CSL-NICD-MAM) transforms CSL into an activator of gene expression. Among the transcriptional targets are members of the *Hes* (Hairy–Enhancer of Split) and *Hey*/*Hrt* (Hes related type) genes, which are bHLH proteins acting as transcriptional repressors of proneural genes (Bertrand et al., 2002; Neves et al., 2013a). The distribution of the Notch receptors is usually widespread, and specificity comes from expression of the different Notch ligands. In Drosophila the two Notch ligands are Delta and Serrate. In vertebrates, more than just two ligands exist: in amniotes members of the *Delta-like* family are analogous to *Delta* and members of the *Jagged* family correspond to *Serrate*. The zebrafish, due to its whole genome duplication, has multiple ligands of both families of which *Delta A*, *B*, and *D* and *SerrateB* (now called *Jagged2b*) are expressed in the ear (Haddon et al., 1998). Notch signaling can act in two different modes: lateral inhibition and lateral induction both of which have been shown to be active in the developing inner ear (Raft and Groves, 2015). In the classical **lateral inhibition model,** progenitors from a competent domain are singled out and differentiate while neighboring cells maintain the progenitor state. High Notch and low Delta maintain the progenitor state by inducing *Hes* and *Hey* genes. Low Notch and high Delta on the other hand permit the expression of proneural genes and thus differentiation. Hallmark of this mode of action of Notch signaling is a speckled expression pattern of the Delta ligand. On the other hand, in **lateral induction** Notch induces expression of its ligand *Serrate* in neighboring cells and thereby leads to a continuous domain of coordinated cell behavior (Neves et al., 2013a). Notch signaling can be modulated by members of the *Fringe* family: Lunatic Fringe (*Lfng*), Maniac fringe (*Mnfg*) and Radical fringe (*Rfng*). These enzymes glycosylate the Notch receptor intra-cellularly during maturation and this potentiates signaling via the Delta ligand (Neves et al., 2013a).



The two modes of operation of Notch.
Lateral induction (**left**) and lateral inhibition (**right**). Adapted from (Neves et al., 2013a).

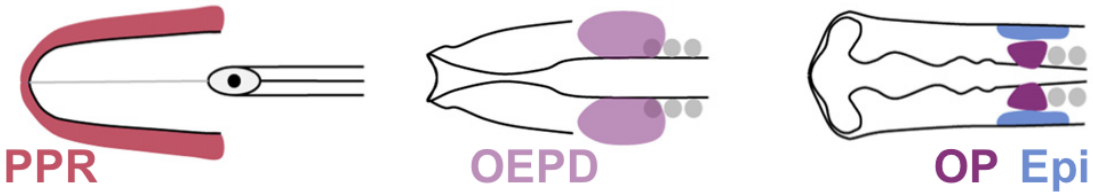
In different tissues the same proneural gene is involved in differentiation of distinct cell types. As an example, *Atoh1* governs the differentiation of inner ear hair cells, cerebellar granule and brainstem neurons, interneurons of the spinal chord and diverse non-neuronal cell types, such as Merkel cells and intestinal secretory lineages (Klisch et al., 2011), (Lai et al., 2011). Until recently only few transcriptional targets of Atoh1 were known. But by now, the new Atoh1 targetome analyses from granule cell precursors and hair cells of the inner ear suggest that Atoh1 regulates the expression of a multitude of genes responsible for diverse biological processes, including cell proliferation, differentiation, migration, metabolism and even housekeeping functions (Cai et al., 2015; Klisch et al., 2011). It will be a matter of future research to determine what precisely are the combinatorial requirements for Atoh1 to trigger specific fates in different temporal and spatial contexts.

1.3 Development of the inner ear

1.3.1 Formation of the otocyst/otic vesicle

From the non-neural ectoderm surrounding the cranial neural plate the otic territory is specified adjacent to the hindbrain at the level of rhombomere 5. This involves integration of multiple signals from the surrounding tissues and the action of a complex and dynamic gene regulatory network at the level of the presumptive otic precursors (see BOX 3 for specification of the otic placode). The otic placode first becomes visible as a thickened epithelium adjacent to the hindbrain and morphogenetic events subsequently transform this simple structure into the mature inner ear with its complex three-dimensional organization. In a first step, in amniotes, the otic placode invaginates and pinches out to give rise to the otocyst. In chick it was demonstrated, that basal extension and apical constriction of the epithelium require FGF mediated remodeling of the cytoskeleton such that actin-myosin II complexes localize apically (Sai and Ladher, 2008).

BOX 3: Induction of otic identity



Otic and epibranchial placode specification from the pre-placodal region.
Schematic dorsal view of chick embryos illustrating the different steps in otic induction. Anterior is to the left. PPR (pre-placodal region), OEPD (otic epibranchial progenitor domain), OP (otic placode), Epi (epibranchial placode). See text for details. Modified from (Chen and Streit, 2013).

The so-called Pre-Placodal Region (PPR) is a horseshoe shaped domain surrounding the anterior neural plate, which is specified by FGF, BMP and Wnt signals from the head mesoderm and the neural plate. These signaling events activate transcription factors of which some serve as competence factors for the expression of PPR specific genes *Eya1/2* and *Six1/4*, and others serve to restrict PPR gene expression. Competence factors include members of the *Ap2*, *Foxi*, *Gata2/3* and *Dlx* gene families, which are activated downstream of BMP signaling. In contrast *Msx1* under the control of BMP signaling and Wnt regulated *Pax3* act to restrict the PPR specific gene expression domain (Chen and Streit, 2013). At early neural plate stages all PPR progenitors have the potential to give rise to any of the cranial placodes, but this potential is lost upon regionalization: for example, the expression domains of the homeobox transcription factors *Otx1* and *Gbx2* initially overlap partially but upon mutual repression resolve to complementary domains which segregate the anterior trigeminal (*Otx1*) and the posterior otic (*Gbx2*) placodes (Steventon et al., 2012). Subsequent fate restrictions can then transform the posterior domain of the PPR into the so-called Otic-Epibranchial-Progenitor Domain (OEPD) of which *Pax2* is one of the earliest markers (Schlosser and Ahrens, 2004). FGFs from the hindbrain and the mesoderm (Fgf3/8 in all vertebrates and additional FGFs such as Fgf10 and/or Fgf19 in terrestrial vertebrates) are central in this process (Ladher et al., 2010). In zebrafish, BMP regulated *Foxi* and *Dlx* become restricted to the posterior PPR. Here they can act either individually or in a concerted manner and with FGF inputs from the hindbrain and mesoderm to activate the OEDP specific genes *Pax2, Pax8,* and *Sox3* (Chen and Streit, 2013; Sun et al., 2007). The OEPD comprises precursors for the otic, epibranchial, and in zebrafish the anterior lateral line placodes and undergoes further steps of lineage restriction to segregate these domains. Here FGF regulates and synergizes with Wnt in the hindbrain, such that graded Wnt activity adjacent to the hindbrain promotes otic fate while low level Wnt more laterally promotes epibranchial fate. While initially FGF signaling is necessary to specify the OEPD, successive steps in otic specification require downregulation of FGF (Freter et al., 2008; Ladher et al., 2010). Furthermore, Wnt signaling activates *Notch1*, *Jagged1* and *Hes1*, and this again favors otic fate decisions in the OEPD. In turn Notch signaling also increases Wnt activity and this interplay of Wnt and Notch is thought to stabilize the otic fate decision (Chen and Streit, 2013). While *Pax2/8* expression is initially uniform in the OEPD, higher levels at in the presumptive otic territory provide a bias later on for otic fate decision in these progenitors (McCarroll et al., 2012). From studies in the zebrafish it became apparent that the same genes that drive otic induction, are also necessary to provide a general competence to generate specific fates: *foxi1* and *dlx3b/4b* become restricted to the OEPD and provide competence to generate neurons and sensory hair cells, respectively. Both rely on BMP signaling and confer identities in an FGF dependent manner (Chen and

Streit, 2013). While inhibition of *dlx3b/4b* abrogates sensory fates early in development with no effect on neurogenesis, loss of *foxi1* abolishes early neuronal specification without impairing sensory development (Hans et al., 2013).

In zebrafish, the otic placode thickens and here hollowing, rather than invagination gives rise to the otic vesicle, but likewise involving apical localization of actin-myosin complexes (Hoijman et al., 2015). Concomitantly with these morphogenetic events the otic epithelium is patterned and cell fate specification takes place.

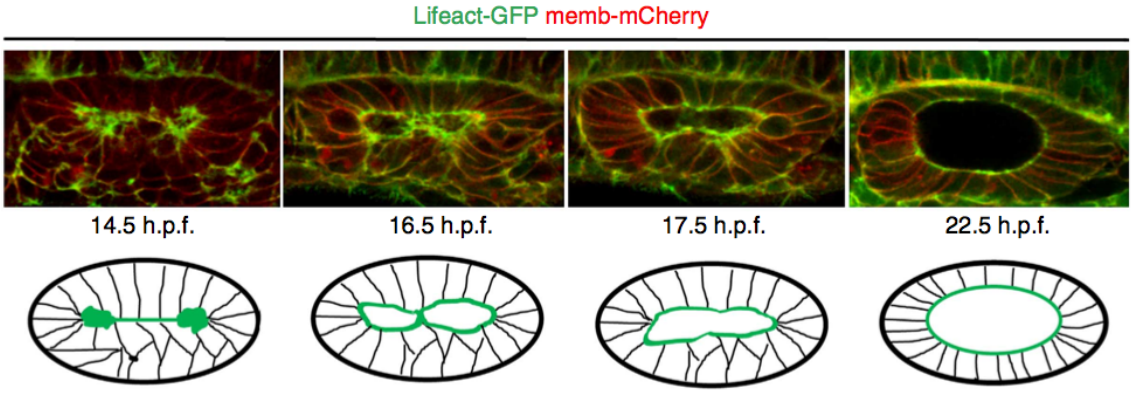


Figure 4. Zebrafish otic lumen formation.
Dynamics of F-actin-polarized distribution during lumen formation and expansion. Schematic representations of F-actin localization are shown below (F-actin in green, membranes in black). Anterior is to the left, dorsal to the top. Adapted from (Hoijman et al., 2015).

1.3.2 Establishment of the neurogenic domain

In the inner ear, molecular asymmetries precede morphological asymmetries and genes encoding transcription factors that are expressed within the early otic primordium are thought to specify the future regions of the ear (Abelló and Alsina, 2007; Fekete and Wu, 2002; Schneider-Maunoury and Pujades, 2007). Patterning of the otic primordium is achieved very early during embryonic development and prefigures the neurogenic and non-neurogenic regions of the otic vesicle. In amniotes, *Lfng* expression foreshadows the neurogenic domain, located in the antero-ventral region of the otocyst, and is excluded from the *Lmx1a* domain, whose cells most probably contribute to the non-neurogenic region (Abelló and Alsina, 2007; Giraldez, 1998; Raft et al., 2004; Vázquez-Echeverría et al., 2008). Otic neuroblasts are generated in the ventral aspect of the otocyst and delaminate from a site defined by the overlapping expression of *NeuroD*

and *Tbx1*. Several reports in different species have suggested that *Tbx1* suppresses neuronal fate (Arnold et al., 2006; Radosevic et al., 2011; Raft et al., 2004), and that *Tbx1* levels play a critical role in restricting the neurogenic domain (Arnold et al., 2006).

Work in chick has revealed the importance of members of the SoxB1 HMG box transcription factor family of genes (Sox1-3) in the establishment of the neurogenic domain: *Sox3* is expressed in the antero-medial aspect of the otic placode and marks together with *Neurog1*, *Delta1* and *Hes5* the neurogenic domain. This region starts to express *Fgf8*, and *Sox3* is maintained in part by this FGF8 signaling (Abelló et al., 2010). *Sox3* also induces the expression of *Sox2* in this territory, which persists until later stages and provides sensory competence (Abelló et al., 2010; Kiernan et al., 2005; Neves et al., 2007). Interestingly, in zebrafish *sox3* expression is under the control of *foxi1* (Sun et al., 2007), but a role for *sox3* in the establishment of the neurogenic domain remains to be investigated.

1.3.3 Making sensory neurons and building the SAG

In the development of otic sensory neurons the Atonal homologs *Neurog1* and *NeuroD* share the proneural functions. The expression of the *Neurog1* in the neurogenic domain specifies cells to the neuronal lineage and upon loss of *Neurog1* no otic neurons are produced (Andermann et al., 2002; Ma et al., 1998). Neurog1 drives expression of *NeuroD,* which irreversibly commits these cells to the neuronal fate. Upon *NeuroD* expression these neuroblasts undergo epithelial to mesenchymal transition (EMT), delaminate from the otic structure and accumulate ventrally to form the so-called statoacoustic ganglion (SAG) in chick and zebrafish or the cochlea-vestibular ganglion (CVG) in mice. *NeuroD* is also important for neuroblasts survival (Kim et al., 2001). While *NeuroD* is the best characterized among the targets of Neurog1, there are other proneural genes present in the inner ear in the different species that appear to have partially redundant functions with *NeuroD* (chick *NeuroM* and zebrafish *NeuroD4* (Park et al., 2003).

Commitment of progenitors involves lateral inhibition (see BOX 2) and *Neurog1* is upregulated in a speckled pattern within the neurogenic domain. Upon inhibition of Notch signaling *Neurog1* is activated throughout this region and an excess of neurons is

produced (Abelló and Alsina, 2007; Daudet and Lewis, 2005; Haddon et al., 1998). Neuroblasts delaminate and migrate a short distance to become situated between the hindbrain and otic vesicle undergoing a transient phase of proliferation to expand the precursor population (Alsina et al., 2004; Bell et al., 2008; Haddon and Lewis, 1996; Matei et al., 2005). This phase, termed transit-amplification, is characterized by co-expression of *NeuroD* and proliferation markers (Camarero et al., 2003). The neuroblasts eventually exit the cell cycle and differentiate into mature neurons.

The resulting SAG is topologically organized. In zebrafish two different neuronal subpopulations are present, one antero-ventral and another postero-medial, which are spatially segregated and innervate specifically the sensory patches: neurons innervating the anterior macula and anterior and lateral cristae are located antero-ventrally to the ear within the anterior part of the SAG, whereas neurons innervating the posterior macula and the posterior crista form a posterior subgroup within the SAG, which is positioned medially to the otic vesicle (Figure 5 ; (Sapede and Pujades, 2010)). However, the molecular and temporal identities of these neurons are not known neither how these neuronal populations are sorted out during development into endorgan-specific subtypes.

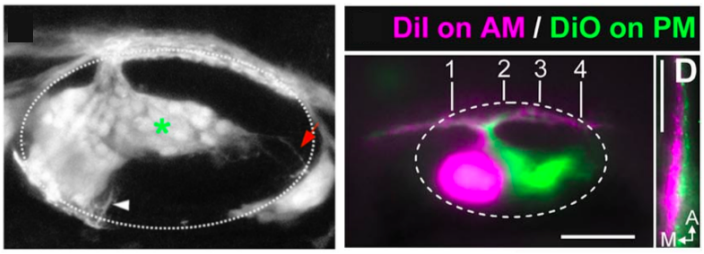


Figure 5. Segregation of the SAG in zebrafish.
(**left**) Maximal projections of confocal z-stack of the SAG by expression of Tg[Isl3:GFP] in differentiated neurons of embryos at 42-48 hours past fertilization. (**right**) DiI and DiO lipophilic dyes were injected in the anterior and posterior macula, respectively. The respective SAG population and the central projections are labeled. (left) and (right) are lateral views with anterior to the left and dorsal to the top. The inset D in (right) is a dorsal coronal section that shows how the central projections from the anterior and posterior SAG are adjacent and segregated. Modified from (Sapede and Pujades, 2010).

The different neuronal populations of the SAG not only innervate different sensory patches but they do project to distinct regions in the hindbrain. Recently we have unveiled that the topographical representation of cranial sensory ganglia is established

by entrance order, with the entry points determined by cell contact between the sensory ganglia cell bodies and the hindbrain (Zecca et al., 2015).

1.3.4 Making hair cells from the prosensory domain

The proneural gene required for hair cell specification is the Atonal homolog *Atoh1* (Bermingham, 1999). The zebrafish genome contains three paralogs of *Atoh1*, of which two are expressed in the inner ear and share the proneural functions.

In zebrafish, *atoh1b* establishes a single prosensory domain during placodal development and subsequently activates Delta-Notch feedback to split the domain into separate utricular and saccular primordia in the nascent otic placode by 12 hours past fertilization (hpf). Lateral inhibition and specification of tether cells occurs by 14 hpf, when *atoh1b* activates expression of *atoh1a*. The first hair cells to arise, the tether cells, do so in pairs at each pole of the structure and serve to tether the otoliths via their kinocilia. In the second phase, beginning soon after formation of the otic vesicle, *atoh1a* expression predominates in the maculae and maintains *atoh1b* in a subset of cells. Moreover, *atoh1a* is primarily responsible for specifying later-forming hair cells and activating Delta-Notch- mediated lateral inhibition (Figure 6; (Millimaki et al., 2007)). Hair cells of the cristae will develop yet later, at around 42 hpf, likewise under the control of *atoh1a* (Figure 6; (Millimaki et al., 2007)).

The development of the sensory patches involves pseudostratification of the otic epithelium (Haddon and Lewis, 1996). The establishment of the arrayed organization of the sensory patches, with a mosaic of hair cells embedded in the supporting cell layer, involves Notch-mediated lateral inhibition. Differentiating hair cells maintain *atoh1* expression, while supporting cells downregulate *atoh1*. Interfering with Notch signaling at this stage, leads to an overproduction of hair cells from the prosensory progenitors at the expense of supporting cells (Daudet et al., 2007; Haddon and Lewis, 1996; Pan et al., 2010; Petrovic et al., 2014; Riley and Grunwald, 1996).

In amniotes, there is a single *Atoh1* gene, which is indispensable for the generation of hair cells. *Atoh1* is expressed in hair cell progenitors and differentiating hair cells, but is downregulated in supporting cells (Bermingham, 1999; Neves et al., 2007; Raft and Groves, 2015).

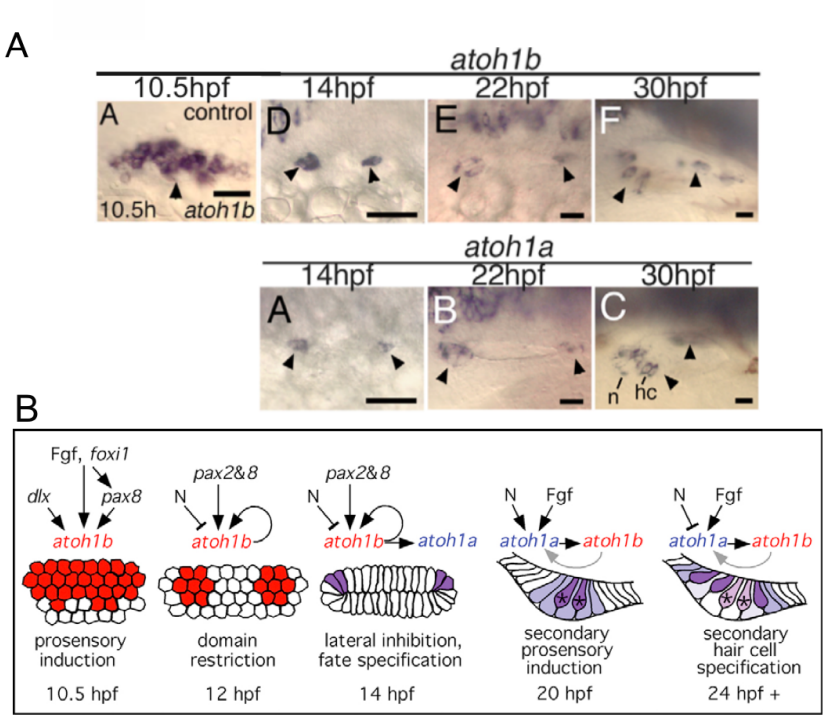


Figure 6. *Atoh* genes in zebrafish hair cell specification.
A) In situ hybridizations of *atoh1b* (top row) and *atoh1a* (bottom row). *atoh1b* is expressed in a broad domain at 10.5 hpf and then is restricted to the poles of the otic vesicle where it is observed in a small subset of cells. *atoh1a* expression starts later than *atoh1b* expression at the poles of the otic vesicle and *atoh1a* domains expand with time. Dorsolateral views with anterior to the left and dorsal to the top. arrows point to the prosensory domain (top row first panel) or to the presumptive maculae (other panels). **B**) Model of *atoh1* regulation and function. *Fgf pax8* and *dlx* pathways induce expression of *atoh1b* (red) in medial preotic cells, specifying the prosensory equivalence group. By 12 hpf, the domain is restricted into two intermediate groups by Dl-N activity, which is activated by *atoh1b* function. Tether cells are specified around 14 hpf as *atoh1a* is activated (blue, coexpression with *atoh1b*, purple). At 20 hpf, N and Fgf activate a wider domain of *atoh1a* associated with later-forming hair cells. Tether cells (asterisks) terminally differentiate. *atoh1a* is required to maintain or activate *atoh1b* in differentiating cells, and *atoh1b* helps maintain high levels of *atoh1a*. At 24 hpf and thereafter, later-forming hair cells begin to differentiate and coexpress *atoh1* genes, and N activity limits *atoh1* expression. Mature tether cells and hair cells downregulate *atoh1* expression. Modified from (Millimaki et al., 2007).

Sox2 is expressed in both amniotes and zebrafish and its role in the process of sensory specification has led to some confusion. In chick in course of establishment of the neurogenic domain *Sox3* activates *Sox2,* and while *Sox3* expression ceases *Sox2* remains and marks the prosensory domain (Abelló et al., 2010; Neves et al., 2007). Upon sensory differentiation *Sox2* expression is lost in hair cells, but is retained in the supporting cell layer (Kiernan et al., 2005; Neves et al., 2007). The loss of *Sox2* in mouse otic vesicles has dramatic consequences for hair cell development: no *Atoh1*

expression is present and differentiated hair cells are consequently absent (Kiernan et al., 2005). Conversely, in chick ectopic expression of *Sox2* can convert non-sensory ear epithelia into sensory ones (Neves et al., 2011). Therefore, *Sox2* maintains the cell renewal state and commits progenitors to neural fate.

Blocking of *sox2* in the zebrafish has no such dramatic effects: zebrafish *sox2* is expressed in the presumptive anterior and posterior maculae, and blockage of *sox2* translation by morpholino does not prevent hair cell production. Instead the rate of accumulation is reduced due to sporadic death of differentiated hair cells. However, regeneration of hair cells upon damage does not occur in Sox2-depleted embryos. These data show that zebrafish *sox2* is required for hair cell survival, as well as for trans-differentiation of supporting cells into hair cells during regeneration (Millimaki et al., 2010). The ability by ectopic *atoh1* expression to generate hair cells is spatially limited. Ectopic co-expression of *atoh1a* and *sox2* greatly enhances this potential, such that ectopic hair cells can then be generated in all non-sensory regions of the ear (Sweet et al., 2011).

In conclusion, some general roles of *Sox2* have been elucidated that apply to amniotes as well as to zebrafish: i) *Sox2* is expressed in the prosensory domain early in development (Millimaki et al., 2010; Neves et al., 2007). ii) *Sox2* is expressed in supporting cells and is downregulated in hair cells (Kiernan et al., 2005; Millimaki et al., 2010; Neves et al., 2007). iii) *Sox2* maintains the self-renewal state and the pluripotency of progenitors (Alsina et al., 2009; Chen and Segil, 1999; Kiernan et al., 2005).

Apart from the described role of Notch signaling in lateral inhibition, there is evidence in mouse and chick that Notch functions in lateral induction in the prospective sensory patches. The Notch ligand *Jagged1* is expressed in a uniform pattern and maintains the expression of *Sox2* in these domains in (Kiernan et al., 2006; Neves et al., 2011). In zebrafish several Notch ligands are expressed in the developing inner ear such as *deltaA/B/D* and *serrateB* (now called *jagged2b*) (Haddon et al., 1998; Lecaudey et al., 2007). *Jag1b* is the zebrafish homolog of the mammalian *Jagged1*. Recent studies in zebrafish have identified a role for *jag1b* in development of the cristae. Here *jag1b* mediates separation of the anterior and lateral cristae from a common prosensory domain in an FGF dependent manner (Ma and Zhang, 2015; Zecchin et al., 2005).

The multiplicity of cell types formed in the otocyst, the fact that these different cell types arise from specific regions of a single epithelium, as well as the complex morphogenesis taking place in this organ, underline the importance of integrating regionalization and cell type specification in the developing ear.

1.3.5 Coordinating cell fate specification

Hair cells and sensory neurons are tightly connected forming the sensory patch, which underlies the function of the inner ear. Both cell types arise early in embryonic development from a simple ectodermal thickening, and in amniotes, hair cell specification starts after neurogenesis has almost ceased. This observation led to a series of questions: does a sequential activation of proneural genes for neurons and hair cells result in sequential fate specification from a common territory? And if this was the case would this involve a common progenitor that sequentially gives rise to all the fates or rather are there segregated populations of progenitors within such common domain? In either of the cases, how could proneural factors interact to mediate their own specific cell fate and what might be the temporal and spatial requirements for such interactions?

The question of whether cells of the distinct anatomical subdivisions in the inner ear share a clonal origin was addressed in chick by the use of retroviral lineage tracing at the time of delamination. It was shown that in few cases neurons of the SAG could be clonally related to hair cells and supporting cells of the utricular macula, and that vestibular and sensory neurons of the SAG share a lineage on a more frequent basis (Satoh, 2005).
A different approach using DiI/DiO lipophilic dye labeling could establish a fate map of the chick otic placode. Commonly, domains labeled with one dye would give rise to a sensory patch and its innervating neurons in the SAG. Also there was found little intermingling of labeled derivatives and unlabeled cells within anatomical subdivisions. This led to the suggestion that there is a spatial segregation of these progenitors already at placode stage: anterior cristae and corresponding neuroblasts are located antero-laterally in the otic placode; medially lie the progenitors of the maculae and their corresponding neuroblasts; and finally, the progenitors of the basilar papilla and the corresponding auditory neuroblasts are located postero-medially. Furthermore,

combined injections with different dyes in two different domains of the placode or sequential injections in a similar domain gave some insights in the temporal sequence of neurogenesis and sensorigenesis. In neurogenesis and sensorigenesis antero-lateral structures are thought to be specified first, followed by medial and then postero-medial structures (Bell et al., 2008).

If hair cells and neurons for the distinct sensory patches are derived from common neurosensensory territories, then how is the switch from neurogenesis to sensorigenesis controlled at the genetic level? It was shown also in mice that neurogenesis precedes sensorigenesis and that these two processes coincide temporally and spatially in the otic vesicle. Genetic tracing using *Atoh1* and *Neurog1* reporter lines in mice could elucidate some of the aspects of proneural gene regulation in this process. First, *Neurog1* derivatives could be found in the vestibular and auditory (spiral) ganglion, in hair cells and supporting cells of the utricular and sacular maculae and of the organ of Corti and also in non-sensory epithelial cells adjacent to these sensory domains. Second, *Neurog1* and *Atoh1* are subject to cross-inhibition, since the knock out of either *Atoh1* or *Neurog1* leads to an expanded expression of the respective other gene. Third, *Atoh1* positively regulates its own expression while *Neurog1* negatively regulates its own expression, and the latter is dependent on Delta-Notch signaling (Figure 7; (Raft et al., 2007)). However, this study could not rule out whether these interactions occur in a common progenitor or intercellularly.

As regarding the question of how the genetic switch from neurogenesis to sensorigenesis could occur, work in chick and cell culture has led to a model involving *Sox2* acting in incoherent feed-forward loop: *Sox2* drives expression of both *Neurog1* and *Atoh1* and also of their respective inhibitors, such as *Hes/Hey* and *Id* genes. Initially neurogenesis is prevailing and *Atoh1* expression keeps being repressed by the action of the repressors and by Neurog1. Later, this balance is shifted such that Atoh1 can accumulate and exert its autoregulatory function that irreversibly commits progenitors to the hair cell fate (Neves et al., 2012; Neves et al., 2013b; Raft and Groves, 2015).

However, as described previously, in zebrafish the action of *sox2* in this context might be slightly different. Despite the fact that there are some divergences between the zebrafish and amniotes, the main mechanisms are largely conserved.

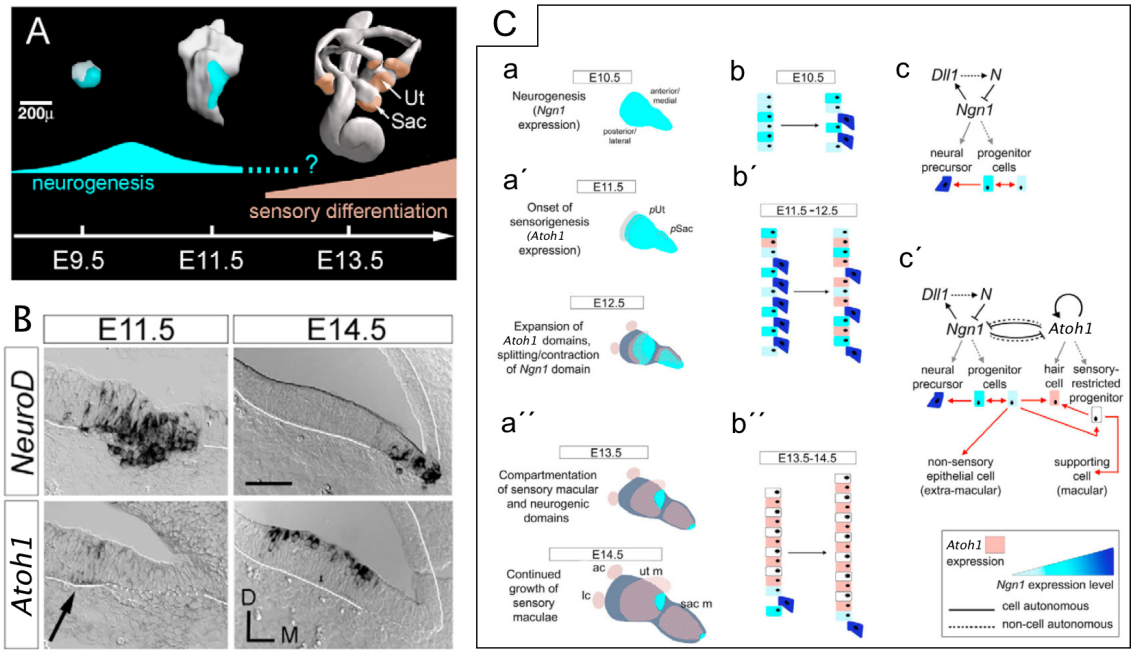


Figure 7. Cross-regulation of Atoh1 and Neurog1 in the mouse inner ear.
A) Temporal-spatial relationships between neurogenesis (cyan) and sensory epithelial differentiation (beige). Activity levels are schematized in color above the time-line. Ut, utricle; Sac, saccule. **B**) Alternating serial sections through the presumptive (E11.5) and definitive (E14.5) utricle, hybridized for *NeuroD* or *Atoh1*. Arrow indicates early *Atoh1* expression. **C**) A model of the transition from neurogenesis to sensory hair cell formation. (**a-a´´**) Tissue-level changes in *Ngn1* expression (cyan), *Atoh1* expression (beige hatching), and regions where *Ngn1* expression has been extinguished (dark blue/gray) from E10.5-14.5. Light gray stripe at E11.5 represents *Bmp4* expression, which marks the prospective anterior and lateral cristae. pUt, presumptive utricular macula; pSac, presumptive saccular macula; ut m, utricular macula; sac m, saccular macula; ac, anterior crista; lc, lateral crista. (**b-b´´**) Changes in gene expression and behavior (delamination) on a cellular scale and over short periods (denoted by arrows) in the neurogenic region of the otocyst (b), presumptive maculae (b´) and definitive maculae (b´´). Shades of blue represent various intensities of *Ngn1* expression (see key). Beige represents *Atoh1*+ cells. White represents cells expressing neither bHLH gene (sensory-restricted progenitors) that can differentiate as either hair or supporting cells. (**c, c´**) Genetic interactions (black lines), gene functions (gray lines) and cell fate transformations (red lines) before (c) and after (c´) the onset of *Atoh1* expression. *Dll1*, delta-like 1; *N*, Notch receptor. Solid gray and black lines indicate cell-autonomous interactions or functions. Dotted gray and black lines indicate non-cell-autonomous interactions or functions. Solid and dotted lines between *Ngn1* and *Atoh1* indicate that either, or both, mechanisms might mediate cross-inhibition. Modified from (Raft et al., 2007).

1.3.6 Extrinsic signals in neurosensory development

Extrinsic signals from the surrounding tissues, important in conferring otic identity early in development, are also in involved in later events such as conferring regional identities, which provide the basis for the correct spatio-temporal formation of hair cells and neurons. Additionally, some extrinsic signals are required directly in the process of cell specification. In the following section, I will do a brief summary of them.

Hindbrain derived FGF is crucial for establishment of the early anteroposterior (AP) patterning of the otic vesicle. Evidence comes from the analysis of *MafB* and related mutants that show defects in hindbrain patterning and defective FGF signaling (Fgf3 and Fgf10 in mouse and Fgf3 in zebrafish). Even though in mouse and zebrafish the *MafB*-related mutations lead to attenuated and increased *Fgf3* expression, respectively, the effects on patterning of the otic vesicle are similar: in mouse, the neurogenic domain is expanded posteriorly and dorsally, as revealed by expression of *Neurog1*, *NeuroD* and *Lfng*, while the non-neurogenic *Lmx1*-domain is smaller (Kwak et al., 2002; Lecaudey et al., 2007; Vázquez-Echeverría et al., 2008). Upon cell specification in the otic vesicle the neurogenic and prosensory domains are converted to FGF signaling centers themselves (Abelló et al., 2010; Alsina et al., 2004; Sweet et al., 2011). At these later stages FGFs might act in a dose-dependent manner to spatially restrict neurosensory territories (Maier and Whitfield, 2014).

The process of neuronal development in the otic vesicle is subject to autoregulation as shown in zebrafish: *fgf5* is expressed in differentiated neuroblasts, and moderate levels of FGF at early times allow continued neuroblasts specification and delamination. As the number of differentiated neurons increases, FGF levels elevate and eventually terminate neuroblasts delamination. Within the SAG Fgf5 levels further have role in balancing the rate of differentiation among the transit amplifying progenitors (Figure 8; (Vemaraju et al., 2012)).

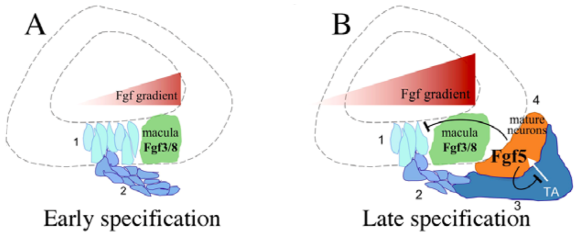


Figure 8. Model for the regulation of SAG development by Fgf.
A) Neuroblast specification at early stages. A moderate level of Fgf3 and Fgf8 in a gradient generated by the utricular macula specifies neuroblasts in the floor of the otic vesicle (step 1), and nascent neuroblasts quickly delaminate from the otic vesicle (step 2). **B**) As development proceeds, neuroblasts establish a pool of transit-amplifying (TA) progenitors (step 3), which eventually differentiate into mature neurons and express Fgf5 (step 4). Rising levels of neuronal Fgf5, combined with Fgf3 and Fgf8 from the growing utricular macula, exceeds an upper threshold that serves to terminate specification of new neuroblasts within the otic vesicle. Neuronal Fgf5 also slows differentiation of progenitors into mature neurons. Diagram of transverse otic sections with lateral to the left and dorsal to the top. Adapted from (Vemaraju et al., 2012).

Retinoic Acid (RA) signals from the posterior mesoderm have been shown to have an effect in AP patterning upon placode formation (Bok et al., 2011). RA is important in specifying the non-neurogenic domain by positively regulating *Tbx1* (Maier et al., 2014; Radosevic et al., 2011). Surprisingly, after placode induction RA appears to favor sensory fates and has been suggested to coordinate together with graded FGF levels the pattern refinement (Maier and Whitfield, 2014).

BMP is expressed not only in the dorsal neural tube, but in all prosensory regions in the chick, and in the prosensory domains of the cristae in mouse and zebrafish. In the development of vestibular hair cells BMP signaling upregulates the expression of *Id* genes and thereby inhibits *Atoh1* expression in these cells. Moreover, BMP signaling reduces proliferation and induces apoptosis in these prosensory domains (Kamaid et al., 2010; Kelley, 2006; Pujades et al., 2006). In the developing organ of Corti in mice BMP is expressed at high levels in the nonsensory epithelium. It has been suggested that BMP specifies sensory and non-sensory fates in a dose dependent manner, where intermediate levels of BMP are responsible for sensory specification (Basch et al., 2015; Ohyama et al., 2010).

Wnt signals from the dorsal neural tube and Shh signals from the ventral neural tube work in an opposing manner in patterning the otic dorso-ventral axis. Shh signals are required for ventral patterning in mice and chick and for posterior patterning in zebrafish (Basch et al., 2015; Raft and Groves, 2015; Riccomagno et al., 2002; Sapede and Pujades, 2010; Schneider-Maunoury and Pujades, 2007). Loss of Shh signaling results in the loss of the CVG and the cochlea in mammals, and in the loss of the posterior macula and the posterior compartment of the SAG in zebrafish (Riccomagno et al., 2002; Sapede and Pujades, 2010). In mammals, Shh signals from the developing spiral ganglion may regulate the timing of differentiation of cochlear hair cells, and *Neurod1* and *Neurog1* might regulate this process indirectly by defining spiral ganglion formation (Basch et al., 2015). Hair cell differentiation in the cochlea normally follows a basal to apical gradient, and inactivation of the Shh receptor Smoothened leads to a shorter cochlea in which progenitors differentiate prematurely in an inverted (apical to basal) gradient. These findings have indicated a role for Shh in promoting proliferation and preventing premature hair cell differentiation in the cochlear duct (Basch et al., 2015).

Wnt signals from the dorsal hindbrain are required for some but not all dorsal otic identities (Basch et al., 2015; Riccomagno et al., 2005). Wnt signaling has also been proposed to play a role in medio-lateral patterning, as fate mapping of Wnt-responsive cells in the dorso-medial otic cup indicated that these cells make broad contributions to the inner ear (Basch et al., 2015; Brown et al., 2015).

Many of the signals that pattern the developing brain have been coopted for regionalization of the otic territory. Even though these signals have been studied extensively, the question of how they integrate in a network with patterning proteins and proneural factors to achieve robust otic development is not very clear just yet. Moreover, these interactions occur in a very dynamic tissue of which, morphogentic movements and growth are the least understood features. It will be important to gather more knowledge about these parameters to integrate information about genetic interactions in spatio-temporal context.

2 AIMS AND SCOPE OF THIS THESIS

A lot of research has been conducted on the genetic requirements for cell fate specification within the sensory organs, and specifically in the inner ear. However, a precise description of the distribution and behavior of progenitors giving rise to these fates in the whole organ context remains elusive. Growth and morphogenetic movements during these processes further complicate the matter as they transform the topologies of the structure at the same time that cells are specified. Extrinsic signals are important in different aspects of otic development but the topological relationships between signaling centers and specific otic domains might change during embryonic development. It has been, and still is, a challenge to dissect early effects of these signals from later ones. Precisely, because we do not know how cells change positions during development and how their behavior might be impacted by manipulations of the system that would then result in collateral effects.

This brought us to specifically concentrate on the following objectives:

1.) To characterize the expression of proneural gene involved in cell fate specification in the zebrafish inner ear.
2.) To assess proneural requirements on hair cell and neuronal fates by functional studies.
3.) To characterize the crude location of progenitors for hair cells and neurons and to investigate their clonal relationship in the zebrafish embryo by the use of photo-convertible tracers.
4.) To establish an experimental pipeline that would allow for in-vivo imaging and of the whole otic structure with cellular resolution during an extended period encompassing neuronal and sensory specification. The aim of this setup was to generate data from which information about lineage relationships and cellular behavior could be extracted.
5.) To generate the map of neurosensory progenitors exploiting the methodologies of the Bioemergences platform for reconstruction of the neurosensory lineages.

6.) To analyze the behavior of progenitors:

- The proliferative behavior of progenitors in context of the sensory domain, and in the whole organ context in control embryos and upon manipulation of proneural gene function.
- The behavior of neuroblasts in the development of the SAG concentrating on proliferative behavior, time and place of delamination and on behavior of these cells within the SAG.

The zebrafish provides some great advantages compared to amniotes in studying otic development: it is small and translucent and develops rapidly. These features make it an ideal model to study progenitor dynamics at a single cell level in the whole organ context by in vivo imaging. Additionally, targeted genomic modifications are becoming more feasible and this will likely yield strategies in which the dynamic gene expression patterns can be assessed by conditional (spatial and temporal) activation or disruption.